Relationship between High Esterase Activity and *In Vitro*
Degradation of ^{14}C -Malathion by Organophosphate-Resistant
and Susceptible Strains of the Kanzawa Spider Mite,
Tetranychus kanzawai KISHIDA (Acarina : Tetranychidae),
and Their Inhibition with Specific Synergists¹

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The relationship between the enzymes which catalyze hydrolysis of β -naphthyl acetate and degradation of malathion was studied in resistant and susceptible strains of the Kanzawa spider mite, *Tetranychus kanzawai* KISHIDA.

The homogenate of resistant mites exhibited higher activity in the degradation of malathion *in vitro* than that of susceptible mites, but such activity was remarkably inhibited by the compound K-1, which also manifested a prominent synergism with malathion to resistant mites. Six esterase bands and three peaks of malathion degradation were resolved by agar-gel electrophoresis. Resistance to malathion was associated with increased esterase activity at E_3 and E_4 bands on which the main peak of malathion degradation was detected. The compound K-1 selectively inhibited the esterase and degradation activities. Compounds K-9 and IBP, on the other hand, selectively inhibited E_1 and E_2 bands which overlapped with the minor peaks of malathion degradation.

INTRODUCTION

Resistance of mites to acaricides, and to organophosphates (OPs) in particular, is a world wide problem. Due to the intensive cultivation system in Japan, extensive and repeated application of OPs have taken place and have resulted in the develop-

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ment of various degrees of resistance in mites and insects.

Several reviews have been published dealing with resistance to malathion, which is easily hydrolyzed by carboxylesterase(s) (SEUME and O'BRIEN, 1960; HEATH, 1961; O'BRIEN, 1967; METCALF, 1967). It has been demonstrated that certain methylcarbamates are selective inhibitors of aliesterases (CASIDA, 1967), some of which have been identified to be carboxylesterases (MAIN and BRAID, 1962). Mixture of these carbamates with certain OPs such as malathion might therefore yield the potentiation of these insecticides. The application of carboxylesterase inhibitors with malathion has actually demonstrated significant synergism on the toxicity to insects and mites (PLAPP et al., 1963; HENNEBERRY and SMITH, 1964; PLAPP and TONG, 1966; O'BRIEN, 1967).

Simultaneous application of some specific aliesterase inhibitors or methylcarbamates with malathion or phenthoate manifested prominent synergism on the toxicity to spider mites (TAKAHASHI et al., 1973; KUWAHARA, 1977). These two insecticides have carboxylester bond as a common feature in their structures. Despite the accumulation of a considerable amount of information in recent years, the exact mechanism by which these synergists inhibit the carboxylesterase enzyme is still a matter of speculation.

This work was initiated in search of a biochemical explanation of potentiation or the greater-than-additive physiological effect of malathion produced by the simultaneous administration of synergists, and to study the *in vitro* degradation of malathion in the Kanzawa spider mite with special reference to its OP resistance.

MATERIALS AND METHODS

Mite strains and toxicity test. Two OP-susceptible strains, Nk and Ns, and two OP-resistant strains, NER and ZoRR, of the Kanzawa spider mite, *Tetranychus kanzawai*, were used in this study. Their origin and susceptibility to chemicals were shown elsewhere (KUWAHARA, 1977). For the acaricide test only female mites were used placing them in kidney bean leaf disks put on 0.4% agar gel in a petri dish. Treatments were made by spraying 3 ml of a chemical emulsion on the mites with a rotary type spray tower. Treated mites were then kept at 25°C and mortality was read 48 hr after treatment. Three replications were made for each concentration. LC_{50} was expressed in ppm of active ingredients.

Electrophoretic separation of esterases. The thin-layer agar gel electrophoretic method described by MIYATA et al. (1976) was used to separate mite enzymes with esterase activity. Fifty mg of mites were homogenized in 0.2 ml of ice-cold phosphate buffer (6.7×10^{-2} M; pH 7.0) using a glass homogenizer. A strip of filter paper (2×60 mm) was immersed in the brei. To test the inhibitory effect of synergists on esterase activity, these brei were incubated for 10 min at 37°C with 2 μ l of synergists in ethanol prior to their absorption with the filter paper. The filter paper was then placed on the gel and kept in a refrigerator for 30 min at 4°C. Then the paper was removed from the agar plate and electrophoresis was run for 60 min using a constant current of 2 mA per cm width. After completion of electrophoresis, the middle portion of the agar gel was sliced transversely into 5×50 mm pieces to test the degrading activity of the insecticides by incubating them with 14 C-malathion. To the remaining portion of the gel, a mixture of 0.1% of β -naphthyl acetate and 2% Fast Blue BB was

sprayed and incubated in order to localize esterase activity.

In vitro degradation of ^{14}C -malathion. The technique used was essentially that described by MIYATA et al. (1976). Twenty mg of mites were homogenized with 1 ml of phosphate buffer (6.7×10^{-2} M; pH 7.0) by glass homogenizer. The homogenate was briefly centrifuged at 1500 g for 10 min and the resultant supernatant was used as the enzyme source. To study the degradation activity of the mite homogenate and its inhibition by synergists, 10 aliquot of ^{14}C -malathion was added simultaneously with the synergist to the reaction mixture containing 0.2 ml of enzyme solution and 0.8 ml of phosphate buffer. The mixture was incubated for 30 min at 37°C with shaking. For the degradation study of ^{14}C -malathion on the electrophoretically separated enzyme bands, each agar piece was incubated with 1.5 ml of 1×10^{-5} M of ^{14}C -malathion and synergist in phosphate buffer. The mixture was incubated at 37°C for 2 hrs with shaking, and the reaction was stopped by the addition of 0.1 ml of 10% trichloroacetic acid. The unchanged substrate was extracted three times with 2 ml portion of chloroform and the products in the water phase determined.

Chemicals. ^{14}C -malathion labelled in methoxy groups with specific activity of 1,500 dpm/ μg was synthesized according to the method of KRUGER and O'BRIEN (1965). The following chemicals were used as synergists for malathion, *i.d.* K-1, K-2, K-9, IBP and TPP. These chemicals have been described as good inhibitors for insect and mite aliesterases (PLAPP et al., 1963; PLAPP and TONG, 1966; OHKAWA et al., 1968; MIYATA et al., 1979; TAKAHASHI et al., 1973). Preparation methods and physical properties of K-1 and K-2 have been previously described (ETO and OSHIMA, 1962; ETO et al., 1963a,b). K-9 (Salithion) was supplied by Sumitomo Chemical Co., Ltd., Osaka. IBP (Kitazin P®) was the gift of Dr. Y. UESUGI, National Institute of Agricultural Sciences. TPP (tri-phenyl phosphate) was purchased from Tokyo Kasei Co., Ltd., Tokyo. These synergists were combined to make an ethanol solution containing 2% TritonX-100 (W/V).

RESULTS

The response of four strains of the mite to malathion and the inhibitory effects of phosphorus esters on the *in vitro* degradation of ^{14}C -malathion by the mite homogenates are shown in Tables 1 and 2, respectively. It is obvious from these data that the more resistant the mite is to malathion, the higher is the degradation activity of the homogenate. Compound K-1 which demonstrated prominent synergism with malathion to resistant strains of the mite (KUWAHARA, 1977) exhibited a distinctive inhibitory effect on the *in vitro* degradation of this chemical. Other esters which demonstrated no significant synergism with malathion, however, exhibited no such inhibitory effect on its degradation.

Table 1. RESPONSE OF FOUR STRAINS OF MITE TO MALATHION

Strain	Dosage mortality regression equation	LC50 (ppm)	Resistant factor
NER	$Y = 5 + 1.873 (X - 3.914)$	8200	414
ZoRR	$Y = 5 + 2.127 (X - 4.033)$	10800	545
Ns	$Y = 5 + 2.086 (X - 1.505)$	32	1.6
Nk	$Y = 5 + 3.072 (X - 1.297)$	19.8	1.0

Table 2. *In vitro* DEGRADATION OF MALATHION WITH AND WITHOUT SYNERGISTS
BY FOUR STRAINS OF KANZAWA SPIDER MITE
(dpm/2.8 mg mite/30 min)

Mixture	Strain			
	ZoRR	NER	Ns	Nk
^{14}C -malathion	772	609	172	121
^{14}C -malathion + K-1	290	220	143	128
^{14}C -malathion + K-2	783	602	183	128
^{14}C -malathion + K-9	787	603	188	125
^{14}C -malathion + IBP	766	578	169	119

Synergist concentration : 1×10^{-5} M

Fig. 1. *In vitro* degradation of ^{14}C -malathion by ZoRR strain enzymes, and the effect of K-1 and IBP on the activity of esterases separated by agar gel electrophoresis.
●—●, ^{14}C -malathion only; ○—○, ^{14}C -malathion with K-1; ◐—◐, ^{14}C -malathion with IBP.

Figs. 1–3 show the *in vitro* degradation of ^{14}C -malathion by mite enzymes separated by the electrophoretic method from the four strains, along with the corresponding zymogram for β -naphthylacetate hydrolysis. Six esterase bands were identified, E_1 to E_6 from cathods to anode (Fig. 1). Although there was no difference in the relative mobility of these esterase bands among the strains tested, there was a qualitative difference in certain bands as judged by the intensity of the color development. Namely, the E_3 and E_4 bands of OP-resistant strains ZoRR and NER developed a moderately clear red color, while those of OP-susceptible strains Ns and Nk developed

Fig. 2. *In vitro* degradation of ^{14}C -malathion by NER strain enzymes, and the effect of K-1 and IBP on the activity of esterases separated by agar gel electrophoresis. Conditions were the same as in Fig. 1.

a faded red. Thus, the resistance to malathion in the Kanzawa spider mite was determined to be associated with an increased aliesterase activity at the E_3 and E_4 bands.

The malathion degrading activity resolved into three peaks (Figs. 1–3). The major peak position coincided with that of esterases E_3 and E_4 , and the two minor positions with those of esterases E_1 and E_2 respectively. The compound K-1, which manifested prominent synergism with malathion to OP-resistant strains, distinctively inhibited the degradation of malathion by E_3 and E_4 bands but did not inhibit its degradation by E_1 and E_2 bands. To the contrary, IBP which manifested no prominent synergism with malathion to these mites did not inhibit the degradation of malathion by E_3 and E_4 bands. This compound did, however, inhibited the degradation by E_1 and E_2 bands.

Three peaks of malathion degradation, though at much lower levels, were also recognized with the OP-susceptible strain Ns, and the relative mobility of these peaks coincided with those of OP-resistant strains. With the Nk strain, however, no peaks of malathion degradation were detected; it is therefore very likely that the degradation activity by aliesterases in this strain is negligible, if present at all.

It should be noted from these results that in mites there are at least three malathion degrading esterases which can be classified into two groups for their distinctive response to synergists.

Figure 4 shows the inhibitory effects of synergists on the esterase activity of the OP-resistant strain ZoRR. The compound K-1, which manifested synergism with malathion, inhibited the activity of E_3 and E_4 bands, but did not inhibit that of E_1 and E_2 bands. On the contrary, IBP did not inhibit the activity of E_3 and E_4 bands

Fig. 3. *In vitro* degradation of ^{14}C -malathion by Ns and Nk strain enzymes, and the effect of K-1 on the activity of esterases separated by agar gel electrophoresis. Conditions were the same as in Fig. 1.

but did inhibit that of E_1 and E_2 bands. Compounds K-2 and TPP, which manifested no prominent synergism with malathion, did not inhibit the esterase activity of any bands.

It is, therefore, likely that the Kanzawa spider mite has esterases active for both β -naphthylacetate hydrolysis and malathion degradation. Synergists such as K-1 and IBP can selectively inhibit these esterases.

DISCUSSION

From the results obtained in the present study, it is difficult to determine whether both reactions were catalyzed by the same enzyme or by two different enzymes, since more than two enzymes can have a common electrophoretic mobility. However, the fact that the compound K-1 inhibited both reactions selectively suggests that the same enzyme is probably responsible for both reactions.

The synergism of K-1 with malathion appears to be due to the inhibition of the carboxylesterase which is responsible for the degradation of malathion. This com-

Fig. 4. Effect of organophosphorus esters on esterase zymogram patterns from ZoRR strain.

pound can, therefore, be used as a tool for assessing malathion specific resistance in mites. The K series of compounds used in this experiment was originally discovered as biological active metabolites of TOCP (tri-*o*-cresyl phosphate) by CASIDA et al. (1961), and the insecticidal activity and chemical properties of analogous cyclic phosphates have been reported by ETO and his colleagues (ETO et al., 1962, 1963; ETO and OSHIMA, 1962a, b). It has been demonstrated that K-2 was a potent inhibitor for aliesterases from the housefly and the leafhopper, and the compound manifested a prominent synergism with malathion to these insects (OHKAWA et al., 1968). In the case of spider mites, however, K-2 does not manifest synergism with malathion (TAKAHASHI et al., 1973; KUWAHARA, 1977) nor, as shown by the present study, does it inhibit the esterases responsible for degradation of malathion. This is in contrast to compound K-1 which manifested synergism with malathion, and also selectively inhibited the esterase activities. These results indicated that properties of the alie-esterases which hydrolyze malathion must differ greatly between insects and spider mites.

It has been generally recognized that the insensitivity of acetylcholinesterase to OP and carbamate is the most important and common mechanism of resistance to these compounds in Acari (SMISSAERT, 1964; MATSUMURA and VOSS, 1964; LEE and BATHAM, 1966). The involvement of an additional mechanism, *i.e.* an increased activity in degradation of the toxicants, has been reported with certain resistant strains (MATSUMURA and VOSS, 1964; VOSS and MATSUMURA, 1964; SCHNITZERLING et al., 1974). A combination of both mechanisms might be responsible for the extensive and intensive resistance observed worldwide in mites. A specific synergist, such as K-1, which inhibits the degradation enzyme may be useful for elucidation of the role

of detoxification in resistance mechanisms of spider mites.

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