# The Susceptibility to Selected Insecticides and Acetylcholinesterase Activity in a Laboratory Colony of Midge Larvae, *Chironomus Tentans* (Diptera: Chironomidae)

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The adult stage of various chironomids (midges) are considered as pests in several areas of the United States (GRODHAUS 1963). Conversely, larval midges are an important, often major, source of food for fresh water fishes. Thus, depending upon the circumstances, midges are considered as target organisms or nontarget organisms in pest control programs.

When a pest, control measures are often directed at the larval aquatic stage. Most of the papers dealing with insecticides described field studies, laboratory experiments directly related to control methods or the influence of formulation on treatment success. Recently, a laboratory procedure for measuring the acute toxicity of unformulated insecticide to midge larvae was published (MULLA and KHASAWINAH 1969). We devised a similar assay procedure and measured the toxicity of a wide variety of technical grade insecticides to midge larvae.

Two major considerations in physiological studies on toxic effects are metabolic detoxication and inhibition of the target enzyme. Most of the newer chemicals for larval midge control are organophosphate and carbamate insecticides which act as poisons by inhibiting acetylcholinesterase (AChase). Metabolic detoxication figures prominently in explanations of differential susceptibility among and within species. One enzyme system, the multifunction oxidase (MFO), catalyzes the degradation of a wide variety of insecticide chemicals (LYKKEN and CASIDA 1969), requires molecular oxygen and may be inhibited by piperonyl butoxide (PBO), thereby preventing the detoxication of certain insecticides (HODGSON and PLAPP 1970). The MFO system is so prominent in the metabolism of carbamates that the synergism of carbamates by PBO in joint action experiments provides indirect evidence for an MFO system (BRATTSTEN and METCALF 1970). The midge larvae are interesting from this viewpoint because many members of this family (Chironomidae) are able to survive in practically anoxic water (WALSHE 1950, CURRY 1962, AUGENFELD 1967) using hemoglobin to supply oxygen internally when dissolved oxygen is low (WALSHE 1950). The study of $O_2$-requiring protective enzymes (MFO system) in such an animal is

particularly intriguing.

We determined the susceptibility of midge larvae to selected insecticides representing markedly different chemical classes and modes of action and investigated physiological aspects of the organism relating to toxicity. Thus, AChase levels in midge larvae homogenates were measured spectrophotometrically and the MFO detoxication system was investigated indirectly by joint action experiments with insecticides and PBO.

## Methods and Materials

Toxicity assay: The larvae were either third or fourth instar Chironomus tentans Fabricius. This species was collected as larvae at the Jackson Pike sewage treatment plant in Columbus, Ohio and reared to the adult stage in the laboratory. When egg masses of these adults hatched, the larvae were fed Hartz Mountain Dog Kisses$^{R}$ and reared according to a described method (BIEVER 1965) using glass wool as a substrate.

The toxicity assays were conducted at 22°C in quart glass jars containing glass wool (AUGENFELD 1967) and conditioned tap water (aged 24 hr with vigorous aeration). A small amount of food, 150 mg powdered Hartz Mountain Dog Kisses$^{R}$, was also required in the test containers to avoid unusually high control mortality.

The exact assay procedure was as follows: Ten larvae were transferred to test containers containing one-half the final volume of insecticide solution, a small amount of glass wool and food. The remainder of each insecticide solution was added to the appropriate container in such a manner to insure complete mixing. The final volume in all cases was 500 ml. The solutions were gently aerated with air stones for the duration of the assay.

All insecticide solutions or suspensions were prepared by adding an appropriate volume of acetone stock solution (2000 ppm) to water. The insecticides, at least 90% pure except for dieldrin (85%), included allethrin (2-allyl-4-hydroxy-3-methyl-2-cyclopenten-1-one ester of 2,2-dimethyl-3-(2-methylpropenyl)-cyclopropanecarboxylic acid), two organochlorine compounds (DDT, dieldrin), three O-P compounds (dichlorvos, chlorpyriphos and malathion) and five carbamate insecticides (carbaryl, carbofuran, Mesurol$^{R}$ = 4-(methylthio) 3,5-xylyl methylcarbamate, propoxur and Zectran$^{R}$ = 4-dimethylamino-3,5-xylyl methylcarbamate.

Allethrin and carbaryl were assayed for synergism by PBO (a-(2-(2-butoxyethoxy)ethoxy)-4,5-methylene=dioxy-2-propyltoluene) by pretreating the larvae in 250 ml of 0.2 ppm PBO for at least 3 hr, then adding 250 ml of the appropriate insecticide solution so that the final concentration of PBO was 0.1 ppm in 500 ml of test

solution (0.1 ppm PBO is just sublethal).

Each insecticide was assayed at least 3 times and mortality was assessed after 24 hr. The results were pooled and plotted as a composite curve on logarithmic-normal paper and the $LC_{50}$ for each insecticide was interpolated by inspection. Corrections for check mortality were made in each case (ABBOTT 1925) and regression coefficients were calculated with values of y and x that were interpolated from the line.

The determination of mortality was not an easy matter and required some skill and subjectivity as pointed out by others (MULLA and KHASAWINAH 1969). Several investigators have used similar criteria for defining mortality (AUGENFELD 1967, STURGESS and GOULDING 1968, MULLA and KHASAWINAH 1969) which included a lack of movement when touched with a probe, the inability to make undulating movements, and color changes. In our assays, emphasis was placed on the motility of the larvae. Swimming motions and general coordinated movement in response to probing with a dissecting needle were considered to be indicative of a healthy animal. Moribund midges, those that moved and responded abnormally, were included in the mortality counts. In several experiments with different insecticides, moribund midges transferred to clean water after the 24 hr exposure died before or during pupation.

Acetylcholinesterase assay: A spectrophotometric technique was used to assay for AChase activity (ROBBINS et al 1958, BIGLEY and PLAPP 1960). Midge brei were prepared by grinding them for 3-5 minutes in 10 ml of phosphate buffer, pH 7.4, in a glass Potter-Elvehjem tissue grinder immersed in ice chips. The homogenate was filtered through glass wool, then rinsed with enough cold buffer to obtain the desired final concentration. The midge homogenates were prepared as needed but house fly head homogenates (1 head/ml) were prepared as above, divided into aliquots, and kept frozen at -20°C until needed. No significant loss of activity occurred in the latter preparation during experimentation.

The conditions were the same as those described (ROBBINS et al 1958) with minor changes: $NaH_2PO_4$ replaced $KH_2PO_4$ in the buffer, the pH was adjusted to 7.4 rather than 7.2 and acetylcholine bromide (AChBr) was substituted for acetylcholine chloride. A fly-head homogenate was included in almost every experiment as a check on the method since the activity of AChase in fly-head preparations is well documented (METCALF et al 1955, BIGLEY and PLAPP 1960).

## Results

Table 1 contains the interpolated $LC_{50}$ and the regression coefficient (slope) of the response curve for

TABLE 1
Toxicity of Selected Insecticides
to Larval Chironomus tentans

| Insecticide | $LC_{50}$, ppb | Regression Coefficient |
|---|---|---|
| allethrin | 7.8 (13.8)[a] | 2.3 (1.9)[a] |
| allethrin + PBO[b] | 3.9 (5.4) | 2.6 (1.3) |
| allethrin[b] | 9.0 (11.5) | 1.3 (1.1) |
| carbaryl | 2.5 (7.0) | 0.8 (0.6) |
| carbaryl + PBO[b] | 3.1 (4.2) | 1.0 (1.3) |
| carbaryl[b] | 1.2 (1.6) | 1.6 (1.4) |
| carbofuran | 0.7 (1.6) | 1.4 (1.7) |
| Mesurol[R] | 0.9 (1.6) | 0.8 (1.9) |
| propoxur | 1.0 (1.7) | 1.5 (1.4) |
| Zectran[R] | 0.8 (1.8) | 1.0 (1.0) |
| chlorpyriphos | 3.7 (6.4) | 1.8 (1.6) |
| malathion | 1.7 (2.0) | 3.5 (4.6) |
| dichlorvos | 3.7 (5.0) | 1.6 (1.7) |
| DDT | 9.5 (19.5) | 2.1 (2.3) |
| dieldrin | 0.7 (0.9) | 2.7 (2.2) |

[a] values in parentheses are corrected for check mortality
[b] simultaneous experiment to assay for synergism (one trial)

each insecticide. The larval stage of Chironomus tentans was very susceptible to representatives of the major classes of insecticides; all of the insecticides were toxic in the part per billion (ppb) range. Corrections for check mortality ($\bar{x} \pm$ S.D.=20 ± 12%; n=45) among all insecticides resulted in higher $LC_{50}$ values but the regression coefficients generally showed little change (Table 1). In essence, the curves shifted to the right toward lowered toxicity estimates. $LC_{50}$ values in experiments with no check mortality were not significantly different from those requiring correction for death in acetone checks.

Dieldrin was the most toxic of all the insecticides assayed ($LC_{50}$, 0.91 ppb).

Of the five carbamate insecticides assayed, the $LC_{50}$ values for carbofuran, Mesurol[R], propoxur, and Zectran[R] fell in a close interval (1.6 to 1.8 ppb); the $LC_{50}$ for carbaryl was 7.0 ppb.

All three O-P insecticides, i.e. malathion (phosphorodithioate), chlorpyriphos (phosphorothioate), and dichlorvos (phosphate) were in the same order of magnitude of toxicity ($LC_{50}$, 2.0 to 6.4 ppb). Malathion

was the most toxic of the three.

The $LC_{50}$ of allethrin, a synthetic pyrethroid, was 13.8 ppb. DDT was the least toxic of all of the insecticides in this series ($LC_{50}$, 19.5 ppb).

Carbaryl was not synergized by PBO; allethrin was synergized to a small degree. The synergistic ratio ($LC_{50}$, insecticide alone/$LC_{50}$, insecticide + PBO) (BRATTSTEN and METCALF 1970) for carbaryl was 0.8; the ratio for allethrin was 2.3.

The initial *in vitro* AChase experiments were designed to determine the optimum substrate concentration for midges using $1X10^{-2}M$ and $2.5X10^{-3}M$ AChBr, the optimum concentration for fly-head AChase (METCALF et al 1955, BIGLEY and PLAPP 1960). The fly-head activities in our experiments agreed with published values at the above substrate concentrations (METCALF et al 1955, BIGLEY and PLAPP 1960) but no midge AChase activity was measured (Table 2).

TABLE 2
Acetylcholinesterase Activity in
Midge Bodies and Fly-Heads

| Initial AChBr, molar | µmoles AChBr/midge body[a] | | µmoles AChBr/ fly-head[a] |
|---|---|---|---|
| | 1 midge/ml | 4 midge/ml | 1 head/ml |
| $2X10^{-2}$ | 0 | ... | 2.40 |
| $1X10^{-2}$ | 0 | ... | 2.40 |
| $5X10^{-3}$ | 0 | ... | 1.96 |
| $4X10^{-3}$ | ... | 0.02 | 3.23 |
| $2.5X10^{-3}$ | 0.17 | ... | 2.00 |
| $1X10^{-3}$ | ... | .06 | ... |
| $7.5X10^{-4}$ | 0.11 | 0.18 | ... |
| $5X10^{-4}$ | 0.41 | 0.78 | ... |
| $2.5X10^{-4}$ | 0.36 | 0.53 | ... |

[a] corrected for non-enzymatic hydrolysis

The lack of AChase activity in the initial series of experiments with midges led to several additional experiments. First, an endogenous inhibitor of AChase may have been liberated when midges were homogenized but experiments with mixed brei (midge and fly-head) failed to reveal any inhibition of fly-head AChase. With AChBr at $5X10^{-3}M$, no enzymatic hydrolysis occurred in the midge brei, 2.10μmoles were hydrolyzed by the fly-head brei ( 1 head/ml) and 2.12μmoles were hydrolyzed in the mixed brei ( 1 fly head/ml; 1 midge/ml).

Next, we found that midge AChase had a lower optimum substrate concentration than fly-head AChase ($2.5X10^{-3}M$ vs. $2.5\text{-}5X10^{-4}M$) and relatively low levels of AChase activity in midge-body homogenates compared to fly-heads (Table 2). When the midge homogenate was concentrated 4-fold, the AChase activity per midge body increased slightly but did not approach that of fly-heads.

## Discussion

The toxicity assays establish the high degree of susceptibility of the midge larvae to representatives of the major groups of organic insecticides. The dearth of literature on the toxicology of larval C. tentans makes comparison difficult, but $LC_{50}$ values determined by similar laboratory assays were located for several O-P insecticides and tetramethrin with three other chironomid species (MULLA and KHASAWINAH 1969): a range of $LC_{50}$ values between 0.42 and 8.0 ppb was reported for five organophosphates with Chironomus sp. 51. These results and ours are in general agreement regarding susceptibility to O-P insecticides among the Chironomid larvae even though a different species and different assay techniques were employed.

One important consideration in interpreting toxicity assays is the slope (regression coefficient) of the dosage-mortality curves. In most cases, the slopes in our experiments were between 1.00 and 2.00 (Table 1). The slopes for dieldrin and DDT were intermediate (2.25 and 2.31 respectively); that for malathion was the highest, 4.63. The slope of the dosage-mortality curve indicates the homogeneity of the population in its response to the toxicants, a shallow slope indicating great variability in susceptibility among the sampled population. Care must be taken when using laboratory data to predict effectiveness of insecticides in pest control situations. While the $LC_{50}$ values demonstrate a high degree of susceptibility, the concentration needed for effective control (e.g. 95% mortality) may be several-fold greater than the $LC_{50}$. Carbaryl, for example, exhibited an $LC_{50}$ of 7.0 ppb and a slope of 0.61; the $LC_{95}$ interpolated from the dosage-mortality

curve was 3 ppm, approximately a 400-fold difference. Conversely, malathion exhibited an $LC_{50}$ of 2.05 ppb with a slope of 4.63; the interpolated $LC_{95}$ was 4.55 ppb, only a 2-fold difference.

The susceptibility of midge larvae to insecticides may, in part, be due to the low activity of protective enzymes such as the multifunction oxidase (MFO). Joint exposures with carbaryl or allethrin and PBO resulted in low synergistic ratios, a good indication that the MFO system has low activity in this species. However, extreme susceptibility to insecticide alone generally corresponds to low synergistic ratios with an MFO inhibitor (BRATTSTEN and METCALF 1970). Further circumstantial evidence for the presence of at least minimal MFO activity is indicated by the minor differences in susceptibility to a phosphorothioate, phosphorodithioate and phosphate insecticide. The first two are oxidized (desulfurated) *in vivo* by MFO in other insects to form more toxic compounds (LYKKEN and CASIDA 1969) and their similar toxicities (Table 1) suggests that this occurred in midges. Otherwise, wider variation in toxicity may be expected among these 3 O-P insecticides. Although this is not a rigorous analysis, it seems that this species, often living in environments with very low dissolved oxygen, may utilize the oxidative MFO system for metabolizing insecticides, at least when adequate dissolved oxygen is present.

The *in vitro* assays for AChase (Table 2) demonstrated low AChase activities in midge homogenates. Two groups of insecticides tested herein (organophosphates, carbamates) are toxic by virtue of AChase inhibition, suggesting that susceptibility to these insecticides may be due to low amounts of the target enzyme. However, formal enzyme kinetics of the inhibitory process must be investigated before formulating conclusions on the sensitivity of AChase to inhibition. Stated otherwise, one cannot assume that preparations with low *in vitro* enzyme activities would require proportionately less inhibitor to effect inhibition.

Past observations have revealed an apparent correlation between physical activity and levels of AChase (METCALF et al 1955). Active insects such as some Diptera, bees, ants, and cockroaches had a maximum of AChase activity while the less active coleopterous and lepidopterous larvae showed a minimum of activity. The chironomid larvae would fit into the latter group of less active organisms (SADLER 1935, WALSHE 1950). In light of these observations the low AChase activity noted for the midge homogenates should not be considered unusual.

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