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Title: NAPHTHALENE RESISTANCE IN A HOUSE FLY STRAIN: ITS INHERITANCE AND CHARACTERISTICS

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A combination of genetical and biochemical methods were utilized in investigating house fly resistance to naphthalene. The inheritance of resistance was determined by crossing a naphthalene resistant strain of Musca domestica L. with a susceptible mutant marker strain. Biochemical comparisons were made on substrains isolated from the progeny of these crosses.

Factors on chromosomes II, III and V were found to be important in resistance when F_2 and backcross progeny were bioassayed with naphthalene vapors. Statistical analysis of the bioassay results indicated that resistance factors on chromosome III were inherited as recessive or incompletely recessive genes, and the factors on chromosomes II and V were of a dominant nature. The factors on chromosomes II and III were most important in resistance.

Toxicological experiments were conducted on the parent strains and the naphthalene tolerant substrains. In in vivo experiments with

these strains, flies with chromosome III from the resistant parent were two to three times more resistant to knockdown by dieldrin, naphthalene, and tributyltin chloride than flies with susceptible alleles on this chromosome. Slow absorption of the toxicants is thought to account for this knockdown resistance.

The oxidative activity of microsomal enzymes was found to be greater in substrains with chromosome II from the resistant parent. Both hydroxylation and epoxidation reactions were measured in these in vitro experiments, using naphthalene and aldrin as substrates. Aldrin epoxidation was more closely aligned with resistance in parent and substrains than naphthalene hydroxylation. Resistance due to factors on chromosome V could not be attributed to increased oxidase activity or to the slow absorption of toxicants.

When the metabolic and non-metabolic factors were combined in a strain, resistance increased nearly three-fold. This complementary effect suggests that slow absorption (chromosome III) and active detoxication of naphthalene (chromosome II) interact, and allow flies to resist larger doses of naphthalene. Resistance due to factors on chromosomes II and III did not account for all of the resistance of the naphthalene strain, indicating that chromosome V probably plays a more important role when combined with other resistance factors.

Naphthalene Resistance in a House Fly Strain: Its Inheritance and Characteristics

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NAPHTHALENE RESISTANCE IN A HOUSE FLY STRAIN: ITS INHERITANCE AND CHARACTERISTICS

I. INTRODUCTION

Many studies of house fly resistance to insecticides have dealt with the metabolic reactions which, directly or indirectly, lead to detoxication of the toxicant. Hook, Jordan, and Smith (1968) state that the most important of these reactions are the oxidative detoxications which occur in the microsomal system. The importance of the microsomal system as a defense mechanism has been assessed in studies with such compounds as aldrin and naphthalene.

Naphthalene was first used to measure oxidative activity in house flies by Arias (1962), who found that microsomes from DDT-resistant flies were more active in naphthalene metabolism than those from susceptible flies. In later studies with microsomes from naphthalene and dieldrin resistant flies (Schonbrod, Philleo, and Terriere, 1965), resistance was further correlated with high levels of microsomal oxidase activity. These strains also resisted naphthalene vapors in dosage-mortality studies, suggesting that high levels of microsomal oxidase activity were characteristic of resistance to naphthalene and other insecticides.

In a more recent investigation, (Schonbrod et al., 1968), the activity of microsomal oxidases was determined in still other house fly strains resistant to naphthalene, and to the chlorinated

hydrocarbon, organophosphorous, and carbamate insecticides. Microsomal hydroxylation seemed well correlated with resistance to insecticides known to be detoxified oxidatively, such as carbaryl and Isolan. However, only medium to low hydroxylation values were found for strains resistant to chlorinated hydrocarbon and some organophosphate insecticides. This indicated that high microsomal oxidase activity was not associated with all types of resistance. Also, when these house fly strains were bioassayed with naphthalene vapors, the correlation between naphthalene resistance and microsomal oxidase activity was not complete. For example, only moderate hydroxylase activity was shown by microsomes from a strain highly resistant to naphthalene vapors, while a strain with high in vitro naphthalene hydroxylase activity was susceptible to naphthalene vapors. These discrepancies suggested that factors other than microsomal detoxication must be involved in naphthalene resistance.

Therefore, a genetical and biochemical investigation of house fly resistance to naphthalene was begun. The objectives of this research were to determine how the factors involved in naphthalene resistance were inherited and to isolate the chromosomes with these factors to determine the nature of their contribution to resistance.

II. LITERATURE REVIEW

Prior to the development of house fly strains with mutant morphological characters, information from the genetical analysis of insecticide resistance was rather indirect and often based on complex dosage-mortality determinations (Tsukamoto, 1964). In recent years, such genetical analyses of resistance in the house fly have been improved by the development of morphological mutants that can be used in crossing experiments (Hiroyoshi, 1960; Hoyer, 1966). With these visible markers, it is possible to obtain more direct information on the inheritance of resistance because each chromosome can be identified and assessed separately for resistance. This "chromosome labelling" then allows flies with single factors for resistance to be isolated from a strain with several for bioassay and biochemical comparisons.

This combination of genetical and biochemical methods has been used in many studies of house fly resistance. Results of these studies have been recently summarized by Milani (1960), Georghiou (1965) and Oppenoorth (1965). A similar summary was not attempted for this literature review because of the extent of the literature and its limited relationship with naphthalene resistance. However, some of the recent literature on the inheritance and mechanisms of resistance in the house fly is reviewed here.

Major factors for resistance to DDT, organophosphate, and carbamate insecticides, conferring from 15 to 2500 fold resistance in house flies, were found to be inherited as dominant and semi-dominant genes on the second chromosome (Oppenoorth, 1959; Franco and Oppenoorth, 1962; Hoyer, Plapp and Orchard, 1965; Plapp and Hoyer, 1967).[1] Minor factors for resistance to these insecticides have been linked with chromosome V (Oppenoorth, 1967; Sawicki and Farnham, 1967; Tsukamoto, Shrivastava and Casida, 1968).

A major factor for dieldrin and gamma-BHC resistance in the flies studied by Oppenoorth and Nasrat (1966) was located on the fourth chromosome. Sawicki and Farnham (1968) found a factor for minor dieldrin resistance on chromosome II.

In most of the studies cited, biochemical factors thought to confer resistance, such as altered esterases or highly active oxidative enzymes, could be linked with chromosomes responsible for the observed resistance. For example, Oppenoorth (1959) showed genes for resistance to diazinon and malathion in house flies to be inherited in the same manner as genes for low ali-esterase activity. Also, Oppenoorth (1967) found that resistance to DDT and diazinon in strain

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[1] The numbering system used in referring to chromosomes is in accordance with the system of Wagoner (1967).

Fc was controlled by chromosome V, as was the factor for the oxidative detoxication of these compounds. Hoyer and Plapp (1966) found factors on chromosomes II and III to cause near immunity to DDT in the Orlando-DDT resistant strain. This resistance was attributed to a second chromosomal dominant gene "Deh," for DDT-dehydrochlorinase production, and to a third chromosomal recessive factor "kdr-o" for resistance to knockdown. Another study, which also illustrates the multifactorial nature of resistance in some strains, was that of Tsukamoto et al. (1968) in which three factors were found to be important for resistance to the carbamates Baygon and Matacil. The factors on chromosomes II and V were important in the oxidative metabolism of these carbamates, and the third chromosomal factor was non-metabolic.

Another physiological factor which may contribute to resistance is decreased cuticular penetration of insecticides. However, Perry (1964) concluded that reduced cuticular permeability was a variable factor, found in both susceptible and resistant house fly strains and probably does not constitute a basic protective mechanism. On the other hand, Forgash, Cook, and Riley (1962) and Farnham, Lord, and Sawicki (1965) reported that reduced cuticular permeability was a factor in house fly resistance to diazinon. El Bashier (1967) and Sawicki and Farnham (1968) found that DDT and dieldrin penetrated the cuticle more slowly in diazinon selected house flies, resulting in

knockdown resistance and two-fold resistance at death.

A gene was described by Hoyer and Plapp (1968) and Plapp and Hoyer (1968) that conferred resistance to organotin compounds and acted as an intensifier of resistance to several insecticides by slowing the absorption of toxicants through the cuticle. When exposed to doses of insecticides lethal to susceptible flies, knockdown occurred more slowly in house flies with this third chromosomal gene. Camp and Arthur (1967) showed differential absorption to be partially responsible for the variable mortality response of four insect species exposed to carbaryl. Sun (1968) found differences in penetration to be one of the main factors that determine the toxicity of insecticides to different species of insects as well as to susceptible and resistant house fly strains.

This review of the pertinent literature on the biochemical genetics of house fly resistance shows that chromosomes II, III, and V may be involved with resistance to a variety of insecticides. The many factors causing resistance may be metabolic or non-metabolic. A metabolic factor, hydroxylation, is known to detoxify naphthalene in house flies (Schonbrod et al., 1965), but this and other possible mechanisms of resistance have not been investigated genetically in a naphthalene resistant strain.

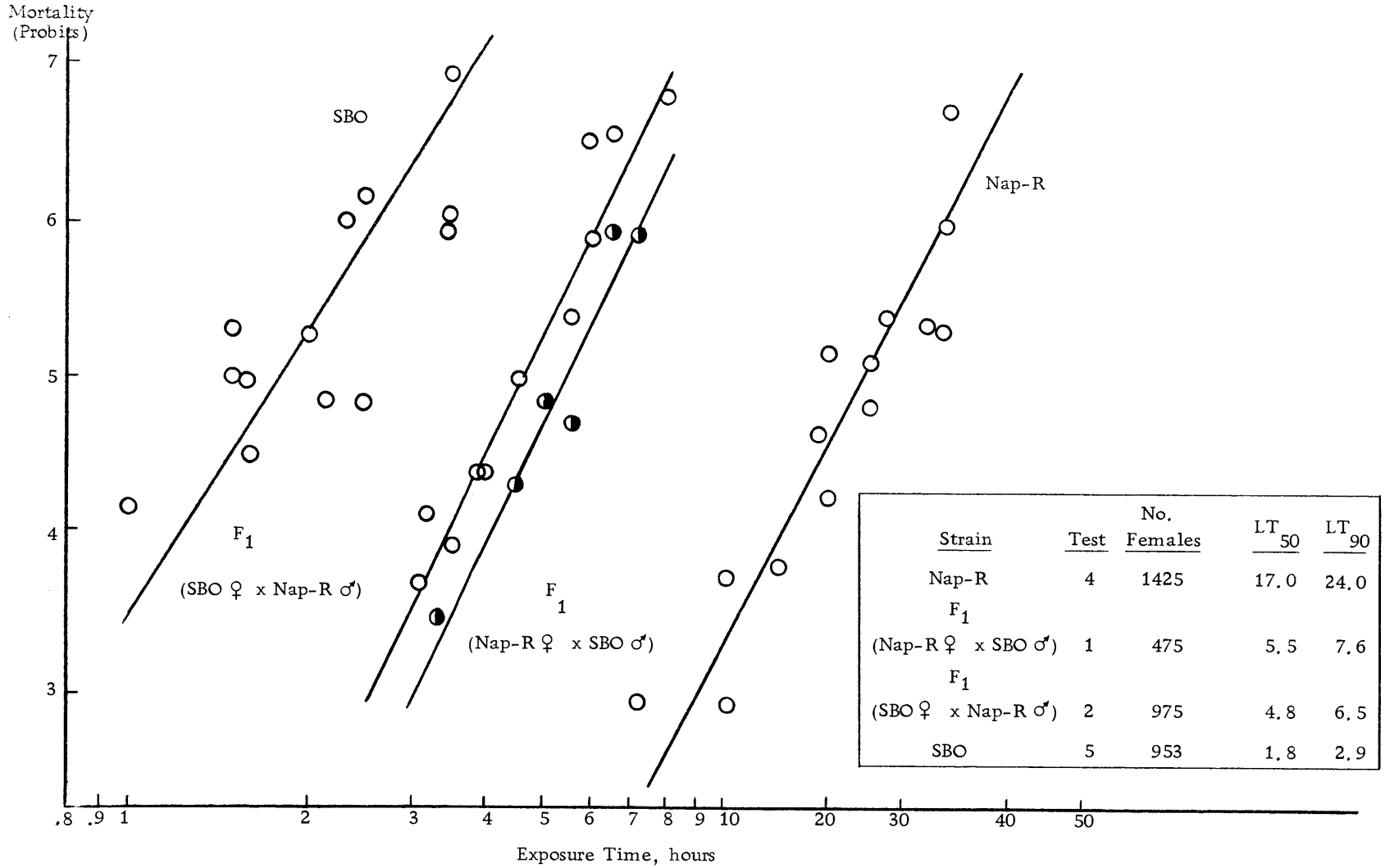
III. METHODS AND MATERIALS

Using the procedures of Tsukamoto (1964), crosses were made between a susceptible mutant house fly strain and a naphthalene resistant strain. By bioassaying the progeny of these crosses with naphthalene vapors and analyzing the data statistically, the inheritance and relative importance of the resistance factors from the parent strain were determined. Substrains possessing resistance factors were isolated and examined by *in vitro* and *in vivo* methods in order to characterize these factors.

House Fly Strains and Their Maintenance

Naphthalene-resistant strain: The Nap-R strain of *M. domestica* was derived from a susceptible strain of house flies obtained from the World Health Organization standard reference colony, Milan, Italy. Naphthalene resistance was developed in this strain by weekly selection of the flies with naphthalene vapors for a two year period. When the genetical experiments were begun, the time of exposure to naphthalene vapors to produce 50 percent mortality (LT_{50}) was 17 hours (Figure 1).

Stubby wing; brown body; ocra eye strain: This strain of house flies was synthesized by R. Hoyer and F. Plapp (USDA, Corvallis, Oregon, 1966) and will be referred to as the SBO strain. The



Strain	Test	No. Females	LT_{50}	LT_{90}
Nap-R	4	1425	17.0	24.0
F_1 (Nap-R ♀ x SBO ♂)	1	475	5.5	7.6
F_1 (SBO ♀ x Nap-R ♂)	2	975	4.8	6.5
SBO	5	953	1.8	2.9

Figure 1. Mortality due to naphthalene vapors in a naphthalene resistant house fly strain (Nap-R), a susceptible multi-marker strain (SBO), and their F_1 progeny.

morphological markers stubby wing, brown body, and ocra eye, are controlled by recessive genes located on chromosomes II, III, and V, respectively. These house flies are quite susceptible to naphthalene vapors, with an LT_{50} value of about two hours (Figure 1).

Rearing and maintenance of house fly colonies: Larval rearing medium consisting of mill-run, alfalfa meal, sawdust, and water was placed in a one gallon jar and seeded with 0.5 cc of viable eggs. Approximately one thousand adults weighing up to 24 mg per fly were obtained per larval rearing jar ten days after seeding. Adults were reared in constant light at approximately 75° F. with 30 to 60 percent relative humidity. They were fed a mixture of powdered milk, sugar, and egg yolk.

Bioassay Methods

Three to five day old sexed house flies were used in the bioassays with naphthalene vapors. The insects were anesthetized by cold or by CO_2 and the bioassays conducted at 70° F. Twenty-five house flies in metal screen cages 8 1/2 x 4 cm were placed in a one gallon jar containing at least thirty grams of crystalline naphthalene. This compound, which is quite volatile at room temperature, was used as a fumigant and flies did not contact the crystalline material. After various exposure times, the house flies were transferred to recovery jars and examined 24 hours later for mortality. Mortality

differences between replications averaged 12 percent when 725 female flies were assayed at several exposure times in one day. In genetical studies, exposure times were chosen so that susceptible phenotypes were killed.

Differences in the cuticular penetration of toxicants were assessed by measuring the extent of knockdown of house flies exposed to three insecticides: naphthalene, dieldrin and tributyltin chloride. Criterion of knockdown was inability of the flies to walk. The number of affected flies was recorded at various time intervals until knockdown was complete.

In tests with naphthalene vapors, three to five day old female house flies were put in screen-covered pint jars which were placed in the one gallon exposure jars. Each test included two replicates of up to twenty flies, each strain being tested three times. There was usually less than ten percent variation in knockdown rate between replicates. The average standard deviation between tests at the fifty percent knockdown level was five minutes.

Knockdown response to dieldrin was measured by treating female flies with one microgram of dieldrin on the dorsal thorax. Two replicates of ten to twenty flies each were tested for all phenotypes. Tests were repeated three to five times. Agreement between tests was not as close as with naphthalene vapors, with an average of 23 percent variation observed between tests at the fifty percent

knockdown level.

Differences in rate of knockdown were also determined using tributyltin chloride (TBTC) as a residue in glass jars. Twenty flies were placed in a pint jar in which 250 micrograms of TBTC had been evenly coated by jar rolling (Plapp et al., 1963). Flies were then observed until knockdown was complete. Ten percent variation within replicates was noted, with little variation at the fifty percent knockdown level, although fewer tests were performed. Considerable variability in knockdown rate above the fifty percent knockdown level was noted in the more resistant flies. Often some of the resistant flies were not affected.

Crossing Procedure

The mass crosses between SBO and Nap-R strains were designed to test for the dominance, recessivity, sex linkage, and maternalism of resistance factors on chromosomes II, III, and V. The mode of inheritance of the major factors was determined by comparing the results of the backcross and F_1 cross. The diagrams in Tables Ia and Ib show the crosses and the expected phenotype ratios. When virgin females were needed for crosses, male and female house flies were separated with 12 hours after emergence until the appropriate crosses were made. F_1 male heterozygotes were used in the backcrosses to reduce crossing over, which is rare

Table Ia. Diagram of Crossing Procedure and Resulting Phenotype Ratios.

Reciprocal Parental Cross

$$\frac{+;+;+}{+;+;+} \times \frac{stw;bwb;ocra}{stw;bwb;ocra} \qquad \frac{stw;bwb;ocra}{stw;bwb;ocra} \times \frac{+;+;+}{+;+;+}$$

(Nap-R ♀) x (SBO ♂) (SBO ♀) x (Nap-R ♂)

$$\frac{+;+;+;}{stw;bwb;ocra} \ (F_1) \qquad \frac{+;+;+;}{stw;bwb;ocra} \ (F_1)$$

Backcross

$$\frac{+;+;+}{stw;bwb;ocra} \times \frac{stw;bwb;ocra}{stw;bwb;ocra} \qquad \frac{+;+;+}{stw;bwb;ocra} \times \frac{stw;bwb;ocra}{stw;bwb;ocra}$$

(F_1 ♂) x (SBO ♀) (F_1 ♂) x (SBO ♀)

Backcross Phenotypes

1/8 +;+;+	1/8 stw;bwb;+
1/8 stw;+;+	1/8 stw;+;ocra
1/8 +;bwb;+	1/8 +;bwb;ocra
1/8 +;+;ocra	1/8 stw;bwb;ocra

Table Ib. Diagram of Crossing Procedure and Resulting Phenotype Ratios.

Reciprocal Parental Cross

$$\frac{+;+;+}{+;+;+} \times \frac{stw;bwb;ocra}{stw;bwb;ocra}$$

(Nap-R ♀) x (SBO ♂)

$$\frac{+;+;+;}{stw;bwb;ocra} \quad (F_1)$$

$$\frac{stw;bwb;ocra}{stw;bwb;ocra} \times \frac{+;+;+}{+;+;+}$$

(SBO ♀) x (Nap-R ♂)

$$\frac{+;+;+;}{stw;bwb;ocra} \quad (F_1)$$

F_1 Cross

$$\frac{+;+;+}{stw;bwb;ocra} \times \frac{+;+;+}{stw;bwb;ocra}$$

(F_1 ♀) x (F_1 ♂)

$$\frac{+;+;+}{stw;bwb;ocra} \times \frac{+;+;+}{stw;bwb;ocra}$$

(F_1 ♀) x (F_1 ♂)

F_2 Phenotypes

27/64 +;+;+	3/64 stw;bwb;+
9/64 stw;+;+	3/64 stw;+;ocra
9/64 +;bwb;+	3/64 +;bwb;ocra
9/64 +;+;ocra	1/64 stw;bwb;ocra

in male house flies (Hiroyoshi, 1960). The original linkage-group numbering system of Hiroyoshi (1960), previously used by most authors, has been revised by the recent linkage map determination of Wagoner (1967). References to chromosome number will follow this new system.

Analysis for dominant factors: The F_1 males resulting from the original resistant:susceptible cross were backcrossed with SBO females to detect chromosomes with dominant resistance factors (Table Ia). The progeny from this cross were exposed to discriminating dosages of naphthalene and the live and dead flies separated, grouped by phenotype visually, and counted.

The presence of a chromosome with resistance factors was indicated by the greater susceptibility of the flies possessing recessive mutant marker chromosomes in place of chromosomes with dominant resistance factors. Thus, by testing at a discriminating dosage and noting the response of the phenotypes, the chromosomes containing genes for resistance could be detected. This cross produced eight phenotypes. A reciprocal cross was also made, using the same procedures, in order to detect maternal influences (Table Ia).

Analysis for recessive factors: To identify the linkage groups carrying recessive factors, F_1 progeny were inter-bred, or selfed, to produce an F_2 population (Table Ib). These phenotypes were

bioassayed, as before, with discriminating dosages of naphthalene. Those house flies with a recessive mutant chromosome replacing a resistance factor were susceptible when compared to resistant flies with other or no mutant linkage groups. This cross also produced eight phenotypes. A reciprocal cross was again made to detect maternalism (Table Ib).

Statistical Analysis

The effect of replacing chromosomes of the resistant parent with susceptible, labelled chromosomes was measured by exposing both male and female flies to naphthalene vapors. The percent survival of each phenotype was transformed into arc-sin units, or mean survival rates (Tsukamoto, 1964). The net effect of a given chromosome was then determined by subtracting the mean survival rates of a mutant from its wild type counterpart. Interactions or synergistic effects were similarly detected by subtracting the survival rate of the double or triple mutants from those of their wild type counterpart. Simultaneous determination of effect values for all phenotypes was possible by arranging the survival means linearly, and adding and subtracting in a prescribed fashion (Yates, 1937).

The analysis of variance was performed by dividing the sums of squares for dosage, phenotype, and net effect by the appropriate

degree of freedom to determine their mean square values. When these were divided by the mean square of error, the resulting variance ratios were compared using an F test for significance. An F value for a chromosome that was significant at the one or five percent level was a good indication that an important resistance factor was associated with that chromosome. Also, if more than additive interactions between chromosomes were involved in resistance, significant F values would be found for such chromosome combinations.

Enzyme Assays

The parent strains, and the substrains from backcross populations which were retained and colonized, were compared for differences in microsomal metabolism of 1-C^{14} naphthalene. Microsomes were prepared by using the procedures of Schonbrod and Terriere (1966), in which house flies, homogenized in a tissue grinder, were differentially centrifuged so that only microsomal tissue was sedimented. The subcell particles, microsomes, were incubated with 1-C^{14} naphthalene for 30 minutes. The reaction was stopped by the addition of 15 cc of ethyl ether. Aliquots of aqueous and ether phases were pipetted onto 2 x 4 cm paper sections, and after the unmetabolized naphthalene was lost by air drying, the paper sections were placed in dioxane counting solution in scintillation vials.

The remainder of the radioactivity, a measure of naphthalene metabolism, was determined with a liquid scintillation counter.

Relative yield of major radioactive metabolites from microsomal metabolism was estimated in the ether portion of the incubates by descending paper chromatography. Paper strips were spotted, then developed in a benzene:acetic acid:water (5:4:1) solvent system. The strips were scanned radiometrically to locate the areas containing the major metabolites and these were cut out and assayed for radioactivity in a scintillation counter. The ratio of the amounts of radioactivity in the active areas indicated differences in metabolism among the strains.

IV. RESULTS AND DISCUSSION

Genetical Experiments

The naphthalene resistant strain (Nap-R), the susceptible marker strain (SBO) and the F_1 populations from the reciprocal crosses were bioassayed with naphthalene vapors. The results of the assays are shown in Figure 1. The Nap-R strain was nearly ten times more resistant to naphthalene than the SBO strain. The F_1 populations were quite similar in their response to naphthalene, and exhibited a three-fold tolerance to the toxicant, indicating that resistance was neither fully dominant nor recessive.

Analysis for Dominant Factors

Over 2000 sexed house flies resulting from the backcrosses SBO♀ x F_1 (SBO♀ x Nap-R♂)♂, and SBO♀ x F_1 (Nap-R♀ x SBO♂)♂ were exposed to naphthalene vapors. Percent survival values for each phenotype were converted to arc-sin units, and the effect values calculated as previously described. Sex-linkage of resistance factors was not evident in these crosses (Figure 1), so female and male survival values were combined to permit more replication in the calculations. Results of these tests are shown in Tables IIa and IIb. The mode of inheritance of the resistance factors and their association

Table II. Analysis for Dominant Factors in Backcross Progeny Exposed to Discriminating Doses of Naphthalene.

a. Cross: stw;bwb;ocra♀ x F_1 (stw;bwb;ocra♀ x Nap-R♂)♂. Analysis based on four replications.

Phenotype	Total No. Tested	Mean Survival Rate θ [a]	R chromosome effect value [a]
+;+;+	249	49.70	178.18
+;bwb;+	217	28.40	69.04
+;+;ocra	199	39.13	32.84
+;bwb;ocra	172	17.54	13.06
stw;+;+	166	23.58	91.36
stw;bwb;+	163	3.83	16.74
stw;+;ocra	124	11.20	10.02
stw;bwb;ocra	99	4.80	13.64

b. Cross: stw;bwb;ocra♀ x F_1 (Nap-R♀ x stw;bwb;ocra ♂)♂. Analysis based on three replications.

Phenotype	Total No. Tested	Mean Survival Rate θ [a]	R chromosome effect value [a]
+;+;+	189	67.72	297.66
+;bwb;+	220	35.55	95.38
+;+;ocra	167	56.23	46.60
+;bwb;ocra	149	27.62	12.10
stw;+;+	154	45.44	76.58
stw;bwb;+	148	23.92	26.18
stw;+;ocra	139	27.13	-8.76
stw;bwb;ocra	101	14.05	-4.88

[a] Descriptions of terms in "Methods."

with chromosomes are shown by the effect values and F values and by changes in these values due to various crosses (Tsukamoto, 1964). The relative contribution of each chromosome, alone and in combination, is shown in the "effect" column. As expected, the wild type house flies had the greatest effect values, while the double and triple mutants, which could have indicated synergistic interaction, had the lowest effect values. The effect values of the chromosomes with the single mutant phenotypes stubby wing (II), brown body (III), and ocra eye (V) were at medium levels.

In the analysis for variance of these data, Tables IIIa and IIIb, the F value for differences between phenotypes was significant (one percent level). Calculation of the F values for the chromosomal effects showed that factors inherited in opposition to the stubby wing, brown body, and ocra eye chromosomes were important to resistance when in the heterozygous condition. According to the F values, the factors on the second and third chromosomes were the most important.

The relative importance of the F values for factors on chromosomes II and III varied between the reciprocal crosses. Progeny with the brown body factor (chromosome III) had a greater F value in one cross than the stubby wing factor, but the F value of the brown body factor in the reciprocal cross was second to the stubby wing factor.

Such a difference in reciprocal crosses not attributed to

Table IIIa. Analysis of Variance of the Backcross Data, Table IIa.

Source of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	F Value[a]
Total	11482.35	31	-----	-----
Phenotypes	7495.27	7	1070.75	25.65**
+;bwb;+	2383.26	1	2383.26	57.08**
+;+;ocra	539.23	1	539.23	12.91**
+;bwb;ocra	85.28	1	85.28	2.04
stw;+;+	4173.32	1	4173.32	99.96**
stw;bwb;+	140.11	1	140.11	3.35
stw;+;ocra	50.20	1	50.20	1.20
stw;bwb;ocra	93.02	1	93.02	2.23
Doses	3110.18	3	1036.72	24.83**
Error	876.90	21	41.75	-----

[a]Levels of significance with 1/21 degrees of freedom: 0.05* - 4.07
0.01**- 7.30

Table IIIb. Analysis of Variance of the Backcross Data, Table IIb.

Source of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	F Value[a]
Total	7581.61	23	-----	-----
Phenotype	6810.44	7	972.92	20.15**
+;bwb;+	3411.50	1	3411.50	70.65**
+;+;ocra	814.33	1	814.33	16.86**
+;bwb;ocra	54.90	1	54.90	1.14
stw;+;+	2199.19	1	2199.19	45.54**
stw;bwb;+	257.02	1	257.02	5.32*
stw;+;ocra	28.78	1	28.78	0.59
stw;bwb;ocra	8.93	1	8.93	0.18
Doses	95.11	2	47.55	0.98
Error	676.06	14	48.29	-----

[a]Levels of significance with 1/14 degrees of freedom: 0.05* -4.60
0.01**-8.86

sex-linked factors has been termed "maternalism" (Gardiner, 1964) and appears to be involved with chromosome III. Similar results were obtained when reciprocal crosses were made with resistant cockroaches and resistant mosquitoes (Cochran, Grayson, and Levitan, 1953; Thomas, 1966). These workers found that the strains of insects from resistant mothers were more insecticide tolerant than progeny from the reciprocal cross. It was suggested that something other than autosomal transmission, perhaps a cytoplasmic factor, was involved in maternalism.

From this analysis, it is concluded that the major dominant factor in the backcross progeny is carried by chromosome II, the stubby wing chromosome. The factor on chromosome III is next in importance, especially when derived from the Nap-R female. The small F value of the ocra eye factor indicates that chromosome V is of minor importance compared to the other two factors involved in this backcross.

Analysis for Recessive Factors

The percent survival data and chromosomal effect values from 16 bioassays of the F_2 progeny (Table Ib) are summarized in Tables IVa and IVb. The brown body factor on chromosome III had a high effect value while the stubby wing (II) and ocra eye (V) effect values were lower. As in the backcross experiments, the F value

Table IV. Analysis for Recessive Factors in F_2 Progeny Exposed to Discriminating Doses of Naphthalene.

a. Cross: F_1 (Nap-R♀ x stw;bwb;ocra♂)♀ x F_1 (Nap-R♀ x stw;bwb;ocra ♂)♂. Analysis based on nine replications.

Phenotype	Total No. Tested	Mean Survival Rate θ[a]	R chromosome effect value[a]
+;+;+	814	52.80	232.58
+;bwb;+	330	27.33	95.98
+;+;ocra	349	44.73	37.60
+;bwb;ocra	81	18.83	4.52
stw;+;+	257	39.87	54.80
stw;bwb;+	77	15.09	6.76
stw;+;ocra	83	26.88	4.46
stw;bwb;ocra	19	7.05	5.38

b. Cross: F_1 (stw;bwb;ocra♀ x Nap-R ♂)♀ x F_1 (stw;bwb; ocra♀ x Nap-R ♂)♂. Analysis based on seven replications.

Phenotype	Total No. Tested	Mean Survival Rate θ[a]	R chromosome effect value[a]
+;+;+	196	43.97	165.16
+;bwb;+	386	16.59	78.20
+;+;ocra	386	37.55	34.74
+;bwb;ocra	119	15.84	11.14
stw;+;+	216	28.34	62.74
stw;bwb;+	55	11.05	19.98
stw;+;ocra	59	11.82	-20.40
stw;bwb;ocra	16	0	0.20

[a]Descriptions of terms in "Methods."

for the F_2 phenotypes indicated that differences in tolerance between phenotypes were significant at a high level. The analysis of variance is shown in Tables Va and Vb. The stubby wing, brown body and ocra eye chromosomes all had significant F values, indicating that factors on these three chromosomes contributed to resistance in the F_2 cross. The F value of the brown body factor was about twice as great as that of the other factors.

The increased importance of chromosome III in the F_2 population is indicative of a recessive factor, which is expressed more fully when in the homozygous state. The F value of the factor on the ocra eye chromosome remains at a low level in both the backcross and F_2 populations. The stubby wing factor fluctuates somewhat, being relatively less important to resistance in the F_2 flies than in the backcross populations.

In the previous statistical analyses, the male and female mortality data were combined to give the greatest scope and replication of data. An analysis of data obtained with each sex was performed to determine if there were sex related differences in contribution to resistance. The F values from this analysis are presented in Table VI. The greatest F values were obtained from the single mutants of brown body, ocra eye, and stubby wing phenotypes. The F values for female house flies were usually much greater than male values. The effect of maternalism, as indicated

Table Va. Analysis of Variance of F_2 Data, Table IVa.

Source of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	F Value[a]
Total	44580.87	71	-----	-----
Phenotypes	15466.13	7	2209.45	15.98**
+;bwb;+	10363.68	1	10363.68	74.98**
+;+;ocra	1413.76	1	1413.76	10.22**
+;bwb;ocra	22.98	1	22.98	0.17
stw;+;+	3378.40	1	3375.40	24.40**
stw;bwb;+	51.41	1	51.41	0.37
stw;+;ocra	22.38	1	22.38	0.16
stw;bwb;ocra	32.36	1	32.56	0.23
Doses	21374.83	8	2671.85	19.33**
Error	7739.91	56	138.21	-----

[a]Levels of significance with 1/56 degrees of freedom: 0.05* - 4.02
0.01**- 7.14

Table Vb. Analysis of Variance of F_2 Data, Table IVb.

Source of Variation	Sums of Squares	Degrees of Freedom	Mean Squares	F Value[a]
Total	21945.71	66	-----	-----
Phenotypes	10672.37	7	1524.62	21.64**
+;bwb;+	5350.83	1	5350.83	75.96**
+;+;ocra	1056.01	1	1056.01	14.99**
+;bwb;ocra	108.59	1	108.59	1.54
stw;+;+	3444.27	1	3444.27	48.90**
stw;bwb;+	349.30	1	349.30	4.96*
stw;+;ocra	364.14	1	364.14	5.16*
stw;bwb;ocra	0.03	1	0.03	.00
Dose	8314.80	6	1385.80	19.67**
Error	2958.54	42	70.44	-----

[a]Levels of significance with 1/42 degrees of freedom: 0.05* - 4.07
0.01**- 7.30

Table VI. Comparison of F Values for Males and Females from F_2 and Backcross Progeny.

Phenotype	Stw;bwb;ocra♀ x F_1(stw;bwb;ocra♀ x Nap-R♂)♂		Stw;bwb;ocra♀ x F_1(Nap-R♀ x stw;bwb;ocra♂)♂	
		Backcross		
	♀	♂	♀	♂ [a]
+;bwb;+	64.38**[b]	40.27**	162.91**	
+;+;ocra	8.58*[c]	11.67*	29.47**	
+;bwb;ocra	4.84	0.13	3.05	
stw;+;+	129.21**	41.31**	57.73**	
stw;bwb;+	2.73	2.52	3.07	
stw;+;ocra	0.00	3.20	0.13	
stw;bwb;ocra	1.89	1.58	0.22	
Phenotypes	30.80**	12.80**	35.56**	

Phenotype	F_1(stw;bwb;ocra♀ x Nap-R♂)♀ x F_1(stw;bwb;ocra♀ x Nap-R♂)♂		F_1(Nap-R♀ x stw;bwb;ocra♂)♀ x F_1(Nap-R♀ x stw;bwb;ocra♂)♂	
		F_2 Cross		
	♀	♂	♀	♂
+;bwb;+	63.96**	4.66	78.20**	19.19**
+;+;ocra	12.66**	0.01	15.97**	0.09
+;bwb;ocra	0.09	1.38	0.20	6.22*
stw;+;+	36.59**	4.46	23.88**	8.59*
stw;bwb;+	3.11	0.72	0.63	16.36**
stw;+;ocra	5.19*	0.13	1.02	1.89
stw;bwb;ocra	0.04	0.02	0.20	0.54
Phenotypes	17.34**	1.79	17.15**	7.56*

[a] Insufficient number of replications to complete analysis.

[b] Indicates significance at the 1% level.

[c] Indicates significance at the 5% level.

by the F values, increased the resistance of both sexes in the F_2 and backcross progeny when the original cross was made with the Nap-R females. Results of Table VI indicate that the factors for resistance reside on the same chromosomes in both male and female houseflies, but expression of the male genotype in terms of resistance is less than in the female.

This genetical study showed factors on three chromosomes to be involved in naphthalene resistance. The factor on chromosome II was important when heterozygous in the backcross progeny, thus behaving as a dominant factor. The third chromosomal factor was more important in the F_2 cross than in the backcross, a characteristic of recessive or incompletely recessive genes. Maternalism appears to be involved with this factor. The fifth chromosomal factor, of a dominant nature, has low statistical significance in both crosses and is the least important of the three chromosomes. These genetical studies indicated which substrains should be studied in further in vivo and in vitro experiments.

Toxicological Experiments

Substrains in which one or more chromosomes from the resistant parent were replaced with homologous chromosomes from the susceptible mutant marker strain were established for further study. The phenotypes +;bwb;ocra, stw;+;ocra, stw;bwb;+, and

+;+;ocra were isolated from the backcross progeny and colonized separately. These strains are referred to as Nap II, Nap III, Nap V, and Nap II + III to indicate the chromosome (s) with resistance factors derived from the Nap-R parent. Each substrain was selected with naphthalene to assure the homozygosity of factors for resistance. Over five hundred females of each strain were tested with naphthalene vapors to determine the LT_{50} and LT_{90} values given in Table VII. The Nap-R house flies were the most resistant strain, followed by the Nap II + III strain. The Nap II, Nap III, and Nap V substrains gave LT_{50} values at least two times that of the SBO susceptible strain. Both in vivo and in vitro tests for specific mechanisms of resistance were performed on these substrains.

Table VII. Toxicity of Naphthalene Vapors to House Flies of Six Strains.

Phenotype	Chromosome from R parent			Strain[a] Designation	LT_{50} Hr.	LT_{90} Hr.
	II	III	V			
+;bwb;ocra	R	-	-	Nap II	3.6	5.2
stw;+;ocra	-	R	-	Nap III	3.9	6.0
stw;bwb;+	-	-	R	Nap V	3.6	7.25
+;+;ocra	R	R	-	Nap II + III	8.0	13.0
stw;bwb;ocra	-	-	-	SBO	1.8	2.9
+;+;+	R	R	R	Nap-R	17.0	24.0

[a]These strain designations used in following tables.

Absorption of Insecticides

Sawicki and Farnham (1968) reported that knockdown resistance to topically applied dieldrin in the SKA strain of house flies was due to slower penetration of the insecticide through the cuticle. In similar work by Plapp and Hoyer (1968), a factor for delayed knockdown due to slow absorption of insecticides was important as an intensifier of resistance in certain house fly strains in their laboratory. When isolated in a strain, this slow absorption factor caused resistance to organotin compounds.

Since knockdown response was a good indicator of absorption rate in several fly strains, the technique was used in the present work. The parent and Nap-R substrains were assayed to determine if a similar factor was involved in naphthalene resistance.

Three insecticides, dieldrin, naphthalene and tributyltin chloride (TBTC), and three methods of application were used in knockdown tests. The organotin-resistant strain (tin-R) studied by Plapp and Hoyer (1968) was included in these assays for comparison with the Nap-R strains.

The times required for fifty percent knockdown of the flies by naphthalene, dieldrin and TBTC are shown in Table VIII. The knockdown resistant Nap III, Nap II + III, and tin-R strains were quite uniform in their response to the three toxicants, as were the

Table VIII. Knockdown Response of House Fly Strains Exposed to Three Insecticides.

Strain	Minutes to 50% knockdown					
	Naphthalene[a]		TBTC[b]		Dieldrin[c]	
	Number Tested	Minutes ± S.D.	Number Tested	Minutes ± S.D.	Number Tested	Minutes ± S.D.
Nap II	100	15 ± 4	170	29 ± 2	80	117 ± 24
Nap III	70	30 ± 0	80	63 + 0	120	161 ± 49
Nap V	70	15 ± 4	170	27 ± 7	120	127 ± 40
Nap II + III	50	39 ± 8	90	76 ± 0	80	165 ± 28
SBO	100	11 ± 4	120	18 ± 8	145	111 ± 30
Nap-R	120	63 ± 14	40	160 ± 0	145	185 ± 50
Tin-R	50	36 ± 3	80	85 ± 1	105	175 ± 33

[a] Exposed to naphthalene vapors in one gallon jar

[b] Exposed to TBTC film at 250 μg/pint jar

[c] Dieldrin applied to thorax at 1 μg/fly

susceptible SBO, Nap II, and Nap V strains. The two strains with the third chromosomal factor, Nap III and Nap II + III, were nearly three times more resistant to knockdown than the other substrains to naphthalene and TBTC. A maximum of six to eight fold difference in rate of knockdown was found between the SBO and Nap-R strains. These data are presented graphically in Figures 2 and 3.

Time-response lines of the tin-R strain and Nap-R flies with the chromosome III factor were similar (Figures 2 and 3). This is the chromosome found in previous studies to be important in the delayed knockdown response of flies because of slow absorption of the toxicant. Thus, it seems likely that the knockdown resistance exhibited by the naphthalene selected flies was due to a similar slow absorption factor on chromosome III.

The factor for delayed knockdown in the Nap-R strains appeared to be semi-specific, as resistance was observed in compounds of high and low vapor pressure, and in both vapor and solid states. However, little protection was noted when the Nap-R flies were tested to a second fumigant, para-dichlorobenzene. The tolerance to TBTC and dieldrin indicates that the factor on the brown body chromosome was probably a non-metabolic resistance factor.

An interesting exception to the general pattern of knockdown rates is the flattened time-response line of the Nap II substrain treated with dieldrin. Perhaps a portion of the flies of this strain

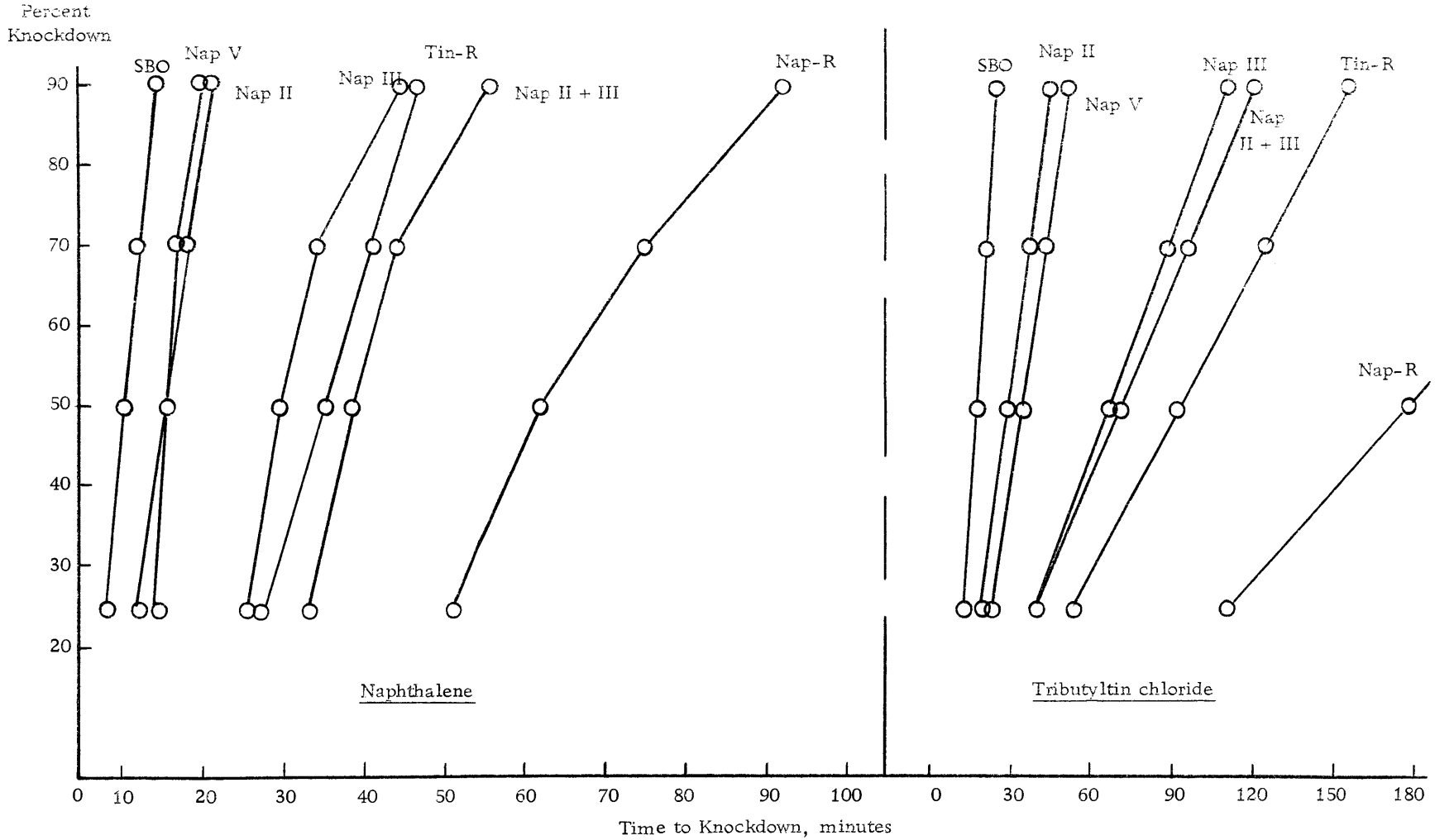


Figure 2. Rate of knockdown of house fly strains exposed to naphthalene vapors and tributyltin chloride residues.

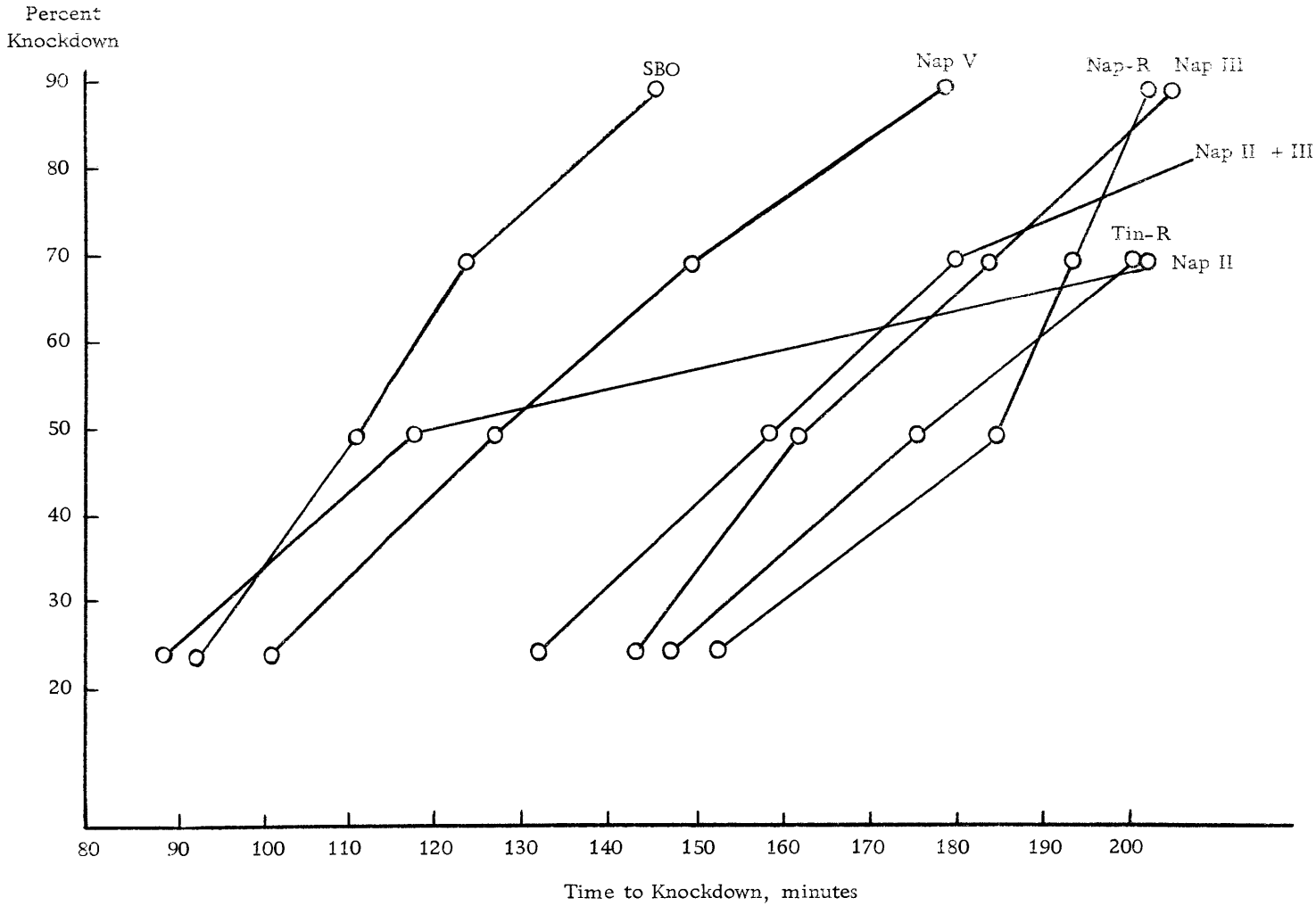


Figure 3. Rate of knockdown of house fly strains treated topically with dieldrin.

have a chemical or physical barrier which protects sensitive nerves from attack by internal dieldrin, and allows some knockdown resistance.

Microsomal Metabolism

The genetical study indicated that three factors are involved in naphthalene resistance. Knockdown assays suggested that slow absorption of the toxicant was one of these. But this factor conferred only part of the total resistance seen in the Nap-R strain (Table VII). Another mechanism, the metabolism of naphthalene, was examined as a second reason for the resistance levels of some strains. This detoxication process was investigated by studying the microsomal hydroxylation of naphthalene.

A measurement of naphthalene metabolism by microsomes from the substrains permitted correlation of the hydroxylative function with genetic make-up and resistance. Results of these experiments are shown in Table IX, where six strains are compared. Because age is a factor in the rate of naphthalene metabolism (Schonbrod et al., 1965), the flies were compared at several ages.

Activity was usually greatest in the Nap-R and SBO parent strains and in the substrains possessing chromosome II from the resistant parent. The activity of the strains generally increased with age. Differences between the Nap-R parents and the substrains

were not as great as expected, and the high oxidase activity of the SBO strain was quite unexpected. These results indicated that microsomes from the susceptible SBO strain and resistant Nap-R strain have quite similar rates of naphthalene metabolism.

Table IX. Hydroxylation of Naphthalene 1-C^{14} by Microsomes Prepared from House Flies of Different Ages.

	mμ Moles hydroxylation products/fly with age as indicated				
Strain	7 da[b]	8 da[a]	15 da[a]	15 da[a]	16 da[b]
Nap II	0.232	0.377	0.880	0.699	0.422
Nap III	0.352	0.290	0.335	0.437	0.234
Nap V	0.217	0.228	0.353	0.320	0.235
Nap II + III	0.226	0.357	0.719	0.518	0.351
SBO	0.375	0.512	0.503	0.933	0.156
Nap-R	0.369	0.428	0.613	0.862	0.297

[a] 30 minute incubation

[b] 15 minute incubation

To explain the oxidase level of the SBO strain, consideration was given to the possibility that endogenous inhibitors of microsomal enzymes, reported by Mathews and Hodgson (1966), were involved. Hook et al. (1968) suggested that enzymatic oxidation rates in insects could differ if amounts of inhibitor changed with age. It was reasoned that the activity of such inhibitors might also differ between strains of flies. For example, if the SBO strain contained

less inhibitor than the Nap-R flies, the microsomal oxidases might appear correspondingly active.

Bovine serum albumen (BSA) was found by Tsukamoto and Casida (1967) to reduce the influence of inhibitory materials released at the time of homogenization and thereby to enhance microsomal activity. The results of an experiment in which BSA was used in the preparation of microsomes are given in Table X. The effect of BSA was to increase the activity of all strains at both ages tested, although the increase among the eight-day-old house fly strains was smaller than in the fifteen-day-old group. At fifteen days, a two-fold increase in activity occurred in the Nap II and Nap II + III strains, and smaller increases were seen in the other strains. Although BSA affected the oxidase activity of the strains, the relative position of the strains did not change. It was concluded from this experiment that inhibitor activities were nearly similar in all the strains and that the SBO strain must possess a naphthalene degrading enzyme system that does not protect the flies in vivo.

House flies possess two enzyme systems which metabolize naphthalene to two primary products (Schonbrod and Terriere, 1966). Differences in the production of 1-naphthol and 1,2-dihydroxy-1,2-dihydro naphthalene (naphthalene diol) in resistant and susceptible flies might indicate which enzyme system was most important in naphthalene resistance. Such differences might also explain why

the SBO strain could hydroxylate naphthalene rapidly, but still remain susceptible to its vapor.

Table X. Effect of BSA Supplement on the Hydroxylation of Naphthalene 1-C^{14} by House Fly Microsomes.

	mμ Moles hydroxylation products/fly with age as indicated	
Strain	8 da	15 da
Nap II	0.575	1.280
Nap III	0.296	0.492
Nap V	0.356	0.378
Nap II + III	0.388	0.848
SBO	0.575	0.616[a]
Nap-R	0.431	0.695

[a]From 8 day old flies.

Differences in the amounts of these metabolites produced by each substrain were assessed by paper chromatography, with results given in Table XI. The Nap-R, Nap II + III and Nap II strains were found to have a naphthalene diol:1-naphthol ratio of 1.5 to 1.7, while the ratio in the SBO, Nap III, and Nap V strains was about 1.0. Therefore, the Nap-R, the Nap II and the Nap II + III strains differed from the other strains by producing at least 50 percent more naphthalene diol than 1-naphthol in the metabolism of naphthalene. Those strains with the increased naphthalene diol production,

Table XI. Paper Chromatographic Resolution of Naphthalene Diol and 1-Naphthol Produced During Hydroxylation of Naphthalene 1-C^{14} by House Fly Microsomes.

Strain	Experiment No.	Fly age, days	Amount present, DPM		Diol/1-naphthol	
			Diol	1-naphthol	Ratio	Average ratio
Nap II	1	15	1539	755	2.04	1.49
	2	15	9687	10807	0.90	
	3	7	1876	1413	1.33	
	4	16	10072	5989	1.68	
Nap III	1	15	766	732	1.05	1.16
	2	15	5185	6697	0.77	
	3	7	4036	2896	1.39	
	4	16	4872	3430	1.42	
Nap V	1	15	515	494	1.04	0.94
	2	15	1100	2107	0.52	
	3	7	2506	3506	0.71	
	4	16	3724	2519	1.48	
Nap II + III	1	15	849	481	1.76	1.57
	2	15	10440	11859	0.88	
	3	7	3213	1977	1.62	
	4	16	7923	3875	2.04	
SBO	2	15	12813	14659	0.87	0.99
	3	7	5619	4201	1.34	
	4	16	938	1423	0.66	
	5	14	9423	8435	1.11	
Nap-R	1	15	195	167	1.17	1.69
	2	15	3565	3936	0.90	
	3	7	6996	4068	1.72	
	4	16	4377	2707	1.67	
	5	14	17740	5824	3.05	

especially the Nap-R and Nap II + III strains, were most resistant to naphthalene vapors (Table VII).

Thus, the hydroxylation of naphthalene by the SBO flies, where equivalent amounts of 1-naphthol and naphthalene diol were formed, did not confer resistance. The association of increased naphthalene diol production and house fly resistance suggests that selection of house flies with naphthalene vapors results in selection of the diol-producing enzyme system, which apparently confers resistance.

To further clarify the role of chromosome II in naphthalene resistance, another oxidative reaction, the conversion of aldrin to dieldrin by epoxidation, was measured with microsomes from the house fly strains. The Milan standard reference strain (SRS) was included as a second susceptible strain. The amount of dieldrin produced by the epoxidase reaction was determined by gas chromatography and compared with naphthalene hydroxylation values obtained at the same time from the same strains. Results are shown in Table XII. The relative hydroxylase activity of the strains was similar to that found previously (Table IX). However, striking differences were seen in epoxidase activity. The Nap-R strain formed dieldrin three times faster than the SBO strain. High epoxidase activity was also found in the Nap II and Nap II + III substrains. Low activity was found in the Nap III, Nap V, Milan (SRS), and SBO strains. Microsomes from substrains with the chromosome II

factor were nearly ten times more active than the substrains without this factor.

Table XII. Microsomal Aldrin Epoxidase and Naphthalene Hydroxylase Activity in Seven House Fly Strains.[a]

Strain	mμ Moles product/fly	
	Naphthalene Hydroxylation	Aldrin Epoxidation
Nap II	0.699	0.257
Nap III	0.437	0.039
Nap V	0.320	0.025
Nap II + III	0.518	0.258
SBO	0.933	0.102
Nap-R	0.862	0.383
Milan (SRS)	0.529	0.060

[a]Age of all strains 15 days.

Thus, differences in hydroxylase values were not a good reflection of the resistance levels of the substrains, but the production of diol and the epoxidation of aldrin did correlate with resistance. The epoxidase enzyme system associated with chromosome II apparently offers more protection to flies than high levels of the "natural oxidase" found in the SBO flies (Table IX). The metabolic detoxication of naphthalene by an oxidative enzyme system is probably the resistance factor the genetics study showed to be important on chromosome II.

The factor on the fifth chromosome did not appear to be

related to slow absorption or directly involved in the hydroxylation of naphthalene. The Nap V strain did not have any unusual epoxidase activity (Table XII). While the results of the genetical study indicated that a minor resistance factor was involved with this chromosome, none of these studies gave clues as to its role in resistance.

Summary and Conclusions

From the preceding discussion it is evident that two factors confer most of the resistance to naphthalene in the Nap-R strain. Flies with either a homozygous recessive condition for the slow absorption factor, or a heterozygous or homozygous condition for the dominant oxidation factor were partially tolerant to naphthalene. When the absorption factor and the oxidation factor were combined in a single strain, the Nap II + III strain, a higher tolerance level was found (Table VII). The resistance of the Nap II + III strain was exceeded only by the Nap-R strain. Apparently, the slow absorption factor and the detoxication system, in combination, contribute more than additive protection against naphthalene vapors.

The factor on chromosome V must also contribute to the resistance of the Nap-R flies because the factors on chromosomes II and III accounted for only one-half of the total resistance. As with chromosome II and III, combining resistance factors on chromosome V with another factor might increase its effect on

resistance. For example, if the chromosome V factor was important in detoxifying products from naphthalene metabolism, its potential effect might not be seen unless it was coupled with the oxidative factor on chromosome II.

Similar results, in which a combination of factors was necessary for maximum resistance, are seen in a genetical and biochemical study by Tsukamoto et al. (1968). Genetical analyses showed several chromosomes to be important in resistance to the carbamates Baygon and Matacil. A factor on chromosome II was important in the oxidation of these insecticides. However, in toxicity studies, when this oxidation factor was not in combination with one of the other chromosomal factors, little was contributed to resistance in terms of survival rate.

In the study by Schonbrod et al. (1968), the in vitro oxidase activity of various resistant strains was not well correlated with observed tolerances to naphthalene vapors, a point which appeared to contradict previous work (Schonbrod et al., 1965). This discrepancy can now be explained by considering the multifactorial nature of naphthalene resistance. As seen, the observed resistance of the Nap-R strain is dependent upon both metabolic and non-metabolic factors. While a detoxication mechanism, such as the microsomal oxidase system, can be an effective defense mechanism in vivo, it protects against naphthalene mainly when coupled with

other factors that may modify the toxic effect of this compound until it is detoxified. Therefore, oxidative ability alone is not a good index of naphthalene resistance because of the importance of non-metabolic factors in conferring a high degree of resistance.

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