Linkage Group Analysis of Carboxylesterase in a Malathion Resistant Strain of the Housefly, *Musca domestica* L. (Diptera: Muscidae)¹

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The Wakamatsu-m strain of housefly was established from a colony collected in Kitakyushu, Japan, and selected for 18 successive generations with malathion. This strain possesses high malathion carboxylesterase activity, high glutathione S-transferase activity and insensitive acetylcholinesterase, as the mechanisms of resistance. The linkage group of the dominant factor controlling malathion carboxylesterase activity was analyzed by the F₁ male backcross method using two susceptible visible marker strains. Malathion carboxylesterase activity was estimated with ¹⁴C-malathion as a substrate. Results of the crossing experiments clearly showed that only the 2nd chromosome was associated with a dominant factor controlling malathion carboxylesterase activity and the other four autosomes did not show any dominant effect on malathion carboxylesterase. These crossing experiments also revealed that the male determining factor, M, was located on the 5th chromosome in Wakamatsu-m strain.

INTRODUCTION

Malathion carboxylesterase, which splits the carboxylester bond of malathion [*O,O*-dimethyl *S*-1,2-bis(ethoxycarbonyl)ethyl phosphorodithioate] is an important mechanism of malathion resistance in many insect species (DYTE and ROWLANDS, 1968; HEMINGWAY, 1982; MATSUMURA and BROWN, 1961; TOWNSEND and BUSVINE, 1969), including houseflies, *Musca domestica* L. (MATSUMURA and HOGENDIJK, 1964; MOTOMYAMA et al., 1980; NIWA et al., 1977; WELLING and BLAAKMEER, 1971; YOEH et al., 1981).

Genetic analysis of resistance genes controlling various resistance mechanisms has been most extensively studied in houseflies. However, no appropriate studies have yet been undertaken on malathion carboxylesterase using malathion itself as a substrate in houseflies. The present paper describes the results of linkage group analysis for the factor controlling malathion carboxylesterase activity of an extremely high malathion resistant strain of the Japanese housefly. Malathion carboxylesterase activities of resistant and susceptible parents, F₁- and B₁-offspring, were estimated *in vitro* utilizing ¹⁴C-malathion as a substrate.

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MATERIALS AND METHODS

Housefly strains. The following strains were used.

Wakamatsu-m: Extremely malathion resistant strain, was established from houseflies collected at a stable of the Wakamatsu Riding Club in Kitakyushu, Japan, and subjected to laboratory selection for 18 successive generations with malathion. The dominant factor for malathion resistance is located only on the 2nd chromosome in this strain (SHONO et al., 1982).

ac; ar; ye: A susceptible strain, of which the 1st, 2nd and 4th chromosomes are marked with the recessive visible mutant markers, ali-curve, aristapedia and yellow eyes, respectively.

ac; ar; bw; ocra: A susceptible strain having the 1st, 2nd, 3rd and 5th chromosomes with the recessive visible mutant markers: ali-curve, aristapedia, brownbody and ocra eyes, respectively.

SRS: A WHO susceptible standard strain, which was supplied every 6 months by Professor R. MILANI, Università di Pavia, Italy.

Chemicals. Malathion (purity 95.6%) was obtained from Mikasa Chemical Co., Amagi, Fukuoka. ^{14}C -malathion [*O,O*-dimethyl *S*-1,2-bis(ethoxycarbonyl)-(1,2- ^{14}C)-ethyl phosphorodithioate] specific activity 4.6 mCi per mmole, was purchased from Amersham, Buckinghamshire, England. Samples of malaoxon, desmethyl malathion, malathion α -acid and malathion β -acid were kindly supplied by Professor M. ETO, Kyushu University. Tepp (tetraethyl pyrophosphate) was synthesized by Dr. H. YOSHIKAWA, Kyushu Kyoritsu University.

Determination of malathion carboxylesterase activity. For the determination of malathion carboxylesterase, whole housefly bodies were homogenized with 0.05 M phosphate buffer, pH 7.4, at the concentration of 5 flies per ml, using a Biotron homogenizer. Only the homogenates filtered through nylon gauze were used for the enzyme preparation, unless otherwise stated. One ml of the housefly homogenates was incubated with 1 μ mole of ^{14}C -malathion. After 30 min incubation at 30°C, unhydrolyzed ^{14}C -malathion was extracted from the reaction mixture with 5 ml chloroform, and radioactivity of aqueous fraction was determined by liquid scintillation counting.

Identification of metabolites. Radioactive metabolites were identified by two dimensional thin-layer cochromatography using precoated silica gel, 60-F254 chromatoplate (20×20 cm, 0.25 mm thickness, E. Merck), developed with the following solvent systems:

System 1: benzene-ethyl acetate-acetic acid (50:50:3)

System 2: benzene-ether-acetic acid (80:20:10)

Thin-layer cochromatography utilized autoradiography to detect ^{14}C compounds and the 2,6-dibromoquinone chlorimide reagent for unlabeled standards (MENN et al., 1957).

Genetic analysis. Only the dominant effect of each chromosome on malathion carboxylesterase was studied in these linkage group analyses. The crossing design for linkage group analysis for the dominant factor of malathion carboxylesterase activity in Wakamatsu-m strain was based on the F_1 male backcross method as follows:

Cross 1: *ac; ar; ye* ♀ × F_1 (*ac; ar; ye* ♀ × Wakamatsu-m ♂) ♂

Cross 2: *ac; ar; bw; ocra* ♀ × F_1 (*ac; ar; bw; ocra* ♀ × Wakamatsu-m ♂) ♂

In Cross 1, where *ac*, *ar* and *ye* were utilized as visible characters, 1st, 2nd and 4th chromosomal factors can be analyzed, and in Cross 2, where *ac*, *ar*, *bwb* and *ocra* were used, 1st, 2nd, 3rd and 5th chromosomal factors can be detected. The dominant effect of all autosomes from 1st to 5th was, therefore, able to be determined from the results of Cross 1 and Cross 2. The progenies resulting from these backcrosses were separated into 8 phenotypes from Cross 1, and 16 phenotypes from Cross 2, and were homogenized and incubated with ^{14}C -malathion for the estimation of malathion carboxylesterase activity.

The crossing designs used here were completely the same as those used for analysis of male determining factor (TSUKAMOTO et al., 1980). The male determining system of Wakamatsu-m strain, therefore, was simultaneously analyzed by those crosses.

RESULTS

Susceptibility and mechanisms of resistance

The malathion LD_{50} and resistance ratio of the Wakamatsu-m and susceptible strains are shown in Table 1. The Wakamatsu-m strain had a resistance ratio of 1,670 due to increased carboxylesterase activity (Table 3), glutathione S-transferase (Table 2) and insensitive acetylcholinesterase (Table 1).

Malathion carboxylesterase assay

A simple method is preferable for the enzyme assay in the linkage group analysis, because the enzyme preparations of many different phenotypes from backcross progenies must be submitted to assay at once. The simplest procedure was selected for malathion carboxylesterase assay in this experiment. Table 2 shows the degradation of malathion by the subcellular fractions from Wakamatsu-m and *ac*; *ar*; *bwb*; *ocra* strains. In the resistant Wakamatsu-m strain, the nuclei plus mitochondrial fraction showed the highest activity for malathion degradation, and also the microsome and soluble fractions degraded malathion without a cofactor. In order to gather up the activities of all subcellular fractions and simplify the procedure of the enzyme assay, only the homogenates of whole bodies of houseflies were used for the enzyme preparations for malathion degradation in the linkage group analysis. The homogenate of the Wakamatsu-m strain metabolized several times more malathion to the water soluble compounds than did the *ac*; *ar*; *bwb*; *ocra* strain.

Table 3 shows that nearly all water soluble metabolites produced by the homo-

Table 1. Toxicity of malathion and anticholinesterase activity of malaoxon to the housefly strains used

Strain	Malathion LD_{50} ($\mu\text{g}/\text{♀}$)	Resistance ratio ^a	Malaoxon I_{50} (10^{-8} M)	Insensitivity ratio ^a
Wakamatsu-m	>750 (8.4) ^b	>1,670	190	76
<i>ac</i> ; <i>ar</i> ; <i>ye</i>	0.45	1.0		
<i>ac</i> ; <i>ar</i> ; <i>bwb</i> ; <i>ocra</i>	0.40	0.89		
SRS	0.45	1.0	2.5	1.0

^a Based on a value of 1.0 for SRS strain.

^b Figure in parenthesis indicates percent mortality at the dose of 750 $\mu\text{g}/\text{♀}$.

Table 2. Malathion degradation by subcellular fraction from resistant Wakamatsu-m and susceptible *ac; ar; bw; ocra* strains of houseflies

Subcellular fraction	Water soluble metabolites	
	Wakamatsu-m	<i>ac; ar; bw; ocra</i>
Homogenates	37.4 ^a	7.0 ^a
Nuclei+mitochondria	20.6	2.5
Microsomes	7.3	2.5
Soluble fraction	9.8	5.0
Soluble fraction (with 4 mM GSH)	34.8	9.3

^a nmol/ fly equivalent/ 30 min.Table 3. Water soluble metabolites produced by the homogenates of Wakamatsu-m and *ac; ar; bw; ocra* strains for 30 min incubation with malathion

Metabolites	<i>Rf</i> value ^a		Wakamatsu-m (%)	<i>ac; ar; bw; ocra</i> (%)
	System 1	System 2		
Desmethyl malathion	0.06	0.12	0.7	0.1
Unknown 1	0.32	0.44	0.4	0.0
Malathion α -monoacid	0.39	0.48	26.9	4.1
Malathion β -monoacid	0.42	0.48	3.9	1.9
Unknown 2	0.67	0.68	0.2	0.6
Total			32.1	6.7

^a TLC solvent system 1: benzene-ethyl acetate-acetic acid (50:50:3), solvent system 2: benzene-ether-acetic acid (80:20:10).

Table 4. Effects of esterase and mixed function oxidase inhibitors on the malathion degradation by the homogenates of Wakamatsu-m houseflies

Inhibitor	Concentration	Inhibition (%)
Tepp	10 ⁻⁵ M	88.8
	10 ⁻⁶	68.6
Triphenyl phosphate	10 ⁻⁵	85.3
	10 ⁻⁶	82.7
Pyperonyl butoxide	10 ⁻³	3.2

genates of the Wakamatsu-m and *ac; ar; bw; ocra* strains were identified as malathion monoacid, resulting from malathion carboxylesterase cleavage of the carboxylester bond. In the resistant Wakamatsu-m strain, the amount of malathion monoacid exceeded 95% of the total water soluble metabolites produced. Malathion diacid was not detected from the preparations of either strain. Metabolites by glutathione S-transferase were desmethyl malathion and possibly unknown 1, of which *Rf* value was similar to that of tetraethyl dithiodisuccinate, an artifact product from desmethyl malathion (NOMEIR and DAUTERMAN, 1978). A small amount of glutathione S-transferase metabolites found in both strains was formed as the result of utilization of

endogenous glutathione in the homogenates. The contribution of glutathione S-transferase was, therefore, almost negligible for the production of water soluble metabolites, unless glutathione was added to the homogenates.

The production of water soluble metabolites by the homogenate of the Wakamatsu-m strain was not affected by a mixed-function oxidase inhibitor, piperonyl butoxide, but was clearly inhibited by esterase inhibitors, tepp and tripheny phosphate, as shown in Table 4. These results mentioned above clearly show that the degradation of malathion to water soluble metabolites by the homogenates of houseflies depends predominantly on malathion carboxylesterase. Then, to simplify the procedure of the enzyme assay, the amount of water soluble metabolites produced by the housefly homogenates was regarded as the same as that of hydrolyzed product produced by malathion carboxylesterase. A thirty minutes incubation period was selected for the enzyme assay, as the relation between the amount of water soluble metabolites produced and the time of incubation was linear over 30 min, as shown in Fig. 1.

The F_1 hybrids between Wakamatsu-m and the two susceptible marker strains hydrolyzed 2.6 times more malathion than the susceptible parents did, and this amount was about 60% of malathion degradation of the resistant Wakamatsu-m strain (Table 5).

Fig. 1. Malathion degradation by the homogenates of resistant Wakamatsu-m and susceptible *ac; ar; bw; ocra* strains of houseflies.

Table 5. Malathion degradation by the homogenates of resistant Wakamatsu-m and susceptible mutant marker strains, and their F_1 progenies

Parent strain or progeny	Water soluble metabolites
Wakamatsu-m	55.4 ^a
<i>ac; ar; ye</i>	13.3
<i>ac; ar; bw; ocra</i>	12.6
F_1 (<i>ac; ar; ye</i> ♀ × Wakamatsu-m ♂)	34.6
F_1 (<i>ac; ar; bw; ocra</i> ♀ × Wakamatsu-m ♂)	33.3

^a nmole/fly/30 min.

Table 6. Relation between phenotypic expression of visible markers and malathion degradation in backcross progenies from Cross 1

[*ac; ar; ye* ♀ × F₁ (*ac; ar; ye* ♀ × Wakamatsu-m ♂) ♂]

Phenotype (1; 2; 4)	Water soluble metabolites
+ ; + ; +	29.7 ^a
<i>ac</i> ; + ; +	29.0
+ ; <i>ar</i> ; +	10.6
<i>ac</i> ; <i>ar</i> ; +	9.2
+ ; + ; <i>ye</i>	36.4
<i>ac</i> ; + ; <i>ye</i>	31.6
+ ; <i>ar</i> ; <i>ye</i>	13.0
<i>ac</i> ; <i>ar</i> ; <i>ye</i>	12.0

^a nmole/fly/30 min.

Table 7. Relation between phenotypic expression of visible markers and malathion degradation in backcross progenies from Cross 2

[*ac; ar; bw; ocra* ♀ × F₁ (*ac; ar; bw; ocra* ♀ × Wakamatsu-m ♂) ♂]

Phenotype (1; 2; 3; 5)	Sex	Water soluble metabolites
+ ; + ; + ; +	♂	37.2 ^a
<i>ac</i> ; + ; + ; +	♂	31.3
+ ; <i>ar</i> ; + ; +	♂	10.7
<i>ac</i> ; <i>ar</i> ; + ; +	♂	10.5
+ ; + ; <i>bw</i> ; +	♂	32.0
<i>ac</i> ; + ; <i>bw</i> ; +	♂	31.3
+ ; <i>ar</i> ; <i>bw</i> ; +	♂	9.6
<i>ac</i> ; <i>ar</i> ; <i>bw</i> ; +	♂	9.6
+ ; + ; + ; <i>ocra</i>	♀	44.8
<i>ac</i> ; + ; + ; <i>ocra</i>	♀	41.4
+ ; <i>ar</i> ; + ; <i>ocra</i>	♀	12.5
<i>ac</i> ; <i>ar</i> ; + ; <i>ocra</i>	♀	10.4
+ ; + ; <i>bw</i> ; <i>ocra</i>	♀	40.4
<i>ac</i> ; + ; <i>bw</i> ; <i>ocra</i>	♀	34.2
+ ; <i>ar</i> ; <i>bw</i> ; <i>ocra</i>	♀	9.8
<i>ac</i> ; <i>ar</i> ; <i>bw</i> ; <i>ocra</i>	♀	8.8

^a nmole/fly/30 min.*Linkage group analysis*

Malathion degradation by malathion carboxylesterase in each phenotype in Cross 1 and Cross 2 is shown in Table 6 and 7, respectively. The homogenates of houseflies possessing the *ar* character produced less than half of water soluble metabolites made by those of non-*ar* houseflies. The results of Crosses 1 and 2 clearly indicate that the dominant factor controlling malathion carboxylesterase activity exists only on the 2nd chromosome.

To confirm the effect of each chromosomal factor and the interaction between these factors controlling malathion carboxylesterase activity, the results in Table 6

Table 8. Factorial analysis of chromosomal effects on malathion carboxylesterase activity using *ac*; *ar*; *ye* multichromosomal mutant strain

Chromosome	Effect	Degree of freedom	Mean square	F value
1	7.9	1	7.8	0.1
2	81.9	1	838.5	7.1 ^a
4	-14.5	1	26.3	0.2
1-2	3.1	1	1.2	0.0
1-4	-3.7	1	1.7	0.0
2-4	-4.1	1	2.1	0.0
1-2-4	-4.5	1	2.5	0.0
Error		7	118.8	

^a Significant at 5% level.Table 9. Factorial analysis of chromosomal effects on malathion carboxylesterase activity using *ac*; *ar*; *bwb*; *ocra* multichromosomal mutant strain

Chromosome	Effect	Degree of freedom	Mean square	F value
1	19.5	1	23.0	0.1
2	210.7	1	2774.7	14.0 ^a
3	23.1	1	33.4	0.2
5	-30.1	1	56.6	0.3
1-2	12.9	1	10.4	0.0
1-3	3.7	1	0.9	0.0
1-5	-5.9	1	2.2	0.0
2-3	10.5	1	6.9	0.0
2-5	-27.9	1	48.7	0.2
3-5	-8.7	1	4.7	0.0
1-2-3	1.1	1	0.1	0.0
1-2-5	-0.1	1	0.0	0.0
1-3-5	7.1	1	3.2	0.0
2-3-5	-4.1	1	1.1	0.0
1-2-3-5	8.9	1	5.0	0.0
Error		15	198.1	

^a Highly significant at 1% level.

and 7 were submitted to factorial analysis (TSUKAMOTO, 1964). The results of these analyses, summarized in Table 8 and 9, again clearly shows that the dominant factor controlling malathion carboxylesterase in the Wakamatsu-m strain exists only on the 2nd chromosome.

Male determining system

Analysis of the male determining system of Wakamatsu-m strain was carried out by Cross 2. In this backcross, all the houseflies having *ocra*-eyes were females and all non-*ocra*-eyes houseflies were males without exception (Table 7). This indicated that the male determining factor M is located on the 5th chromosome, i.e., the male determining system of the Wakamatsu-m strain was the V^M type. In Cross 1, however, it was

reasonable that flies of each phenotype consisted of both sexes, because no 5th chromosomal marker was involved in this backcross.

DISCUSSION

OPPENORTH (1976) advocated that gene *a* on the 2nd chromosome controlled not only the activity of phosphatase, which degraded the oxygen analogs of phosphorothioate insecticides, but also the activity of malathion carboxylesterase. The dominant factor controlling the malathion carboxylesterase activity on the 2nd chromosome of Wakamatsu-m strain is possibly identical to the gene *a*. However, for the confirmation of the relation of the gene *a* and the gene controlling the malathion carboxylesterase, further genetic study is necessary to map the location of the genes on the chromosome.

It was shown in the previous publication (SHONO et al., 1982) that malathion resistance of Wakamatsu-m is incompletely dominant, although being very close to completely dominant, and the dominant factor responsible to resistance was located only on the 2nd chromosome. This dominant factor for malathion resistance consists of at least 3 genes, each of which controls the different mechanism of resistance. First, one gene controls the activity of malathion carboxylesterase, and a second one, gene *g*, contributes to the high activity of glutathione S-transferase (Table 2), which degrades malathion to desmethyl malathion. This gene is located on the 2nd chromosome (LEWIS and SAWICKI, 1971; OPPENORTH et al., 1972). The third gene is *AChE-R*, which decreases acetylcholinesterase sensitivity to inhibition by organophosphorus and carbamate insecticides. Decreased sensitivity to the inhibition by malaoxon was observed in Wakamatsu-m strain (Table 1). Gene *AChE-R* is also located on the 2nd chromosome (DEVONSHIRE and SAWICKI, 1974).

Extremely high level of malathion resistance observed in the Wakamatsu-m strain is due to the 2nd chromosomal dominant factor, which consists of the 3 genes controlling the 3 different mechanisms of malathion resistance, the high activities of malathion carboxylesterase and glutathione S-transferase, and insensitive acetylcholinesterase.

The Wakamatsu-m strain possessed only one male factor located on the 5th chromosome (V^M). Three autosomal male factors, located on the 1st, 2nd and 3rd chromosome, were detected in the original Wakamatsu colony (TSUKAMOTO et al., 1980). However, the 5th chromosomal male factor may still exist in the Wakamatsu colony, because not all males of the colony were investigated. The V^M factor of Wakamatsu-m probably resulted from the random drift of the autosomal male factor caused by the small amount of sampling of the offspring from generation to generation, although the possibility of this effect being due to the malathion selection itself, cannot be ruled out.

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