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<th>Title</th>
<th>Mechanism of the Selective Toxicity of Organophosphorus Compounds in the Armyworm, Leucania separata Walker. Port I. Topical Toxicity and Anticholinesterase Activity of Certain Organophosphorus Compounds</th>
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<tr>
<td>Author(s)</td>
<td>SINCHAISRI, Neungpanich; MIYATA, Tadashi; SAITO, Tetsuo</td>
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Kyoto University

Armyworm, *Leucania separata* Walker is one of the important pests of corn, *Zea mays* L. and rice, *Oryza sativa* L. Many ecological and physiological works have been reported so far but it seems that publications concerning with the toxicological study on this species are still lack. The toxicity test with 31 insecticides to the armyworm larvae indicated that most of organophosphorus (OP) insecticides were highly toxic whereas carbamates were not and endrin and dieldrin, the only two organochlorine compounds gave relatively high effect.

It is generally acknowledged that OP insecticides kill animals by inhibiting cholinesterase (ChE). Most of them are rather poor inhibitors of ChE *in vitro*. They owe their potency *in vivo* to the fact that they are converted in the body to give compounds which are presumed to be the direct inhibitors.

Thus, as the first approach to clarify the mechanism of selective toxicity of four OP compounds, methyl parathion, phenthoate, diazinon and fenitrothion against the armyworm, topical toxicity of them and anticholinesterase activity of their oxygen analogs *in vitro* were investigated.

Despite the measurement of ChE activity by the method described Hestrin showed the unsatisfactory result in the preliminary procedure due to the strong interference of melanization effect in the armyworm enzyme, the radioisotopic assay, exercised in this study, offered much advantages and quite desirable for the micro-volume of solution in the measurement of ChE activity.

**Materials and Methods**

**Insect**

The colony of the armyworm larvae was obtained from the University Farm, Nagoya University, Togo, Aichi prefecture in 1974. The larvae were reared at 25°C in the plastic box, 24×30×5cm, fed with fresh corn leaves as their basic food (partly fed with the leaves of Italian Rye grass to keep the successive generations during winter season). The mass rearing technics were essentially the same as the previous described. The 5th-instar, 2-3-day larvae were provided for experiments.

**Chemicals**

Over 97% purity of four organophosphorus compounds were used. Methyl parathion and...
fenitrothion were received from Sumitomo Chemical Co., Ltd., phenthoate was obtained from Nissan Chemical Industry Ltd., diazinon was obtained from Nihon Kagaku Co., Ltd.

Cholinesterase inhibitors, P=O analogs of these 4 OP compounds were synthesized by an oxidation of P=S compounds in bromine solution and purified by thin layer chromatography (t.l.c.).

Acetyl [1- 14C]-choline chloride (14C-AChCl), 13.7 mCi/m mole, was purchased from Amersham Co., England. The Amberlite CG-120, type 2, resin sodium salt was a product of Organo Co., Tokyo. Chemicals for the preparation a scintillation solution, for instances, naphthalene (special grade), 2,5-diphenyloxazole (PPO), dimethyl POPOP and 1,4-dioxane were products of Katayama Chemical Co., Ltd., Osaka.

Toxicity test
The evaluation of contact toxicity of 4 OP compounds was accomplished by topical treating the 5th-instar larvae of the armyworm with 0.7 µl of acetone solution containing amount of OP compounds. In control, insects were treated with the same volume of acetone. Insects were fed with fresh corn leaves and kept in a constant temperature chamber of 25°C. After 72hr, mortality counts were taken. Badly affected or moribund larvae were justified as dead insects according to the method of Ando and Sherman11). At least 4 replicates of 10 insects at 5-8 dosage levels were provided for each insecticide in this test. The values of LD50 and LD95 were calculated by the method of Finney12) and toxic index was evaluated by the method of Sun13).

Radioisotopic assay of ChE activity
Substrate solution.--Stock solution of 14C-AChCl, 8 x 10^-3M, containing approximate 3, 300, 000 cpm/ml was prepared by dissolving the contents of AChCl from a ampoule in distilled water. Stock solution of non-labelled AChCl, 0.55M, was simultaneously prepared. The mixture of labelled and non-labelled AChCl was provided to obtain higher concentrations than 8 x 10^-4M of AChCl with highly enough radioactivity for the experiments. The mixture were always kept under -20°C.

Source of enzyme.--Three kinds of homogenate, head (H), whole body (WB) and brain & ventral nerve cord (B & VNC), from the 5th-instar larvae of the armyworm were prepared separately. Homogenates of H and WB were centrifuged at 900g for 5 min. The supernatants were used as enzyme sources throughout the study. B & VNC homogenate was directly applied as the enzyme source without centrifugation.

Buffer's solution.-Phosphate buffer's solution, 1/15M, pH 7.4, was mainly used in the routine works. In the experiment of an effect of pH on the ChE activity, buffer of Chadwick et al.8) was used: NaCl, 26.30g; KH2PO4, 3.85g; NaOH, 1.00g; water to make one litre. With all suspensions, pH was adjusted to the desired value by addition of 0.1N NaOH or 0.1N HCl. The range of pH values adjusted would cover enough in the acidic and alkaline conditions from pH 6.1-9.2.

Amberlite resin-dioxane mixture.--Preparation was essential the same as the method described by Siakotos et al.14). Ten grams of dried resin was put into 100ml of dioxane.

Scintillation solution.--It was a modified Bray's Cocktail15) containing 100g naphthalene, 4g PPO, 200mg POPOP and 100ml absolute methanol, and then made to 1 litre with 1,4-dioxane.

Assay procedure
With an equal volume of 0.05ml, the mixture of enzyme and 14C-AChCl solution was put in a 20ml-tube, and the content of the tube was well mixed by shaking and incubated for 10 min at 37°C. Non-enzymatic hydrolysis of the substrate was measured with the solution containing of 0.05ml of buffer instead of enzyme. ChE activity was expressed as µ mole of the substrate hydrolyzed/hr/g.

The proceeding steps of reaction were assayed as previously described by Siakotos et al.14). In order to stop the reaction, an approximately 5ml of the suspended resin-dioxane mixture was added. Then, the enzyme-resin-dioxane solution was brought to an exact volume of 10ml with dioxane. The tube was tightly capped, and mixed by inversion or shaking and allowed it to stand. The resin was allowed to settle by gravity for at least 2 hr. Five millilitres of the supernatant solution was transferred to a scintillation counting vial, and 10ml of the modified Bray's scintillation solution was added. Special care must be taken
to avoid transferring resin particles into the vial. The samples, total volume of 15ml, were counted the radioactivity of the hydrolysis product in a liquid scintillation spectrometer (Tricarb Model 3320 Liquid Scintillation Spectrometer) which has an efficiency about 60% at the optimized 14C setting.

For the inhibition study, inhibitors (P=O analogs of OP compounds) were dissolved in acetone in widely different concentrations. With the volume of 0.01ml, an inhibitor solution was delivered by means of a microsyringe into the 20ml tube and allowed acetone to evaporate absolutely, 0.05ml of enzyme solution, then after, was added and pre-incubated at 37°C for 30 min and then, 0.05ml of 14C-substrate was added, incubated at 37°C for 20 min. The next procedure for ChE activity measurement was carried out by the same manner as described above.

Results

Toxicity test
LD50 and LD95 values of 4 OP compounds were shown in Table 1. Methyl parathion gave the highest toxicity to the 5th instar larvae of the armyworm. The relative toxicities of fenitrothion, phenthoate and diazinon were lower than methyl parathion, a standard, as expressed by toxic index value of 297, 383 and 1080 respectively.

Measurement of ChE activity
Substrate concentration.- The ChE activities of three homogenates were shown in Fig. 1. From the bell shaped curves in Fig. 1, it is likely to presume that the maximum enzymatic activities of H and WB are quite similar to each other at 2.5×10⁻⁴M final concentration of the substrate, but it needs higher concentration of 1.0×10⁻³M to produce the maximum activity for tissue homogenate of B & VNC. The data of this experiment provided an additional evidence that ChE in the armyworm is true ChE.

Enzyme concentration.- From Fig. 2, enzymatic

Table 1. LD50, LD95, regression equation of dosage-mortality and the relative toxicity of 4 organophosphorus insecticides topically treated on the 5th instar larvae of the armyworm.

<table>
<thead>
<tr>
<th>Insecticides</th>
<th>Regression equation</th>
<th>LD50 (µg/g)</th>
<th>LD95 (µg/g)</th>
<th>Toxic index (LD50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl parathion</td>
<td>( Y = 5 + 3.9893(x - 0.9916) )</td>
<td>0.97</td>
<td>2.51</td>
<td>100</td>
</tr>
<tr>
<td>Phenthoate</td>
<td>( Y = 5 + 2.5934(x - 1.5729) )</td>
<td>3.72</td>
<td>15.85</td>
<td>383</td>
</tr>
<tr>
<td>Diazinon</td>
<td>( Y = 5 + 3.1467(x - 2.0263) )</td>
<td>10.48</td>
<td>34.75</td>
<td>1080</td>
</tr>
<tr>
<td>Fenitrothion</td>
<td>( Y = 5 + 1.7571(x - 1.4684) )</td>
<td>2.88</td>
<td>25.20</td>
<td>297</td>
</tr>
</tbody>
</table>

\( Y = \) mortality in probit, \( x = \) log dosage (µg x 10)
activity was in the proportional rate to enzyme concentration up to 10% in H, 20% in WB and 4% in B & VNC enzymes. Higher enzyme concentrations yielded non-linear results.

Time course of the reaction.—As illustrated in Fig. 3, the time course of the substrate hydrolysis by enzymes was demonstrated. Absolute linearity was found in ChE activities of all homogenates up to 20 min incubation. The hydrolysing rates decrease with the time thereafter. The sharp decrease occurred from 60 min in H and WB but slow decrease was found in B & VNC homogenates.

Optimum pH.—Based on the suitable condition obtained from the previous experiments, the measurement of ChE activity as a function of pH was carried out and the obtained results were illustrated in Fig. 4. The effect of pH in the reaction of enzyme and substrate caused different enzymatic activity. The optimum pH of H and B & VNC homogenates were obtained in slight alkaline condition at the range of 8.0-8.6 while of WB homogenate was almost neutral at the range of 7.2-7.4 (Fig. 4 and Table 2).

The peaks of the curves in Fig. 4 would represent the maximum ChE activity of the armyworm from different enzyme sources which were
Table 2. The different properties among the sources of enzyme in the armyworm larvae

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Optimum</th>
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<tbody>
<tr>
<td></td>
<td>AChCl</td>
<td>pH</td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>2.5×10⁻⁴M</td>
<td>8.0-8.3</td>
<td></td>
</tr>
<tr>
<td>Whole body</td>
<td>2.5×10⁻⁴M</td>
<td>7.2-7.4</td>
<td></td>
</tr>
<tr>
<td>Brain &amp; ventral nerve cord</td>
<td>1.0×10⁻³M</td>
<td>8.3-8.6</td>
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Table 3. Cholinesterase activity in the armyworm larvae

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>ChE activity (µmole AChCl hydrolyzed/hr/g)</th>
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</thead>
<tbody>
<tr>
<td>Head</td>
<td>53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole body</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain &amp; ventral nerve cord</td>
<td>265</td>
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shown in Table 3. ChE activities were 53 µmoles AChCl hydrolyzed/hr/g in H, 15 µmoles in WB, and the highest activity was found in B & VNC at 265 µmoles AChCl hydrolyzed/hr/g.

Anticholinesterase activity
P=O analogs or oxon isomers of 4 OP compounds were applied as ChE inhibitors in the ChE inhibition test. The results were tabulated in Table 4. Methyl paraoxon was given as a standard compound in the comparative inhibitory activities. The activity was expressed as 50% inhibition (IN₅₀) and 80% inhibition (IN₈₀) by the molar concentrations of the inhibitors.

Diazoxon and fenitroxon gave lower inhibitory activities than methyl paraoxon which, by all cases, were well corresponding with their P=S toxicities. But phenthoate-oxon showed an exceptional result where it exhibited its anti ChE activity about 5 times higher than methyl paraoxon while its corresponding P=S toxicity was 3.8 times lower.

Discussion
The basic properties of ChE in the armyworm enzymes from H, WB and B & VNC homogenates seemed to be the typical characteristic of true
ChE but it needs much lower concentration of substrate (2.5×10^-5 M) to produce a maximum enzymatic activity, particularly, in head and whole body homogenates than the other insects tissue homogenates (5×10^-4 M-1.0×10^-2 M). This obtained optimum substrate concentration strongly confirms the failure in the measurement of ChE activity by the colorimetric method described by Hestrin. As well as the interference of melanization effect of enzyme solution, too low concentration will give a narrow range in activity variation and subsequently much larger error appears causing a difficulty in enzymatic activity measurement. The optimum pH in H and B & VNC homogenates were obtained at the range of 8.0-8.6 which was also found in the enzyme of developing grasshopper eggs, house fly head, and cat brain. Stegwee, working with central nerve tissue of beetle, Hydrophillus and roach, Periplaneta, recorded rather sharp optima at pH 7.4 which is quite similar to the result with whole body homogenate in this experiment.

It is evident from previous works that interpreting the selective toxicity of different insecticides is difficult because the toxicity data are the end-product of the complex interaction of many factors. According to Saito and others, factors responsible for selectivity of insecticides were divided into 3 steps; differences of distribution of toxicants in insect body, differences of susceptibilities of insect ChE to toxicants and differences of activation and detoxification metabolism of toxicants. Metcalf and March demonstrated the strong relationship between the in vitro antiChE activity and the in vivo toxicity of diisopropyl paraaxon in mouse, honey bee, and cockroach. The similar relationship between house fly and mouse was reported in the diethyl substituted phenyl phosphate compounds.

The correlation coefficient (r) of toxicity and antiChE activity among 4 OP compounds were calculated as shown in Table 5. From r values, seemingly, there is a weak correlation among all four compounds but positive correlation is obtained among three compounds except phenthoate.

Considering the toxic index of phenthoate is 383 and antiChE activity ratio of phenthoate-oxon is 0.12-0.37 in Inso and Inso of all three sources of enzyme, the mechanism of selective toxicity of phenthoate might associate with other factors being different from those of other 3 OP compounds.

In some cases two factors may cancel each other out. This has been shown for OP compound, famphur which is degraded eight times faster by mice than by milkweed bugs, yet is 20 times better against mouse than against milkweed bug ChE in vitro. The combination of two factors led to a very small selective toxicity, famphur is only 1.5 times more toxic to milkweed bugs than to mice. The other work supports this evident

<table>
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<tr>
<th>Sources of enzyme</th>
<th>Correlation coefficient (r)</th>
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<tbody>
<tr>
<td></td>
<td>LDso-INso</td>
</tr>
<tr>
<td>Head</td>
<td>r = 0.1844</td>
</tr>
<tr>
<td></td>
<td>r1 = 0.8306</td>
</tr>
<tr>
<td>Whole body</td>
<td>r = 0.3114</td>
</tr>
<tr>
<td></td>
<td>r1 = 0.9019</td>
</tr>
<tr>
<td>Brain and ventral nerve cord</td>
<td>r = 0.4087</td>
</tr>
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<td>r1 = 0.7508</td>
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</table>

r values were calculated from the logarithmic values of LDso×10, LDso×10 INso×10^6, and INso×10^6; r1 is the values of correlation coefficient calculated without phenthoate.
as the data are enable an explanation of unusual features of the toxic action of dimethoxon on Periplaneta americana L. comparing with diazoxon. From a small restriction of both compounds to penetrate into the central nervous system (CNS), dimethoxon was found 200 times less active than diazoxon (both against cockroach ChE and in electrophysiological test against the CNS of cockroach). Yet, this weak neurotoxicity, however, is compensated for by relatively high stability to detoxication and allowing sufficient to accumulate in insect to render it almost as toxic to cockroaches as a more active antiChE but more rapidly being detoxified like diazoxon.

In order to clarify the mechanism of selective toxicity of these 4 OP compounds, other factors (metabolism, penetration and etc.) will be contributed to the further outline of experiments.

Summary

As the first step to clarify the selective toxicity of 4 OP compounds in the armyworm, Leucania separata Walker, the bioassay and in vitro antiChE activity were studied. The compounds were treated topically to the 5th-instar, 2-3-day larvae. Comparing their relative toxicities by LD_{50} values, it was found that the toxic index of fenitrothion, phenthoate and diazinon to methyl parathion were 297,383 and 1080 respectively.

Cholinesterase (ChE) activity was measured by radioisotopic method using ^14C-acetylcholine chloride (AChCl) as a substrate. ChE of the armyworm shows the typical characteristic of true ChE having an optimum substrate concentrations: $2.5 \times 10^{-4}$M with tissue homogenates of head and whole body and $1.0 \times 10^{-4}$M with brain and ventral nerve cord homogenate.

The results of in vitro antiChE activity with oxon isomers of the compounds were orderly accomplished; phenthoate-oxon $>$ methyl paraoxon $>$ diazoxon $>$ fenitrooxon.

The correlation between the toxicity of the OP compounds and the antiChE activities of their oxygen analogs was examined. Statistically, basing on the correlation coefficient values ($r$), it seems that there is poor correlation between toxicity and antiChE activity data among 3 compounds, methyl parathion, fenitrothion and diazinon. Unexpectedly, phenthoate was an exception where its antiChE activity was the highest, exhibiting about 5 times higher inhibitory activity whereas it was 3.8 times less toxic than that of methyl parathion, a standard compound. This might indicate that the mechanism of selective toxicity of phenthoate against the armyworm is attributable to the different feature from those of other three OP compounds.

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15) Kanehisa, K.: Bull. of Lab. of Appl. Ent. and Nema. Faculty of Agri. Nagoya Univ. Aichi,


