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SENSITIVITY OF ACETYLCHOLINESTERASE IN SPIDER MITES TO ORGANO-PHOSPHORUS COMPOUNDS*

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Abstract—Field-collected susceptible and organophosphorus-resistant mite strains were examined for sensitivity of their AChE to O–P compounds. A very good correlation was generally found between resistance and insensitivity of AChE to malaoxon, malathion, dichlorvos and phosphamidon. The degree of insensitivity of AChE from the resistant strains was lower towards dichlorvos than towards the other compounds tested. The implications of these differences are discussed. Reduced AChE activity was found in three resistant mite strains. However, a highly resistant strain contained elevated AChE activity, indicating that no general correlation exists between depressed AChE activity and resistance. Insensitivity of AChE to inhibitors seems to be a widespread phenomenon in O–P resistance in mites.

THE ACETYLCHOLINE (ACh)–acetylcholinesterase (AChE) system exists in the nervous tissue of many arthropod species.[1] The toxic action of organophosphorus (O–P) compounds is generally attributed to inhibition of AChE present at nerve endings.[2]

In insects, resistance to O–P compounds is correlated to degradation of the toxicant.[3] A similar mechanism has also been reported in one resistant mite strain.[4] Another O–P resistant mite strain contained a modified AChE, insensitive to these inhibitors.[5] This enzyme was also less active than AChE of sensitive mite strains.[6] A similar pattern of O–P resistance was found in another acarine, the cattle tick *Boophilus microplus*.[7] However, no AChE, insensitive to O–P compounds, has been reported in resistant insects.

In order to find out whether the mechanism of mite resistance manifested by a modified AChE is a widespread phenomenon in the genus *Tetranychus*, the sensitivity to O–P compounds of AChE from malathion resistant and sensitive spider mite strains in Israel was examined. Generally a good correlation was found between malathion resistance and insensitivity of their AChE's to the O–P compounds.

MATERIALS AND METHODS

Materials. Acetylcholine chloride was obtained from Koch–Light Laboratories; acetylthiocholine iodide from Calbiochem Chemical Company; 5,5-dithio-bis-2 nitrobenzoic acid from Aldrich Chemical Co.; purified malathion [S-(1,2-dicarboethoxyethyl) phosphorodithioate] and malaoxon [S-(1,2-dicarboethoxyethyl)phos-

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phorothiolate] from American Cyanamid Co., Princeton, N.J.; purified phosphamidon (1-chloro-1-diethylcarbamoyl-1-propen-2-yl dimethyl phosphate) and purified dichlorvos (2,2-dichlorovinyl dimethyl phosphate) from CIBA Ltd., Basle, Switzerland.

Mite strains. Eight strains of the carmine spider mite *Tetranychus cinnabarinus* syn *T. telarius* were collected from various regions in Israel and reared in the laboratory. Methods of rearing and testing for sensitivity to acaricides are reported elsewhere.[8]

Five of the strains were resistant to malathion and three were susceptible (Table 1).

TABLE 1. RESISTANCE *in vivo* TO MALATHION OF VARIOUS MITE STRAINS

	Sensitive strains		Resistant strains				
Amirim	Wadi Ara	Kfar Sattaf	Neve Ur III	Par'od	Ayeleth Hashahar I	Ayeleth Hashahar XII	Neve Ur I
LD_{50} and LD_{80} in mg/cm² leaf area	Resistance index*						
LD_{50} = 5·3	1·2	1·4	43	143	127	190	250
LD_{80} = 7·5	1·3	2·4	133	280	290	333	400

(Data from Tahori and Raccah[8].)

* $\frac{LD_{50} \text{ of resistant strain}}{LD_{50} \text{ of Amirim strain}}$

Preparation of homogenate. 500 mites were collected in 2·5 ml NaK phosphate buffer 0·067 M pH 7·5, ground thrice in a Potter–Elvehjem glass homogenizer for 1 min, and cooled on ice between grindings. 0·5 ml of the homogenate was taken for AChE determination.

Preparation of particulate or soluble AChE. The homogenate was centrifuged at 9000 *g* at 2° for 10 min. 0·5 ml of the supernatant or the precipitate (resuspended in 2·5 ml of the buffer solution) was taken for the determination of soluble or particulate AChE.

Solubilization of particulate AChE. The particulate suspension was sonicated in an ice-cooled container for 30 sec with the immersed probe of the 'Sonifer' oscillator of Branson Instruments Inc. (Stamford, Conn., U.S.A.) set at 2·5 A. The sonicate was then centrifuged at 9000 *g* for 10 min and the supernatant used for AChE determination.

Determination of AChE. AChE was measured by the method of Ellman *et al.*[9] The reaction was carried out in 1 ml cells at 25° for 1 hr. The reaction mixture (total volume 1 ml) contained 3×10^{-4} M acetylthiocholine iodide, 3×10^{-4} M 5,5-dithiobis-2-nitrobenzoic acid, 0·1 M K phosphate buffer pH 7·5 (instead of pH 8·0 in the original method), 3% sodium chloride and 0·5 ml of enzyme. Phosphamidon was added in 0·1 ml of water. Malathion, dichlorvos and malaoxon were added in 0·1 ml of 5% acetone. The controls contained 0·1 ml of 5% acetone. Unless otherwise stated, the reaction mixture minus substrate was preincubated with the O–P inhibitor for 10 min. Protein was determined by the method of Lowry *et al.*,[10] using crystalline bovine serum albumin as reference.

RESULTS

Inhibition of AChE from sensitive or resistant mite strains by O–P compounds

The relative resistance of various mite strains to malathion is listed in Table 1. Malathion is a comparatively weak inhibitor of AChE of mites *in vitro*: the AChE of the sensitive Amirim strain was 800 times less sensitive to malathion than to malaoxon (Table 2). It is known that malathion is oxidised to the more potent AChE inhibitor malaoxon in the living insect[11] or mite.[12] When homogenates of mites of the sensitive

TABLE 2. INHIBITION OF AChE FROM PARTICLES OF SENSITIVE AND RESISTANT MITE STRAINS BY O–P COMPOUNDS

Sensitive Amirim strain		Insensitivity index of AChE*						
Compound	ID_{50} and ID_{80} in M	Kfar Sattaf	Wadi Ara	Neve Ur III	Par'od	Ayeleth Hashahar I	Ayeleth Hashahar XII	Neve Ur I
malathion	$ID_{50} = 2{\cdot}2 \times 10^{-5}$			1·7				†
	$ID_{80} = 6{\cdot}8 \times 10^{-5}$			2·1				
malaoxon	$ID_{50} = 3 \times 10^{-8}$	1·1	1·6	2·7	270	330	400	650
	$ID_{80} = 8{\cdot}5 \times 10^{-8}$	1·1	1·4	5·5	280	470	570	900
dichlorvos	$ID_{50} = 3{\cdot}4 \times 10^{-8}$				25		53	38
	$ID_{80} = 10^{-7}$				30		52	46
phosphamidon	$ID_{50} = 7{\cdot}3 \times 10^{-6}$			1·9				550
	$ID_{80} = 2{\cdot}4 \times 10^{-5}$			2·9				800

ID_{50} and ID_{80} concentration of compound causing 50 and 80 per cent inhibition of AChE respectively. The figures for malaoxon are averages of 5–7 experiments, for other compounds 3–4.

* $\frac{\text{ID of AChE of resistant strain}}{\text{ID of AChE of Amirim strain}}$

† 2×10^{-3}M malathion gave only 30 per cent inhibition.

The reaction mixture minus substrate was preincubated with the O–P inhibitor for 10 min. The reaction was carried out at 25° for 1 hr.

Amirim strain were preincubated with malathion for 1 hr, instead of 10 min, the inhibition of AChE increased from ID_{50} $2{\cdot}2 \times 10^{-5}$ only to 8×10^{-6} M. Thus, assuming that the total increase in inhibition was caused by malaoxon production, not more than 0·2–0·3 per cent of malathion is converted to malaoxon during the one hour of preincubation, since the ID_{50} for malaoxon is 3×10^{-8} M.

Malaoxon and dichlorvos, in contradistinction to malathion and phosphamidon, are effective inhibitors of mite AChE (Table 2). The experiments reported in Table 2 were performed with particles, the specific activity of which was twice that of whole homogenates (see Table 5). The results with whole homogenates were essentially the same (ID_{50} for malaoxon in homogenates of Amirim strain $3{\cdot}2 \times 10^{-8}$ M; of Neve Ur I strain $2{\cdot}2 \times 10^{-5}$ M) as those obtained with particles (see Table 2), indicating that no malaoxon degradation occurred in the whole homogenate.

Since malaoxon and not malathion is the active AChE inhibitor *in vivo* and the observed inhibition of AChE by malathion might have been caused by a 0·1% malathion content, malaoxon instead of malathion was used to correlate AChE inhibition with toxicity. A good correlation between resistance to malathion and the insensitivity of AChE to malaoxon was found in seven out of the eight strains examined

(compare Tables 1 and 2). The only exception was the Neve Ur III strain, which was forty-three times more resistant to malathion, but its AChE was only three times less sensitive to malaoxon, as compared with the sensitive Amirim strain. Resistance of the Neve Ur III strain declined steadily during the course of this investigation, until its LD_{50} reached the low level of 20 mg of malathion/cm² leaf area. This final toxicity correlated well with the AChE insensitivity index. However, it is possible that degradation was also involved in the initially relatively higher resistance of this strain.

In order to make sure that particles of the resistant strains do not contain malaoxon-degrading enzymes which would inactivate the O–P inhibitor, the particles of the high-resistant Neve Ur I strain were preincubated with $2{\cdot}5 \times 10^{-7}$ M of malaoxon for 20 min and then particles of the sensitive Amirim strain were added. This amount of malaoxon only slightly inhibits the AChE of the resistant strain, while it completely inhibits AChE of the sensitive strain (Table 4). As expected, the inhibition of AChE of the 1:1 mixture was half the value for the Amirim strain alone (Table 4). Since the AChE inhibitory properties of the O–P compound were retained after 20 min pre-incubation, no degradation of malaoxon by particles of the resistant Neve Ur I strain took place during this period.

The AChE of the malathion resistant Neve Ur I strain were, in addition to malaoxon and malathion, also insensitive to phosphamidon and to a lesser extent to dichlorvos (Table 2). Some of the experiments performed with particulate AChE (Table 2) were repeated with a solubilized AChE preparation from these particles (Table 3). Essentially, similar results were obtained for both preparations, stressing again the difference in sensitivity of AChE from the resistant strains to malaoxon and dichlorvos.

TABLE 3. INHIBITION OF AChE FROM SONICATED PARTICLES BY MALAOXON AND DICHLORVOS SENSITIVE AMIRIM AND RESISTANT AYELETH HASHAHAR XII MITE STRAINS

compound	ID_{50} or ID_{80} in M	Time of preincubation with inhibitor			
		5 min		10 min	
		sensitive strain	resistant strain	sensitive strain	resistant strain
malaoxon	ID_{50}	$4{\cdot}5 \times 10^{-8}$	$1{\cdot}4 \times 10^{-5}$	$3{\cdot}5 \times 10^{-8}$	$1{\cdot}2 \times 10^{-5}$
	ID_{80}	$1{\cdot}1 \times 10^{-7}$	$4{\cdot}3 \times 10^{-5}$	$7{\cdot}0 \times 10^{-8}$	$3{\cdot}3 \times 10^{-5}$
dichlorvos	ID_{50}	$6{\cdot}3 \times 10^{-8}$	$2{\cdot}1 \times 10^{-6}$	$4{\cdot}6 \times 10^{-8}$	$1{\cdot}8 \times 10^{-6}$
	ID_{80}	$1{\cdot}8 \times 10^{-7}$	$7{\cdot}6 \times 10^{-6}$	$9{\cdot}3 \times 10^{-8}$	$5{\cdot}3 \times 10^{-6}$

Activity of AChE in mites. Seventy per cent of the AChE activity present in the whole homogenate of mites is recovered in the particles precipitated at 9000 *g*. The specific activity (in mμmole acetylthiocholine hydrolyzed/mg protein/hr) of the particulate fraction is about twice of that of the whole homogenate (Table 5). With mite particles, as source of AChE, the rate of reaction is almost constant during 1 hr, whereas with the homogenate the rate declines significantly after 30–45 min. In a few samples the AChE of mites was determined by the method of Hestrin,[13] with ACh as a substrate, and the activities found were about 60 per cent higher than those obtained by the Ellman *et al.*[9] method (Table 5). For example, the AChE activity as

determined by the method of Hestrin was 55 mμmoles/100 mites/hr or 480 mμmoles/mg protein/hr in particles of the Wadi Ara strain.

Three of the four resistant mite strains had an AChE activity lower than the sensitive strains. However, the highly resistant Neve Ur I strain contained a high AChE activity (Table 5).

When the particles were sonicated and centrifuged at 9000 *g*, about 80 per cent of

TABLE 4. STABILITY OF AChE-INHIBITORY PROPERTIES OF MALAOXON AFTER INCUBATION WITH PARTICLES FROM A RESISTANT MITE STRAIN

Origin of particles added initially	Origin of particles added after 20 min	AChE activity in mμmoles acetylthiocholine hydrolysed/100 mites/hr		
		no malaoxon	with $2{\cdot}5 \times 10^{-7}$M malaoxon	% inhibition
Neve Ur I	Neve Ur I	52	42	19
Amirim	Amirim	38	0	100
Neve Ur I	Amirim	44	20	55

0·25 ml of a particulate suspension mentioned above was preincubated in reaction mixture at 25° with malaoxon for 20 min, then 0·25 ml of the appropriate particulate suspension was added. Acetylthiocholine was added 10 min later. Preincubation mixture contained all the components described in Materials and Methods except substrate. Particulate protein present finally: for Amirim 0·12 mg; for Neve Ur I 0·14 mg; and for Neve Ur I–Amirim 1:1 mixture 0·125 mg.

TABLE 5. ACTIVITY OF AChE IN VARIOUS MITE STRAINS

Strains	AChE activity expressed as amount of acetylthiocholine hydrolysed						
	mμmole/100 mites/hr			mμmole/mg protein/h ± S.D.*			
	homogenate	particulate	soluble	homogenate	particulate	soluble	whole†
Amirim		33	14		372 ± 44	81 ± 17	173 ± 21
Wadi Ara	53	34	13	220 ± 21	304 ± 38	63 ± 10	147 ± 13
Neve Ur III	44	35	13	195 ± 33	286 ± 28	67 ± 12	143 ± 16
Par'od		22	9		193 ± 19	41 ± 7	94 ± 9
Ayelet Hashahar I		29	10		268 ± 57	46 ± 9	122 ± 24
Ayelet Hashahar XII		21	8		207 ± 51	40 ± 6	94 ± 10
Neve Ur I	59	34	13	247 ± 47	383 ± 46	78 ± 12	183 ± 18

* S.D. (Standard deviation), data are average of six to eight experiments.

† Calculated by summing up activity of particulate and soluble fractions over total protein. For reaction mixture see Materials and Methods.

the original AChE activity was recovered in the supernatant. The specific activity of AChE in the supernatant was twice that of whole particles (compare Table 5 with data in Table 6 for AChE activity at the 3×10^{-4} M acetylthiocholine concentration). The Michaelis constants (K_m) and maximal velocities (V_{max}) for AChE of the various mite strains were determined with this soluble AChE preparation. No significant difference between the AChE's of sensitive and resistant strains was found with respect to their K_m or V_{max} values (Table 6).

TABLE 6. ACTIVITY OF AChE IN THE SUPERNATANT OF SONICATED PARTICLES FROM VARIOUS MITE STRAINS

Concn of acetylthiocholine in M	Sensitive strains			Resistant strains		
	Amirim	Kfar Sattaf	Wadi Ara	Par'od	Ayelet Hashahar XII	Neve Ur I
	(mμmole acetylthiocholine hydrolised/mg protein/hr)					
5×10^{-5}	310	403	310	220	219	298
1×10^{-4}	494	605	499	343	351	468
$1{\cdot}5 \times 10^{-4}$	603	725	616	423	437	603
3×10^{-4}	794	885	768	494	593	743
4×10^{-4}	850	900	802	554	654	833
6×10^{-4}	910	960	868	562	729	951
1×10^{-3}	990					
2×10^{-3}	1045					
6×10^{-3}	1075					
V_{max}	1130	1090	1050	700	920	1140
$K_m(10^{-4}M)$	1·3	0·8	1·05	1·05	1·6	1·4

Enzyme extract was 0·04 mg protein in 1 ml of reaction mixture. Results are average of three to four experiments.

K_m and V_{max} were obtained by plotting concentration/activity (S/V) against concentration (S) in the range of 5×10^{-5}M to 6×10^{-4}M, according to the method of Lineweaver and Burk, *J. Am. Chem. Soc.* **56**, 658 (1934)

DISCUSSION

Two different mechanisms of resistance to O–P compounds have been reported for mites: resistance caused by increased degradation of the pesticide to non-toxic products,[12] and resistance caused by insensitivity of the target enzyme to the inhibitor.[5, 14, 14] Insensitivity at the site of action has been reported for fluoroacetate in a house fly strain resistant to this compound,[15] presumably because of the difficulty to metabolise fluoroacetate. Up till now no other case of such a mechanism of resistance is known in insects and degradation is therefore the major mechanism of resistance.[3]

The 800-fold higher sensitivity of mite AChE to malaoxon as compared with malathion (Table 2) is in agreement with the concept (refs. 11 and 12) that in arthropods malathion is converted *in vivo* to the strong AChE inhibitor malaoxon. We, therefore, used malaoxon rather than malathion in the sensitivity tests of AChE from malathion resistant spider mites. In four out of the five resistant mite strains tested, a very good correlation was found between the *in vivo* resistance to malathion and insensitivity of their AChE to malaoxon (Tables 1 and 2). On the other hand we found no evidence for the inactivation of malaoxon by particles of the resistant Neve Ur I strain (Table 4). Thus, the O–P-insensitivity of AChE from this strain *in vitro* is not caused by an increased degradation of malaoxon. Moreover, the insensitivity to malaoxon of the AChE in the resistant strain is sufficient to account for the high *in vivo* resistance to malathion. Our results, and findings by others (refs. 5 and 14), indicate that insensitivity of AChE to O–P compounds is the major mechanism in the resistance of mites to O–P compounds. It is noteworthy that this mechanism of resistance was also found in another member of the order Acarinae, the cattle tick *Boophilus microplus*.[7] It would, therefore, be interesting to explore whether O–P-insensitive AChE is a widespread mechanism of O–P resistance in Acarina

AChE from mites is twenty to forty times less sensitive to phosphamidon than

AChE from aphids and Mediterranean fruit flies *Ceratitis capitata*. This is in accordance with the fact that phosphamidon is a satisfactory economic control for aphids but not for mites.[17] The relative insensitivity of mite AChE to phosphamidon might be due to steric factors.[16]

The differences in sensitivity of AChE between malathion-sensitive and resistant mite strains are much greater for malaoxon and phosphamidon than for dichlorvos (Table 2). A similar decrease in interstrain difference was also reported when *O,O*-dimethyl analogues of malaoxon were substituted with *O,O*-dialkyls of a higher molecular weight.[6] However, the latter compounds were also weaker inhibitors of AChE. On the other hand, dichlorvos is a strong inhibitor of mite AChE and this compound, in contrast to malaoxon, inhibits appreciably the AChE of the highly resistant Neve Ur I strain (Table 2 and 3). Following this trend with other O–P compounds, an O–P acaricide may be found for resistant mite strains and some information may be gained on the active site of the malaoxon-insensitive AChE.

The O–P insensitive AChE of resistant mites or ticks display lower enzymatic activity when compared with the AChE of sensitive strains (refs. 5 and 7). It was suggested that the AChE of the resistant Leverkusen strain of the two-spotted spider mite *T. urticae*, possesses a weak esteratic site because of lowered affinity for substrate as well as for inhibitor.[6] With our carmine spider mite strains no consistent correlation between diminished AChE activity and resistance was observed; while three resistant strains had a lower AChE activity than three sensitive strains, the highly resistant Neve Ur I strain had the highest AChE activity found (Tables 5 and 6). Moreover, the K_m and V_{max} values of the AChEs of resistant and sensitive strains did not differ significantly (Table 6). These results show that the low AChE activity of resistant mite strains is a side-effect as already suggested by Smissaert[5] and therefore not necessarily connected with resistance or O–P insensitivity of the modified enzyme.

REFERENCES

1. L. E. Chadwick, *Cholinesterases and anticholinesterase agents* (Ed. G. Koelle), p. 741 (1963).
2. R. D. O'Brien, *Toxic Phosphorus Esters*, p. 143. Academic Press, New York (1960).
3. A. W. A. Brown, *Bull. Ent. Soc. Am.* **14**, 3 (1968).
4. G. Voss and F. Matsumura, *Nature, Lond.* **202**, 319 (1964).
5. H. R. Smissaert, *Science* **143**, 129 (1964).
6. G. Voss and F. Matsumura, *Can. J. Biochem.* **43**, 63 (1965).
7. R. M. Lee and P. Batham, *Ent. exp. appl.* **9**, 13 (1966).
8. A. S. Tahori and B. Raccah, *J. econ. Ent.* in press (1969).
9. G. L. Ellman, D. K. Cortney, V. Anders, Jr. and R. M. Featherstone, *Biochem. Pharmac.* **7**, 88 (1961).
10. O. H. Lowry, A. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* 193, 265 (1951).
11. H. R. Krueger and R. D. O'Brien, *J. econ. Ent.* **52**, 1063 (1959).
12. F. Matsumura and G. Voss, *J. econ. Ent.* **57**, 911 (1964).
13. S. Hestrin, *J. biol. Chem.* **180**, 249 (1949).
14. G. H. Ballantyne and R. A. Harrison, *Ent. exp. appl.* **10**, 231 (1967).
15. M. Zahavi, A. S. Tahori and J. Mager, *Biochim. biophys. Acta* **153**, 787 (1968).
16. R. D. O'Brien, *Ann. Rev. Entomol.* **11**, 369 (1966).
17. U.S. Department of Agriculture, Agriculture Handbook No. 313 (1966).