respectively in 11 and 13 generations of selection.

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***In Vitro* Degradation of ^{14}C-Methyl Malathion by Organophosphate Susceptible and Resistant Smaller Brown Planthopper, *Laodelphax Striatellus* Fallén[1]**. Tadashi Miyata, Hachiro Honda, Tetsuo Saito, Kozaburo Ozaki* and Yoshitaka Sasaki* (Laboratory of Applied Entomology and Nematology, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya and* Division of Phytopathology and Entomology, Kagawa Agricultural Experimental Station, Takamatsu, Kagawa) Received July 29, 1975. *Botyu-Kagaku* 41, 10, 1976.

3. 有機リン剤感受性および抵抗性ヒメトビウンカによる *in vitro* での ^{14}C-methyl malathion の分解について 宮田 正，本多八郎，斎藤哲夫，*尾崎幸三郎，*佐々木善隆（名古屋大学農学部害虫学教室，名古屋市千種区不老町，*香川県農業試験場病虫部，香川県高松市） 50. 7. 29 受理

感受性 (LE)，malathion 抵抗性 (R_m)，fenitrothion 抵抗性 (R_f) ヒメトビウンカをもちい，*in vitro* における ^{14}C-methyl malathion の代謝をしらべた．LE，R_m，R_f 系統ヒメトビウンカ雌成虫に対する malathion の24時間後の LC_{50} 値は，それぞれ 159，2,190，637 ppm であった．ヒメトビウンカの全虫体まさい液による *in vitro* での ^{14}C-methyl malathion の分解をしらべたところ，R_m および R_f 系統による分解は LE 系統のそれぞれ7倍，5倍であった．また各系統の代謝物は殆んどカルボキシルエステラーゼによるもので，フォスファターゼによるものは殆んど認められなかった．*In vitro* での ^{14}C-methyl malathion の分解におよぼす各種阻害剤，共力剤，金属イオン，補酵素等の影響についてしらべたところ，10^{-6}M の K-2 および 10^{-5}M の dichlorvos 添加により分解は殆んど100%阻害された．寒天ゲル電気泳動法によりヒメトビウンカの酵素を分離し，エステラーゼ泳動帯と ^{14}C-methyl malathion の分解作用についてしらべたところ，抵抗性に関連があると考えられている E_7 泳動帯を中心に分解作用が認められたのみで，他の泳動帯には分解作用は全く認められなかった．

Malathion resistance in the smaller brown planthopper, *Laodelphax striatellus* Fallén, was first found in Hiroshima and Okayama Prefecture, Japan in 1964[1], and since then many examples of the smaller brown planthopper resistance in other parts of the country were reported[2,3].

According to Ozaki and Kassai[4], there is a good correlation between the resistance level of the smaller brown planthopper to malathion and β-naphthyl acetate hydrolyzing activity of the E_7 band separated by thin layer agar-gel electrophoresis. And it was suggested that both esterase activity of the E_7 band and malathion resistance depend on the same factor.

In this paper, *in vitro* metabolism of ^{14}C-methyl malathion, and the relationship between β-naphthyl acetate hydrolyzing activity and ^{14}C-methyl malathion degrading activity of the E_7 band were studied.

Materials and Methods

Insect ; Female adults of the smaller brown planthopper were used. Malathion resistant (R_m) and fenitrothion resistant (R_f) strains were obtained from the susceptible (LE) strain through

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a continuous selection with either malathion or fenitrothion[5]. All planthoppers were reared on rice seedlings in a rearing bottle under the controlled condition of 25°C and 16 hr illumination per day, with weekly supply of fresh rice seedlings.

^{14}C-Methyl malathion; The synthesis of ^{14}C-methyl malathion was accomplished according to the method of Krueger and O'Brien[6]. In a 10m*l* flask, were placed 4 mM of anhydrous methyl alcohol containing 0.5 mCi of ^{14}C-methyl alcohol (12.8 mCi/m mole), 2 m*l* of anhydrous toluene, and 1 mM of phosphorus pentasulfide. The mixture was refluxed at 80-90°C for 2 hr. After cooling the mixture, 2 mM of diethyl maleate was added to the flask. The mixture was again refluxed at 80-90°C for additional 2 hr. The solvent was evaporated at 40°C, and the oily residue was dissolved in 30 m*l* of chloroform. The chloroform solution was first washed with an equal volume of 10% sodium carbonate and was again washed with water. The chloroform extract was dried over anhydrous sodium sulfate over night. The chloroform solution was concentrated to about 2 m*l* at 40°C and was purified by thin layer chromatography with a solvent mixture of acetone: hexane=1 : 4 (V/V) as a mobile phase. The portion of ^{14}C-methyl malathion on the plate was scraped, and was extracted with acetone. The acetone was evaporated at 40°C. The stock solution of ^{14}C-methyl malathion was prepared by dissolving it in absolute ethyl alcohol, and was kept in a refrigerator. The purity of the synthesized ^{14}C-methyl malathion exceeded 98% with an activity 1,500 d.p.m./μg as determined by an Aloka LSC-653 liquid scintillation spectrometer.

Measurement of resistance to malathion and fenitrothion; Resistance to malathion and fenitrothion was measured by a spraying method as follows: Fourty per cent solution of malathion and fenitrothion in a mixture of acetone: benzene: Newcol 863 = 3 : 3 : 1 (V/V) were diluted to a suitable concentration with distilled water. Ten females were anesthetized with carbon dioxide, and 3m*l* of the insecticide solution was applied with a rotary spray tower (Mizuho Scientific Co., Nagoya) at 280 mm Hg. Treated insects were transferred into 2.5×20 cm glass tubes containing rice seedlings, and allowed to stand at 25°C. Mortality was measured at 24 hr after treatment. At each concentration, the experiment was repeated for 3-5 times. From these results, LC_{50} values were calculated[7].

Electrophoresis; The thin layer agar-gel electrophoresis method reported by Ozaki and Kassai[4] was modified as follows: The supporting agar was prepared by adding 0.7 g each of PVP (polyvinyl pyrolidone, K-90, Katayama Chemical, Osaka) and agar powder (Wako Pure Chemical Ind., Osaka) to 100 m*l* of phosphate buffer (pH 6.8, ionic strength 0.015 μ). Ten (R_m, R_f) to twenty (LE) female planthoppers were crushed with a glass rod in 0.04 m*l* distilled water on a glass plate, and a strip of filter paper (Toyo filter paper No. 2, 2×60 mm) was immersed in the brei. The strip of filter paper was placed on the origin of the gel plate and kept in a refrigerator (4-5°C). After 30 min. the strip was removed from the agar plate, and the agar plate was connected by filter paper with buffer solution (pH 6.8, ionic strength 0.05 μ) in the electrode vessels. Electrophoresis took about 90 min. using a constant current of 2 mA per cm width. For the detection of esterases, β-naphthyl acetate was used with diazo blue B as a coloring reagent for the end product of hydrolysis of β-naphthol. A part of the agar plate was stained for esterases with β-naphthyl acetate, and the other was tested for the degradation activity for the insecticide by first transversely sectioning agar bed into 35-40 pieces and by incubating them with ^{14}C-methyl malathion.

In vitro degradation of ^{14}C-methyl malathion; Female planthoppers were homogenized with 1/15M phosphate buffer at pH 7.4 by using a Potter-Elvehjem glass homogenizer. The homogenate was briefly centrifuged at 1,500 × g for 10 min., and the resulting supernatant was used as the enzyme source. To study the direct degradation activity of the planthopper homogenate, a 10 μ*l* aliquot of ^{14}C-methyl malathion (10^{-3}M) in absolute ethyl alcohol was added to the reaction mixture containing 0.5 m*l* of the enzyme solution (homogenate containing 0.8 and 0.16% weight by volume of the planthopper for

LE and R strains, respectively), and 0.5 m*l* of the standard buffer (1/15 M phosphate buffer, pH 7.4). To study the properties of degradation enzymes of the homogenate, 0.2 m*l* of the standard buffer with metal ions, inhibitors, EDTA, NADPH, GSH or synergists was added to make the reaction mixture 1.0 m*l*. The system was incubated for 1 hr at 37°C with shaking.

For degradation study of ^{14}C-methyl malathion on the electrophoretically separated enzyme bands, the methods described by Miyata and Matsumura[8] was modified as follows: The agar piece (5× 50mm) separated from the agar bed was transferred to a test tube containing 1.5 m*l* of the standard buffer with ^{14}C-methyl malathion (10^{-5}M). The system was incubated at 37°C for 2 hr with shaking.

The incubated material was extracted with an equal volume of chloroform three times, and the radioactivity in the aqueous was determined for the degradative products with Bray's counting solution[9] by an Aloka LSC-653 liquid scintillation spectrometer. To separate carboxylesterase products and phosphatase products, the incubate was first extracted with an equal volume of chloroform twice, and the pH of the aqueous phase was lowered by adding 0.3 m*l* of 2.5% trichloroacetic acid and extracted twice with an equal volume of chloroform[10].

Results

Resistance levels to malathion and fenitrothion for female adults of the planthopper are presented in Table 1. The LC_{50} values of R_m and R_f strains to malathion were about 13.8 and 4 times higher than that of LE strain. In case of fenitrothion, the resistance levels of R_m and R_f strains to LE strain were about 4-fold.

Table 1. Toxicities of malathion and fenitrothion to LE, R_m and R_f strains of the smaller brown planthopper.

Insecticide	Strain	Dorsage-mortality[a] regression line	LC_{50}[b] (ppm)	Index of resistance
Malathion	LE	$Y = 5+1.931\ (X-2.201)$	159	1
	R_m	$Y = 5+3.314\ (X-3.340)$	2,190	13.8
	R_f	$Y = 5+1.907\ (X-2.804)$	637	4.0
Fenitrothion	LE	$Y = 5+2.082\ (X-1.931)$	85.3	1
	R_m	$Y = 5+2.092\ (X-2.517)$	329	3.9
	R_f	$Y = 5+2.227\ (X-2.532)$	340	4.0

[a] Y = probit of death rate.
X = insecticide concentration (ppm).
[b] Determined 24 hr after spraying.

Table 2. *In vitro* degradation activity of ^{14}C-methyl malathion in LE, R_m and R_f strains of the smaller brown planthopper.

Enzyme source[a]	Degradation activity (%)			Degradation ratio
	Carboxylesterase products	Phosphatase products	Total	
Buffer only	1.2	2.3	3.5	—
LE	32.4	3.0	35.4	1
R_m	44.1	3.2	47.3	6.9
R_f	32.4	3.0	37.4	5.3

The enzyme solution containing 10^{-5} M of ^{14}C-methyl malathion was incubated at 37°C for 1 hr.
[a] LE : 0.4% final enzyme solution.
R_m and R_f : 0.08% final enzyme soiution.

In vitro degradation of ^{14}C-methyl malathion by the homogenate of the planthopper was studied (Table 2). Most metabolites were carboxylesterase products. Total degradative activity of R_m and R_f strains were 6.9 and 5.3 times higher than that of LE strain. Table 3 shows the effects of metal ions, inhibitors, EDTA, NADPH, GSH and synergists on the degradation of ^{14}C-methyl malathion. K-2 (2-phenoxy-4H-1,3,3-benzodioxaphosphoric 2-oxide) and dichlorvos significantly inhibited the degradation of ^{14}C-methyl malathion at the concentration of 10^{-6} and 10^{-5}M, respectively. At the concentration of 10^{-5}M, EPN inhibited the degradation moderately, but the concentration of 10^{-3}M it completely inhibited the degradation. Cu^{++} and S-421 (octachlorodipropyl ether) also inhibited the degradation at 10^{-3}M. Addition of NADPH or GSH did not stimulate the reaction;rather they inhibited the degradation of ^{14}C-methyl malathion.

Table 3. Effects of certain additives on the *in vitro* degradation of ^{14}C-methyl malathion in LE, R_m and R_f strains of the smaller brown planthopper. [a)]

Additives (final concentration)	Enzyme source		
	LE	R_m	R_f
Ca^{++} (1×10^{-3}M)	11.6	−0.2	5.5
Mn^{++} (1×10^{-3}M)	9.8	6.8	5.6
Cu^{++} (1×10^{-3}M)	79.6	85.0	89.3
EDTA (1×10^{-3}M)	22.2	7.5	22.6
NADPH (2×10^{-4}M)	5.7	22.0	3.6
GSH (4×10^{-3}M)	13.0	10.0	6.1
K-2 (1×10^{-6}M)	98.8	98.3	99.0
Dichlorvos (1×10^{-5}M)	96.4	98.9	98.6
EPN (1×10^{-5}M)	46.6	19.0	53.7
EPN (1×10^{-3}M)	100	100	100
Dimethoate (1×10^{-3}M)	34.9	19.3	67.4
S-421 (1×10^{-3}M)	80.4	90.0	92.9

[a)] Data expressed as percent inhibition of normal enzyme degradation activity.

To correlate the above enzyme activities to the esterase positions on the electrophoresis plate, the agar pieces corresponding to esterase bands were separated and were incubated with ^{14}C-methyl malathion. Results shown in Figs. 1 to 3 indicate that the peak of malathion degrading activity coincide with band position around the E_7 band.

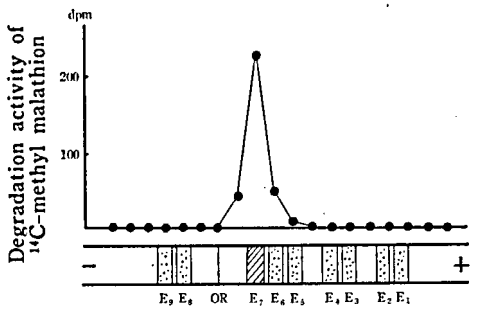

Fig. 1. Degradation of ^{14}C-methyl malathion by the smaller brown planthopper enzymes of LE strain separated by agar-gel electrophoresis. The agar plate was sliced into 5 × 50 mm pieces and each agar piece was incubated at 37°C for 2 hr with 10^{-5} M of ^{14}C-methyl malathion to assess its degradation activity. The corresponding zymogram for β-naphthyl acetate indicates the relative positions of esterase bands against malathion degrading enzyme.

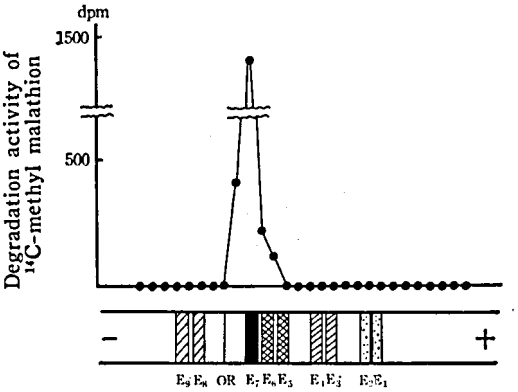

Fig. 2. Degradation of ^{14}C-methyl malathion by the smaller brown planthopper enzymes of R_m strain separated by agar-gel electrophoresis.

Discussion

The physiological mechanism of organophosphorus insecticide resistance are classified as follows: a reduction in penetration[11,12,13], an increase in detoxification of insecticides[14,15,16,17] and a reduction in the susceptibility of the action point to insecticides[18,19,20,21].

Considering the resistance level of the planthopper to malathion (Table 1) and the *in vitro* degradation of ^{14}C-methyl malathion (Table 2),

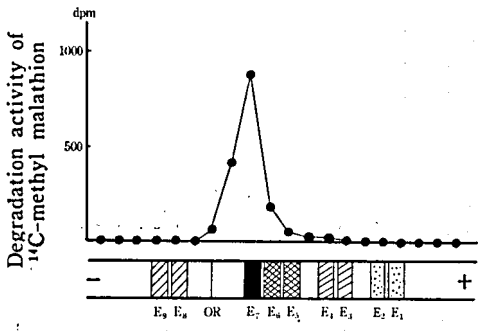

Fig. 3. Degradation of ^{14}C-methyl malathion by the smaller brown planthopper enzymes of R_f strain separated by agar-gel electrophoresis.

enhanced degradation of malathion seems to be one of the most important mechanisms in the smaller brown planthopper resistance to malathion as already reported in other insects[15,16,22,23].

It has been found that carboxylesterase is highly sensitive to inhibition by dichlorvos[24] and K-2[23]. ^{14}C-Methyl malathion degradation *in vitro* was also highly sensitive to dichlorvos and K-2 in the smaller brown planthopper.

Ozaki and Kassai[4] reported that β-naphthyl acetate hydrolyzing activity of malathion resistant planthopper was higher than that of susceptible one, and that most resistant planthoppers had middle and high activity of the E_7 band. The esterases hydrolyzing β-naphthyl acetate of the planthopper were separated into nine different bands by thin layer agar-gel electrophoresis, and R_m and R_f strains showed remarkably higher activity of the E_7 band than LE strain as already reported by Ozaki and Kassai[4]. There was a good correlation between β-naphthyl acetate hydrolyzing activity and ^{14}C-methyl malathion degrading activity of the E_7 band. Though more than two malathion degrading enzymes were detected on the agar plate separated by electrophoresis in the American cockroach[25], house fly[23] and rat liver[8] homogenate, only one malathion degrading peak was detected in the smaller brown planthopper homogenate under the experimental condition. It is not easy to determine whether the E_7 band represents only one enzyme or more, for there could always be a possibility that a number of enzymes come to occupy the same location in an electrophoresis experiment. Therefore, the E_7 band merely represents the location of malathion degradation enzymes.

Summary

In vitro metabolism of ^{14}C-methyl malathion by susceptible (LE), malathion resistant (R_m) and fenitrothion resistant (R_f) strains of the smaller brown planthopper was studied. LC_{50} values of LE, R_m and R_f strains of the planthopper to malathion were 159, 2,190 and 637 ppm, respectively. ^{14}C-Methyl malathion was degraded more than about 7 and 5 times by the homogenate of R_m and R_f strains as to that of LE strain. Most metabolites of each strain were carboxylesterase products, and the small amounts of phosphatase products were detected. Among tested materials, *in vitro* degradation of ^{14}C-methyl malathion was inhibited almost 100% by K-2 (10^{-6}M) and dichlorvos (10^{-5}M). Enzymes of the smaller brown planthopper were separated by agar-gel electrophoresis, and esterase pattern and degradation of ^{14}C-methyl malathion was studied. Only one peak of malathion degradation was detected at the E_7 band.

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Changes in Blood Carbohydrate and Protein Titers During Morphogenesis of Silkworms. Masaji S. Nishimura and Ayako Ohsawa (Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka, 553 Japan) Received August 1, 1975. *Botyu-Kagaku* 41, 15, 1976.

4. カイコの形態形成にともなう血中炭水化物とタンパク質の変動

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カイコでは，血中トレハロースは雌雄とも5令および蛹の前半期に増加し，蛹化および羽化前に減少した．一方，去勢雌では成虫化前に正常個体のような減少はみられず，これより同時期でのトレハロースは卵形成にある程度使用されると思われる．去勢雄のトレハロースは正常個体のそれと変化なかった．

カイコの雌の血中タンパク質はトレハロースの場合と同様の変動を示した．一方，去勢雌では蛹化後期にも高い値がつづき，タンパク質もトレハロースと同様に，雌の卵形成に消費されるものと推定される．雄のタンパク質は熟蚕期から蛹化直前にかけて高い値を保っていた．雌雄間でタンパク質の量的な性差はみられなかった．

血中グルコースは雌雄共に殆んど検出されなかった．

エリサンの血中トレハロースは雌雄共量的に同じ変動を示した．ただエリサンでは蛹化時に別に一つのピークがみられた．

エリサンの血中タンパク質は，5令の初期から増加しはじめ，蛹化時に一旦減少し，以後再び増加して成虫化直前まで高い値のままとどまった．その間，性差はみられなかった．

除脳休眠蛹の場合，除脳後180日を経た個体でさえ，血中炭水化物，タンパク質共に蛹化時の値と変化なかった．

Introduction

Since Wyatt and Kalf demonstrated that the major blood sugar in insects is α-trehalose[1,2], a non-reducing dimer of α-glucose, many reports have appeared on the physiological role of trehalose as the circulating form of carbohydrate cellular food and as a biochemical characteristic substance of insects[3,4,5].

In holometaborous insects, the concentration of blood trehalose changes during normal physiological process. The protein concentration in