Relation of AChE and AliE Inhibition of Anti-AChE Insecticides, TIA-230, Fenitrothion and Carbaryl, to Their Insecticidal Activity in *Spodoptera litura* Larvae (Lepidoptera: Noctuidae)

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Insecticidal activities of three AChE inhibiting insecticides, TIA-230, fenitrothion and carbaryl, were compared in *Spodoptera litura* larvae. Inhibitory activities of the insecticides to AChE of isolated nerve cords and to AliE of fat body and haemolymph were also investigated. By injection, TIA-230 was the most insecticidal with an LD₅₀ 1/7.5 that of carbaryl. Fenitrothion was the least active, but its activated form, fenitroxon, showed the same level of activity as TIA-230. There was no correlation between insecticidal activity and AChE inhibition. Fenitroxon showed very high affinity to AliE, so sequestering of this compound by AliE seemed to be one of the degradation factors. In TIA-230, the sequestering was not a degradation factor because of its low affinity to AliE. These results explained well why TIA-230 showed potent activity and fenitrothion did not. As to carbaryl, although oxidation and sequestering were ruled out as main degradation factors, the main factor could not be identified in these studies.

INTRODUCTION

An *O*-ethyl *S*-*n*-propyl phosphorothiolate, TIA-230, shows potent insecticidal activity against Lepidopteran insect pests, especially against *Spodoptera* spp. and *Heliothis* spp. which are rather tolerant to *O*, *O*-dialkyl phosphorothioates.

TIA-230 itself is a weak acetylcholinesterase (AChE) inhibitor, but is oxidatively activated to a potent inhibitor in the nervous system of insects to exhibit insecticidal activity (KONO et al., 1983 a, b). TIA-230 has other characteristics showing inhibitory activity against non-specific esterases (AliE) of insects, based on its $P=O$ structure, but its activity varies among insect species. In a previous paper (KONO and MANABE, 1983) it was demonstrated that the insecticidal activity of TIA-230 was negatively correlated with its inhibitory activity against AliE, e.g., insect species having AliE less susceptible to TIA-230 were more susceptible to it. This suggests that sequestering TIA-230 by the AliE is one of the degradation factors in the insect body (DEVONSHIRE and MOORES, 1982).

In the present paper, susceptibility of *Spodoptera litura* larvae to TIA-230, fenitrothion and carbaryl, all of which demonstrate their activity through AChE inhibition in the insect nervous system, were compared and inhibition of these insecticides against AChE and AliE of the larvae was tested in order to know the difference in modes of action.

MATERIALS AND METHODS

1. *Insects.* Sixth instar larvae of *Spodoptera litura* were selected from a stock culture maintained in our laboratory and used for the experiments.

2. *Chemicals.* TIA-230: *O*-[1-(4-chlorophenyl)-4-pyrazolyl] *O*-ethyl *S*-propyl phosphorothiolate and fenitroxon were synthesized in our laboratories, purified by column chromatography and characterized by infrared and NMR spectra. Fenitrothion (99.0% purity), piperonyl butoxide (PB) and IBP were purchased from Wako Chemicals Industries, Ltd. Carbaryl used was a technical material of 98% purity.

3. *Test of insecticidal activity.* Topical application and injection were adopted for the test of insecticidal activity. One microliter per larva acetone solution including various doses of insecticides was applied in both methods. To test the synergistic action of PB and IBP, 30 μ g/larva of each synergist was topically applied 30 min prior to the application of the insecticides. Dead insects were counted 1 day after the application (20 larvae in each dosage, 25°C) and LD₅₀s were calculated from the mortality by computer (IPSEN and FEIGL, 1970).

4. *Measurement of AChE activity.* AChE activity was measured by the method of ELLMAN et al. (1961); details were given in a previous paper (KONO et al., 1983 b).

5. *Inhibition of AChE in the nerve cord (in situ measurement).* The nerve cord from the metathoracic ganglion to the terminal abdominal ganglion was isolated from the final instar larvae of *Spodoptera*. Four isolated nerve cords were incubated in 1 ml of LOCKE's saline containing 10^{-7} – 10^{-5} M insecticides for 15 min at 25°C. After rinsing with the saline, the nerve cords were homogenized in 2 ml of the ringer solution (0.9% NaCl) containing 10^{-3} M acetylcholine iodide to prevent the further inhibition of AChE in later processes. AChE activity of the homogenate was measured by the method previously noted. Percent inhibition of AChE activity was calculated by comparing with the activity of the control without inhibitors.

6. *Measurement of AliE activity.* AliE of the fat body and haemolymph were separated by polyacrylamide gel electrophoresis (7.5% gel, pH 7.3), using glycine-rutidine buffer (pH 8.3) as a running fluid. Fat bodies gathered from 5 individuals were homogenized in 0.3 ml ringer and centrifuged for 10 min at 3,000 rpm. Ten microliters of the supernatant was mounted on a gel column (0.3 cm in diameter and 6 cm in length). As a haemolymph sample, 5 μ l of gathered haemolymph from 5 individuals was mounted.

After the electrophoresis, the gel discs were incubated in a phosphate buffer solution (pH 6.8) containing 10^{-6} or 10^{-4} M insecticides at room temperature (ca. 25°C) for 20 min. Then, the discs were incubated in the buffer with α -naphthylacetate and fast blue RR salt at 30°C for 30 min for the detection of AliE activity. The gel discs colored were scanned by a densitometer (Gelman Sciences, DCD-16) at 525 nm wavelength. Inhibitory activity of insecticides was quantitatively estimated by comparing the height of density peaks in the treated and control discs.

Measurement of AChE and AliE activities was duplicated and standard deviations were calculated.

RESULTS

1. *Insecticidal activity*

The results of topical application of the three insecticides to final instar larvae of

Table 1. Insecticidal activity of TIA-230, fenitrothion and carbaryl against the final instar larvae of *Spodoptera litura*, and influence of piperonyl butoxide (PB) and IBP on the activity

Compound	LD ₅₀ and (95% confidence limit) $\mu\text{g}/\text{insect}$				A/B
	Topical application			Injection	
	Alone (A)	+ PB ^a	+ IBP ^a	Alone (B)	
TIA-230	2.49 (1.72–3.62)	2.50 (1.78–3.50)	2.70 (1.89–3.86)	0.71 (0.55–0.91)	3.2
Fenitrothion	15.60 (9.14–26.60)	28.28 (18.98–42.16)	15.90 (8.68–29.14)	9.97 (6.86–14.48)	1.6
Fenitroxon	8.98 (6.67–12.03)			0.99 (0.59–1.66)	9.1
Carbaryl	107.4 (64.78–176.86)	97.1 (28.84–327.17)	86.9 (50.60–149.37)	5.49 (2.77–10.91)	19.5

^a 30 $\mu\text{g}/\text{larva}$ of PB or IBP was applied 30 min prior to the insecticide application.

Table 2. AChE inhibition by TIA-230, fenitroxon and carbaryl in the nerve cord of *Spodoptera* larvae (*in situ*^a) and *in vitro*

Compound	Conc. (M)	% inhibition <i>in situ</i> mean $\pm$ S.D.	I-50 <i>in situ</i> (M)	I-50 <i>in vitro</i> (M)
TIA-230	10 ⁻⁷	0 $\pm$ 4.7	2.0 $\times$ 10 ⁻⁶	1.0 $\times$ 10 ⁻⁴
	10 ⁻⁶	36.3 $\pm$ 0.7		
	10 ⁻⁵	82.2 $\pm$ 3.5		
Fenitrothion	10 ⁻⁵	48.9 $\pm$ 3.1	1.0 $\times$ 10 ⁻⁵	4.0 $\times$ 10 ⁻³
Fenitroxon	10 ⁻⁷	11.1 $\pm$ 6.4	1.3 $\times$ 10 ⁻⁶	5.0 $\times$ 10 ⁻⁷
	10 ⁻⁶	47.3 $\pm$ 6.3		
	10 ⁻⁵	82.9 $\pm$ 12.7		
Carbaryl	10 ⁻⁷	23.2 $\pm$ 0	6.5 $\times$ 10 ⁻⁷	5.1 $\times$ 10 ⁻⁷
	10 ⁻⁶	60.7 $\pm$ 2.5		
	10 ⁻⁵	80.9 $\pm$ 0.3		

^a The nerve cords were incubated in the insecticide solution.

Spodoptera with and without synergists, PB, a mixed function oxidase inhibitor, and IBP, an AliE inhibitor, are shown in Table 1. LD₅₀s of TIA-230, fenitrothion and carbaryl were 2.5, 15.6 and 107.0 $\mu\text{g}/\text{insect}$, respectively. The insecticidal activity of carbaryl was very weak in this method of application. When PB was added, insecticidal activity of fenitrothion was obviously decreased, but that of the other insecticides was not affected. Synergistic action was not observed in the addition of IBP to the insecticides.

When the insecticides were injected into the abdominal body cavity of the larvae, the activity was stronger than that in topical application (Table 1); carbaryl especially showed much higher activity. Fenitroxon, an activated form of fenitrothion, showed ten times higher activity than its mother compound, fenitrothion.

Poisoning symptoms by carbaryl injection, hyperactivity and convulsion, appeared quickly within 10 min following the application, but some individuals recovered from the poisoning in a day. Symptoms also appeared soon after the injection of fenitroxon. On the other hand, symptoms of excitation by the injection of TIA-230 and fenitrothion appeared after 30 min.

Rates of LD₅₀ in topical application to LD₅₀ in injection were as follows: TIA-230: 3.2, fenitrothion: 1.6, carbaryl: 19.5, fenitroxon: 9.1. A high rate of carbaryl indicated that the penetration process was a difficult barrier for this insecticide.

2. AChE inhibition

Measured *in vitro*, carbaryl was a potent AChE inhibitor (I-50: 5×10^{-7} M), but TIA-230 and fenitrothion showed only weak inhibitory activity (Table 2) with I-50s of over 10^{-4} M. Fenitroxon showed activity as high as carbaryl.

When the isolated nerve cords were incubated in the saline solution containing insecticides (*in situ* measurement), intensity of inhibition to AChE differed from the results of *in vitro* inhibition. TIA-230 showed high inhibitory activity, and its I-50 was

Table 3. Inhibition of separated AliE activity from fat body and haemolymph of *Spodoptera litura* larvae by insecticides

Compound	Conc. (M)	% inhibition (S.D)			
		Fat body		Haemolymph	
		F 1	F 2	H 1	H 2
TIA-230	10^{-6}	11.76 (4.29)	32.70 (0.83)	2.25 (3.18)	5.00 (7.07)
	10^{-4}	45.10 (6.93)	43.10 (0.28)	10.45 (8.41)	21.25 (5.30)
Fenitroxon	10^{-6}	55.65 (1.63)	64.75 (6.01)	24.60 (3.90)	7.10 (4.61)
	10^{-4}	89.70 (1.98)	83.30 (2.55)	100.0 (0)	93.00 (0.52)
Carbaryl	10^{-6}	12.60 (17.80)	16.65 (23.50)	12.75 (—)	1.97 (—)
	10^{-4}	72.95 (1.48)	73.05 (3.89)	32.89 (—)	44.12 (—)

Fig. 1. Scanning chart of AliE activity separated from fat body (top, F1, F2) and haemolymph (bottom, H1, H2) by polyacrylamide gel electrophoresis. F: front, O: origin. α -Naphthyl acetate was used as a substrate.

2.0×10^{-6} M. This result indicates the activation of TIA-230 in the nerve cord, as already mentioned. Carbaryl and fenitroxon strongly inhibited the nerve AChE *in situ*. I-50 of carbaryl was close to that of *in vitro* measurement, but that of fenitroxon was larger than the I-50 *in vitro* measurement. Fenitrothion did not show high inhibitory activity (49.8% at 10^{-5} M) *in situ* compared with TIA-230 (82.2% at 10^{-5} M), though fenitroxon did show strong inhibition.

3. AliE inhibition

Inhibitory activity of the insecticides against fat body and haemolymph AliE which had been separated by electrophoresis were measured. Inhibition percent of AliE by each insecticide (TIA-230, fenitroxon and carbaryl) is summarized in Table 3.

Two AliE activity bands (F1, 2 in Fig. 1) were always separated from the fat body homogenate supernatant of the larvae; F2 esterase was the main one. The gel with separated AliE was incubated in the buffer solution containing 10^{-6} and 10^{-4} M insecticides. TIA-230 inhibited less than 50% of the activity of both AliE at 10^{-4} M. Fenitroxon, to the contrary, showed strong inhibitory activity, inhibiting more than 50% of the activity at 10^{-6} M. At 10^{-4} M, most of the activity was inhibited. Carbaryl hardly inhibited the activity at 10^{-6} M, but inhibited 70% of the activity at 10^{-4} M.

Two AliE activity bands (H1, 2 in Fig. 1) were also separated from the haemolymph. Fenitroxon showed strong inhibition against these bands. Most of the activity of both of the AliE was inhibited at 10^{-4} M of this compound. AliE of haemolymph were slightly inhibited by 10^{-4} M of TIA-230 and weakly by carbaryl.

Fenitrothion did not show any inhibitory activity against AliE of this insect.

DISCUSSION

The three insecticides used in the present experiments, TIA-230, fenitrothion and carbaryl have been known to exhibit their insecticidal activity through AChE inhibition in the central nervous system. However, their detailed modes of action, especially the way of activation in the insect body are different. TIA-230 is oxidatively activated in the central nerve as mentioned previously (Table 2; Kono et al., 1983 b), and the oxidation of this compound in other organs or tissues may act degradatively because of the unstability of its oxidated metabolite, phosphinyloxy sulfonate, in water (Kono et al., 1985). To the contrary, fenitrothion is mainly activated in tissues outside of the nervous system, fat body and midgut, and the activated form, fenitroxon, is transported through the haemolymph to the nerve tissue, the target site. In fact, the process from activation to AChE inhibition of fenitrothion in the nerve cord is inefficient (Table 2). Carbaryl is an inhibitor of AChE itself and in this compound, oxidation processes in all tissues are degradative.

As to anti-AChE activity, carbaryl was shown to be the strongest in the incubation with the nerve cords as well as *in vitro* inhibition. The inhibitory action of TIA-230 was not strong *in vitro* and that of an activated form, phosphinyloxy sulfonate, was shown to be lower than fenitroxon (Kono et al., 1985). However, inhibition of TIA-230 *in situ* was at the same level as fenitrothion and I-50 of this compound was estimated as 2×10^{-6} M. This value accorded with the critical concentration necessary to cause nervous excitation in the *Spodoptera* nerve cord in the electrophysiological experiment (Kono et al., 1983 b). I-50 of each of the chemicals *in situ*, therefore, can be considered

Table 4. Calculation of insecticide doses when the concentration is supposed to be I-50 value against AChE in the larval body cavity of *Spodoptera litura*

Compound	Molecular weight	I-50 (M) against AChE in nerve cord incubation	Dose ($\mu\text{g/insect}$) calculated (C)	LD ₅₀ ($\mu\text{g/insect}$) (D)	D/C
TIA-230	360.5	2.0×10^{-6}	0.32	0.71	2.2
Fenitroxon	261	1.3×10^{-6}	0.15	0.99	6.6
Carbaryl	201	6.5×10^{-7}	0.059	5.49	93.1

the critical concentration for excitation of the nerve.

If the insecticides were uniformly distributed in the body cavity of the larvae (averaged weight: 450 mg) after the injection, critical dosages could be calculated from I-50 against AChE *in situ* as shown in Table 4. The gap between the estimated dosage and the real LD₅₀ (D/C in the Table) must be attributable to the degradation capacity during dispersion through the haemolymph against the respective insecticides. In the case of injection, the main factors degrading the chemicals must exist in the haemolymph and fat body, and they must act quickly. The conjugation of insecticides with AliE, called sequestering and first proposed in the mechanism of insecticide resistance in the peach aphid (DEVONSHIRE and MOORES, 1982), seems to be one of the degradation factors. From the experimental results (Table 2), AliE of fat body and haemolymph of this insect obviously showed high affinity to fenitroxon at concentrations of 10^{-6} – 10^{-5} M which is the critical level for nervous excitation. Conjugation, therefore, occurs in a moment and shows that buffer action prevents the increase of fenitroxon concentration in the haemolymph and consequently in the nerve. These processes do not occur in TIA-230 because of its weak affinity to AliE of the larvae. This is one of the reasons why the gap (D/C) of fenitroxon was larger than that of TIA-230, and why fenitrothion topically applied showed only low insecticidal activity. At the fat body activation site of fenitrothion, AliE can trap the oxidated product, fenitroxon, efficiently because of their very high affinity.

Related observations have been made on the variation of susceptibility to fenitrothion in the silkworm, *Bombyx mori*. Silkworm strains having high AliE activity which were inhibited by the application of fenitrothion were shown to be relatively tolerant to this insecticide (WATANABE and KOBARA, 1967). Negative correlation between susceptibility of insects to TIA-230 and their AliE affinity to the insecticide was also elucidated (KONO and MANABE, 1983).

Capacity of AliE to conjugate insecticides is another important factor affecting the sequestration. Insecticide resistant strains of *Myzus persicae* (DEVONSHIRE and MOORES, 1982) and *Nephotettix cincticeps* (MOTOYAMA et al., 1984) were shown to have an amount of AliE to sequester insecticides comparable to that degraded by other factors such as hydrolysis. In *Spodoptera litura*, respective experiments are now being pursued.

Failure of IBP synergistic action in *Spodoptera* larvae can be explained by its slight inhibitory activity against AliE of this insect (KONO and MANABE, 1983).

In carbaryl treatment, there existed a great gap between the calculated dose to cause 50% inhibition of AChE activity and LD₅₀ in the injection. Because of the lack of effect of PB in the insecticidal activity and the low affinity of AliE to carbaryl, the gap cannot be explained either by oxidative degradation or by AliE sequestering. Other factors must be functioning, but these were not identified in the present paper.

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