INCIDENCE AND PHYSIOLOGICAL NATURE OF MALATHION RESISTANCE IN FOUR SPECIES OF STORED PRODUCTS COLEOPTERA FROM GRAIN PRODUCING STATES

by

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ABBREVIATIONS

∝NA	∝-naphthyl acetate
BSA	bovine serum albumin
cpm's	counts per minute
HPLC	high performance liquid chromatography
IMM	Indian meal moth (*Plodia interpunctella*)(Hübn.)
LGB	lesser grain borer (*Rhizopertha dominica*)(F.)
LSC	liquid scintillation spectroscopy
MeCN	acetonitrile
N	normal
NC strain	North Carolina strain
$O.D._{560}$	optical density at 560 nanometers
PTU	phenyl-thio urea
RFB	red flour beetle (*Tribolium castaneum*)(Herbst)
RW	rice weevil (*Sitophilus oryzae*)(L.)
SDS	sodium lauryl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
USGMRL	United States Grain Marketing Research Laboratory
UV	ultraviolet

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INTRODUCTION

Since its registration as a residual insecticide for direct treatment of post-harvest grain (Anon. 1958), malathion has become the principle chemical used for this purpose in the United States. Resistance to malathion in stored products insects is not a new phenomenon (Parkin, et al., 1962; Parkin, 1965; Speirs, et al., 1967; Champ, 1968). In a global survey conducted by the Food and Agriculture Organization (FAO) encompassing 61 countries, (Champ and Dyte, 1977), 8.5% of 623 strains of Sitophilus spp., 31.6% of 158 strains of lesser grain borer (LGB) (Rhizopertha dominica F.) and 86.9% of 505 strains of red flour beetle (RFB) (Tribolium castaneum Herbst) were found to be resistant to malathion. Other surveys have shown resistance to be present in Nigeria (Parkin, 1965), Egypt (Toppozada et al., 1969), Australia (Champ and Campbell-Brown, 1970), Burma, India, Tanzania, and the Sudan (Dyte and Blackman, 1970), Malawi (Pieterse et al., 1972), and Canada (Mensah and Watters, 1979). Little work has been done in the United States on the resistance levels to malathion in beetles infesting stored products, with the exception of the RFB and confused flour beetle (CFB) (Tribolium confusum J. duV.). Speirs et al. (1967) found resistance in RFB collected from stored peanuts, corn meal and citrus pulp in Georgia and Florida. Vincent and Lindgren (1967) found low level resistance in the RFB in Iowa, New York, and Kansas. Strong et al. (1969) found no resistance in a survey of RFB in California. Bansode and Campbell (1979) pooled strains of RFB from North Carolina and found the resultant strain to be 43.9 fold more resistant than the laboratory strain. Zettler (1974, 1975, 1982) found resistance to malathion in RFB in Louisiana, Texas, Alabama, Georgia, and Florida.

Champ and Campbell-Brown (1970) discussed two types of resistance found in the RFB in Australia: a malathion specific resistance , suppressible with triphenylphosphate (TPP), and a non-specific resistance, not suppressible with TPP. Malathion is a carboxylester, and hydrolysis of this ester detoxifies malathion. The presence of an enzyme specifically hydrolyzing malathion is the basis of the malathion-specific resistance. Plapp and Eddy (1961) discovered the synergistic effect of TPP, a car-

boxylesterase inhibitor, with malathion. TPP suppressibility is therefore a convenient test for the malathion-specific resistance.

This study examines in detail for the first time, the status of malathion resistance in the RFB, LGB, Sitophilus spp., and Cryptolestes spp. in the major grain producing regions of the United States.

MATERIALS AND METHODS

Technical grade (95%) malathion was donated by NOVA Products Inc., Kansas City, Kansas. TPP was purchased from Eastman Kodak, Rochester, New York. The vehicle for carrying the malathion was a mixture of hexanes (b.p. 68°-70°C), acetone, and sunflower oil (3:1:1 by volume). Whatman #1 7 cm filter paper discs were used for the exposure surface. Glass rings for confining the insects on the discs were cut in 2.5 cm lengths from 5 cm diameter glass tubing. Bioassay discs were placed on a glass plate, and confinement rings covered with fine mesh screens to prevent escape.

Corn, wheat, and oats in randomly selected bins on farms in 26 states had been sampled in the summer of 1980, under a project coordinated by the Agriculture Stabilization and Conservation Service for the purpose of assessing the quality of farm stored grain reserves. All samples containing live adult coleoptera were sieved to remove the adults. Upon finding a minimum of five adults of a genus (mean $\sim$ 50), the adults were cultured for reproduction in the following media: Sitophilus spp. in whole grain wheat with a small amount of dockage added, Cryptolestes spp. in a mixture of oatmeal, flour, and corn meal (2:1:1 by volume); LGB in whole grain wheat; and RFB in whole wheat flour with 5% by weight brewers yeast added.

All laboratory strains (susceptible) were maintained at the U.S. Grain Marketing Research Laboratory (USGMRL), with the exception of Cryptolestes ferrugineus (Steph.), which was obtained from the Stored Products Laboratory, Dept. of Entomology, Kansas State University. C. ferrugineus was used as the representative of the genus for determining the discriminating dose. It was chosen for its relatively high LD_{100} to avoid false positive results in the screening. The rice weevil (RW) (Sitophilus

oryzae L.) was the representative from the genus Sitophilus in determining the discriminating dose. Strong et al. (1967) showed the RW as the most tolerant to malathion of the three major species in the genus. Cultures were kept in mason jars with a screen and filter paper top to allow air exchange. Jars were maintained in incubators at 28°C and 70% relative humidity.

Malathion was dissolved in the carrier solution at the desired concentration. The test solution was applied to the filter papers (0.5 ml/disc) and the papers were allowed to stand overnight, permitting the solvents to evaporate and the oil to entirely permeate the filter papers. Twenty-five insects were confined on each filter paper for 48 hours. Adults 2-8 weeks old were used in all experiments. Insects were considered dead if they were ataxic and unable to right themselves. The basic method used is that of Stringer (1949) and Blackith (1950), which is the standard method recommended by the FAO (Anon. 1974). Dilute concentrations (less than 20 mg/ml) of the test solutions were made fresh every two weeks. A 5:1 ratio by weight of TPP:malathion was used in the tests for suppression of resistance.

A discriminating dose technique was used to screen the field strains for resistance. Log probit analyses of data were done according to Finney (1952) for the lab strains to determine an approximate discriminating dose and LD_{95}. The LD_{95} was then used in calculating resistance factors. The LD_{100} of a laboratory strain of each species was determined empirically. The lowest possible LD_{100} was chosen to detect low-level resistance. For LGB and RFB, survivors of the initial screening were cultured and progeny retested at the discriminating dose and higher doses to determine levels of resistance and selectability.

One hundred-fifty insects were used for each test (six replicates of 25 insects each). The Sitophilus spp. were the only group exhibiting mortality in the controls, and this never exceeded 4%. Therefore, the data were not corrected for mortality in the controls.

RESULTS AND DISCUSSION

Discriminating doses for the respective species are shown in Table 1. The results of log probit analyses of data for the lab (susceptible) strains is also provided in Table 1.

Sitophilus spp. were the only group tested showing no significant resistance to malathion. Figure 1 shows the origin of the strains. One strain from Iowa exhibited a low level tolerance which did not increase after selection, even for eight generations (Beeman, personal communication). This type of response might be better labeled "vigor tolerance". The other 21 strains from eight states showed little or no resistance.

The genus Cryptolestes was comprised of 42 strains from 12 states. Five of the 42 strains showed resistance (Table 2). Low levels of resistance were detected from Minnesota and Illinois. Less than 50% mortality was recorded in three strains, from Georgia, North Dakota, and Idaho, in increasing order of resistance. Figure 2 shows the distribution and resistance levels of the strains.

Of the 13 LGB strains tested, only one showed a high level of resistance, strain CO-1 (Table 3). The strains with the next four highest levels of resistance all originate in Minnesota. Once selected colonies of the six resistant strains were retested at the discriminating dose and at the higher doses of 5 mg and 15 mg of malathion on the filter paper discs. Results of this test are also shown in Table 3. In four of the six strains (MN-1, MN-2, MN-4, and MT-1) the level of resistance increased significantly after one generation of selection. The CO-1 strain, while it did not seem to respond to selection, remained the most resistant strain. Figure 3 dipicts the geographic origin and resistance levels found in the LGB.

Widespread resistance was detected in the RFB, making it unique among those tested. Of 36 strains tested from ten states, only five strains suffered 100% mortality at the discriminating dose (Table 4). A wide range of resistance was present in the remaining 31 strains. Fifteen of the most resistant strains were tested at the discriminating dose and at higher doses after one selection (Table 4). Strains tested at the higher doses

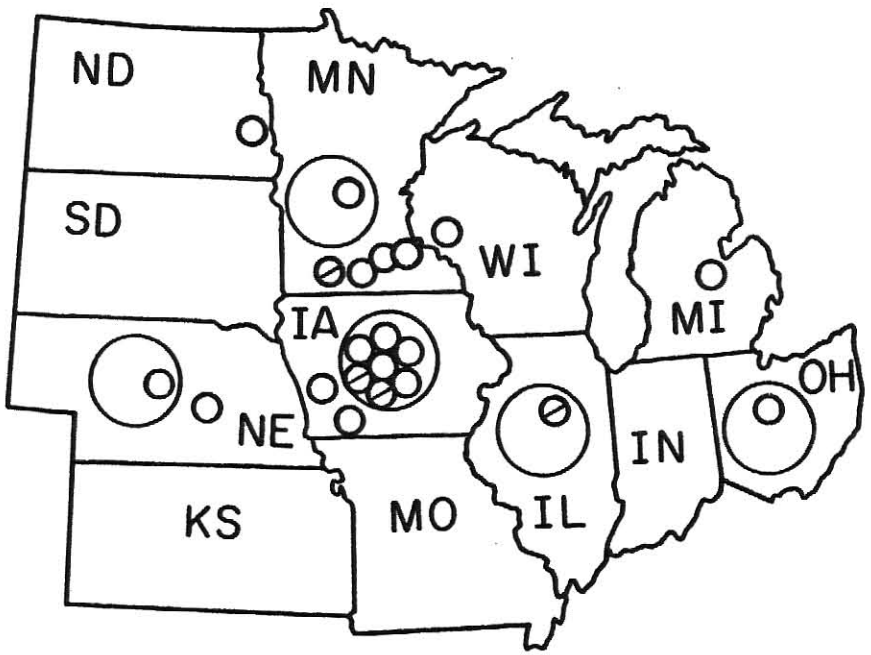
TABLE 1. PROBIT ANALYSIS & DISCRIMINATING DOSES FOR MALATHION AGAINST LAB SUSCEPTIBLE STRAINS OF 4 SPECIES OF COLEOPTERA.[A]

SPECIES	LD_{50}[B]	LD_{95}[B]	SLOPE	LD_{100}
SITOPHILUS SPP.	.10(.06-.14)	.17(.13-1.58)	6.89	.15
CRYPTOLESTES SPP.	.14(.10-.18)	.48(.31-1.19)	3.08	1.0
RHYZOPERTHA DOMINICA	.6(.53-.66)	1.50(1.30-1.85)	4.12	1.75
TRIBOLIUM CASTANEUM	.06(.04-.07)	.12(.10-.19)	5.08	.15

[A]VALUES EXPRESSED AS MG/DISC FOR EACH SPECIES, PROBIT ANALYSIS BASED ON 3-6 DOSAGE LEVELS USING ~100 BEETLES PER DOSE. VALUES FOR DISCRIMINATING DOSES (LD_{100}) WERE DETERMINED EMPIRICALLY.

[B]95% CONFIDENCE LIMITS GIVEN IN PARENTHESES.

FIGURE 1. Geographic origin and resistance levels in Sitophilus spp. Data points enclosed by large circles indicate pooled strains from many counties or from scattered locations within the state. Data points not enclosed by large circles indicate county of origin, or the county contributing the largest number of samples in the case of composite strains from adjacent counties.



SITOPHILUS

SPP.

MORTALITY (%)

- ⊘ 95 - 98
- O 98 - 100

TABLE 2. MORTALITY (%) IN STRAINS OF CRYPTOLESTES SPP. TREATED WITH DISCRIMINATING DOSES OF MALATHION.[A]

STRAIN[B]	MORTALITY	R-FACTOR[C]
ID1	17.3 ± 1.9	>2.1
ND1	24.0 ± 4.9	>2.1
GA1	48.0 ± 21.7	>2.1
IL1	85.0 ± 12.4	~2
MN1	94.67 ± 3.8	~2

[A]VALUES FOR % MORTALITY ARE MEANS ± SD OF 6 INDEPENDENT DETERMINATIONS OF 25 BEETLES EACH.

[B]STRAIN IDENTIFICATIONS INDICATE THE STATE OF ORIGIN.

[C]THE RESISTANCE FACTOR. IT IS THE RATIO OF THE LD_{95} VALUE FOR THE FIELD STRAIN TO THAT FOR THE LAB-S STRAIN.

FIGURE 2. Geographic origin and resistance levels in Cryptolestes spp. Data points enclosed by large circles indicate pooled strains from many counties or from scattered locations within the state. Data points not enclosed by large circles indicate county of origin, or the county contributing the largest number of samples in the case of composite strains from adjacent counties.

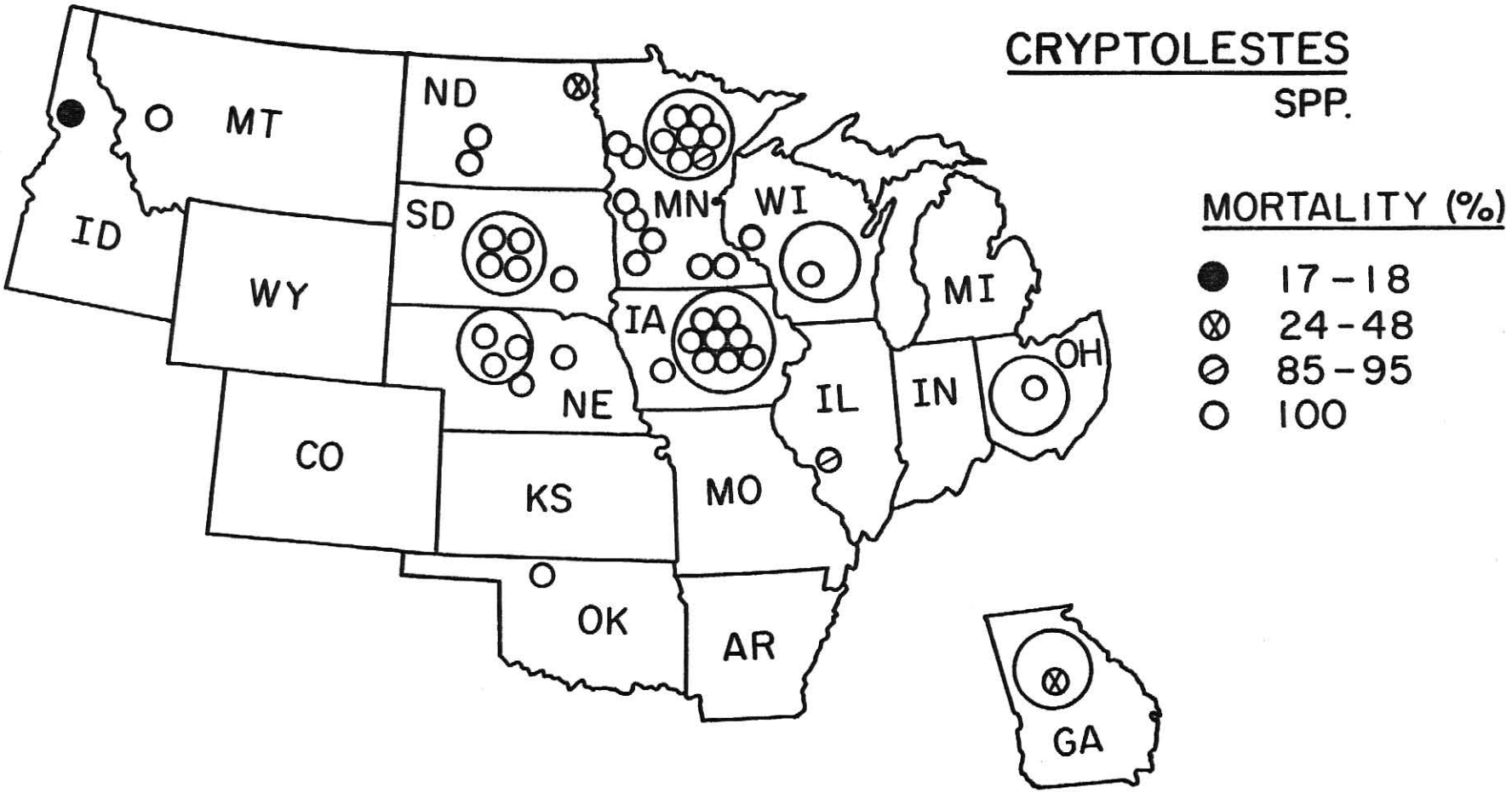

CRYPTOLESTES
SPP.
MORTALITY (%)
17-18
24-48
85-95
100
MT
ND
MN
WI
MI
ID
SD
WY
IA
OH
NE
IL
IN
CO
KS
MO
OK
AR
GA

TABLE 3. MORTALITY (%) IN STRAINS OF RHYZOPERTHA DOMINICA TREATED WITH DISCRIMINATING DOSES OF MALATHION.[A]

	MORTALITY (%) AT INDICATED MALATHION DOSE (MG/DISC)				
	BEFORE SELECTION[C]	AFTER SELECTION[C]			R-
STRAIN[B]	1.75	1.75	5	15	FACTOR[D]
CO-1	6.7 ± 6.0	21.3 ± 5.0	44.0 ± 8.6	93.3 ± 2.3	~10
MN-1	73.0 ± 10.6	25.3 ± 3.8	84.0 ± 6.5	100 ± 0	~4
MN-2	89.3 ± 6.0	70.7 ± 5.0	86.7 ± 1.9	100 ± 0	~4
MN-3	92.0 ± 6.5	86.7 ± 3.8	100 ± 0	100 ± 0	~2
MN-4	94.0 ± 5.5	40.0 ± 8.6	76.0 ± 14.2	100 ± 0	~4
SD-1	94.7 ± 7.9				
MT-1	96.0 ± 3.3	60.0 ± 9.8	100 ± 0	100 ± 0	~2
SD-2	97.3 ± 4.4				
NE-1	98.0 ± 3.0				
ND-1	98.0 ± 3.0				
SD-3	98.0 ± 3.0				
SD-4	100 ± 0				
SD-5	100 ± 0				
LAB-S	100 ± 0				≡1.0

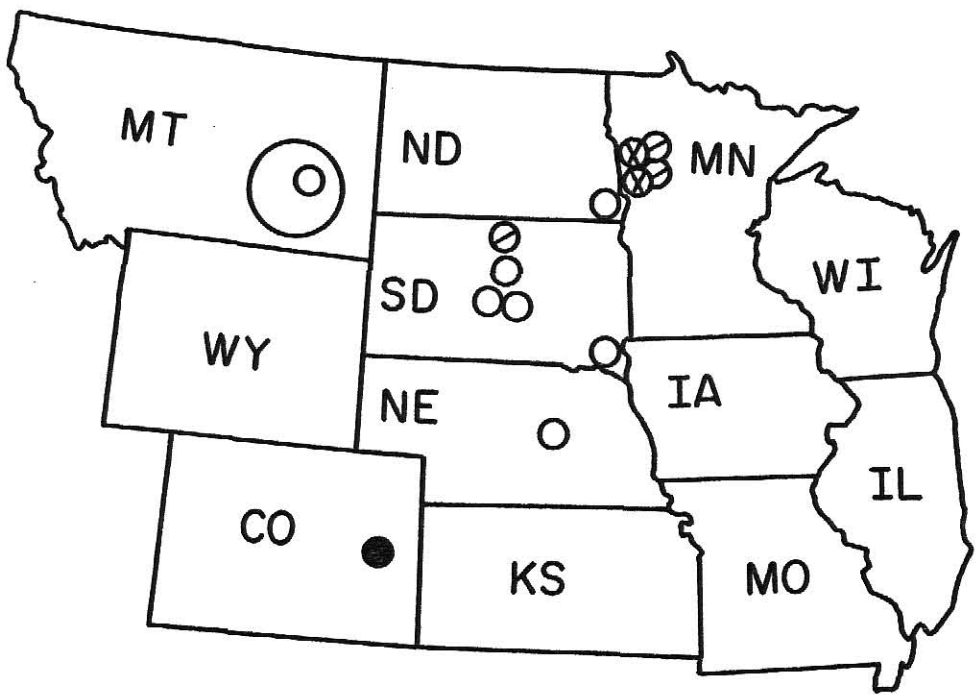
[A]VALUES FOR % MORTALITY ARE MEANS ± SD OF 6 INDEPENDENT DETERMINATIONS OF 25 BEETLES EACH.

[B]STRAIN IDENTIFICATIONS INDICATE THE STATE OF ORIGIN.

[C]SURVIVORS OF THE FIRST TEST (DOSE = 1.75 MG) WERE USED AS SEEDING STOCK FOR "SELECTED" CULTURES. PROGENY WERE USED FOR SUBSEQUENT TESTS WITHOUT FURTHER SELECTION.

[D]THE RESISTANCE FACTOR REFERS TO ONCE-SELECTED STRAINS. IT IS THE RATIO OF THE LD_{95} VALUE FOR THE FIELD STRAIN TO THAT FOR THE LAB-S STRAIN.

FIGURE 3. Geographic origin and resistance levels in LGB. Data points enclosed by large circles indicate pooled strains from many counties, or from scattered locations within the state. Data points not enclosed by large cirlces indicate the county of origin, or the county contributing the largest number of samples in the case of composite strains from adjacent counties.



RHYZOPERTHA DOMINICA

MORTALITY (%)

- ● 0-7
- ⊗ 73-90
- ⊘ 92-95
- ○ 96-100

TABLE 4. MORTALITY (%) IN STRAINS OF *TRIBOLIUM CASTANEUM* TREATED WITH DISCRIMINATING DOSES OF MALATHION.[A]

	MORTALITY (%) AT INDICATED MALATHION DOSE (MG/DISC)				
	BEFORE SELECTION[C]	AFTER SELECTION[C]			R-
STRAIN[B]	0.15	0.15	2.5	10	FACTOR[D]
GA-1	0 ± 0	1.3 ± 1.9	4.0 ± 3.3	77.3 ± 4.6	>83
KS-1	0 ± 0				
MN-1	2.7 ± 1.9	12.0 ± 5.6	48.0 ± 20.0	100 ± 0	>20
IA-1	12.0 ± 9.5	6.7 ± 1.9	84.0 ± 8.6	100 ± 0	>20
IA-2	13.5 ± 8.5	3.3 ± 2.7	66.7 ± 5.0	100 ± 0	>20
MN-2	15.3 ± 7.1	4.5 ± 2.8	68.0 ± 5.6	100 ± 0	>20
MN-3	16.7 ± 2.7	8.7 ± 1.9	74.7 ± 10.5	100 ± 0	>20
NE-1	20.0 ± 7.6	13.3 ± 6.8	74.7 ± 9.4	100 ± 0	>20
IA-3	22.0 ± 11.5	2.0 ± 2.0	82.7 ± 5.0	100 ± 0	>20
IA-4	27.3 ± 7.4	15.3 ± 4.3	84.0 ± 3.3	100 ± 0	>20
NE-2	29.3 ± 6.8	12.7 ± 5.8	76.0 ± 4.0	100 ± 0	>20
NE-3	32.0 ± 3.3	21.3 ± 6.4			
IA-5	34.0 ± 3.8	13.3 ± 7.2	64.0 ± 3.3	98.7 ± 2.3	>20
IA-6	35.3 ± 7.1	14.7 ± 5.0	92.0 ± 8.0	100 ± 0	~20
IA-7	35.3 ± 7.1	7.3 ± 6.3	90.7 ± 5.0	100 ± 0	~20
MN-4	36.0 ± 16.4	16.7 ± 5.8			
NE-4	38.0 ± 6.8	23.3 ± 9.1			
IA-8	51.3 ± 10.2	12.7 ± 4.3	77.3 ± 3.8	100 ± 0	>20
SD-1	51.3 ± 16.4	26.0 ± 11.2			
NE-5	55.3 ± 16.4	26.0 ± 11.2			
MN-5	57.3 ± 15.4	17.3 ± 1.9			
ND-1	62.7 ± 9.7	13.3 ± 5.5	82.7 ± 10.0	100 ± 0	>20
IA-9	65.3 ± 12.4	31.8 ± 10.8			

TABLE 4. CONTINUED.

WI-1	70.6 ± 6.4	26.7 ± 4.4	
MN-6	76.0 ± 12.4	20.7 ± 7.4	
SD-2	83.3 ± 9.6	19.3 ± 8.1	
SD-3	86.0 ± 3.8	35.3 ± 7.8	
SD-4	87.3 ± 5.4	33.3 ± 11.0	
IA-10	94.0 ± 7.2	100 ± 0	
OH-1	95.3 ± 4.5	28.7 ± 7.1	
SD-5	99.3 ± 1.5		
MN-7	100 ± 0		<1.25
ND-2	100 ± 0		<1.25
ND-3	100 ± 0		<1.25
SD-6	100 ± 0		<1.25
MI-1	100 ± 0		<1.25
LAB-S	100 ± 0		≡1.0

[A]VALUES FOR % MORTALITY ARE MEANS ± SD OF 4 INDEPENDENT DETERMINATIONS OF 85 BEETLES EACH.

[B]STRAIN IDENTIFICATIONS INDICATE THE STATE OF ORIGIN.

[C]SURVIVORS OF THE FIRST TEST (DOSE = 0.15 MG) WERE USED AS SEEDING STOCK FOR "SELECTED" CULTURES. PROGENY WERE USED FOR SUBSEQUENT TESTS WITHOUT FURTHER SELECTION.

[D]THE RESISTANCE FACTOR REFERS TO ONCE-SELECTED STRAINS. IT IS THE RATIO OF THE LD_{95} VALUE FOR THE FIELD STRAIN TO THAT FOR THE LAB-S STRAIN.

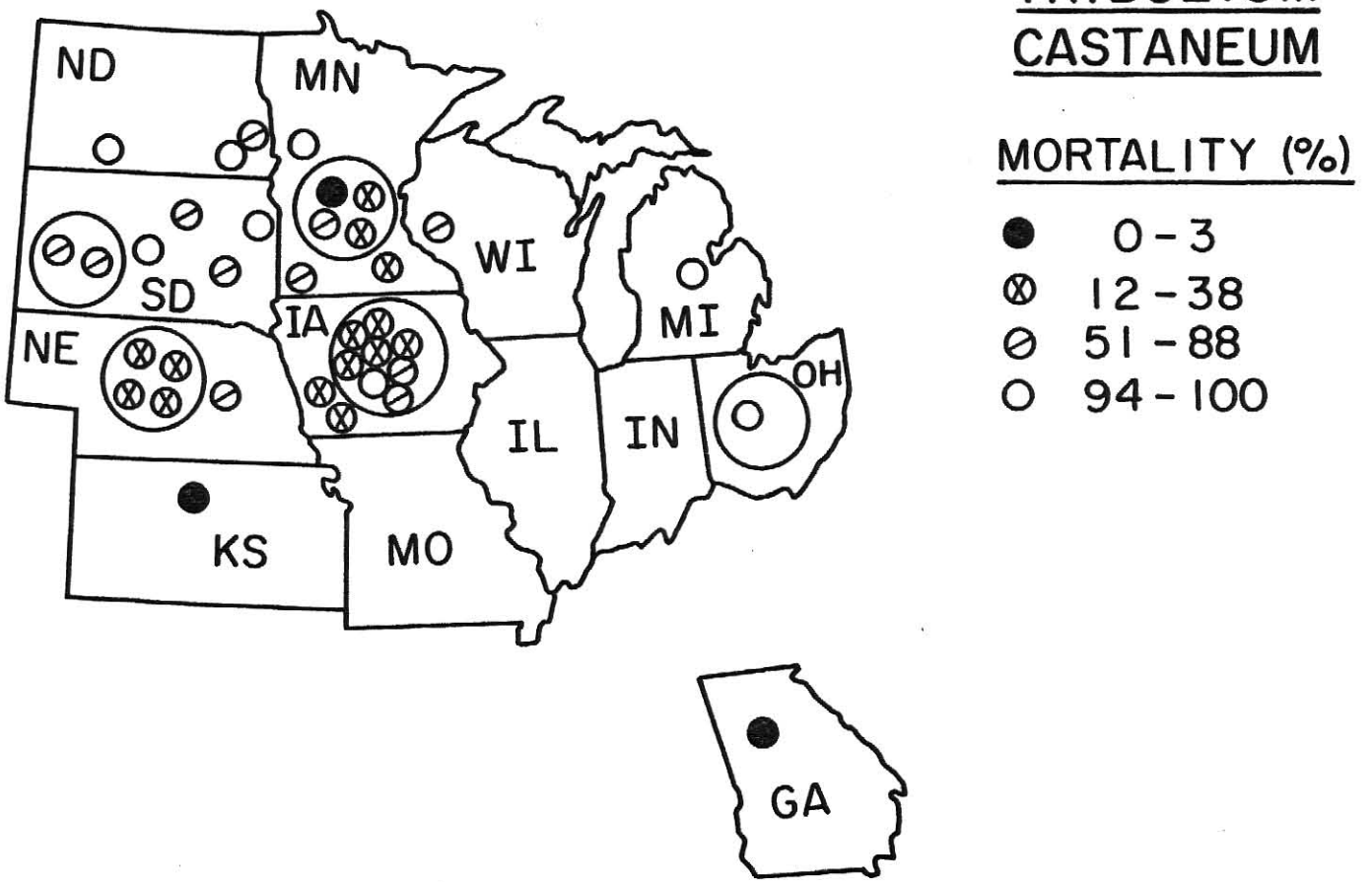
had resistance factors of greater than 20 fold, and one strain from Georgia had a resistance factor of greater than 83 fold. The decreases in mortality after one selection show the response to selection in this species is rapid. Figure 4 illustrates the geographic distribution and resistance levels found in the RFB. Since more than one mechanism of resistance is known in the RFB, 13 of the more resistant strains were tested for TPP synergism. Insects having the malathion-specific resistance mechanism become susceptible upon exposure to TPP (Plapp, et al., 1963). Those strains showing high mortality would be considered to have predominantly the carboxylesterase type of resistance mechanism. Strains showing lower mortality may have a non-specific resistance mechanism. The significant tolerance of strains IA-3, IA-4, and IA-7 suggests that some mechanism other than the malathion-specific one may play a role in resistance in these strains (Table 5).

Evidence presented in this survey indicates that in the U.S. grain belt, resistance to malathion in these four species of stored grain coleoptera (RFB, LGB, Cryptolestes spp., and Sitophilus spp.) is only sporadic with the exception of the RFB, which shows widespread TPP suppressible resistance. Isolated cases of resistance were also found in the LGB and Cryptolestes spp.

Before Zettler (1975), there were no published accounts of resistance to malathion in RFB developing in commodities other than peanuts. His records show resistance to malathion in the RFB in rice in Texas and Louisiana may have developed there. This survey was conducted on farm storage bins, largely outside of the peanut producing area. Resistant RFB detected in this survey probably didn't migrate from peanut or rice storage facilities. I therefore propose the resistance encountered in this survey developed in the commodities sampled, corn, wheat, and oats, as a result of malathion use in bins. This is not surprising, considering the more than 20 years of selective pressure put on an insect with the capacity to develop resistance to malathion (Champ and Campbell-Brown, 1970). Future control of RFB with malathion seems to be limited. As the resistance spreads and increases, malathion will no longer effect control. This may already be the case in parts of the southeastern U.S.

The future use of malathion in stored products is assurred for the time being, since no resistance seems to be present in the Sitophilus spp.,

FIGURE 4. Geographic origin and resistance levels in RFB. Data points enclosed by large circles indicate pooled strains from many counties, or from scattered locations within the state. Data points not enclosed by large circles indicate the county of origin, or the county contributing the largest number of samples in the case of composite strains from adjacent counties.



TRIBOLIUM CASTANEUM

MORTALITY (%)

- ● 0 - 3
- ⊗ 12 - 38
- ⊘ 51 - 88
- ○ 94 - 100

TABLE 5. SUPPRESSION BY TRIPHENYLPHOSPHATE OF MALATHION RESISTANCE IN STRAINS OF *TRIBOLIUM CASTANEUM*.[A]

STRAIN	MORTALITY (%) FOLLOWING EXPOSURE TO		
	MALATHION (0.15 MG)	TPP (0.75 MG)	MALATHION + TPP (0.15 MG + 0.75 MG)
GA-1	1.3 ± 1.9	0 ± 0	99.0 ± 1.7
MN-1	12.0 ± 5.6	0 ± 0	96.0 ± 4.9
IA-1	6.7 ± 1.9	0 ± 0	100 ± 0
IA-2	3.3 ± 2.7	0 ± 0	100 ± 0
MN-2	4.5 ± 2.8	0 ± 0	100 ± 0
MN-3	8.7 ± 1.9	0 ± 0	100 ± 0
NE-1	13.3 ± 6.8	0 ± 0	96.2 ± 2.5
IA-3	2.0 ± 2.0	0 ± 0	77.8 ± 7.3
IA-4	15.3 ± 4.3	0 ± 0	80.0 ± 7.2
IA-5	13.3 ± 7.2	0 ± 0	99.0 ± 1.7
IA-6	14.7 ± 5.0	0 ± 0	100 ± 0
IA-7	7.3 ± 6.3	0 ± 0	65.0 ± 9.9
IA-8	12.7 ± 4.3	0 ± 0	92.0 ± 4.9
LAB-S	100 ± 0	0 ± 0	

[A]VALUES FOR % MORTALITY ARE MEANS ± SD OF 4 INDEPENDENT DETERMINATIONS OF 25 BEETLES EACH.

and mostly low levels in the LGB and Cryptolestes spp. As resistance levels in these species appear and increase, without new control agents or methods, the future capability of insect control in stored grain and other products may be in jeopardy.

INTRODUCTION

Resistance to malathion is often highly specific for this insecticide (Welling, et al., 1974). This is true for some strains of the housefly (Musca domestica L.) (Devonshire, 1973; Ohkawa, et al., 1968), the potato-peach aphid (Myzus persicae Sulz.) (Needham and Sawicki, 1971; Devonshire, 1973,1977), the green rice leafhopper (Laodelphax striatellus Fallen) (Beranek and Oppenoorth, 1977), a mosquito (Culex tarsalis) (Matsumura and Brown, 1961), and a blowfly (Chrysomya putoria) (Oppenoorth, 1975). Malathion-specific resistance is also present in the Indian meal moth (IMM) (Plodia interpunctella Hubn.) (Beeman, et al., 1982). Dyte and Rowlands (1968) report the possibility of malathion-specific resistance in the RFB. Dyte and Blackman (1970, 1972) discuss the presence of malathion-specific resistance in the RFB from different parts of the world. In all species examined, malathion-specific resistance is associated with increased malathion carboxylesterase activity (Devonshire, 1973; Oppenoorth, 1975; Beeman and Schmidt, 1982), and can be suppressed with TPP (Plapp and Eddy, 1961).

Increased esterase activity towards α-naphthyl acetate (αNA) is associated with malathion-specific resistance in some species (Matsumura and Brown, 1963). There are also cases in which resistance is associated with low esterase activity towards αNA (Oppenoorth, 1975; Beeman and Schmidt, 1982).

This work initiates a biochemical study of the enzyme(s) involved in malathion-specific resistance in a strain of the RFB. Stability, titers during development, substrate preferences, and activity relative to a susceptible strain are examined, and partial purification is accomplished by ammonium sulfate precipitation and gel filtration.

MATERIALS AND METHODS

Two resistant and two susceptible strains of RFB were used. The two susceptible strains were the laboratory strain from the USGMRL, and a black mutant strain from the Tribolium Stock Center, California State

College, San Bernadino, California. The two resistant strains were the GA-1 strain from the survey (chapter one of this thesis), and a strain from North Carolina (referred to as the NC strain) described by Bansode and Campbell (1979). The resistant strains were selected for four generations giving a resistance factor in excess of 416.

In vitro radiocarbon enzyme assays were conducted using the lab strain and the NC strain. Radiolabelled (1,2-^{14}C) malathion (side chain ethyl label), 4.6 mCi/mM was purchased from Amersham, and was purified by Beeman and Schmidt (1982). Last instar larvae and untanned pupae were used for the in vitro assays. Insects were homogenized in phosphate buffer (0.1 M pH 7 with 20% sucrose by weight) with Wheaton glass/teflon homogenizing tubes and an Eberbach Con-Torque power unit. Homogenate was centrifuged at 20,000XG for 20 minutes in a Sorvall RC-5 Superspeed Refrigerated Centrifuge. Aliquots of .5 ml of the supernatant were diluted with 1 ml of buffer. Four microliters of a .182 uM solution of malathion (4.6 uCi/uM) were added to each assay tube. Tubes were incubated at 32°C for 30 minutes. Enzyme was kept at 0°C prior to incubation. Reaction was stopped by chilling in ice followed by addition of 2 ul of TPP (100 mg/ml EtOH). The reaction products (the α and β monoacids of malathion) were isolated by a modification of the SEP-PAC® method of Beeman et al. (1982). The monoacids were eluted with 2 ml of 35% aqueous acetonitrile (pH 7). Aliquots of .5 ml of the eluate were added to 10 ml of scintillation fluid (see appendix) and counted by liquid scintillation spectroscopy (LSC) on a Searle Isocap/300 Liquid Scintillation System.

A Waters high pressure liquid chromatograph (HPLC) system was used to determine α:β monoacid ratios. Aliquots of .95 ml of the 35% SEP-PAC fractions containing the monoacids were acidified with five drops of 1 N HCl to a pH of about 1.8, extracted with 1 ml of chloroform, dried under air, and redissolved in HPLC solvent (see appendix). Prior to extraction with chloroform, the fractions were spiked with 80 ul of a monoacid standard (10% α and 90% β 1 mg/ml in acetone) to make UV peaks visible for collection. Peaks were collected manually and quantitated by LSC.

For purification of malathion carboxylesterase(s), a partially refined homogenate was applied to the gel filtration columns. Insects (6 g of NC strain larvae and pupae) were homogenized in 20 ml of buffer (with sucrose)

centrifuged (20,000XG), and fractionated by ammonium sulfate precipitation. The second fraction (20%-40%), containing the activity was put through a .45 um Acrodisc® filter, and ultrafiltered to reduce the volume to 2 ml, using a Diaflo® PM10 ultrafiltration membrane in an Amicon unit. Gel column chromatography was conducted to achieve greater purification of the enzyme. A Sephacryl® S-200 superfine column was run first to give a preliminary idea of protein separation. An S-300 column was then run to achieve greater resolution of the enzyme from other proteins. Buffer used on the S-200 column was .1 M phosphate, pH 7. Buffer used on the S-300 column was .1 M phosphate with 20% sucrose by weight, pH 7. The volume put on the column was 2 ml. Fractions were collected from the column using a Gilson Microfractionator. Fractions of 4.5 ml each were collected and assayed for protein and malathion carboxylesterase. Protein was determined by UV absorption on a Unicam SP1750 Ultraviolet spectrophotometer. Malathion carboxylesterase was assayed by taking 1.5 ml aliquots, adding the 4 ul of radiolabelled malathion, and incubating for 30 minutes at 32°C. Monoacid products were then isolated on a SEP-PAC and quantified by LSC as described earlier. Column fractions exhibiting activity were combined, ultrafiltered to 2 ml, diluted with electrophoresis sample buffer (see appendix), and electrophoresed in polyacrylamide gels using a Bio-Rad model 220 dual vertical gel slab cell with 3% stacking gel (pH 6.8) and 9% running gel (pH 8.8). A system of non-denaturing gels based on Laemmli (1970) was used. The 1.5 mm gels were electrophoresed at 4°C with constant current of 10 mAmps/gel stacking and 30 mAmps/gel running. Strips of the gel were stained with fast red TR salt (see appendix) to determine αNA esterase activity. The remainder of the gels were bathed in washes of distilled water at 0°C for 1 hour prior to cutting to remove tris buffer. Gels were cut into approximately 1 cm strips based on the staining. Strips were homogenized with a Tekmar model SDT homogenizer in 5 ml of buffer with sucrose, allowed to soak at 0°C for seven hours to remove protein, centrifuged, and 1.5 ml of the supernatant assayed for malathion carboxylesterase activity.

Assay for αNA esterase activity was run on four strains of RFB. The assay used ten larvae/ml of phosphate buffer. Homogenate was centrifuged at 20,000XG and supernatant was diluted to 1/20 concentration with buffer.

Reaction mixture consisted of 2.35 ml phosphate buffer, 75 ul substrate (15.6 mg αNA in 6 ml acetone), and 70 ul diluted homogenate. This was incubated for 30 minutes at 32°C, then color reagent added and allowed to develop. Absorption was read after 15 minutes at $O.D._{560}$ to quantify the hydrolysis of αNA.

Four strains of RFB were assayed for αNA esterase activity by crude homogenate electrophoresis in 3% stacking and 9% running gels. Fifteen larvae were homogenized in 1 ml of phosphate buffer with sucrose, and centrifuged. Added to 40 ul of supernatant were 10 ul of concentrated sample buffer, for a total volume of 50 ul applied to the gel. Gels were fixed with fast red TR salt and fixed for photography (see appendix).

The stability of the enzyme was studied in eight carrier solutions by in vitro radiocarbon assay. Results of a fresh assay were used to compare with enzyme held at -15°C for eight days and two months. All enzyme came from the same culture of insects at the same time. A single homogenization was conducted, with aliquots of the supernatant introduced to the carriers to give the desired concentration. Carriers were divided into three aliquots; one for fresh assay and two for delayed assays.

RESULTS AND DISCUSSION

The NC strain had 12 fold higher malathion carboxylesterase activity than the lab strain. The α:β monoacid ratio in the NC strain remained about the same with increasing concentration. Evidence suggests the α:β monoacid ratio in the lab strain increases with concentration. No substrate inhibition was present in either strain (Table 6).

Stability test results are in Table 7. Enzyme in 4 M NaCl seemed to be inhibited, while 20% sucrose seemed to have a stabilizing effect on the enzyme over time. Activity of fresh enzyme in the sucrose was not as high as some other carriers, but after eight days it had the highest activity. After two months, 58% of the activity was intact in the sucrose while no activity was present in the other seven carriers.

Figure 5 shows the absorption spectra and malathion carboxylesterase

TABLE 6. ACTIVITY OF α AND β MALATHION CARBOXYLESTERASES IN TWO STRAINS OF RFB AS A FUNCTION OF CONCENTRATION.[1]

	(MALATHION) 10 M	200 M	600 M
LAB α	140	2940	15000
β	138	1730	3960
TOTAL	278	4670	18960
NC α	7865	111,400	253,080
β	2640	39,080	90,780
TOTAL	10,505	150,480	343,860

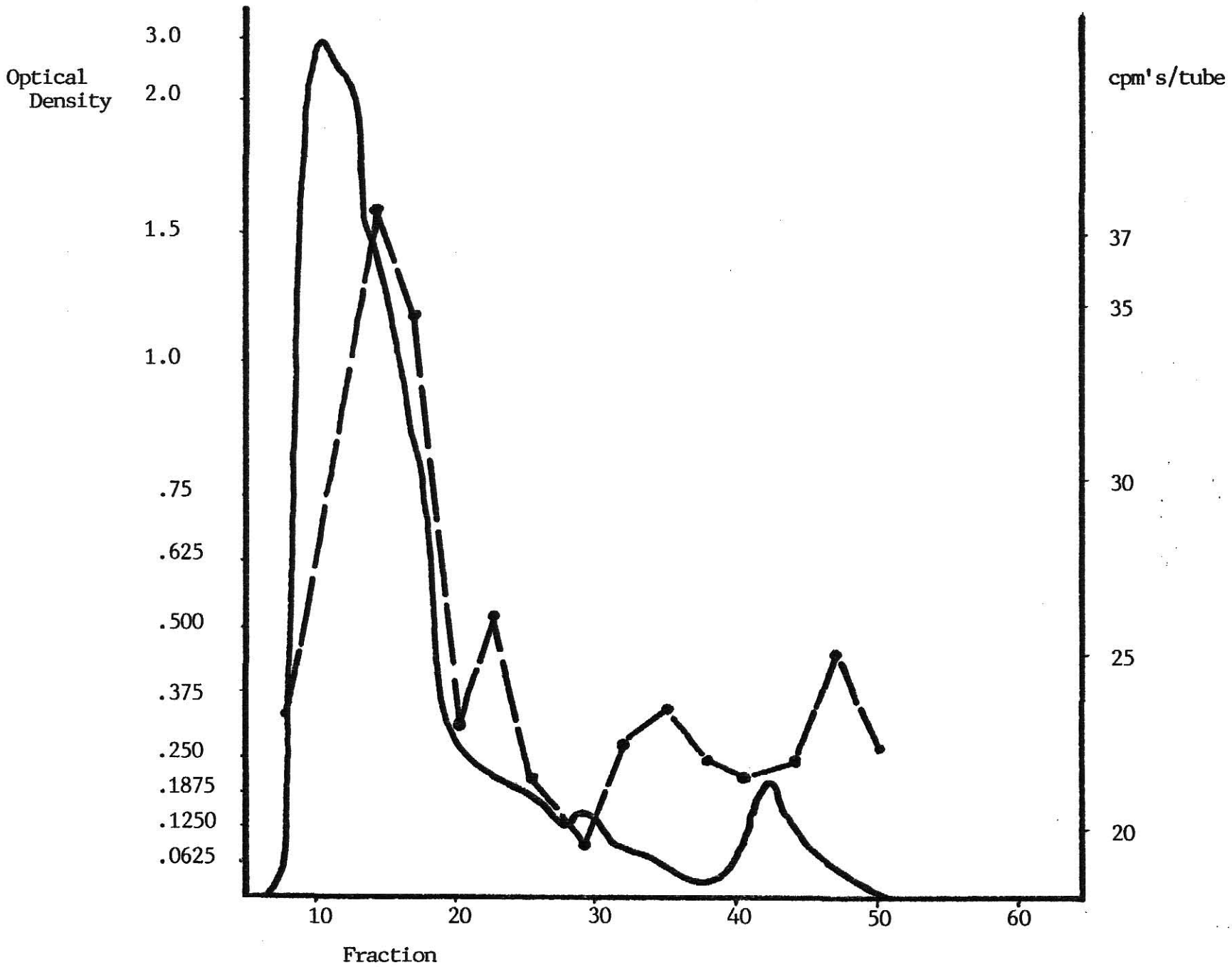
[1]UNITS ARE COUNTS PER MINUTE.

TABLE 7. RESULTS OF IN VITRO CARBOXYLESTERASE STABILITY ASSAY.

	FRESH		8 DAY OLD		2 MONTH OLD	
CARRIER[1]	CONT.	ACTIVE	CONT.	ACTIVE	CONT.	ACTIVE
BUFFER	45.05	313.0	38.50	167.95	44.55	57.25
BSA (.1G/ML)	51.95	275.0	52.05	175.98	47.15	49.32
BSA (1.0G/ML)	42.00	252.72	75.72	123.88	36.22	33.65
BSA (10.0G/ML)	45.69	311.72	52.05	66.95	27.82	29.38
SUCROSE (20% W/V)	43.16	214.48	43.35	207.55	30.95	131.15
GLYCEROL (20% V/V)	53.71	206.52	69.92	88.78	35.92	33.65
4M NACL	47.06	133.75	54.60	63.92	51.08	38.70
PTU (SATURATED)	53.38	268.80	50.05	157.22	33.90	38.18

[1]ALL CARRIERS WERE SOLUTIONS IN .1M SODIUM PHOSPHATE BUFFER, THE FIRST CARRIER.

FIGURE 5. Absorption spectra and carboxylesterase activity in S-200 column fractions. Solid line indicates absorption spectra and broken line indicates carboxylesterase activity.


Optical Density
3.0
2.0
1.5
1.0
.75
.625
.500
.375
.250
.1875
.1250
.0625
cpm's/tube
37
35
30
25
20
10
20
30
40
50
60
Fraction

activity from the fractions of the S-200 column. The separation of esterase activity from major protein peaks was insufficient for our needs, but gave a general idea of where the esterase activity may be found. Much better resolution of protein was observed from the S-300 (Figure 6). Fractions containing the activity were electrophoresed and the gel slices assayed for malathion carboxylesterase activity. The highest activity was found in the second slice (Figure 7 and Table 9). The first slice also had relatively high malathion carboxylesterase activity. Low levels were present in slices three and six.

The resistant strains were significantly different from the sensitive strains in hydrolysis of αNA in vitro (Table 8). Electrophoresis of crude homogenates of the four strains also showed the resistant strains with higher αNA esterase activity (Figure 7).

The difference in α:β monoacid ratios of the lab and NC strains with concentration suggests there may be a mutant enzyme present. No sound conclusion may be made from the high αNA esterase activity in the resistant strains. There are examples of both cases: high αNA esterase activity and malathion-specific resistance (Beranek and Oppenoorth, 1977), and low αNA esterase activity and malathion-specific resistance (Beeman and Schmidt, 1982). The electrophoresis of column fractions (NC strain) shows a single band not found in any of the other strains tested, occurring in gel slice two. This band also appears when crude homogenate is electrophoresed (Figure 7, slice 2, NC strain). This may be the mutant enzyme mentioned previously. Two other slices of gel, one and six showed significant activity, and also stained heavily for αNA esterase activity. This is evidence for increased synthesis of carboxylesterase in the NC strain, since the bands are also present, but did not stain as dark in both sensitive strains.

Both mechanisms of malathion-specific resistance may be operating in the NC strain of the RFB, a mutant esterase, and increased synthesis of already present carboxylesterase. Furthur testing will be needed to determine if the increased synthesis is due to gene duplication or to a mutation in a regulatory gene.

FIGURE 6. Absorption spectra and carboxylesterase activity in S-300 column fractions. Solid line indicates absorption spectra and broken line indicates carboxylesterase activity.

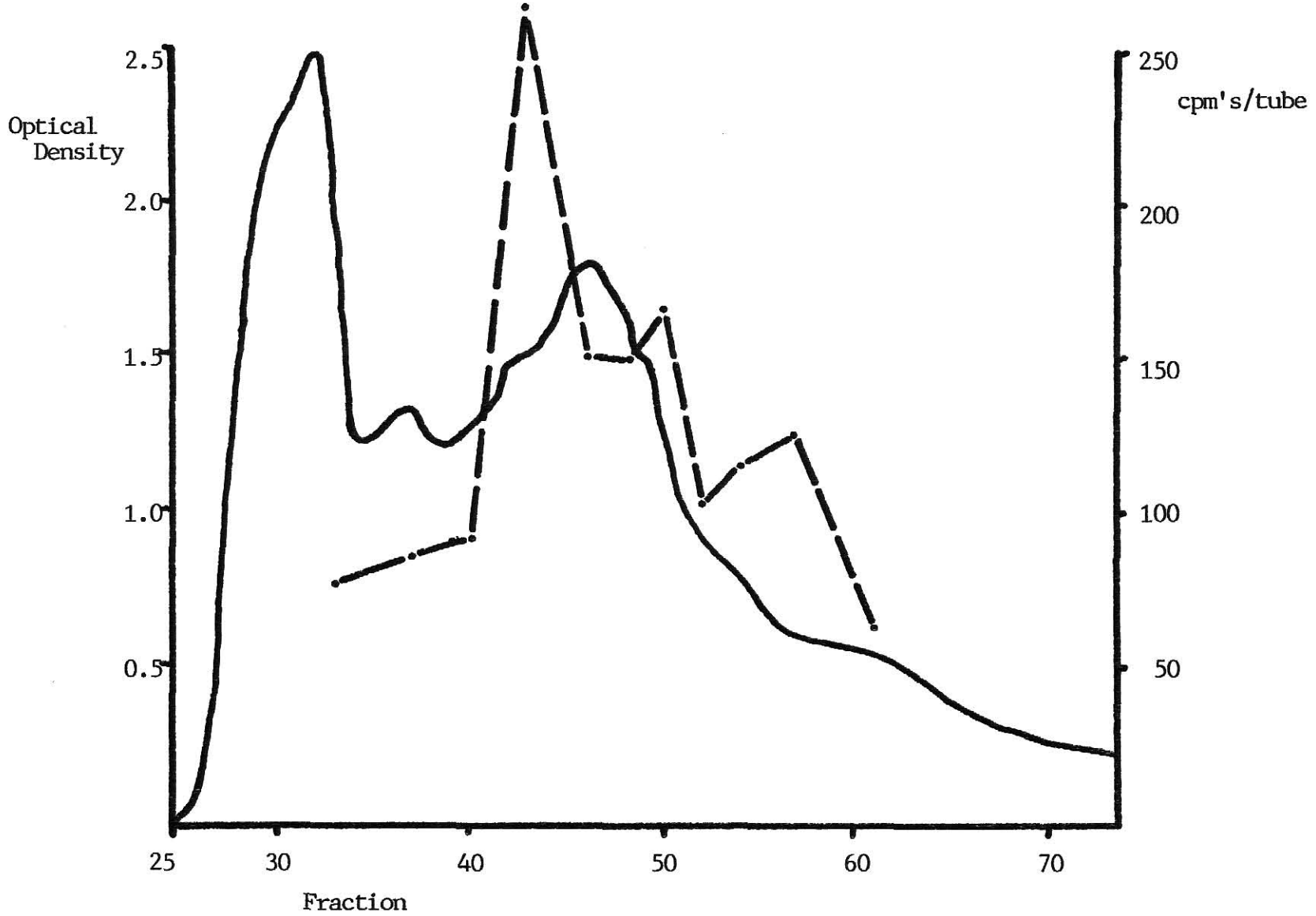

Optical Density
2.5
2.0
1.5
1.0
0.5
250
cpm's/tube
200
150
100
50
25
30
40
50
60
70
Fraction

FIGURE 7. Electrophoresis of NC strain, GA-1, mutant, and lab strain homogenates with stain for αNA esterase activity. Numbers indicate bands of activity.

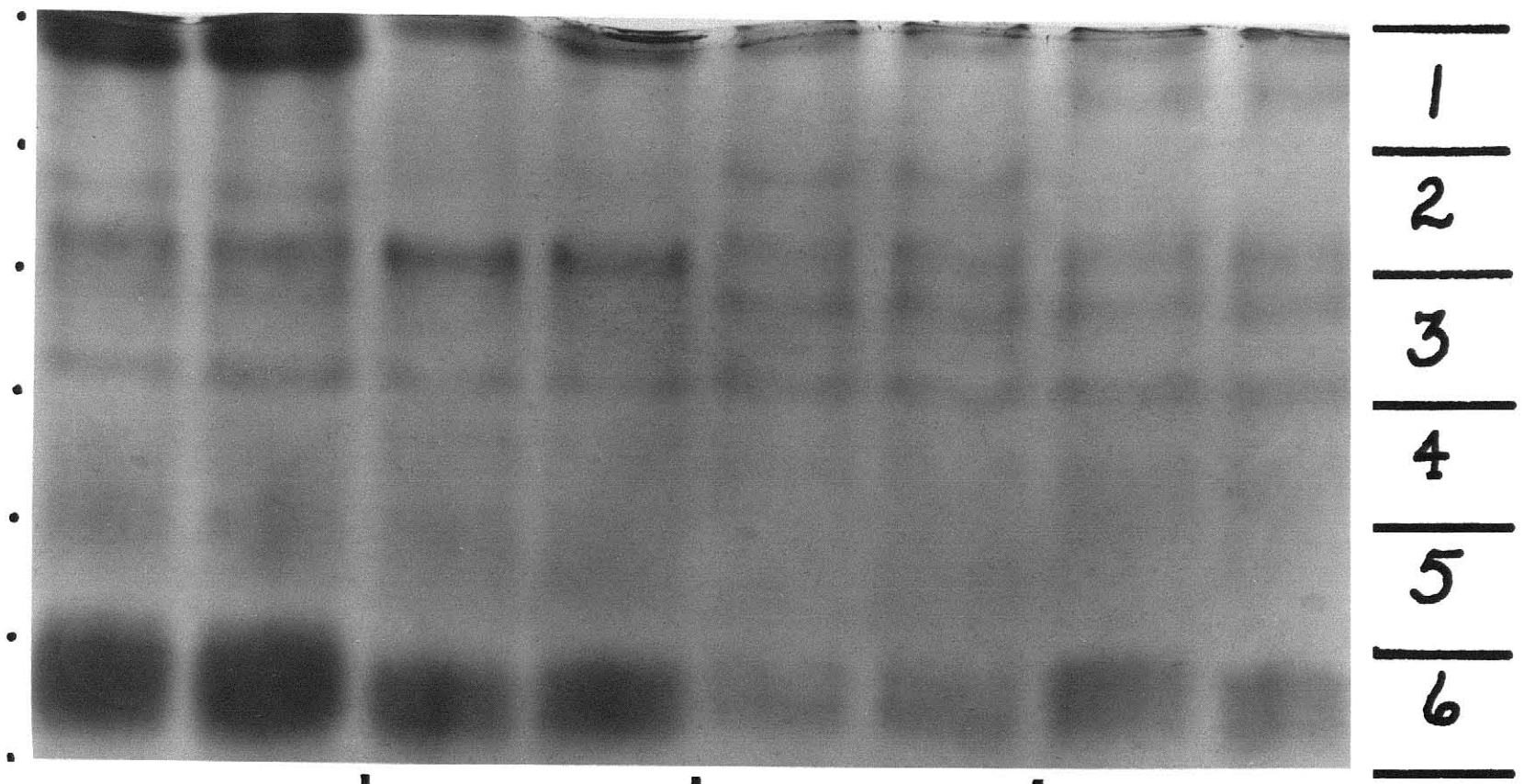

1
2
3
4
5
6
NC STRAIN
GA-1 STRAIN
MUTANT STRAIN
LAB STRAIN

TABLE 8. ACTIVITY OF CARBOXYLESTERASE WITH α NA AS A SUBSTRATE IN 4 STRAINS OF TRIBOLIUM CASTANEUM (HERBST).

STRAIN	ACTIVITY ($O.D._{560}$)[1]
LABORATORY	.225 ± .039
MUTANT	.242 ± .009
GA-1	.320 ± .027
NC STRAIN	.462 ± .029

[1]MEAN, ± S.D. OF 4 INDEPENDENT DETERMINATIONS.

TABLE 9. RELATIVE NET ACTIVITY OF ELECTROPHORESIS GEL SLICES.

SLICE	ACTIVITY[1]
1.	75
2.	100
3.	25
4.	0
5.	0
6.	35

[1]UNITS ARE CPM'S

APPENDIX

MIXTURES, SOLUTIONS AND STAINS

Buffer	.1M sodium phosphate pH 7.00 with sucrose added, 20% by weight/volume
Hot malathion	375ul of .0213ug/ml ^{14}C malathion 4.6uCi/uM side chain ethyl label 1.75ml 60mg/ml malathion (all of above in ethanol 95%)
Scintillation fluid	1500ml toluene 1500ml ethylene glycol monomethyl ether 12g preblend 2a70 (Research Products International Corp.)
Gel stain for α-naphthyl acetate	150ml .1M sodium phosphate buffer pH 7.00 14.0mg α-naphthyl acetate in 1:1 acetone: water (2ml) 0.5mg/ml fast red TR salt (75mg/150ml)
Gel fixing solution	200ml water:methanol:glacial acetic acid (5:5:1)
Color reagent	100mg fast garnet GBC 20ml 10% sodium lauryl sulfate
HPLC solvent	1:1 acetonitrile and 0.1% acetic acid

ELECTROPHORESIS PREPARATIONS

STOCK SOLUTIONS

Acrylamide:BIS (30:0.8)	30g acrylamide 0.8g N'N'-BIS methylene acrylamide adjust to 100ml with distilled water filter and store in dark at 4°C
1.5M tris-Cl pH 8.8	18.15g tris base 50ml distilled water adjust to pH 8.8 with 1N HCl adjust to 100ml with distilled water
0.5M tris-Cl pH 6.8	3.0g tris base 25ml distilled water adjust to pH 6.8 with 1N HCl adjust to 50ml with distilled water
Sample buffer	58ml distilled water 10.0ml 0.5M tris-Cl pH 6.8 10.0ml glycerol 2.0ml .05% (w/v) bromophenol blue
Electrode buffer	6.0g tris base 28.8g glycine adjust to 1 liter with distilled water

GEL PREPARATION

3% stacking gel .125M tris-Cl pH 6.8 (for 2 1.5mm x 3cm x 14cm gels)
12.8ml distilled water
5.0ml 0.5M tris-Cl pH 6.8
2.0ml acrylamide:BIS (30:0.8)
0.2ml 10% ammonium persulfate (fresh)
10.0ul TEMED

6% separating gel .375M tris-Cl pH 8.8 (for 2 1.5mm x 10cm x 14cm gels)
32.8ml distilled water
15.0ml 1.5M tris-Cl pH 8.8
12.0ml acrylamide:BIS (30:0.8)
0.2ml 10% ammonium persulfate (fresh)
15.0ul TEMED

9% separating gel .375M tris-Cl pH 8.8 (for 2 1.5mm x 10cm x 14cm gels)
26.8ml distilled water
15.0ml 1.5M tris-Cl pH 8.8
18.0ml acrylamide:BIS (30:0.8)
0.2ml 10% ammonium persulfate (fresh)
15.0ul TEMED

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INCIDENCE AND PHYSIOLOGICAL NATURE OF MALATHION RESISTANCE IN FOUR SPECIES OF STORED PRODUCTS COLEOPTERA FROM GRAIN PRODUCING STATES

by

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B. S., University of Delaware, 1980

AN ABSTRACT OF A MASTER'S THESIS

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More than 140 strains of red flour beetle (Tribolium castaneum Herbst), lesser grain borer (Rhizopertha dominica F.), Sitophilus spp. and Cryptolestes spp. were collected from grain bins on farms in 14 states. Strains were screened for resistance to malathion by the discriminating dose technique using impregnated filter papers. Resistance was widespread only in the red flour beetle. Of 36 strains tested, 31 showed detectable levels of resistance. This level of resistance increased after one generation of selection in most cases. At least 15 strains from six states were more than 20 fold resistant at the LD_{95} level after one generation of selection. Resistance was suppressed by triphenylphosphate in every case. Four of 13 strains of lesser grain borer, and five of 42 strains of Cryptolestes spp. showed measurable levels of resistance. No significant resistance was detected in the 22 strains of Sitophilus spp. tested.

A malathion resistant strain of red flour beetle from North Carolina had 12 fold higher malathion carboxylesterase activity than a susceptible laboratory strain. The enzyme, which was present in larvae, pupae, and adults was particularly unstable in adult whole body homogenate. Among eight treatments attempted, only sucrose greatly increased the stability of the larval enzyme. Analyses of monoacid product in resistant and sensitive strains showed a preference for formation of monoacid in both strains. The ratio of : monoacids varied little with concentration in the NC strain. The ratio increased with concentration in the laboratory strain. This evidence suggests the NC strain enzyme may be a mutant enzyme. Partial purification of the enzyme is achieved by ammonium sulfate fractionation and gel column chromatography.