Insensitivity of the Acetylcholinesterase from the Organophosphate-Resistant Kanzawa Spider Mite, *Tetranychus kanzawai* KISHIDA (Acarina : Tetranychidae), to Organophosphorus and Carbamate Insecticides

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The AChE of susceptible and OP-resistant strains of the Kanzawa spider mite was examined for resistance and sensitivity to OPs and carbamates. The selection procedure resulted in almost similar end response levels to OPs and carbamates in the selected strains, and a correlation between the low AChE activity and resistance was observed. In each of the strains the activity was slightly lower toward acetylthiocholine than toward acetylcholine. Homogeneity of sensitivity of the AChE to OPs and carbamates was recognized both in the susceptible and resistant mite. Generally, a good correlation was recognized between *in vivo* resistance to toxicants and *in vitro* insensitivity of the AChE to corresponding inhibitors, while no correlation was recognized to malaoxon, indicating the involvement of detoxification in resistance to malathion in resistant mite.

INTRODUCTION

There have been demonstrated two different systems of mechanism for resistance to organophosphates (OPs) in spider mite. One is the biochemical alteration of the toxicants to non-toxic or less-toxic products (MATSUMURA and VOSS, 1964; VOSS and MATSUMURA, 1964), and the other is an altered site of action or insensitivity of the target enzyme, acetylcholinesterase (AChE), to inhibition by OPs and carbamates (SMISSAERT, 1964; VOSS and MATSUMURA, 1965; ZAHAVI and TAHORI, 1970).

There have been, however, no reports on the relationship between OP-resistance and sensitivity of the AChE in the Kanzawa spider mite to toxicants. Therefore, as a preliminary step toward AChE characterization in this mite, the toxicant sensitivity of the AChE from laboratory-selected and field-collected strains of the Kanzawa spider mite was examined.

MATERIALS AND METHODS

Mite strains. Four OP-resistant strains (NsD, NsP, NsM and ZoR) and one reference strain (NsPs) of the Kanzawa spider mite were used for this study. The origin and response of these strains to some OP compounds and the methods used in rearing and treating the mite were described elsewhere (KUWAHARA, 1977; KUWAHARA et al., 1981).

Toxicants and inhibitors. Dichlorvos was obtained from Nippon Soda Co. Ltd.; malathion was from Sumitomo Chemical Co. Ltd.; phenthoate was from Nissan Chemical Co. Ltd.; formetanate was generously provided by Dr. Y. TAKAHASHI, Mitsubishi Chemical Ind. Ltd. These compounds with more than 99% purity were formulated as 25% (W/V) E. C. (25:30:20:10:10:5; AI:acetone:dimethylformamide:monochlorobenzene:xylene:Sorpol® 9838 emulsifier). Methidathion was a commercial product (Supracide® 40% E. C.).

The following oxygen analogues were used to study the kinetics of inhibition of AChE *in vitro*. Malaoxon, phenthoate-oxon and methidathion-oxon were generously provided by Prof. I. YAMAMOTO and Dr. S. KIMURA, Tokyo University of Agriculture. Dimethoate-oxon was generously provided by Dr. N. MOTOYAMA, Chiba University. These compounds were purified by TLC (silica gel GF254) with a benzene-acetone (10:1) solvent system.

Insecticide treatment. Female mites were sprayed with an aqueous suspension of insecticides via a rotary type of spray tower. LC50 values were determined graphically after 48hr as previously described (KUWAHARA, 1977). Results were expressed as resistance factors, i.e. ratios of LC50 of each strain to that of reference strain NsPs.

Measurement of the AChE activity. The AChE activity of the homogenate was determined by the method of ELLMAN et al. (1961), unless otherwise stated, with acetylthiocholine as substrate. Mites were collected by brushing them from kidney bean leaves and were used regardless of their sex and developmental stage. Homogenates were prepared in ice-cold glass-distilled water (100 mg mite/ml) using a glass homogenizer and were diluted with 0.067M phosphate buffer (pH 7.0) to contain 1.43 mg mite/ml.

The reaction mixture (total volume 1 ml) was as follows: 0.9 ml of homogenate, 0.025 ml of the substrate mixture and 0.075 ml of eserine. The substrate mixture was prepared by mixing 1 part of acetylthiocholine iodide (32 mg/ml buffer), 2 parts of DTNB solution [54.5 mg of 5,5'-dithiobis(2-nitrobenzoic acid), and 20.6 mg of sodium bicarbonate/10 ml buffer]. The system was incubated at 30°C for 30 min and shaken intermittently. Thereafter, eserine was added to give a final concentration of 0.9×10^{-3} M and the activity was measured at 412 nm. The amount of cholinester hydrolyzed was proportional to the homogenate concentration and reaction period up to 60 min.

Inhibition of AChE activity in vitro. Inhibitors were dissolved in ethanol containing 2% Triton X-100 and added to homogenate in the ratio of 1:100. Inhibition of the AChE was achieved by incubating the reaction mixture with inhibitors at 30°C for various times. The residual AChE activity toward acetylthiocholine was measured. The bimolecular rate constants (k_i) were calculated according to the method of ALDRIDGE (1950).

RESULTS

The selection of a susceptible laboratory strain Ns with various organophosphorus insecticides showed a very similar increase in resistance. However, the mites had apparently reached a resistant plateau in 5 to 6 times of selection and it has not been possible to increase this resistance in over 5 generations of further selection. It is of interest that the selection procedure employed in this study resulted in almost sim-

ilar end response levels in all the selected strains (Table 1).

The resistant strains extended some appreciable cross resistance to a carbamate, formetanate. A comparison of sensitivities of the AChE from five strains of mite to carbamates, eserine and propoxur, is given in Table 2. It is obvious from the data that resistant mite AChE manifested lower sensitivity to carbamate inhibition than susceptible AChE.

The AChE activities of the five strains toward acetylcholine and acetylthiocholine are shown in Table 3. The hydrolysis rate for both substrates in the NsPs strain was

Table 1. Response of the NsPs strain to various toxicants and resistance factors in the resistant strains of mite

Toxicant	LC50 NsPs strain (ppm)	Resistance factor ^a			
		NsD	NsM	NsP	ZoR
dichlorvos ^b	19.1	35.6	28.7	27.2	32.3
malathion ^b	4.2	2590	3900	3290	3010
phenthoate	2.4	1090	833	1375	1120
methidathion	0.85	64.9	58.4	63.7	55.3
dimethoate	7.0	40.5	32.9	51.4	47.7
formetanate	4.9	6.0	4.7	5.7	5.3

^a Ratios of LC50 of resistant strain to that of NsPs strain.

^b Data from KUWAHARA et al. (1982).

Table 2. AChE sensitivity to carbamates from five strains of mite

Strain	I ₅₀ (M)	
	Eserine	Propoxur ^a
NsPs	3.3×10^{-9}	0.25×10^{-5}
NsD	4.9×10^{-8}	0.45×10^{-5}
NsM	4.4×10^{-8}	0.50×10^{-5}
NsP	5.2×10^{-8}	0.75×10^{-5}
ZoR	5.0×10^{-8}	0.73×10^{-5}

^a Propoxur : 2-isopropoxyphenyl *N*-methylcarbamate.

Table 3. Rate of acetylcholine and acetylthiocholine hydrolysis by AChE from various strains of mite

Strain	Acetylcholine hydrolyzed (A) ^a (μ moles/g/hr)	Acetylthiocholine hydrolyzed (B) (μ moles/g/hr)	Ratio A/B
NsPs	65.6	53.8	1.22
NsD	48.1 (0.73)	40.4 (0.75)	1.19
NsP	50.6 (0.77)	42.5 (0.79)	1.19
NsM	57.5 (0.88)	47.9 (0.89)	1.20
ZoR	45.1 (0.69)	39.8 (0.65)	1.13

^a Activity was determined according to the method of Hestrin (1949) at 30°C for 30 min with 2×10^{-3} M acetylcholine as substrate. Enzyme concentration : 20 mg mite/ml buffer (0.067 M, pH 7.0).

Figures in parentheses show the ratio of activity in resistant strains to that of reference strain NsPs.

Fig. 1. *In vitro* inhibition of mite acetylcholinesterase in the homogenase of susceptible NsPs (●) and OP-resistant NsM (○) strains by various inhibitors.

A : dichlorvos, B : methidathion-oxon, C : malaxon, D : phenthoate-oxon, E : formetanate.

higher than those of the resistant strains, indicating the higher AChE activity in the susceptible strain than those in the resistant strains. In each of the strains the activity was from 13 to 22% lower toward acetylthiocholine than toward acetylcholine.

The relationship between time of incubation with inhibitors and the percentage of initial AChE activity in the homogenates of the susceptible NsPs and OP-resistant NsM strains is shown in Fig. 1. The inhibition of the AChE activity in the homogenates by OPs gave a linear relationship between time of inhibition and residual AChE activity (log scale), indicating first-order reaction rates (ALDRIDGE, 1950) for the irreversible inhibition of enzyme activity of the homogenates by the inhibitors. The inhibition obtained with formetanate, on the other hand, gave some modification of such rates for the reversible inhibition of the enzyme activity. These lines cut the ordinate at about 100% activity in homogenates of both strains for all inhibitors. These results indicated the homogeneity in sensitivity of the AChE to OPs and carbamates.

The bimolecular rate constants (k_i) for the reaction between various inhibitors and the mite AChE are given in Table 4. It is obvious from the data that relative insensitivity of the AChE from the resistant strain to inhibition was clearly demonstrated. The difference in sensitivity of the AChE to inhibitors between susceptible and resistant strains varied from inhibitor to inhibitor. The comparison of the k_i values for susceptible and resistant AChE showed that the greatest difference was recognized when the oxygen analogues of phenthoate and malathion were inhibitors, then followed in descending order by dimethoate, methidathion, dichlorvos and formetanate. The result could, therefore, be interpreted as an indication of a direct relation between inhibitory activity and the degree of interstrain differences.

The relation between the resistance factors for the toxicants and relative insensitivity of the AChE to corresponding inhibitors depicted from the values in Tables 1 and 4 is shown in Fig. 2. Relative insensitivity of the AChE to inhibitors is expressed as the ratio of rate constants: $k_i(\text{NsPs})/k_i(\text{resistant strain})$. Generally a good correlation was recognized between *in vivo* resistance to toxicants and *in vitro* insensitivity of the AChE to corresponding inhibitors: The greater the resistance factor is to the inhibitor, the more is the relative insensitivity of the AChE in the homogenate of resistant strains.

On the other hand, however, no correlation was recognized for malathion resistance. To check the possibility of the enzymic degradation of malaoxon by the tissue homogenate of resistant strains which would result in the inactivation of the

Table 4. Bimolecular rate constants (k_i) for the inhibition of AChE from various strains of mite

Inhibitor	Strain				
	NsPs	NsD	NsM	NsP	ZoR
malaoxon	2.47×10^6	2.33×10^3	3.88×10^3	2.51×10^3	3.38×10^3
formetanate	2.22×10^{6a}	0.40×10^{6a}	0.52×10^{6a}	0.48×10^{6a}	0.44×10^{6a}
phenthoate-oxon	8.00×10^6	0.77×10^4	0.69×10^4	1.00×10^4	0.80×10^4
dichlorvos	4.88×10^5	1.16×10^4	1.33×10^4	1.33×10^4	1.30×10^4
methidathion-oxon	1.05×10^7	1.00×10^5	1.00×10^5	1.08×10^5	1.47×10^5
dimethoate-oxon	2.34×10^6	2.67×10^4	2.11×10^4	3.87×10^4	2.09×10^4

^a These values are approximations only, due to the reversibility of inhibition by formetanate.

Fig. 2. Relationship between relative insensitivity of the acetylcholinesterase from OP-resistant strains and resistance factors to various inhibitors.

A : formetanate, B : dichlorvos, C : dimethoate-oxon, D : methidathion-oxon,
E : phenthoate-oxon, F : malaoxon.

Table 5. Loss of AChE activity in the homogenate of the NsPs strain by inhibitors

Inhibitor	% activity remaining
5×10^{-8} M malaoxon	45.2
malaoxonized NsM homogenate	48.9
malaoxonized ZoR homogenate	46.3

Figures reflect an average of six replications.

inhibitor, the homogenate prepared to contain 1.43 mg mite/ml in 0.067 M buffer (pH. 7.0) was incubated with 5×10^{-6} M of malaoxon at 30°C for 10 min. This amount of malaoxon slightly inhibited the AChE of the two resistant strains (Fig. 1 and Table 4). Thereafter, the malaoxonized homogenate was added to the homogenate of the susceptible strain NsPs at the ratio of 1:100. As might be expected, there was no appreciable loss of AChE activity in the homogenate of the NsPs strain (Table 5). Therefore, it is reasonable to assume that no degradation of malaoxon by resistant homogenate was recognized.

DISCUSSION

The results concerning the response of the selected strains to toxicants showed that the OP-resistant strains may be characterized as showing marked cross resistance to OPs but low cross resistance to carbamates (Tables 1 and 2). The result is in good accordance with the finding of VOSS and MATSUMURA (1964) who demonstrated low cross resistance to carbamates and low sensitivity of the AChE to carbamate inhibition in OP-resistant strains of two-spotted spider mite, *Tetranychus urticae*. It is of interest that the selection procedure employed in this study resulted in almost an similar end response level to the toxicants. This pattern within these compounds suggests that the mechanisms for resistance to these toxicants are closely related.

From the inhibition study of the AChE by various inhibitors, homogeneity of sensitivity of the AChE to OPs and carbamate was recognized both in susceptible and OP-resistant strains (Fig. 1). These results are in good accordance with the findings of others (SMISSAERT, 1964; VOSS and MATSUMURA, 1965; ZAHAVI and TAHORI, 1970), who demonstrated only one type of AChE in the homogenate of resistant mite. In contrast to the results obtained with spider mite, heterogeneity of sensitivity of the AChE to OP and carbamate compounds has been demonstrated in insect (TAKAHASHI et al., 1978) and tick (LEE and BATHAM, 1966; ROULSTON et al., 1967).

There was a good correlation between the *in vivo* resistance obtained using female mite and *in vitro* resistance found when AChE inhibition rates were compared. These results indicate that lowered sensitivity of the AChE is the major mechanism of resistance to these toxicants in the Kanzawa spider mite as already demonstrated in another species of spider mite (SMISSAERT, 1964; VOSS and MATSUMURA, 1965; ZAHAVI and TAHORI, 1970).

On the other hand, however, no correlation was recognized between the *in vivo* resistance to malathion and *in vitro* sensitivity of the AChE from the resistant strains to malaoxon (Fig. 2). The OP-resistant strains of the Kanzawa spider mite manifested increased esterase and malathion-hydrolyzing activity as compared with that of the susceptible strains, and a correlation between esterase activity and synergism of a mixture of malathion and K-1, a malathion specific synergist, has been shown (KUWAHARA et al., 1981, 1982). These esterases were resolved electrophoretically.

They manifested higher activity in the decomposition of malathion *in vitro*, and they were very sensitive to K-1, indicating the possibility of the involvement of detoxification of malathion in OP-resistant strains. However, the decomposition of malaoxon by resistant homogenates was not recognized in this study (Table 5), as it has been demonstrated in the carmine spider mite, *Tetranychus cinnabarinus* (ZAHAVI and TAHORI, 1970). Thus, the inactivation of malaoxon did not take place in the resistant strains of the Kanzawa spider mite. These results are not consistent with the finding of MATSUMURA and VOSS (1964) who demonstrated the definitely higher activity for hydrolyzing malaoxon in the malathion-resistant strains of the two-spotted spider mite as compared with susceptible strains. Accordingly, the increased esterase activity, at least in part, in the OP-resistant strains of the Kanzawa spider mite is directly connected with the decomposition of malathion, which resulted in partial modification of activity of malathion *in vivo*. In contrast to these results, ZAHAVI and TAHORI (1970) demonstrated a good correlation between resistance to malathion and insensitivity of the AChE from the field collected strains of carmine spider mite to malaoxon, indicating no involvement of detoxification mechanism in these strains.

These discrepancies in mechanism of resistance might be due to the difference in genetic background.

It has been shown that even if the factors for resistance might not play an important role in resistance when they alone are present, they enhance resistance not additively but multiplicatively (GEORGHIOU, 1971). Thus, a combination of insensitivity of the AChE to inhibitors and the involvement of additional mechanism, *i.e.*, an increased activity in degradation of toxicants, might be responsible for the extensive and intensive resistance in spider mite.

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