

Biochemical and Genetic Aspects of Malathion-Specific Resistance in the Indianmeal Moth (*Lepidoptera: Pyralidae*)¹

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ABSTRACT

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Malathion resistance in a strain of *Plodia interpunctella* (Hübner) was highly specific for malathion and was suppressed by nontoxic carboxylesterase inhibitors. Fifth instars of the resistant strain had 33 times as much malathion carboxylesterase activity but only 0.30 times as much α -naphthyl acetate esterase activity as larvae of a susceptible strain. Resistance was controlled by a single autosomal gene or closely linked set of genes. Resistance and malathion carboxylesterase were inherited as dominant and codominant factors, respectively, and were genetically linked. Thus, malathion resistance in this insect is due to an altered esterase, just as is true for several dipterans. Comparison of 10 α -naphthyl acetate esterase isozymes resolved by electrophoresis revealed seven interstrain differences.

Malathion resistance in insects is frequently highly specific for this insecticide (Welling et al. 1974). In dipterans and coleopterans, strains exhibiting this type of resistance usually have increased activity of a malathion-degrading carboxylesterase (Matsumura and Brown 1961, Dyte and Rowlands 1968, Ohkawa et al. 1968, Townsend and Busvine 1969) and, in dipterans, reduced esterase activity toward certain unrelated substrates (Openoorth 1959, Townsend and Busvine 1969). We report here the first similar case in a lepidopteran.

Malathion is the primary insecticide used for direct treatment of stored grain in this country. Numerous cases of malathion-specific resistance have been reported in strains of the Indianmeal moth, *Plodia interpunctella* (Hübner), from several countries (Attia et al. 1979, Dyte 1979, Zettler 1982). This species is an important pest of stored products worldwide. We investigate the biochemistry and inheritance of malathion-specific resistance in a strain of Indianmeal moth collected in Illinois.

Materials and Methods

Chemicals

All insecticides used for bioassay were technical or analytical grade and were donated by the manufacturers. [1,2-¹⁴C]malathion (side chain ethyl label), 4.6 mCi/mmol, was purchased from Amersham, and was purified on silica gel thin-layer chromatography (TLC) plates by using benzene:n-hexane:ethyl acetate (47:4:13) as the mobile phase.

Moth Strains

The resistant (R) strain was originally collected from a farmer's corn bin in Illinois in 1972 and has been reared in the laboratory since that time (J. L. Zettler, personal communication). Resistance has been maintained by occasional selection with technical grade malathion. Another resistant strain, NE-1, was collected in Nebraska in 1980 and has never been subjected to selection pressure with malathion in the laboratory. The

susceptible (S) strain has been maintained at the U.S. Grain Marketing Research Laboratory for many years. The moths were reared on a ground wheat larval diet fortified with wheat germ, yeast, honey, and glycerol. Wandering-stage 5th instars (15 to 18 mg) were used in all experiments.

Before any experimentation, the R strain was found to be 94-fold resistant with respect to the S strain. It was used without further selection for studies of cross-resistance and enzyme localization. Before genetic analysis, the R strain was purified by continuous rearing for five consecutive generations on diet containing 250 ppm of malathion. This dose kills 100% of S larvae. The NE-1 strain was virtually immune to malathion (2% mortality at a dose of 320 μ g/larva, resistance factor > 273) and was used for comparative esterase assay.

Moths were mass-crossed to produce all hybrid and backcross strains. To test for linkage of genes for resistance and high activity of malathion carboxylesterase, the R strain was repeatedly backcrossed to the S strain, resistant recombinants being selected before each successive backcross, as shown in the following protocol:

- (R δ \times S ϕ) δ \times S ϕ = B-1
- B-1 δ (selected) \times S ϕ = B-2
- B-2 δ (selected) \times S ϕ = B-3
- B-7 δ (selected) \times S ϕ = B-8

Bioassays

Larvae of both sexes were anaesthetized with CO₂ and treated topically with technical or analytical grade insecticides as described by Zettler et al. (1973). Mortality was assessed after 2 days. No mortality occurred in the controls. Probit analysis was conducted by the method of Finney (1952).

Carboxylesterase Assays

We used two methods to assay for malathion carboxylesterase. Method A was a modification of the radiometric method of Matsumura and Brown (1963). Larvae were homogenized in 0.067 M sodium phosphate buffer pH 7.2 with a Tekmar model SDT homogenizer. Homogenates were centrifuged at 2,000 \times g for 10 min,

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and the supernatant was used directly as enzyme source. Enzyme was incubated for 30 min at 32°C with 50 µg (0.014 µCi) of ¹⁴C-malathion + 5 µl ethanol in a total volume of 1.0 ml. The reaction was stopped by adding 3 ml of 0.6% trichloroacetic acid in universal buffer pH 2.1. Then the mixture was extracted twice with 2-ml portions of chloroform, the combined chloroform extracts (containing malathion and carboxylesterase products) were back-extracted with 2 ml of universal buffer pH 7.0, and the aqueous extract (containing the carboxylesterase products) was washed with 2 ml of chloroform to remove the last trace of malathion, reacidified to pH 1.8 with 4 drops of 10% concentrated HCl, and extracted with 2 ml of chloroform. The final chloroform extract was taken to dryness and counted directly by liquid scintillation spectroscopy.

For method B, larvae were homogenized (glass-tenlon) in groups of 3 in 4.5 ml of 4 M NaCl buffered at pH 7.5 with 50 mM Tris. Homogenates were centrifuged at 10,000 × g for 15 min, and the supernatant was used as the enzyme source. Enzyme was incubated for 20 min at 32°C with 50 µg (0.001 µCi) of [¹⁴C]malathion in a total volume of 1.0 ml. The reaction was stopped with 100 µg of triphenylphosphate and 5 µg of malthion monoacid. Carboxylester hydrolysis products were isolated on reverse-phase mini-columns by a rapid and efficient procedure described in detail elsewhere (Beeman et al. 1982). The method gave ~90% recovery of malathion monoacids and <0.1% carryover of malathion. The reaction rate was linear with respect to enzyme concentration.

To assay for α-naphthyl acetate esterase, we used the spectrophotometric method of Hipps and Nelson (1974). Enzyme was prepared as described in method B, except that each homogenate contained four larvae in 6 ml. Reaction tubes contained 4 M NaCl, 50 mM Tris buffer (pH 7.5), 0.419 mM α-naphthyl acetate, 3% acetone, and enzyme in a total volume of 2.5 ml. Tubes were incubated for 20 min at 32°C. The reaction was stopped by the addition of 0.5 ml of Fast Garnet GBC (0.075 g in 15 ml of 10%, wt/vol, sodium lauryl sulfate). Absorbances were read at 560 nm on a Unicam SP 1750 UV-vis spectrophotometer. The reaction rate was linear with respect to enzyme concentration.

Electrophoresis

For electrophoresis, 5th instars were homogenized in groups of 10 (5 of each sex) in 2.5 ml of 0.05 M Tris buffer (pH 6.8) by a Tekmar model SDT homogenizer. Homogenates were centrifuged at 20,000 × g for 10 min, and the supernatants were diluted fivefold with an appropriate sample buffer containing glycerol and bromophenol blue.

Zone electrophoresis was performed on polyacrylamide gels, using a Bio-Rad model 220 dual vertical gel slab cell. We used a system of nondenaturing gels based on the procedure of Laemmli (1970). The 6% acrylamide running gels, pH 8.8, were overlaid with 3% stacking gels (pH 6.8). The 1.5 mm gels were electrophoresed at 4°C, at a constant current of 10 mamp per gel stack-

Table 1.—Cross resistance patterns in malathion-resistant Indian meal moths*

Insecticide	LD ₅₀ (µg)		Resistance factor
	S	R	
Malathion	1.17	110	94
Malaoxon	0.82	3.6	4.4
Phenthoate	0.26	0.87	3.3
Chlorpyrifos methyl	0.30	0.60	2.0
Methoprene	0.0074	0.0088	1.2
Cypermethrin	0.90	0.83	0.92

*Insecticides were administered topically to 5th instars of the susceptible (S) and resistant (R) strains. Mortality was assessed after 5 days. In the case of methoprene, mortality was defined as failure of adults to emerge from pupae after 1 month. Resistance factor = LC₅₀(R) ÷ LD₅₀(S). LD₅₀ values were estimated graphically.

ing and 30 mamp per gel running. After electrophoresis, the gels were stained for α-naphthyl acetate esterase by soaking at room temperature in 0.1 M phosphate buffer, pH 6.0, containing 0.05% (wt/vol) Fast Red TR salt (Sigma Chemical Co.) and 0.0093% (wt/vol) α-naphthyl acetate, the latter added with 1.3 ml of 50% acetone/100 ml.

Results and Discussion

Table 1 shows the cross-resistance spectrum for the malathion-resistant (R) strain. The observed resistance was highly specific for malathion. The R strain had little or no cross-resistance to chemically unrelated insecticide esters such as methoprene or cypermethrin, and only twofold cross-resistance to the organophosphate insecticide chlorpyrifos-methyl. There was a low order of cross-resistance to substances closely related to malathion, including a resistance factor of ×4.4 for malaoxon and ×3.3 for phenthoate.

Malathion resistance in the R strain was totally suppressed by pretreatment of the insects with either of the nontoxic carboxylesterase inhibitors triphenyl phosphate or S,S,S-tributyl phosphorotrithioate (data not shown) (Plapp and Eddy 1961). These results implied that the resistance is likely to be the malathion-specific type, involving increased levels of a malathion-degrading carboxylesterase.

Table 2 shows the toxicity of malathion to the resistant (R), susceptible (S), hybrid, and backcross strains. Probit analysis of the data for the S strain gave the following results (95% confidence intervals in parentheses): LD₅₀ = 1.17 µg (1.06 to 1.29), LD₉₅ = 3.65 µg (2.93 to 5.06), and slope = 3.32. For the R strain, mortality did not exceed 1% at doses up to 160 µg. The resistance factor was subsequently found to be >240.

The resistance was dominant. F-1 hybrids of the S and R strains were not affected by malathion at doses up to 200 µg (Table 2). When the F-1 hybrids were backcrossed to the S strain. The offspring segregated into two distinct groups: about half were susceptible (killed by 5 µg of malathion) and half were highly resistant (not killed by 160 µg of malathion (Table 2).

The dominance of resistance and the absence of partially resistant recombinants in the backcross indicate that malathion resistance in this strain is primarily con-

Table 2.—Toxicity of malathion to strains of the Indianmeal moth^a

Dose ($\mu\text{g}/\text{larva}$)	Mortality (%)			
	S	R	S♀ × R♂	S♀ × (S♀ × R♂)♂
2	87.0			37.6
5	100			45.6
10	100		1.0	
20	100	0		48.0
40		0	1.0	58.9
50		0		
60		0		
80		0		47.0
160		1.0		51.9
200			0	

^aEach value represents a single determination based on ~100 larvae. The criteria for death were convulsions and knockdown.

trolled by a single dominant gene or closely linked set of genes. The plateau at 50% mortality in the backcross population (Table 2) indicates that the gene for resistance behaves as a simple Mendelian factor.

The resistance was not sex linked. Father-son inheritance was demonstrated by repeated selection of resistant males followed by backcross to susceptible females for eight consecutive generations as illustrated above (data not shown). Mother-daughter inheritance was similarly demonstrated for eight consecutive generations. This result precludes the possibility of sex linkage (Robinson 1971).

As expected, resistant insects had greatly increased activity of a malathion-degrading carboxylesterase over that of their susceptible counterparts (Table 3). On the

average, R insects had ca. 33-fold higher carboxylesterase activity than S insects with malathion as substrate. The results were essentially identical in either sex and in either of two resistant strains (R and NE-1) of widely different origins. The results were the same using larvae in either day 1 or day 2 of the wandering stage, and therefore, were not an artifact due to age-related changes in enzyme titer.

Resistance was genetically linked to high malathion carboxylesterase activity. This was shown by the inseparability of the two after eight consecutive generations of selection and backcross to the susceptible strain (Table 3, strain B-8).

Fat body was the tissue highest in malathion carboxylesterase activity in terms of both specific activity and

Table 3.—Inheritance of malathion carboxylesterase and α -naphthyl acetate esterase activities in strains of the Indianmeal moth^a

Strain ^b	Sex	Malathion carboxylesterase activity ^c (pmol/min per mg of whole larva)	
		Day 1	Day 2
S	♂		6.9 ± 3.5 (5)
R	♂	231 ± 22 (10)	227 ± 20 (7)
S♀ × R♂	♂	111 ± 15 (5)	117 ± 20 (10)
B-8	♂		130 ± 26 (5)
S	♀	6.8 ± 4.8 (2)	
NE-1	♀	221 ± 58 (8)	
S♀ × NE-1♂	♀	106 ± 17 (8)	
		α -Naphthyl acetate esterase activity (nmol/min per mg of whole larva) ^c	
S	♂	181 ± 16 (5)	
R	♂	55 ± 13 (5)	
S♀ × R♂	♂	135 ± (5)	

^aSubstrate concentrations were 0.419 mM and 0.303 mM for α -naphthyl acetate and malathion, respectively. Method B was used for malathion carboxylesterase assay.

^bStrain B-8 was synthesized (see text) to introduce into the S strain the single chromosome from the R strain which contains the gene for resistance.

^cValues for enzyme activity are means ± SD of *n* independent determinations of three larvae each (four larvae each for α -naphthyl acetate esterase, *n* given in parentheses). Day 1 larvae were taken from synchronized cultures <24 h after the first appearance of the wandering-stage. Day 2 larvae were taken from the same culture jars 24 h later.

Table 4.—Tissue distribution of malathion carboxylesterase activity in malathion-resistant Indianmeal moths^a

Tissue	Sp act (μg of malathion/mg of protein)		Total activity (μg of malathion/insect)	
	δ	♀	δ	♀
Hemolymph	0.18	0.14		
Gut	3.71	2.68	0.29	0.28
Fat body	7.34	9.15	1.31	2.87
Carcass	1.51	2.35	0.62	1.17
Dissecting medium	0.41	0.27	0.14	0.12
Total	—	—	2.36	4.44
Whole insect	2.20	1.77	2.48	2.82

^aFor each determination, tissues from three insects were pooled before homogenization. The Bio-Rad protein assay method was used, with bovine serum albumin as standard. Method A was used for malathion carboxylesterase assay.

total activity (Table 4). Whole gut also had significant quantities of the enzyme.

The whole-body titer of malathion carboxylesterase activity was inherited in a semidominant fashion. In particular, the inheritance of the R and S enzymes was additive (codominant), i.e., the F-1 hybrids had an enzyme titer exactly intermediate between the titers of the two homozygous parents (Table 3). The semidominance of this enzyme coupled with the dominance of malathion resistance implies that the enzyme activity of the R strain is considerably more than necessary to confer a high level of resistance.

Accompanying the greatly increased esterase activity toward malathion in the R strain was a sharply reduced esterase activity toward α -naphthyl acetate (Table 3). Enzyme activity toward the latter substrate also showed semidominant inheritance, but the enzyme activity of the F-1 hybrid was somewhat closer to that of the S parent than is predicted by strictly additive inheritance. In both the S and the R strains, the rate of hydrolysis of α -naphthyl acetate was several orders of magnitude greater than that for malathion (note the different units used for enzyme activities in Table 3).

By the "mutant aliesterase" theory first proposed by Oppenoorth and van Asperen (1960), this type of resistance arises by a mutation at an esterase gene locus, resulting in the biosynthesis of a chemically altered, or "mutant" enzyme. The mutant esterase has an altered substrate specificity, including enhanced activity toward the insecticide ester (in this case malathion), but decreased activity toward other substrates such as α -naphthyl acetate (e.g., Townsend and Busvine 1969). Increased malathion carboxylesterase activity accompanied by reduced α -naphthyl acetate esterase activity in the R strain of the Indian meal moth suggests that a mutant esterase may be responsible for resistance in this strain. This conclusion is supported by our finding (unpublished data) that malathion carboxylesterase in whole homogenates of R and S strains of the Indianmeal moth differ in K_m and V_{max} . Reduced esterase activity toward α -naphthyl acetate is a general phenomenon among malathion-resistant populations of the Indianmeal moth throughout the midwestern United States (Beeman et al. 1982).

The codominant inheritance of malathion carboxylesterase activity in the Indian meal moth can be taken as

evidence that the mutation has occurred in a structural gene or a regulatory gene and not in a gene concerned with posttranslational processing, since the former are almost always codominant, whereas the latter tend to be completely dominant or recessive (Paigen 1979). However, the mode of inheritance does not reliably differentiate between structural and regulatory mutations.

Separation of the isozymes of malathion carboxylesterase and interstrain comparison of their physical and biochemical properties will be necessary to resolve the uncertainties just discussed. We have made initial attempts to examine the mutant esterase system of the Indianmeal moth in greater detail by electrophoretic resolution of isozymes followed by esterase-specific staining. Figure 1 shows the resulting esterase zymograms obtained from mixed-sex, whole-body homogenates of R, S, and F-1 hybrids. At least 10 bands of esterase activity were resolved on the gels. The bands fell into four mobility groups, labelled A to D in Fig. 1. In confirmation of the *in vitro* esterase assay, the esterase zymograms showed two major bands of activity in the S strain (A2 and A3) which were greatly reduced or absent

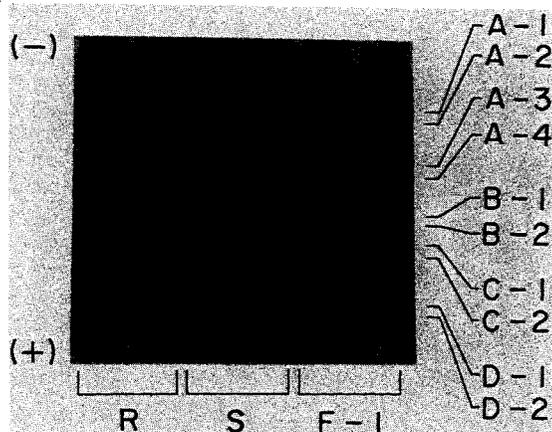


FIG. 1.—Esterase zymograms prepared from whole-body homogenates of S, R, and F-1 hybrid larvae of the Indianmeal moth. Two groups of 10 pooled larvae were analyzed for each strain. F-1 hybrids were $S\text{♀} \times R\text{♂}$.

in the R strain. Inspection of the zymograms revealed visible differences in staining intensity between the S and R strains for most of the resolved bands. Certain bands (A2, A3, B1, and C2) were stained more intensely in the S strain, whereas others (A4, B2, and D1) were stained more intensely in the R strain. Zymograms of F-1 hybrids revealed that bands A2, A3, A4, and possibly others were inherited in a semidominant or codominant fashion. This is again consistent with the in vitro data. It remains to be determined which, if any, of these electrophoretic esterase variants contribute to malathion inactivation in resistant moths. The degree of polymorphism at the various esterase loci could not be determined in the present work, since single individuals were not examined.

ADDENDUM

Genetic data have recently been reported for a malathion-resistant strain of the Indianmeal moth from Australia [Attia, F. I., E. Shipp, and G. J. Shanahan, 1981. Inheritance of resistance to malathion, DDT and dieldrin in *Plodia interpunctella* (Lepidoptera: Pyralidae). J. Stored Prod. Res. 17: 109-115].

REFERENCES CITED

- Attia, F. I., E. Shipp, and G. J. Shanahan. 1979. Survey of insecticide resistance in *Plodia interpunctella* (Hübner), *Ephestia cautella* (Walker) and *E. kuehniella* Zeller (Lepidoptera: Pyralidae) in New South Wales. J. Aust. Entomol. Soc. 18: 67-70.
- Beeman, R. W., W. E. Speirs, and B. A. Schmidt. 1982. Malathion resistance in Indianmeal moths (Lepidoptera: Pyralidae) infesting stored corn and wheat in the North Central United States. J. Econ. Entomol. 75: 950-954.
- Dyte, C. E. 1979. The importation of insecticide-resistant strains of stored-product pests. Ann. Appl. Biol. 91: 414-417.
- Dyte, C. E., and D. G. Rowlands. 1968. The metabolism and synergism of malathion in resistant and susceptible strains of *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae). J. Stored Prod. Res. 4: 157-173.
- Finney, D. J. 1952. Probit analysis, 2nd ed. Cambridge University Press, London. 38 pp.
- Hipps, P. P., and D. R. Nelson. 1974. Esterases from the midgut and gastric caecum of the American cockroach, *Periplaneta americana* (L.) Isolation and characterization. Biochim. Biophys. Acta 341: 421-436.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680-685.
- Matsumura, F., and A. W. A. Brown. 1961. Biochemistry of malathion resistance in *Culex tarsalis*. J. Econ. Entomol. 54: 1176-1185.
1963. Studies on carboxylesterase in malathion-resistant *Culex tarsalis*. Ibid. 56: 381-388.
- Ohkawa, H., M. Eto, Y. Oshima, F. Tanaka, and K. Umeda. 1968. Two types of carboxylesterase degrading malathion in resistant house-flies and their inhibition by synergists. Botyu-Kagaku. 33: 139-145.
- Oppenoorth, F. J. 1959. Genetics of resistance to organophosphorus compounds and low aliesterase activity in the housefly. Entomol. Exp. Appl. 2: 304-319.
- Oppenoorth, F. J., and K. van Asperen. 1960. Allelic genes in the housefly producing modified enzymes that cause organophosphate resistance. Science 132: 298-299.
- Paigen, K. 1979. Acid hydrolases as models of genetic control. Annu. Rev. Genet. 13: 417-466.
- Plapp, F. W., Jr., and G. W. Eddy. 1961. Synergism of malathion against resistant insects. Science 134: 2043-2044.
- Robinson, R. 1971. Lepidoptera genetics. Pergamon Press, Braunschweig. 687 pp.
- Townsend, M. A., and J. R. Busvine. 1969. The mechanism of malathion-resistance in the blowfly *Chrysomya putoria*. Entomol. Exp. Appl. 12: 243-267.
- Welling, W., A. W. de Vries, and J. Voerman. 1974. Oxidative cleavage of a carboxylester bond as a mechanism of resistance to malaonoxin in houseflies. Pestic. Biochem. Physiol. 4: 31-43.
- Zettler, J. L. 1982. Insecticide resistance in stored-product insects infesting peanuts in the southeastern United States. J. Econ. Entomol. 75: 359-362.
- Zettler, J. L., L. L. McDonald, L. M. Redlinger, and R. D. Jones. 1973. *Plodia interpunctella* and *Cadra cautella*: resistance in strains to malathion and synergized pyrethrins. Ibid. 66: 1049-1050.