In Vitro Degradation of 14\(^\text{C}\)-Methyl Malathion by Organophosphate Susceptible and Resistant Smaller Brown Planthopper, Laodelphax Striatellus Fallén

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respectively in 11 and 13 generations of selection.

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References

In Vitro Degradation of 14C-Methyl Malathion by Organophosphate Susceptible and Resistant Smaller Brown Planthopper, Laodelphax Striatellus Fallen\textsuperscript{1}.

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3. 有机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 14C-methyl malathion の分解について

感受性 (LE), malathion 抵抗性 (Rm), fenitrothion 抵抗性 (Rr) ヒメトビウンカをもらい, in vitro における 14C-methyl malathion の代謝をしらべた。LE, Rm, Rr 系統ヒメトビウンカ雌成虫に対する malathion の24時間後の LC\textsubscript{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找出液による in vitro での 14C-methyl malathion の分解をしらべたところ、Rm および Rr 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どカルボキシルエステラーゼによるもので、フェンスファターゼによるもののは殆ど認められなかった。In vitro での 14C-methyl malathion の分解におよぼす各誘導剤、共力剤、金属イオン、酵素等の影響についてしらべたところ、10\textsuperscript{-4} M の K-2 および 10\textsuperscript{-4} M の dichlorvos 添加によ り分解は殆ど100\%阻止された。案外メルク電気泳動法によりヒメトビウン卡の酵素を分離し、エステラーゼ泳動帯と 14C-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\textsubscript{r} 継続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 Fallen, was first found in Hiroshima and Okayama Prefecture, Japan in 1964\textsuperscript{1}, and since then many examples of the smaller brown planthopper resistance in other parts of the country were reported\textsuperscript{2,3}.

According to Ozaki and Kassai\textsuperscript{3}, there is a good correlation between the resistance level of the smaller brown planthopper to malathion and \textbeta-naphthyl acetate hydrolyzing activity of the E\textsubscript{r} band separated by thin layer agar-gel electro-

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phoresis. And it was suggested that both esterase activity of the E\textsubscript{r} band and malathion resistance depend on the same factor.

In this paper, in vitro metabolism of 14C-methyl malathion, and the relationship between \textbeta-naphthyl acetate hydrolyzing activity and 14C-methyl malathion degrading activity of the E\textsubscript{r} band were studied.

Materials and Methods

\textit{Insect}: Female adults of the smaller brown planthopper were used. Malathion resistant (Rm) and fenitrothion resistant (Rr) strains were obtained from the susceptible (LE) strain through
a continuous selection with either malathion or fenitrothion. 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.

**14C-Methyl malathion;** The synthesis of 14C-methyl malathion was accomplished according to the method of Krueger and O'Brien. In a 10ml flask, were placed 4 mM of anhydrous methyl alcohol containing 0.5 mCi of 14C-methyl alcohol (12.8 mCi/m mole), 2 ml 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 ml 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 ml 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 14C-methyl malathion on the plate was scraped, and was extracted with acetone. The acetone was evaporated at 40°C. The stock solution of 14C-methyl malathion was prepared by dissolving it in absolute ethyl alcohol, and was kept in a refrigerator. The purity of the synthesized 14C-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: Forty 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 3ml 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, LC50 values were calculated.

**Electrophoresis;** The thin layer agar-gel electrophoresis method reported by Ozaki and Kassai 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 ml of phosphate buffer (pH 6.8, ionic strength 0.015 µ). Ten (Rm, Rr) to twenty (LE) female planthoppers were crushed with a glass rod in 0.04 ml distilled water on a glass plate, and a strip of filter paper (Toyofilter 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 14C-methyl malathion.

**In vitro degradation of 14C-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 µl aliquot of 14C-methyl malathion (10-2M) in absolute ethyl alcohol was added to the reaction mixture containing 0.5 ml 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 ml of the standard buffer (1/15 M phosphate buffer, pH 7.4). To study the properties of degradation enzymes of the homogenate, 0.2 ml of the standard buffer with metal ions, inhibitors, EDTA, NADPH, GSH or synergists was added to make the reaction mixture 1.0 ml. The system was incubated for 1 hr at 37°C with shaking.

For degradation study of 14C-methyl malathion on the electrophoretically separated enzyme bands, the methods described by Miyata and Matsumura was modified as follows: The agar piece (5 x 50mm) separated from the agar bed was transferred to a test tube containing 1.5 ml of the standard buffer with 14C-methyl malathion (10⁻⁶M). 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 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 ml of 2.5% trichloroacetic acid and extracted twice with an equal volume of chloroform.

### Results

Resistance levels to malathion and fenitrothion for female adults of the planthopper are presented in Table 1. The LC₉₀ values of Rₘ and Rᵣ strains to malathion were about 13.8 and 4 times higher than that of LE strain. In case of fenitrothion, the resistance levels of Rₘ and Rᵣ strains to LE strain were about 4-fold.

### Table 1. Toxicities of malathion and fenitrothion to LE, Rₘ and Rᵣ strains of the smaller brown planthopper.

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Strain</th>
<th>Dorsage-mortality a) regression line</th>
<th>LC₉₀ b) (ppm)</th>
<th>Index of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malathion</td>
<td>LE</td>
<td>Y = 5+1.931 (X-2.201)</td>
<td>159</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rₘ</td>
<td>Y = 5+3.314 (X-3.340)</td>
<td>2,190</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>Rᵣ</td>
<td>Y = 5+1.907 (X-2.804)</td>
<td>637</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y = 5+2.082 (X-1.931)</td>
<td>85.3</td>
<td>1</td>
</tr>
<tr>
<td>Fenitrothion</td>
<td>LE</td>
<td>Y = 5+2.062 (X-2.517)</td>
<td>329</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Rₘ</td>
<td>Y = 5+2.227 (X-2.532)</td>
<td>340</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Rᵣ</td>
<td>Y = 5+2.517 (X-2.532)</td>
<td>340</td>
<td>4.0</td>
</tr>
</tbody>
</table>

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

### Table 2. In vitro degradation activity of 14C-methyl malathion in LE, Rₘ and Rᵣ strains of the smaller brown planthopper.

<table>
<thead>
<tr>
<th>Enzyme source a)</th>
<th>Carboxylesterase products</th>
<th>Phosphatase products</th>
<th>Total Degradation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer only</td>
<td>1.2</td>
<td>2.3</td>
<td>3.5</td>
</tr>
<tr>
<td>LE</td>
<td>32.4</td>
<td>3.0</td>
<td>35.4 1</td>
</tr>
<tr>
<td>Rₘ</td>
<td>44.1</td>
<td>3.2</td>
<td>47.3 6.9</td>
</tr>
<tr>
<td>Rᵣ</td>
<td>32.4</td>
<td>3.0</td>
<td>37.4 5.3</td>
</tr>
</tbody>
</table>

The enzyme solution containing 10⁻⁶ M of 14C-methyl malathion was incubated at 37°C for 1 hr.  
a) LE : 0.4% final enzyme solution.  
Rₘ and Rᵣ : 0.08% final enzyme solution.

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In vitro degradation of ¹⁴C-methyl malathion by the homogenate of the planthopper was studied (Table 2). Most metabolites were carboxylesterase products. Total degradative activity of Rm and Rr 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 ¹⁴C-methyl malathion.

Table 3. Effects of certain additives on the in vitro degradation of ¹⁴C-methyl malathion in LE, Rm and Rr strains of the smaller brown planthopper. *a*

<table>
<thead>
<tr>
<th>Additives (final concentration)</th>
<th>Enzyme source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LE</td>
</tr>
<tr>
<td>Ca++ (1×10⁻⁵M)</td>
<td>11.6</td>
</tr>
<tr>
<td>Mn++ (1×10⁻⁵M)</td>
<td>9.8</td>
</tr>
<tr>
<td>Cu++ (1×10⁻⁵M)</td>
<td>79.6</td>
</tr>
<tr>
<td>EDTA (1×10⁻⁵M)</td>
<td>22.2</td>
</tr>
<tr>
<td>NADPH (2×10⁻⁵M)</td>
<td>5.7</td>
</tr>
<tr>
<td>GSH (4×10⁻⁵M)</td>
<td>13.0</td>
</tr>
<tr>
<td>K-2 (1×10⁻⁴M)</td>
<td>98.8</td>
</tr>
<tr>
<td>Dichlorvos (1×10⁻⁴M)</td>
<td>96.4</td>
</tr>
<tr>
<td>EPN (1×10⁻⁵M)</td>
<td>46.6</td>
</tr>
<tr>
<td>EPN (1×10⁻⁴M)</td>
<td>100</td>
</tr>
<tr>
<td>Dimethoate (1×10⁻⁴M)</td>
<td>34.9</td>
</tr>
<tr>
<td>S-421 (1×10⁻⁴M)</td>
<td>80.4</td>
</tr>
</tbody>
</table>

*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 ¹⁴C-methyl malathion. Results shown in Figs. 1 to 3 indicate that the peak of malathion degrading activity coincide with band position around the E7 band.

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 ¹⁴C-methyl malathion (Table 2),
Fig. 3. Degradation of 14C-methyl malathion by the smaller brown planthopper enzymes of Rf 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\(^ {22}\) and K-2\(^ {22}\). 14C-Methyl malathion degradation \textit{in situ} was also highly sensitive to dichlorvos and K-2 in the smaller brown planthopper.

Ozaki and Kassai\(^ {19}\) reported that \(\beta\)-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 \(\beta\)-naphthyl acetate of the planthopper were separated into nine different bands by thin layer agar-gel electrophoresis, and R\(_m\) and R\(_r\) strains showed remarkably higher activity of the E\(_7\) band than LE strain as already reported by Ozaki and Kassai\(^ {19}\). There was a good correlation between \(\beta\)-naphthyl acetate hydrolyzing activity and 14C-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\(^ {22}\), house fly\(^ {22}\) and rat liver\(^ {3}\) 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**

\textit{In situ} metabolism of 14C-methyl malathion by susceptible (LE), malathion resistant (Rm) and fenitrothion resistant (Rr) strains of the smaller brown planthopper was studied. LC\(_{50}\) values of LE, R\(_m\) and R\(_r\) strains of the planthopper to malathion were 159, 2,190 and 637 ppm, respectively. 14C-Methyl malathion was degraded more than about 7 and 5 times by the homogenate of R\(_m\) and R\(_r\) 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, \textit{in situ} degradation of 14C-methyl malathion was inhibited almost 100\% by K-2 (10\(^{-4}\)M) and dichlorvos (10\(^{-4}\)M). Enzymes of the smaller brown planthopper were separated by agar-gel electrophoresis, and esterase pattern and degradation of 14C-methyl malathion was studied. Only one peak of malathion degradation was detected at the E\(_7\) band.

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**References**

Changes in Blood Carbohydrate and Protein Titers During Morphogenesis of Silkworms.

4. カイコの形態形成にもとづく血中炭水化物とタンパク質の変動 西村将司, 大沢文子（塚野義製薬研究所）50. 8. 1 受理

カイコでは, 血中トレハロースは雌雄とも5日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の正常個体のようないずれもみられず, これより同期間でのトレハロースは卵形成度に対応を使うと思われる。成虫雌のトレハロースは正常個体のそれと変化なかった。

カイコ雌の血中タンパク質はトレハロースの場合に同様の変動を示した。一方, 去勢雌では蛹化後半期に高い値がつまった。タンパク質もトレハロースと同様、雌の卵形成に消費されるものと推定される。雌のタンパク質は孵化直前から増加直後にかけて高い値を保っていた。雌雄間でタンパク質の質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ど検出されなかった。

エリザンの血中トレハロースは雌雄共に前半期に変動を示した。ただエリザンでは蛹化時に別にピーキな変動がみられた。

エリザンの血中タンパク質は、雌の初めから増加している。蛹化時に一度減少し、以後再び増加して蛹化直前に再び高い値を示した。その間、性差はみられなかった。

除蛹体眠の場合、蛹化後180日を経た個体ですでに、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, 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.

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