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Effects of Organophosphorus, Carbamate, Pyrethroid and Organochlorine Pesticides, and a Heavy Metal on Survival and Cholinesterase Activity of *Chironomus riparius* Meigen

H. Ibrahim,[1] R. Kheir,[2] S. Helmi,[1] J. Lewis,[2] M. Crane[2]

[1]Department of Environmental Studies, institute of Graduate Studies and Research, Alexandria University, Alexandria, Egypt
[2]School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK

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Organophosphorus and carbamate compounds are very widely-used insecticides (Hassall 1990). They are toxic because they inhibit acetylcholinesterase (AChE), the enzyme that is responsible for hydrolysing and so deactivating acetylcholine in the nervous system. AChE-inhibiting pesticides can contaminate surface waters through unintentional drift of aerial spraying for agricultural use, through watershed drainage or accidental spillage, and even through intentional application. Freshwater species can thus be exposed to pesticide concentrations ranging from sublethal to lethal. However, despite the widespread use of AChE-inhibiting pesticides in Europe, there are no standard biological techniques for detecting their presence and monitoring their effects in freshwater biota.

The inhibition of AChE has been used extensively for the assessment of terrestrial wildlife poisoning after the application of pesticides to farmland (Greig-Smith 1991). Inhibition of AChE activity in fish has also been used as a tool for diagnosing organophosphorus pesticide pollution (Lockhart et al. 1985), but fish are able to detoxify these substances more easily than invertebrates. Because of this, significant AChE inhibition in fish can only be detected at relatively high concentrations, which makes fish less suitable than invertebrates as biomonitors for these substances. However, until recently there were few published studies on the use of cholinesterase measurements to assess the effect of pesticides on non-target aquatic invertebrates (Edwards and Fisher 1991). Recent studies (Crane et al. 1995; Kuhn and Streit 1994) show that cholinesterase inhibition in the freshwater amphipod *Gammarus pulex* L. is a useful bioassay for organophosphorus pesticides in both the laboratory and field. Several authors have shown that freshwater dipteran larvae from the genus *Chironomus* can be used in a similar way (Day and Scott 1990; Detra and Collins 1986, 1991; Karnak and Collins 1974). Chironomids may be a particularly useful test species for developing such assays because they can be used to test both water and sediment and may therefore allow an investigator to identify the environmental compartment responsible for any cholinesterase inhibition.

The effect of chemicals on AChE can be investigated by two main approaches. *In vitro* studies are rapid and involve the incubation of the test sample with cholinesterase. Despite being a direct measure of the inhibitory potential of a substance, this approach does not take into account the uptake mechanisms and physiological processes that may mediate bioavailability and toxic effects in

Correspondence to: M. Crane

living organisms. *In vivo* studies are more time-consuming, but take these factors into account. However, several factors other than the presence of cholinesterase-inhibiting pesticides have been implicated in the reduction of cholinesterase activity *in vivo* (Mineau 1991). Hence it is important to assess these before the use of AChE activity is recommended as a sublethal endpoint in aquatic bioassays. In particular, the specificity of the response to organophosphorus and carbamate pesticides needs to be established if AChE inhibition is to have a diagnostic value.

The specific objectives of this study were:

1. to test the effects on chironomid cholinesterase activity both *in vivo* and *in vitro* of five compounds: two of them known cholinesterase inhibitors (the organophosphate pirimiphos-methyl and the carbamate carbofuran), two other pesticides (the pyrethroid permethrin and the organochlorine lindane), and one heavy metal (zinc);

2. to examine the relationship between *in vivo* and *in vitro* inhibition of cholinesterase activity, and between these endpoints and the onset of death.

MATERIALS AND METHODS

Fourth instar *Chironomus riparius* were exposed to several concentrations of test compound in dechlorinated tap water. Chironomids were exposed to 0, 4, 8, 16, 32, 64, 128, 256 and 512 µg/L of pirimiphos-methyl, carbofuran, permethrin, and lindane (Greyhound Chemicals). Exposure to zinc chloride (Sigma Chemicals, Analar) was at concentrations of 0, 1, 10, 20, 40, 80, 160, 320, 640 and 1280 mg/L. Ten animals were exposed in each of three replicates at each concentration over a period of 24-h. Test animals were selected at random from culture and added randomly to glass vessels containing 250 mL of exposure medium. Each vessel was aerated and maintained at 20 °C ± 1°C. Observations on the survival of the chironomids were made after 3, 6, 12 and 24-h. Test animals were considered to be dead if they did not respond to gentle prodding with a probe.

Surviving chironomids from the above experiment were assayed for AChE activity immediately after exposure. Three replicates were assayed for each compound at 0, 4, 8, 16 and 32 µg/L for pirimiphos-methyl, carbofuran and permethrin. Lindane was tested at an additional concentration of 64 µg/L because sufficient animals survived at this concentration. Zinc chloride was tested at 0, 1, 10, 20, 40 and 80 mg Zn/L. The Ellman et al. (1961) method was used to assay for AChE. Samples for AChE analysis were prepared from 5-8 chironomids per replicate. The animals were homogenised in a hand-held homogeniser for 30 seconds in 0.50 mL of a solution consisting of sucrose (0.25M) and Triton-X 100 in phosphate buffer pH 7.4. Triton-X, a non-ionic detergent is used in this assay to solubilise membrane-bound AChE and sucrose is used to maintain the osmotic potential during the assay (Anderson et al. 1972). The homogenate was then centrifuged for 10 minutes at 10,000 rpm in an Eppendorf centrifuge to provide a clear supernatant to avoid problems of light scattering during the assay. The resulting supernatant was used for both the AChE assay and a protein content assay.

Since the catalytic activity of acetylcholinesterase is, like most enzymes, temperature dependent, the bioassay was carried out in a 20°C constant temperature room. Throughout the assay all reagents were kept on ice to maintain their stability. Reagents were added to a 1 mL cuvette in the following order: 0.83 mL phosphate buffer, 0.03 mL 10 mM 5, 5' dithiobis-2-nitrobenzoate (DTNB) in phosphate buffer, 0.10 mL 10 mM acetylthiocholine iodide (ATCI) and 0.03 mL chironomid supernatant. The rate of formation of colour was measured on a Cecil CE 393 Digital Grating Spectrophotometer (Series 2). The spectrophotometer was first set to zero using a blank consisting of all the reagents except the supernatant. The supernatant was then added, the cuvette gently shaken to ensure thorough mixing and the absorbance read at 412 nm on the spectrophotometer every 30 seconds over a period of 7 minutes to allow a kinetic interpretation of the results. Analysis of the amount of protein in each sample was carried using the bicinchoninic acid technique (Pierce) to standardise the AChE activity results for differences in chironomid mass.

Short-term *in vitro* assays were run at high compound concentrations to check that any observed *in vivo* AChE inhibition was a direct result of toxicant exposure. The test compounds were preincubated with cholinesterase extracted from unexposed 4th instar chironomids as described above. The pirimiphos methyl was preincubated at room temperature for 1-h to allow the formation of the more toxic oxon metabolite. The other four compounds were preincubated for five minutes. Three replicates were run for each compound at 0, 0.1, 0.5, 1, 1.5, 2 and 2.5 µM for pirimiphos-methyl and carbofuran. Permethrin, lindane and zinc chloride were tested at 0, 1, 10, 50, 100, 150 and 200 µM. The AChE assay was started by adding 30 µL of 0.01M DTNB and 100 µL 10 mM ATCI.

RESULTS AND DISCUSSION

The 24-h LC10, LC50 and LC80 estimates and 95% confidence limits for each of the test compounds are presented in Table 1.

Table 1. 24-h LC estimates and 95 % confidence interval (in parentheses) for the toxicity of several compounds to *C. riparius.* Data are µg/L for all compounds except zinc chloride (mg/L) Estimates were calculated using a complementary log-log analysis.

Test compound	LC10	LC50	LC80
Carbofuran	0.11	27.2	318.4
	(0.02-0.77)	(15.7-46.9)	(153.1-662.3)
Pirimiphos-methyl	0.46	63.8	579.8
	(0.09-2.23)	(39.1-104.0)	(253.3-1326.9)
Permethrin	0.47	16.6	81.8
	(0.16-1.39)	(11.1-24.8)	(56.1-119.3)
Lindane	4.8	45.3	123.9
	(2.6-8.7)	(34.7-59.2)	(92.0-166.9)
Zinc chloride	3.7	94.3	401.5
	(1.6-8.4)	(66.8-132.9)	(275.4-585.4)

The acute lethal toxicity of the four pesticides was very similar after 24-h exposures, and reasonably precise estimates of both LC10 and LC50 were obtained from the data. Karnak and Collins (1974) found 24-h LC50 values for

third to fourth instar *C. tentans* exposed to organophosphate, carbamate, organochlorine and pyrethroid insecticides in the range 0.9-19.5 µg/L. Fisher et al. (1993) found 24-h LC50 values for cholinergic pesticides and fourth instar *C. riparius* of between 1.2 and 141.3 µg/L (the 24-h LC50 for carbofuran was 7. l-9.6 µg/L). Our results suggest that the acute sensitivity of *C. riparius* to these classes of insecticide in this study was similar.

In vivo cholinesterase activity in chironomids declined with increasing dose when the animals were exposed to pirimiphos-methyl and carbofuran, with effects apparent at the lowest concentrations tested (Figure 1). Day and Scott (1990) suggest that a 20% inhibition of AChE indicates exposure to AChE-inhibiting pesticides. Fifty per cent inhibition is considered diagnostic of mortality in wildlife studies (Grue et al. 1991). Twenty per cent AChE inhibition in this study occurred at estimated concentrations of approximately 1 µg/L pirimiphos-methyl and 4 µg/L carbofuran. At these concentrations an estimated 10-30% of the test population died in the tests. Fifty per cent inhibition occurred at estimated concentrations of 3 µg/L pirimiphos-methyl and 12 µg/L carbofuran. At these concentrations an estimated 20-40% of the test population died in the tests. Since only surviving animals were assayed for AChE activity, it is likely that these results are skewed towards the more tolerant members of the test population.

It would be interesting in future experiments to determine the time course of AChE inhibition for organophosphorus and carbamate insecticides in this species. Detra and Collins (1991) found that 88% inhibition of AChE in *C. riparius* occurred after approximately 4-h exposure to 20 µg/L or 40-min exposure to 400 µg/L of the organophosphate parathion. They were able to develop predictive equations that related exposure time, pesticide concentration and AChE inhibition to visible signs of intoxication. Measurable inhibition of AChE early during the time course of toxic effects may be a useful predictor of other toxic effects. However, it is likely that in most environmental studies the period between the onset of measurable AChE inhibition and the onset of behavioural effects or death will be brief. The most useful role for AChE analysis is therefore as a diagnostic tool for determining the possible cause of poisoning in the field.

To be effective as a diagnostic tool, AChE inhibition should be specific to organophosphate and carbamate pesticides. However, a small, but statistically significant effect on *in vivo* AChE activity was also measured in chironomids exposed to permethrin (Figure 1). A linear model was the best fit for these data. The equation for the line was $y = 2.9824 - 0.011x$ and the slope differed significantly from zero ($t_{14} = 199.63$, $p<0.001$, $r^2 = 0.92$). The mean percentage AChE inhibition at the maximum exposure concentration of 32 µg permethrin/L was 12.2 % of the control. No consistent negative effects on *in vivo* AChE activity were apparent when chironomids were exposed to lindane ($t_{17} = 1.12$, $p=0.28$) or zinc chloride ($t_{17} = 1.63$, $p=0.12$), even at relatively high concentrations. The mean AChE activity for chironomids exposed to lindane remained within 0.6% of the controls. The mean AChE activity for chironomids exposed to zinc chloride remained within 1.3 % of the controls.

The *in vitro* AChE results for the organophosphate and carbamate were very similar to those obtained *in vivo.* Activity declined with dose when cholinesterase extracted from chironomids was exposed to pirimiphos-methyl and carbofuran, with effects apparent at the lowest concentrations tested (Figure 2). Twenty per

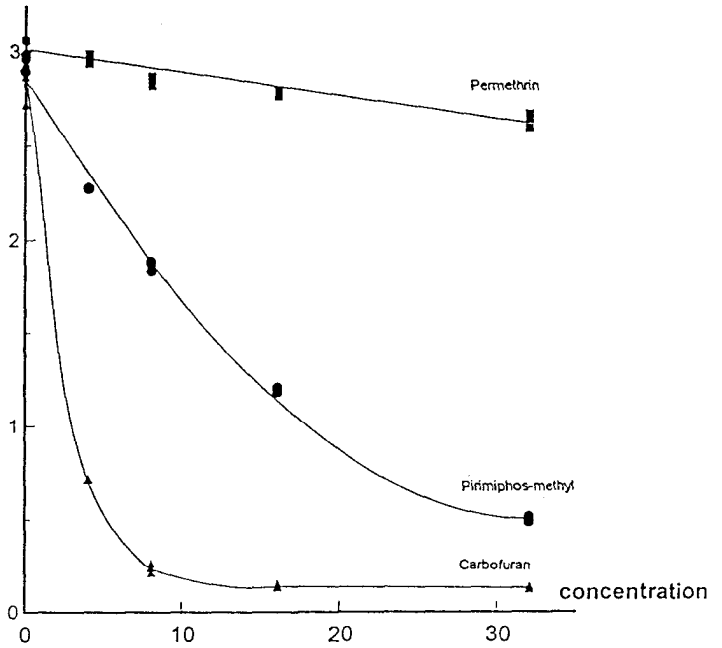

Figure 1. *In vivo* ChE activity (µM AChI hydrolysed/min/mg protein^{-1}) in *C. riparius* exposed to pirimiphos-methyl, carbofuran or permethrin for 24-h (concentrations in µg/L).

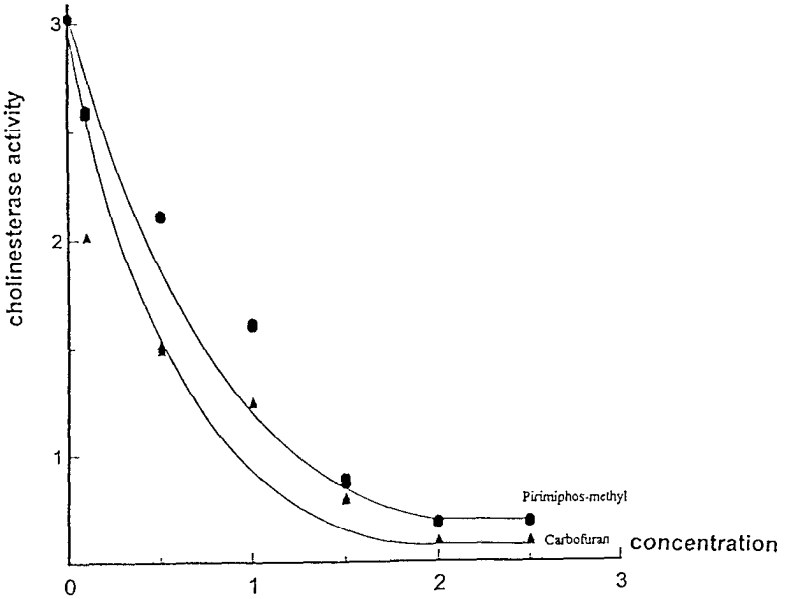

Figure 2. *In vitro* ChE activity (µM AChI hydrolysed/min/mg protein) after preincubation of *C. riparius* cholinesterase with pirimiphos-methyl or karbofuran (concentrations in µM).

cent AChE inhibition occurred at estimated concentrations of 0.19 µM pirimiphos-methyl and 0.06 µM carbofuran. Fifty per cent inhibition occurred at 0.84 µM pirimiphos-methyl and 0.36 µM carbofuran. These results confirmed that the organophosphate and carbamate pesticides directly inhibited AChE, as expected. In toxicity tests, such as those reported here, *in vitro* incubation can be for a short period because high concentrations of test sample are available for use. This accelerates the manifestation of toxic effects by 'replacing' exposure duration with toxicant concentration. If environmental samples such as surface waters were to be bioassayed in a similar way, the investigator would need to consider either concentrating the sample, or extending the duration of incubation to maximise bioassay sensitivity.

There was no evidence *in vitro* for a reduction in activity when chironomid cholinesterase was incubated with permethrin: mean AChE activity remained within 0.2% of controls at all concentrations. The *in vitro* assay was therefore useful in demonstrating that some factor other than direct AChE inhibition by permethrin was responsible for the inhibition observed *in vivo* when chironomids were exposed to this pesticide. Rao and Rao (1995) found that both permethrin and cypermethrin inhibited AChE in rat brain. They speculated that these highly hydrophobic pyrethroids interacted with the hydrophobic aromatic surface region of AChE. This would lead to reduced acetylcholine binding space and hence reduced AChE activity. However, our results suggest that it may be a metabolite of permethrin produced during detoxification that is responsible for AChE inhibition *in vivo* but not *in vitro*. Insects produce many different metabolites when detoxifying permethrin (Shono et al. 1978) and it is possible that one of these was cholinergic. Further work is necessary to determine the metabolic pathways for permethrin in *C. riparius.*

No effects on *in vitro* AChE activity were measured after incubation of chironomid cholinesterase with lindane or zinc chloride: mean AChE activity remained within 0.1% of controls at all concentrations for both compounds.

This study has shown that cholinesterase activity in *C. riparius* declines with dose when animals are exposed to pirimiphos-methyl and carbofuran *in vivo*. A much smaller, but still significant inhibition of AChE can also be measured in chironomids exposed to permethrin *in vivo*. No consistent negative effects on AChE activity are apparent when chironomids are exposed to lindane or zinc chloride. *In vitro* AChE results are very similar to those obtained *in vivo*, except that there is no evidence *in vitro* for a reduction in activity when chironomid cholinesterase is incubated with permethrin. This suggests that any apparent *in vivo* inhibition of AChE in chironomids exposed to permethrin is an indirect, rather than a direct effect. Further work with both Type I and Type II pyrethroids is required to discover whether, and why, *in* vivo inhibition occurs when chironomids are exposed to different members of this class of insecticides. *In vivo* measurement of AChE inhibition in chironomid larvae is potentially useful as a diagnostic tool for contaminated freshwater habitats. Work is currently under way to develop AChE as part of a suite of biochemical biomarkers in *C. riparius* that can be used for *in situ* determinations of water and sediment quality. The results from the present study show that the interpretation of *in vivo* data obtained from field-deployed animals may be assisted by parallel *in vitro* studies.

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