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I am submitting herewith a thesis written by Carla Ann Gunderson entitled "Comparison of the Toxicity of Pulegone in Larvae of Spodoptera eridania and S. frugiperda: Possible Involvement of the Glutathione S-Transferase System." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Lena B. Brattsten, Major Professor

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Graduate Studies and Research

COMPARISON OF THE TOXICITY OF PULEGONE IN LARVAE OF *SPODOPTERA ERIDANIA* AND *S. FRUGIPERDA*: POSSIBLE INVOLVEMENT OF THE GLUTATHIONE S-TRANSFERASE SYSTEM

A Thesis

Presented for the

Master of Science

Degree

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Carla Ann Gunderson

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DEDICATION

This thesis is dedicated to my mother and to my father, who passed away during its preparation. Without their love and support, my education would not have been complete.

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ABSTRACT

The acute and chronic toxicities of pulegone and menthofuran to larvae of the southern and fall armyworms (Spodoptera eridania and S. frugiperda) were compared.

The chronic effects of pulegone and menthofuran were more severe in the fall armyworm. Menthofuran was chronically more toxic than pulegone in both species. Acute toxicities exhibit the reverse of these trends. Possible explanations are discussed.

Glutathione S-transferase activity was investigated as a likely factor in differential pulegone detoxification. Endogenous activity was determined in the fatbody, malpighian tubules and testes of both species, and in the gut of both species throughout the last larval instar. Induction of enzymatic activity by dietary chemicals, including several monoterpenes, was studied.

Glutathione S-transferase activity was very similar in the gut and testes in both species, and fairly constant in the gut throughout the last instar. Fatbody and malpighian tubules, however, had higher activity in the southern armyworm. Probably the most significant species difference was the inducibility of activity. Southern armyworm transferase activities were induced to consistently higher levels than were those of the fall armyworm.

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CHAPTER I

GENERAL INTRODUCTION

The interactions and adaptions between insect herbivores and their host plants have been under a great deal of scrutiny since Fraenkel (1959) brought out the importance of plant "secondary substances" both in plant defense against herbivores and in host plant selection by the insects. Many of these compounds which appear to have little or no function in the plant's primary metabolism are now considered to be adaptions to the various pressures of herbivory (Beck and Reese, 1976; Fraenkel, 1959; Whittaker and Feeny, 1971; Rhoades, 1979). Secondary plant substances, now appropriately called allelochemicals in recognition of their primary ecological importance, may adversely affect the feeding and/or reproductive behaviors of the insects. They may also exert actual toxic effects by disrupting essential developmental or physiological processes (Beck and Reese, 1976). Underlying biochemical lesions may result in acute toxicity, or in chronic toxicity due to decreased growth, fecundity, and survival in the insect population (Rhoades, 1979; Waldbauer, 1968).

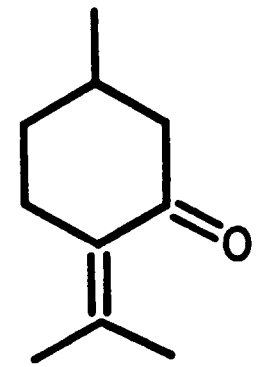
Insect populations, of course, have had the same opportunities for biochemical adaptations as have the plants and, during the long period of their coexistence,

the adaptive mechanisms of the insects have not been idle. Many of the initially repellent allelochemicals have been exploited as specific feeding attractants, feeding cues, and oviposition stimuli. Such specializations may actually benefit the plant, as in the case of pollination attractants. They may also be used to render the insect unpalatable or toxic by sequestration, or may even be used as building blocks for pheromones and hormones (Beck and Reese, 1976; Blum, 1978; Duffey, 1980; Fraenkel, 1959; Pliske, 1975; Rhoades, 1979).

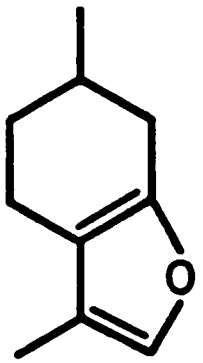
The metabolic and physiological defenses of plants have been, in many cases, similarly defused by their predators. An entire battery of biochemical mechanisms now exists for the detoxification of foreign compounds by insects as well as by other herbivores (Brattsten, 1979; Scheline, 1978). Metabolic degradation of a toxicant to excretable products may take place in several steps, involving more than one enzyme system, and may change the substance by such mechanisms as oxidation, reduction, hydrolysis, or conjugation to any of a number of polar molecules (Brattsten, 1979). Just as plant allelochemicals are not uniformly distributed throughout the kingdom, the biochemical defenses of herbivores vary a great deal among the various taxa. It is this variation in detoxifying ability between species with which this thesis is concerned.

A major class of plant allelochemicals known to affect herbivores is the terpenoids (Mabry and Gill, 1979; Mann, 1978; Scheline, 1978). Monoterpenes, the 10-carbon members of this group of mevalonate-derived compounds, are often found as volatile components of the "essential oils" and act to deter or attract animals by their odor (Mabry and Gill, 1978). Many plants of the family Labiatae (the mints) contain large amounts of monoterpenes (Clark and Menary, 1981; Leung, 1980; Masada, 1976). The monoterpene pulegone (Figure 1) occurs in particularly high concentration in pennyroyal, Mentha pulegium (L.) (Masada, 1976), a volatile oil consisting of 85% pulegone (Zalkow et al., 1979). Peppermint (M. piperita) generally contains less of this compound, but may have high levels of the closely related monoterpene, menthofuran (Figure 1) (Clark and Menary, 1981; Masada, 1976). Both pulegone and menthofuran are undesirable in commercial mint production and in some cases the essential oils require fractionation to remove them (Clark and Menary, 1981).

The pennyroyal plant has a long history of use in herbal folk medicine, both as a flea, mosquito, and tick repellent and for induction of uterine contractions; in fact, the essential oil is reported to induce miscarriage (Padus, 1982; Rose, 1972). Folklore thus suggests both the insect deterrence and the toxicity of pulegone. This potential was borne out in a study



Pulegone



Menthofuran

Figure 1. Structures of pulegone and menthofuran.

with a polyphagous herbivore, the larva of the fall armyworm, Spodoptera frugiperda (J. E. Smith), Noctuidae. Zalkow et al. (1979) found oil of pennyroyal to be highly toxic to second and third instar larvae and to inhibit feeding in sixth instar larvae. Interestingly, however, subsequent studies in our laboratory showed that a close relative, the southern armyworm, S. eridania Cramer, was not affected to nearly the same degree (Gunderson et al., 1983). This difference in biological activity is the subject of the current investigation.

There are, as with other xenobiotic compounds, several possible metabolic detoxification mechanisms for pulegone. The cytochrome P-450-dependent polysubstrate mono-oxygenase, PSMO (nomenclature recommended in 1980, formerly known as mixed function oxidase, or MFO), enzymes are the most important factor in the primary metabolism of lipophilic xenobiotics (Brattsten, 1979). They are also an important step in pulegone metabolism. Brattsten et al. (1983) report that southern armyworm microsomal preparations metabolize pulegone largely to 10-hydroxypulegone and menthofuran, a rearrangement product of 9-hydroxypulegone. Menthofuran, however, can hardly be considered a detoxification product, as it has been found to inhibit growth in the southern armyworm much more than does pulegone, the parent compound (Gunderson et al., 1983). Nor do PSMO-inducing

diets decrease acute pulegone toxicity (Gunderson et al., 1983) as they would if this system detoxified pulegone as it does many insecticides (Brattsten and Wilkinson, 1973; Brattsten et al., 1977).

Another enzyme system must therefore be involved in pulegone detoxification. A strong candidate is glutathione S-transferase, a cytosolic enzyme complex involved in the detoxification of numerous pesticides (Fukami, 1980; Motoyama and Dauterman, 1978), alkylating agents, carcinogens, and other xenobiotics with electrophilic centers by conjugation to glutathione (Chasseaud, 1979). This enzyme system is examined here as a possible explanation of the selectivity of pulegone toxicity in the fall and southern armyworms.

CHAPTER II

TOXICITY OF PULEGONE AND MENTHOFURAN: EFFECTS ON GROWTH AND SURVIVAL

Introduction

Plant allelochemicals are, by definition, compounds produced by one species which "affect the growth, health, behavior, or population biology of organisms of another species" in a manner other than nutritional (Whittaker and Feeny, 1979). If the interaction benefits the "receiving organism" (here, the insect predator), the compound is referred to as a kairomone; if the producer (here, the host plant) maintains the advantage, the compound is designated an allomone (Beck and Reese, 1976; Reese, 1979). An allomone, e.g., pulegone or menthofuran, may act against the potential consumer by repelling or deterring feeding, reducing digestibility, inhibiting developmental processes, or by exerting direct toxicity (Beck and Reese, 1976; Reese, 1979; Zalkow et al., 1979).

Repellents and deterrents (as well as the kairomonal attractants) are often critical in host plant selection (Fraenkel, 1959; Pliske, 1975; Whittaker and Feeny, 1971). Perception of these distasteful compounds is usually accomplished by the senses of olfaction and gustation.

Stimulating chemicals reach the chemosensory sensilla either from direct contact with the plant surface, or as volatile components in the air. Avoidance behavior in response to such a sensation is usually innate (Chapman and Blaney, 1979; Pliske, 1975).

Feeding preferences in insects can be quantified in a number of ways, including comparisons of food mass consumed (Waldbauer, 1968), mass consumed per time involved in chewing (Rausher, 1981), or percent of leaf area consumed (Zalkow et al., 1979). By determining the number of individual insects finishing a treated leaf disc of a given size in 8 minutes, Gunderson et al. (1983) showed that pulegone itself did not deter feeding by the southern armyworm at concentrations of up to 4% of the mass of the leaf tissue, although higher concentrations were inhibitory. Zalkow et al. (1979) showed a strong anti-feedant activity of pulegone toward the fall armyworm; leaf discs dipped in an acetone solution of oil of pennyroyal inhibited feeding at concentrations of 0.1% and higher.

Even allelochemicals which are readily accepted in food may exert toxic effects. Adverse effects on the health, growth, survival, or reproduction of herbivores are widespread among plant-insect interactions, as insects do not always recognize toxic compounds as distasteful (Fraenkel, 1959). Although more common in vertebrate

herbivores, acutely toxic reactions to plant secondary compounds, e.g., pyrethrins and some glycoalkaloids, are sometimes found in insects (Fraenkel, 1959; Whittaker and Feeny, 1971).

More significant, however, are the chronic toxicities of allelochemicals which interfere with insect growth and development. Such allomones, when ingested as a component of foliage or in artificial diets, often reduce insect growth (measured as weight gain over time) to below normal (optimal) levels (Fraenkel, 1959; Soo Hoo and Fraenkel, 1966; Waldbauer, 1968). Decreased food consumption or inability to utilize (digest and/or convert to body tissue) the ingested food may cause a chronic growth reduction (Beck and Reese, 1976; Scriber, 1981; Soo Hoo and Fraenkel, 1966; Waldbauer, 1968).

Growth rate is not the only parameter affected by allelochemicals. Maximum size attained, survival over prolonged periods, development (including pupation and emergence of adults), and fecundity can all be influenced (Applebaum and Birk, 1979; Fraenkel, 1959). The effects of pulegone on each of these factors have been demonstrated in the southern armyworm (Gunderson et al., 1983). In the present study the effects of pulegone (and of its oxidation product, menthofuran) on growth (change in fresh weight) and survival from the fourth larval instar to pupation have been examined and compared in the two species

of Spodoptera. Acute toxicity of the two monoterpenes to sixth instar larvae of both species has also been determined to obtain a better understanding of the relative in vivo effects in the two species.

Materials and Methods

Chemicals. Ingredients for the insect diets were obtained from the following sources: Bacto-agar from Difco Laboratories, Detroit, MI; sorbic acid, cholesterol (USP), vitamin free casein, and linolenic acid from United States Biochemical Corp., Cleveland, OH; brewer's yeast and Vanderzant's vitamin mixture from Bioserv, Inc., Frenchtown, NJ; salt mixture W and alphacel from ICN Nutritional Biochemicals, Cleveland, OH; and ascorbic acid from Fisher. Pinto beans, sucrose (sugar), and Kretschmer wheat germ (International Multifoods, Minneapolis, MN) were purchased at the supermarket. Bean leaf powder was prepared in a Waring Blendor from lyophilized leaves of kidney and lima beans (Phaseolus vulgaris and P. lunatus) grown under fluorescent lights.

(+)Pulegone was purchased from Aldrich Chemical Co., Milwaukee, WI, and menthofuran from Eastman Kodak, Rochester, NY.

Insects. A stock colony of the southern armyworm was maintained on the foliage of lima and kidney beans

as described elsewhere (Brattsten et al., 1980), using eggs originally obtained from Dr. C. F. Wilkinson, Cornell University, and Niagara Chemical Division, Food Machinery Corp., Middleport, NY. Larvae were collected for experiments from this colony at the appropriate life stage--late third to early fourth instar larvae for the growth studies and newly molted sixth instar larvae for the acute toxicity assays.

A colony of the fall armyworm was maintained in the laboratory in large (14 cm) plastic petri dishes on cubes of an artificial diet (Shorey and Hale, 1965). Eggs were originally obtained from Dr. P. B. Martin, Tifton, GA. Larvae of appropriate ages were collected from this colony when needed as from the southern armyworm colony.

Experimental diets. Growth curves for the fourth through sixth instar larvae were obtained by raising the larvae on artificial diets. The control diet for the southern armyworm consisted of a modification of the semi-defined diet described by Brattsten and Wilkinson (1973). The diet contained 2.0% bean leaf powder, 0.6% alphacel, casein, and sucrose; 0.1% vitamin mixture, wheat germ, cholesterol, salt mixture, and linolenic acid; and 0.75% agar, on a weight to volume basis. Various concentrations of pulegone or menthofuran were added to the basic diet on a percent fresh weight basis.

A modification of the basic diet described by Shorey and Hale (1965) was used as a control diet for the fall armyworm. Sorbic acid was the only mold inhibitor included. Either pulegone or menthofuran was incorporated in test diets during blending.

Weight determinations. Individuals of each species were isolated from the stock culture as molting third instar or newly molted fourth instar larvae. Twenty-five insects were weighed individually on a top-loading Mettler PC 200 balance and placed in a 14 cm diameter petri dish. In the case of the southern armyworm, the center of the lid was replaced with a 10 cm circle of nylon screening to allow air circulation.

Larvae were provided with diet ad libitum (fresh each day) and individually weighed and counted each day. As they grew, the larvae were separated into smaller groups in a sufficient number of petri dishes to avoid crowding. When the larvae began to void their guts and form prepupae they were provided with Bacto autoclaved potting soil for burrowing and were no longer weighed daily. Each point on the growth curve thus represents an average weight for the larvae still alive and not burrowing.

Acute toxicities. Newly molted sixth instar larvae were collected and fed standard control diet for 2 days.

The larvae, in groups of 10, were isolated in individual compartments of glass-covered ice cube trays without food for 1 hour. The larvae were then fed, individually, discs of lima or kidney bean leaf cut with a number 5 cork borer, either plain (as a control) or treated with pulegone or menthofuran. The monoterpenes were applied to the leaf discs, either neat or in ethanol solution, in amounts corresponding to doses ranging from 250 µg/g to 20,000 µg/g of larval body weight. The test compounds were applied with a glass syringe in a Burkard Arnold hand microapplicator. After 24 hours the larvae were observed for mortality. Median lethal dose values (LD_{50}'s) were estimated from eye-fitted lines on log-dose probit paper (Finney, 1971).

Results

Acute toxicities. The LD_{50}'s of pulegone and menthofuran (Table 1) were determined from experiments in which hungry larvae were given, as their only food, leaf discs treated with measured aliquots of the monoterpene. Since these compounds have relatively low acute toxicities and deter feeding at high concentrations, the LD_{50}'s do not represent strictly oral doses but rather doses obtained through a combination of oral, dermal, and vapor phase exposure. The larvae tended not to eat the entire leaf disc presented for doses greater than 5000 µg/g of larval

Table 1. Acute Toxicity of Pulegone and Menthofuran to Sixth Instar Fall and Southern Armyworm Larvae.

Species	LD_{50} (µg/g)** Pulegone	Menthofuran
Spodoptera frugiperda	6167 (7600,5800,5100)	7633 (6000,8000,8900
Spodoptera eridania*	1480 (1520,1420,1500)	2172 (2000,2060,2460)

*Gunderson et al., 1983.

**Mean (individual values).

weight and the larger volumes (up to 8 µl) of monoterpene were not absorbed by the leaf tissue. It rather remained on the surface where the larvae contacted it topically. Larvae exposed in this manner frequently writhed and tried to clean themselves, often dying within less than an hour at the highest concentrations.

In acute exposures, menthofuran was slightly less toxic than pulegone to both species, and both monoterpenes were substantially less toxic to S. frugiperda than to S. eridania (Table 1).

Growth curves. Figures 2 through 5 represent the change in weight (growth) of armyworm larvae from the fourth through sixth instars until the larvae began to pupate. Each point represents the mean fresh weight of 25 (or less, when less survived) individuals. The standard errors of the means are indicated by bars on the control curves. Means and their standard errors for all data points used in Figure 4 are given in Table 2. Additional repetitions of these growth experiments yielded essentially similar curves (not presented here).

Figure 2 shows that the growth of southern armyworm larvae was not inhibited, but actually stimulated, by both 0.01% and 0.1% pulegone in the diet. Larvae fed freely and developed at essentially the same rate as larvae raised on control diet. The maximal fresh weights

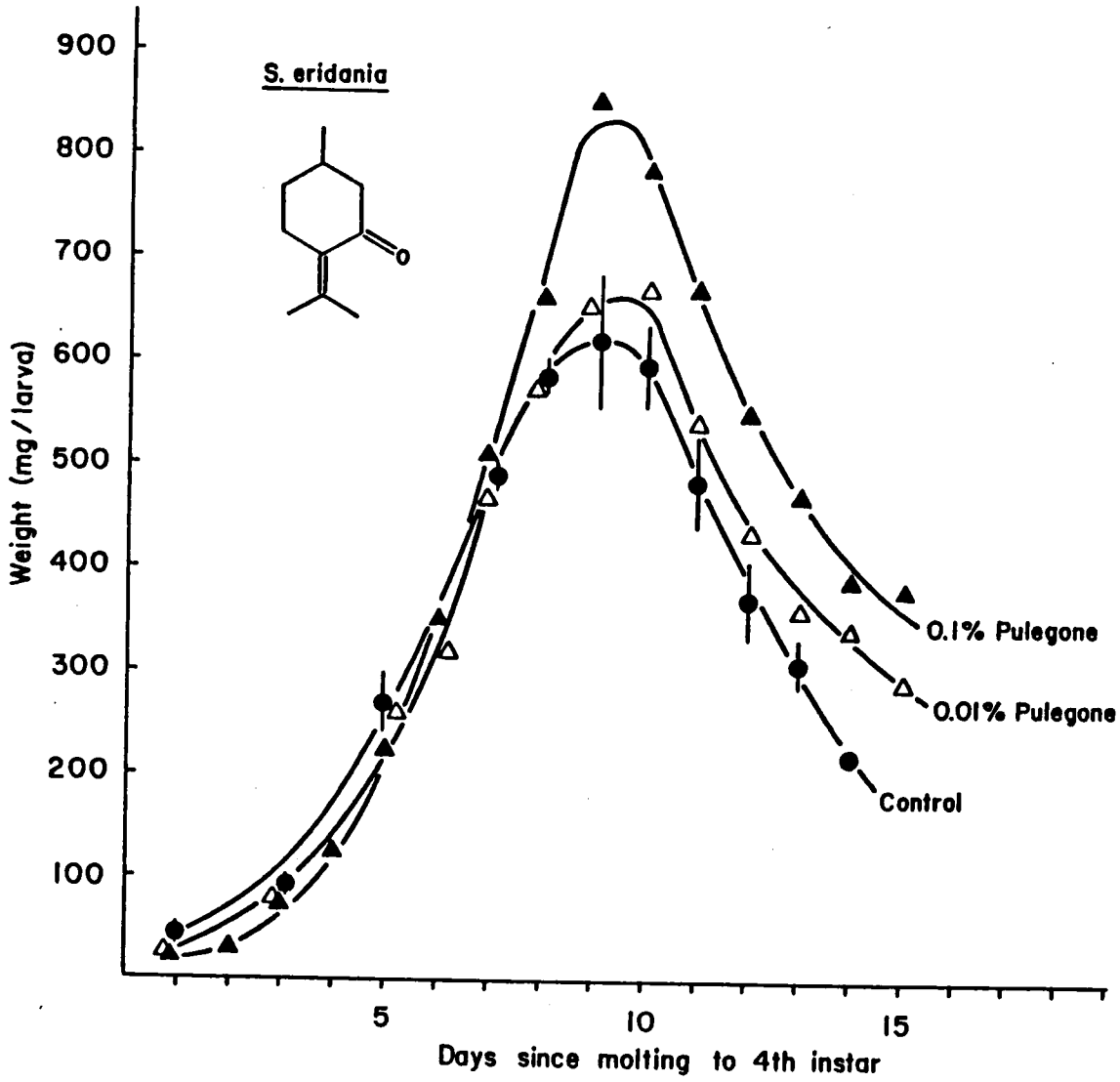


Figure 2. Growth of Spodoptera eridania on control and pulegone-containing diets from the fourth through sixth instars.

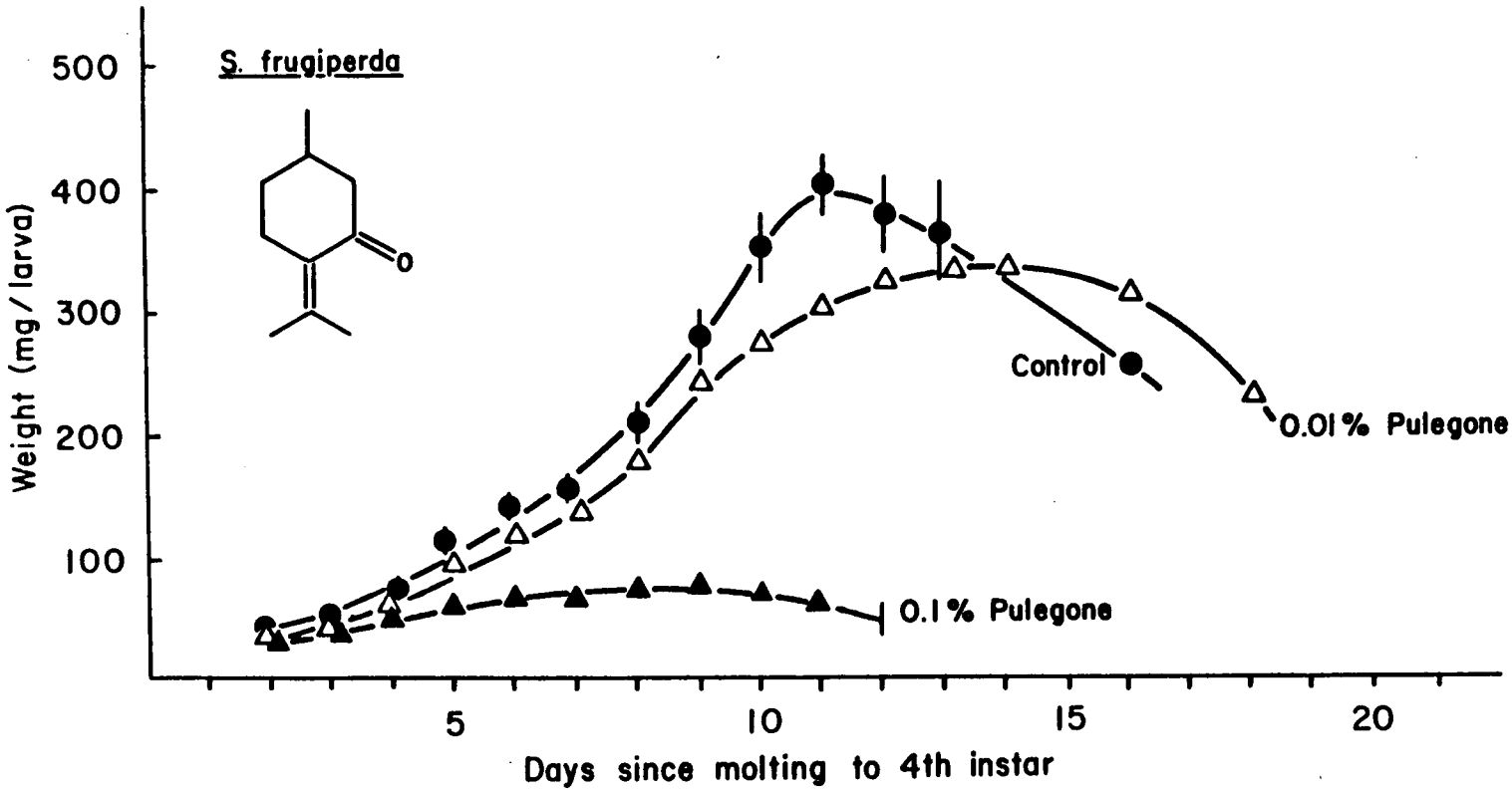


Figure 3. Growth of Spodoptera frugiperda on control and pulegone-containing diets from the fourth through sixth instars.

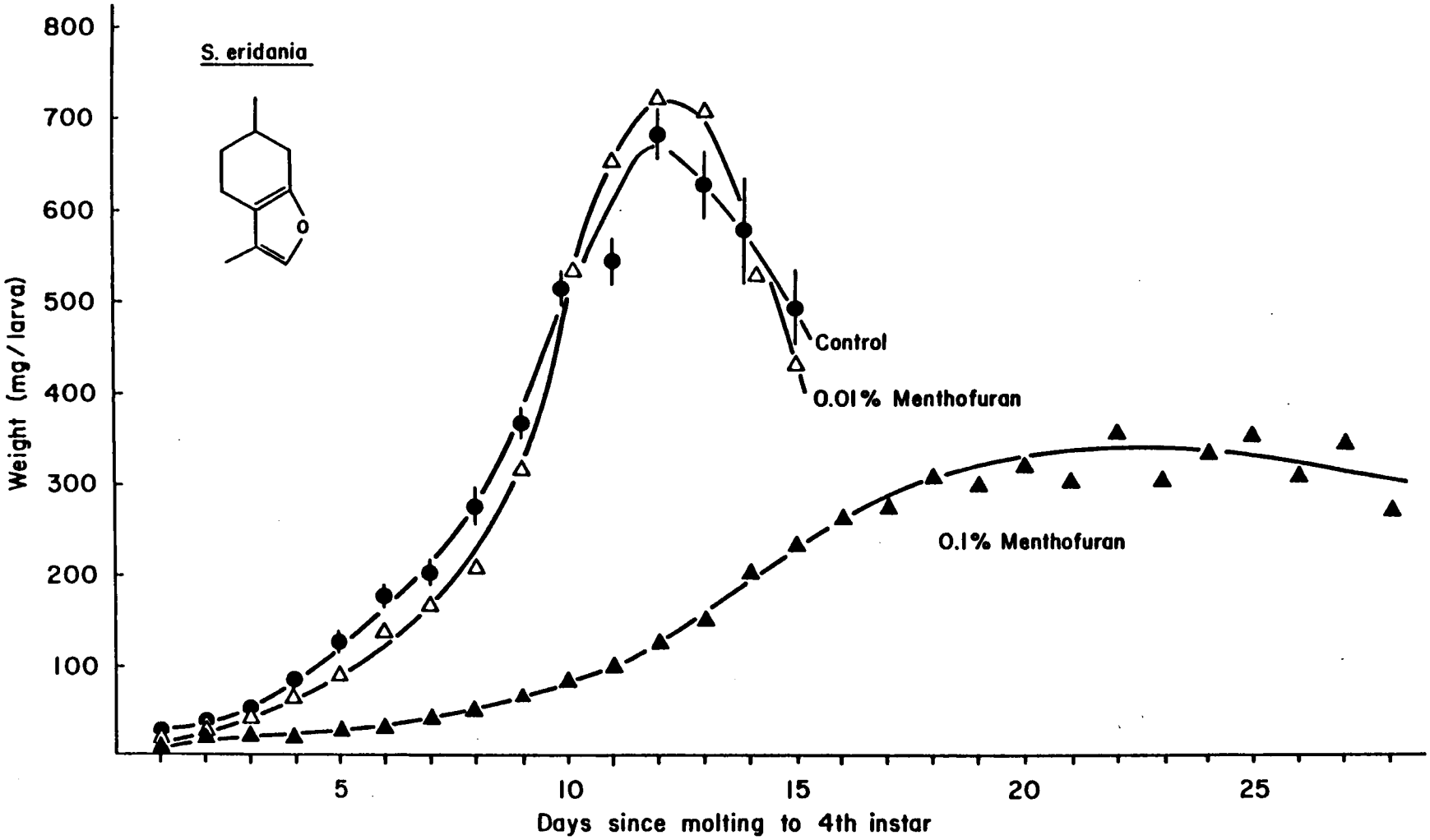


Figure 4. Growth of Spodoptera eridania on control and menthofuran-containing diets from the fourth through sixth instars.

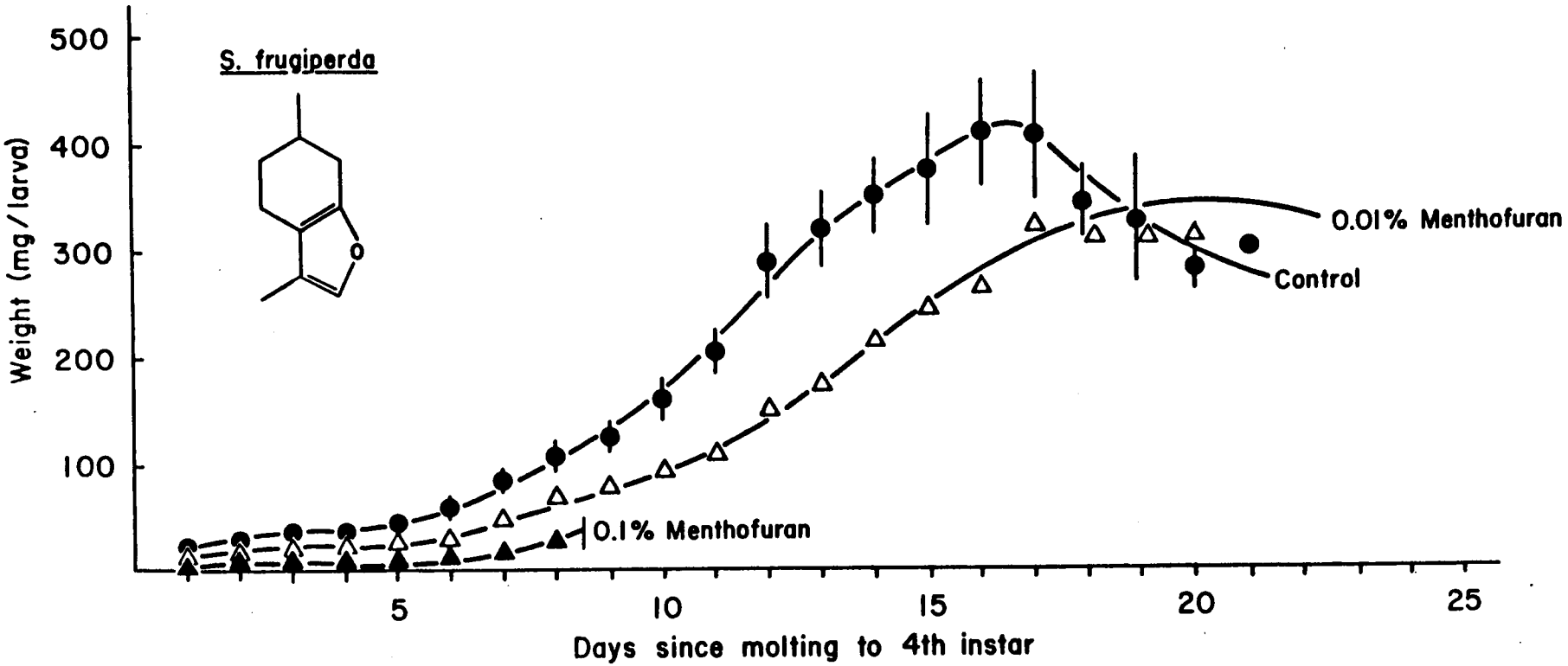


Figure 5. Growth of Spodoptera frugiperda on control and menthofuran-containing diets from the fourth through sixth instars.

Table 2. Mean Weights Used in Figure 4 ± Their Standard Errors.

Days Since Molting to Fourth Instar	Weight (mg/larva)		
	Control	0.01% Menthofuran	0.1% Menthofuran
1	22.6 ± 1.2	19.7 ± 1.0	18.4 ± 0.7
2	36.9 ± 1.7	34.0 ± 1.5	21.2 ± 2.6
3	48.4 ± 3.0	42.7 ± 2.8	20.2 ± 1.8
4	85.3 ± 6.7	67.5 ± 5.4	22.4 ± 1.8
5	128.0 ± 8.7	97.2 ± 5.9	27.7 ± 2.5
6	172.4 ± 9.1	139.1 ± 9.1	31.8 ± 3.7
7	200.7 ± 12.3	169.1 ± 7.1	39.1 ± 5.2
8	275.3 ± 20.4	218.6 ± 18.8	52.7 ± 9.8
9	362.1 ± 21.2	314.5 ± 21.2	65.1 ± 12.2
10	510.3 ± 22.5	529.9 ± 25.4	80.4 ± 15.9
11	538.1 ± 20.4	651.1 ± 26.6	97.1 ± 23.3
12	684.5 ± 28.3	719.3 ± 25.8	125.5 ± 31.6
13	633.2 ± 38.3	712.2 ± 29.2	146.4 ± 38.8
14	574.5 ± 52.8	529.2 ± 30.5	201.6 ± 52.8
15	489.1 ± 40.3	429.3 ± 30.3	232.0 ± 57.5
16			257.7 ± 65.5
17			272.5 ± 71.2
18			305.6 ± 78.9
19			297.3 ± 67.5
20			323.0 ± 61.7
21			301.5 ± 79.5
22			355.8 ± 85.9
23			306.8 ± 97.5
24			337.8 ± 105.3
25			352.8 ± 110.8
26			314.0 ± 122.5
27			345.7 ± 140.5
28			269.0 ± 128.0

of the pulegone-fed insects were significantly greater than those of the controls at $P < 0.002$ (Gunderson et al., 1983), assessed by using Student's T-test.

Fall armyworm larvae, however, showed slightly delayed weight gain and pupation at 0.01% dietary pulegone (Figure 3) and, at 0.1% pulegone, failed to grow or develop properly. At this concentration, 100% of the population died within 12 days.

The PSMO metabolite, menthofuran, had an appreciably more toxic effect on the southern armyworm than did pulegone itself in extended exposures. As shown in Figure 4, 0.01% menthofuran had little effect, but 0.1% inhibited the growth rate and reduced the maximal weight attained. Only 32% of the population survived to the day of peak weight (day 22).

Figure 5 shows that menthofuran is also more toxic to the fall armyworm in prolonged exposures. The lower concentration noticeably slowed growth and development, but 0.1% menthofuran was 100% lethal within 8 days.

Discussion

Both pulegone and menthofuran exhibited allomonal properties in the two species of insect larvae tested. Although the acute toxicities show these monoterpenes to be only slightly acutely toxic at this life stage compared to typical pesticides (Ware, 1983), it is

interesting to note that the relative acute toxicities do not parallel the chronic effects. Whereas the acute toxicity of menthofuran is slightly less than that of pulegone, the chronic growth disruption is much more pronounced, particularly in the southern armyworm. Both allelochemicals are 3.5- to 4-fold less toxic to the fall armyworm in acute doses, yet this species is far more sensitive to chronic exposure.

The biological mechanisms for the toxicity of these monoterpenes are not known, either for the acute or the chronic effects. It is not unusual for a toxic compound to have two unrelated target sites and modes of action for chronic and acute exposure; this is, for example, the case for lead poisoning (Doull et al., 1980) and for cyanide poisoning (Baumeister et al., 1975). If that is the case with these two monoterpenes, then the relative acute and chronic susceptibilities of the two species could easily be reversed.

Another possibility is that either or both of these terpenoids might be metabolized to some extent by one or more enzymes, perhaps at different rates. If this is the case, then chronic exposure to these compounds might result in induction of a number of detoxifying enzymes, including the cytochrome P-450-dependent polysubstrate mono-oxygenases and the glutathione S-transferases (Brattsten, 1979; Chasseaud, 1979). Induction of enzymes by dietary chemicals

has been shown to increase the rate of their metabolism (Morello, 1965; White et al., 1979), and enzyme induction, either by xenobiotic compounds or by components of host plants ingested, has been shown to alter the susceptibility of insects to pesticides (Brattsten and Wilkinson, 1973; Brattsten and Wilkinson, 1977; Brattsten et al., 1977; Wood et al., 1981; Yu, 1982a,b). The two species could easily differ in their biochemical response to induction and this could reverse the degree of susceptibility to the toxic compounds.

It is apparent that acute toxicity, though quickly and easily determined in the laboratory, may not adequately reflect the toxic interaction. A chronic feeding study more closely approximates a natural situation that might result in the field. Repeated sampling of a food plant, like chronic exposure in the diet, would allow time for insect enzyme induction, if any, and chronic, low level exposure. An innate feeding preference might or might not be overcome with time.

The chronic interferences with larval growth shown here in the fresh weight growth curves are thus more indicative of the allomonal relationships between plant and insect than are the LD_{50}'s. Pulegone exposure in ingested pennyroyal would undoubtedly be more harmful to the fall than to the southern armyworm despite the higher LD_{50}, and a mint with high menthofuran content would

be more detrimental to health, growth, and behavior of either species than would one high in pulegone.

These differences in susceptibility between two closely related species are unexplained, but may result in part from differences in detoxifying capability. Enzymatic differences do exist between the two species, and the differences in one enzyme system, the glutathione S-transferase system, are investigated here.

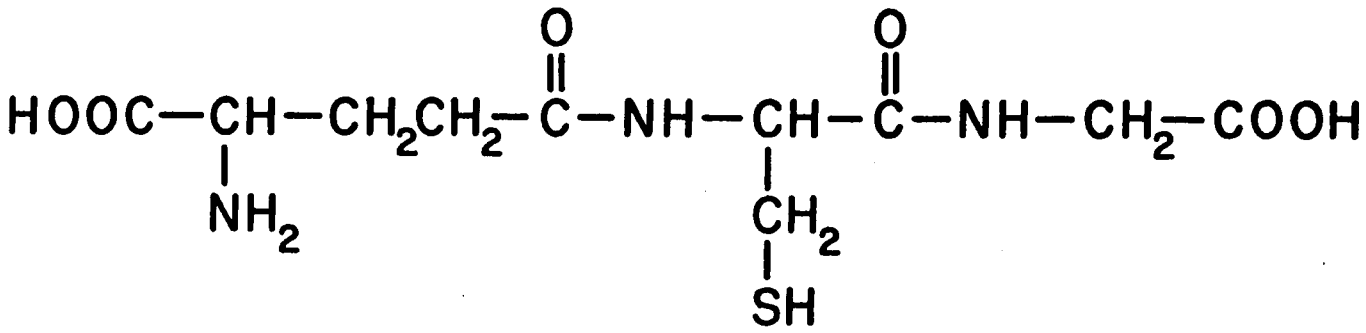
CHAPTER III

GLUTATHIONE S-TRANSFERASE ACTIVITY

Introduction

Glutathione S-transferases (EC 2.5.1.18) are cytosolic group transfer enzymes catalyzing the conjugation of the tripeptide glutathione (γ-glutamylcysteinylglycine) (Figure 6) to any of a large number of xenobiotic compounds with electrophilic centers and their primary metabolites (Brattsten, 1979; Chasseaud, 1979; Doull et al., 1980; Keen et al., 1976). Nucleophilic attack by the thiol (-SH) group of glutathione (GSH) on such compounds reduces their reactivity by neutralizing their electrophilic sites and renders them more water soluble by addition of the tripeptide (Dixit et al., 1981; Habig et al., 1974; Motoyama and Dauterman, 1978).

Although the precise biological role of the GSH S-transferases is not known (Hales et al., 1980), the resulting thioether conjugate is generally substantially less toxic (Chasseaud, 1979; Fukami, 1980; Motoyama and Dauterman, 1978). Occasional exceptions to this include the liberation of hydrogen cyanide in organic thiocyanate conjugation (Fukami, 1980) and a report by van Bladeren et al. (1980) that the primary GSH adduct of 1,2-dibromoethane is responsible for its mutagenic effects. Nevertheless,



Glutathione (GSH)

Figure 6. Structure of glutathione.

this family of enzymes plays an important role in drug metabolism and protection of the cell by detoxification of potentially damaging xenobiotics and their reactive intermediates (Baars et al., 1978; Chasseaud, 1979; Habig et al., 1974), as well as in their elimination from the body (Fukami et al., 1980; Pinkus et al., 1977). Not only is the GSH-conjugate itself more water soluble, but it can be further metabolized by cleavage of the glutamate and glycine residues, followed by acetylation to a mercapturic acid (Chasseaud, 1979; Fukami, 1980; Habig et al., 1974). Most of the conjugates are excreted in the urine or bile either as mercapturic acids or as cysteine derivatives (Boyland and Chasseaud, 1967).

Early studies of GSH S-transferase activity noted an apparent multiplicity of enzymes as well as a wide range of electrophilic substrates. Since these studies were conducted with crude or partially purified enzymes, interest focused mainly on the nature of the second substrate and the enzymes were named according to the type of substrate for which they showed a preference, e.g., S-aryl-, S-alkyl-, S-alkene-, and S-epoxidetransferase (Chasseaud, 1979; Fukami, 1980). Subsequent purification of the enzymes from rat liver revealed at least six enzymes with broad and overlapping specificities; each catalyzed reactions with several second substrates (Chasseaud, 1979; Habig et al., 1974; Pabst et al., 1974).

Purified enzymes are now more often identified by their elution patterns or isoelectric points.

GSH S-transferase activity has been found in the livers of all mammalian species tested (Chasseaud, 1979), as well as in birds, reptiles, amphibians, and fish (Chasseaud, 1979; Fukami, 1980). Activity is also present in the lung, kidney, heart, and spleen (Chasseaud, 1979); in the intestine (Clifton and Kaplowitz, 1977; Pinkus et al., 1977); and to some extent in the skin, blood, adrenal, muscle, brain, and placenta (Chasseaud, 1979); and in the testes (Hales et al., 1980; Mukhtar et al., 1978; Robaire and Hales, 1982).

GSH S-transferase is also present in insects and other invertebrates (Chang et al., 1981; Gould and Hodgson, 1980; Hayaoka and Dauterman, 1982; Tate and Herf, 1978; Yu, 1982b), as well as in plants (Frear et al., 1972). In insects the enzyme has largely been studied in isolated midguts (Chang et al., 1981, Gould and Hodgson, 1980); whole flies (Hayaoka and Dauterman, 1982); fly abdomens (Ottea and Plapp, 1981); and/or head-thorax sections (Motoyama et al., 1978). Cohen and Smith (1964), however, report activity in the fatbody, malpighian tubules, gastric caecae, mid- and hind-gut, integument, foregut, blood, and crop fluid of the locust.

Most of the second substrates demonstrated for GSH S-transferase are synthetic chemicals (Chasseaud, 1979),

but some are naturally occurring, e.g., some α,β-unsaturated compounds (Boyland and Chasseaud, 1967). Kinetic studies of the reactions indicate that despite the wide range of substrates involved, the reactions all proceed via nucleophilic attack of enzyme-bound GSH on the electrophilic center of the second substrate (Keen et al., 1976). Three classes of nucleophilic reactions have been described: addition, addition-elimination, and displacement, e.g., of nitro groups or halides. These include reactions with alkenes, aryl and alkyl hydrocarbons, aralkyl compounds, and epoxides (Fukami, 1980) as well as some reactions with electrophilic centers other than carbon (Chasseaud, 1979; Keen et al., 1976).

Some of the substrates commonly used in assays for GSH S-transferase activity are [^{14}C] methyl iodide (for "alkyltransferase" activity), 1,2-dichloro-4-nitrobenzene (Figure 7) and 1-chloro-2,4-dinitrobenzene ("aryltransferase"), benzylchloride ("aralkyltransferase"), 1,2-epoxyethyl benzene or 1,2-epoxy-3-(p-nitrophenoxy)propane ("epoxidetransferase"), and diethyl maleate ("alkenetransferase") (Fukami, 1980; Tate and Herf, 1978). Most of the common assay methods are spectrophotometric, measuring change in absorbance of a given wavelength with time required for product formation or substrate reaction. Disappearance of GSH has also been used to measure conjugation by reacting the remaining GSH with the thiol reagent

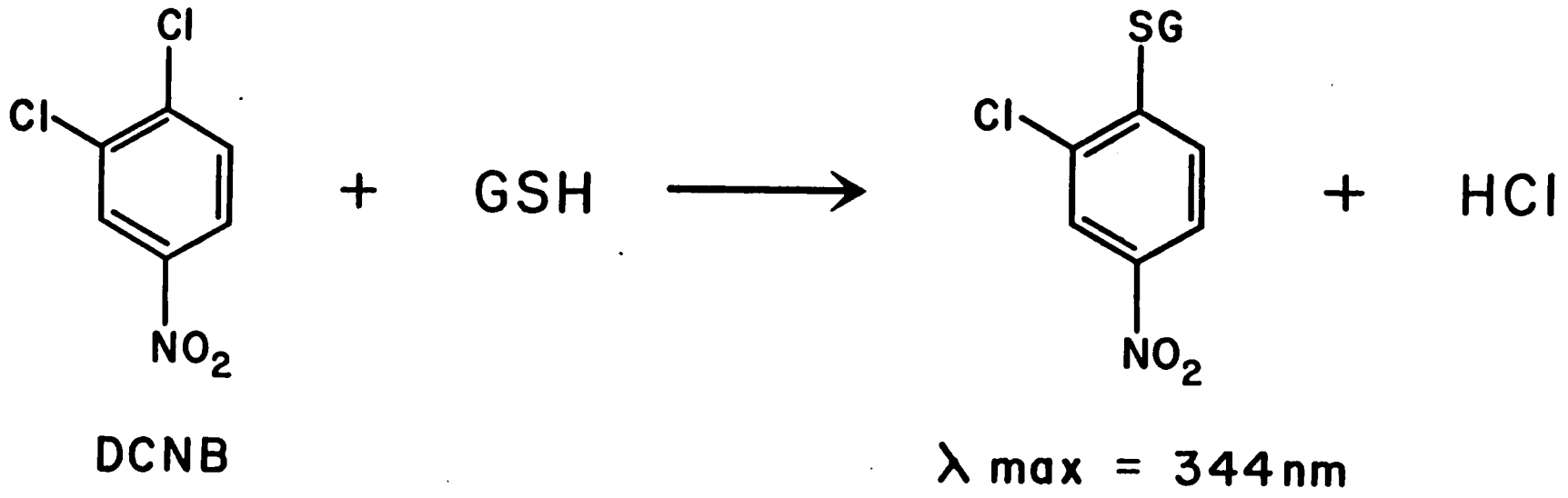


Figure 7. Reaction of 1,2-dichloro-4-nitrobenzene with glutathione.

5,5'-dithiobis-(2-nitrobenzoate) (DTNB) (Baars et al., 1978; Boyland and Chasseaud, 1967; Dixit et al., 1981; Ellman, 1959). Other assays, including titrimetric, colorimetric, and radioisotopic methods are reviewed by Chasseaud (1979).

Induction of GSH S-transferase activities by such typical PSMO inducers as 3-methylcholanthrene (Moran et al., 1979) and phenobarbital was at first not detected, perhaps because of insufficient treatment with the inducer (Chasseaud, 1979). It is now generally accepted, however, that the GSH S-transferases are inducible by phenobarbital, polycyclic hydrocarbons, and certain organochlorine compounds (Chasseaud, 1979). For example, Baars et al. (1978), Clifton and Kaplowitz (1977), Kulkarni et al. (1980), and Pinkus et al. (1977) all found rat tissue GSH S-transferases to be induced by phenobarbital, 3-methylcholanthrene, TCDD, benzo(a)pyrene, and several pesticides, at least in some tissues, and toward some substrates. Induction of house fly transferases by phenobarbital and various pesticides has also been shown (Hayaoka and Dauterman, 1982; Ottea and Plapp, 1981). Additionally, some host plant induction has been noted in the fall armyworm (Yu, 1982b).

It has also been found that GSH S-transferases can be inhibited <u>in vitro</u> by certain compounds, for example, catecholamines and quinones (Kulkarni et al., 1978), such as those found endogenously in house fly abdomens

(Motoyama et al., 1978). Other inhibitors are also known and have been reviewed (Chasseaud, 1979).

Since the GSH S-transferases are active toward a wide range of second substrates and work to detoxify many biologically active compounds, it is quite possible that they would be involved in pulegone metabolism. Pulegone, or especially any of its oxidation products--which include several hydroxypulegones and menthofuran--may present an electrophilic center such that GSH S-transferase may attack, e.g., by addition to one of the double bonds.

Materials and Methods

Chemicals. Pentamethylbenzene, (+)pulegone, and α-pinene, all used as diet additives for enzyme induction, were purchased from Aldrich Chemical Co., Milwaukee, WI. Coumarin was purchased from Sigma Chemical Co., St. Louis, MO, and menthofuran from Eastman Kodak, Rochester, NY. Control diet ingredients are listed in Chapter II.

Bovine serum albumin and reduced glutathione were purchased from Sigma Chemical Co. The substrate, 1,2-dichloro-4-nitrobenzene was purchased from Eastman Kodak.

Insects. Southern and fall armyworm stock colonies were maintained as described in Chapter II. Larvae used for most enzyme assays were selected from the colonies as newly molted sixth instars, except as noted. Larvae

were then fed for 3 days on the artificial diets described in Chapter II, with or without the addition of a test compound at a concentration of 0.2% (w/v) or as tolerated by the insects. Larvae of other stages, as well as pupae and adults, were used in some experiments.

Enzyme preparation. Larval midguts and, in some cases, fatbodies (see Brattsten et al., 1980), malpighian tubules, and testes were isolated from groups of 15 to 40 larvae and cleaned and homogenized in ice cold 0.2 M potassium phosphate buffer, pH 7.8, containing 1mM EDTA, as described by Brattsten and Gunderson (1981). The post-microsomal (210,000 g) supernatant was obtained using the same rapid centrifugation scheme described in the paper. Protein concentrations in the supernatants were estimated by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. When necessary, supernatants were diluted to approximately 2 mg protein per ml.

Enzyme assays. GSH S-transferase activity was measured in the supernatants using a modification of the procedure of Gould and Hodgson (1980). The standard reaction mixture consisted of 5 mM glutathione (GSH) and 0.875 mM 1,2-dichloro-4-nitrobenzene (DCNB) in 0.1 M potassium phosphate, pH 8.0, plus supernatant equal to approximately 0.2 mg protein, in a total volume of 3 ml. The standard assay was conducted as follows. The two

substrates (0.5 ml of 30 mM GSH and 15 µl of 175 mM DCNB per assay) were mixed in a tube or flask with 2.4 ml buffer per assay. A 2.9 ml aliquot of this mixture was added to each of two quartz cuvettes which were then allowed to temperature equilibrate in an Aminco DW2 UV-VIX recording spectrophotometer. After a baseline was drawn, 0.1 ml of supernatant (approximately 0.2 mg protein) was added to the sample cuvette and mixed. Activity was measured as the rate of increase in absorbance of the conjugate at 344 nm, using an extinction coefficient of 10 mM^{-1} cm^{-1}. Replicate assays and assays of other supernatants were carried out with additional aliquots of reaction mixture, using the same reference cuvette.

Assays in which substrate concentration, protein concentration, and pH were varied were also performed, mixing one cuvette (and its reference) at a time.

Results

Optimum assay conditions. Optimum conditions for the *in vitro* conjugation of GSH with 1,2-dichloro-4-nitrobenzene (DCNB) were established using the cytosolic fraction from the gut tissue homogenate from southern armyworms raised on 0.2% pentamethylbenzene. Activity in nanomoles of conjugate produced per minute was linear with respect to protein concentration up to 0.6 mg protein in the 3.0 ml incubation mixture. Routine assays

were performed using approximately 0.2 mg protein per incubation, as this was well within the linear range.

The optimal concentration of GSH in the reaction was determined to be 5 mM, since above this concentration the specific activity no longer increased linearly. By varying the DCNB concentration with the GSH concentration held at 5 mM, it was found that 0.875 mM DCNB gave the highest activity within the linear range.

The pH of the reaction mixture was varied from 6.0 to 8.0 using potassium phosphate buffer, and from 8.0 to 9.0 using Tris-HCl. Figure 8 shows the pH-activity profile of DCNB conjugation in supernatants from pentamethylbenzene-induced southern armyworms. Subsequent assays were performed with potassium phosphate buffer, pH 8.0.

The reaction proceeded linearly for at least 5 minutes at optimum substrate and protein concentrations. The initial 2 minutes were used for calculating the reaction rate.

Developmental stages versus activity. Conjugation of GSH with DCNB was monitored throughout the sixth instar in larvae of both species. Figure 9 shows the age versus activity curve for transferases in the gut on control-fed larvae of both species. Activity was also detected in other life stages, including fourth and fifth instar

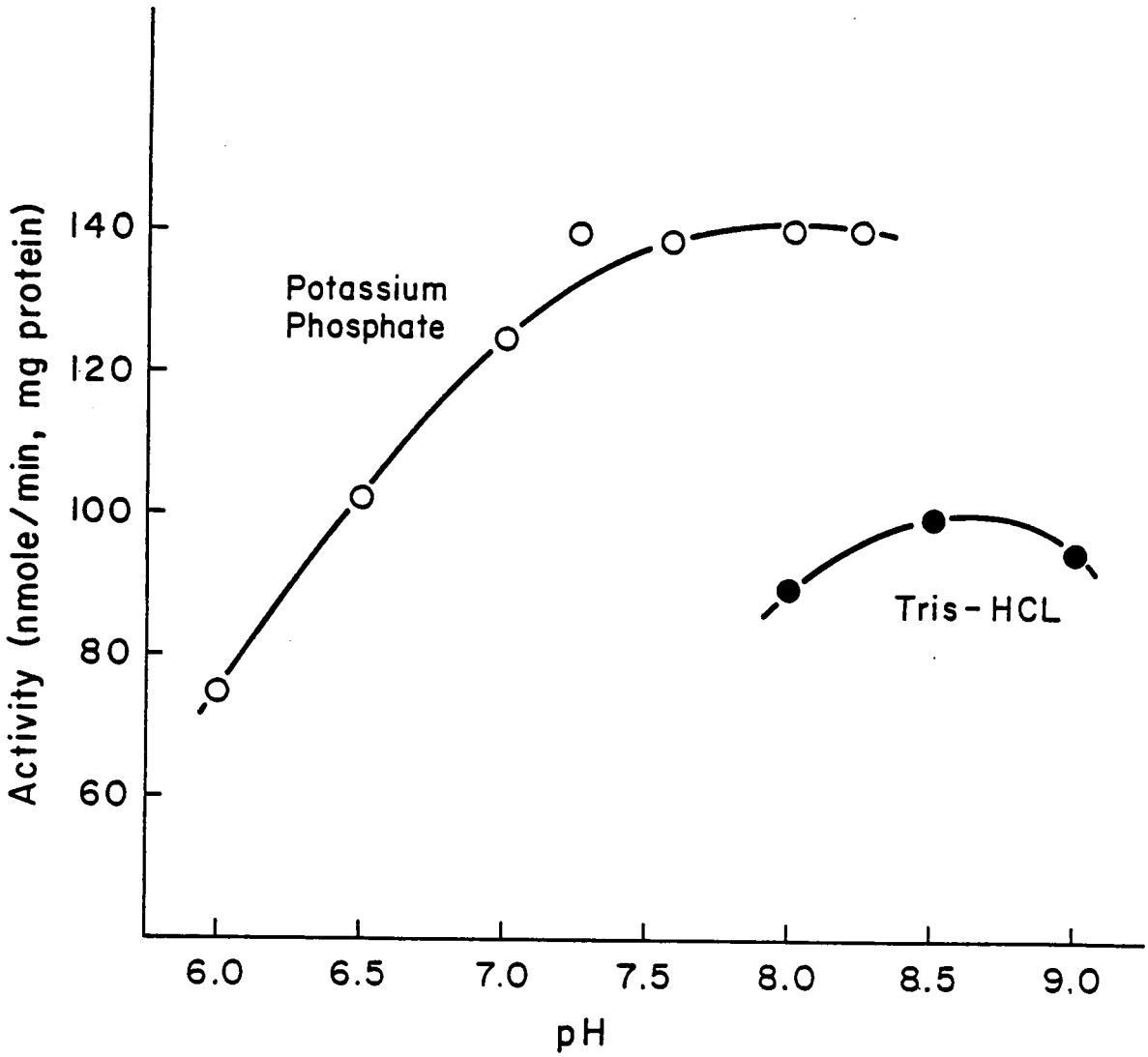


Figure 8. GSH S-transferase activity toward DCNB versus pH of the incubation mixture.

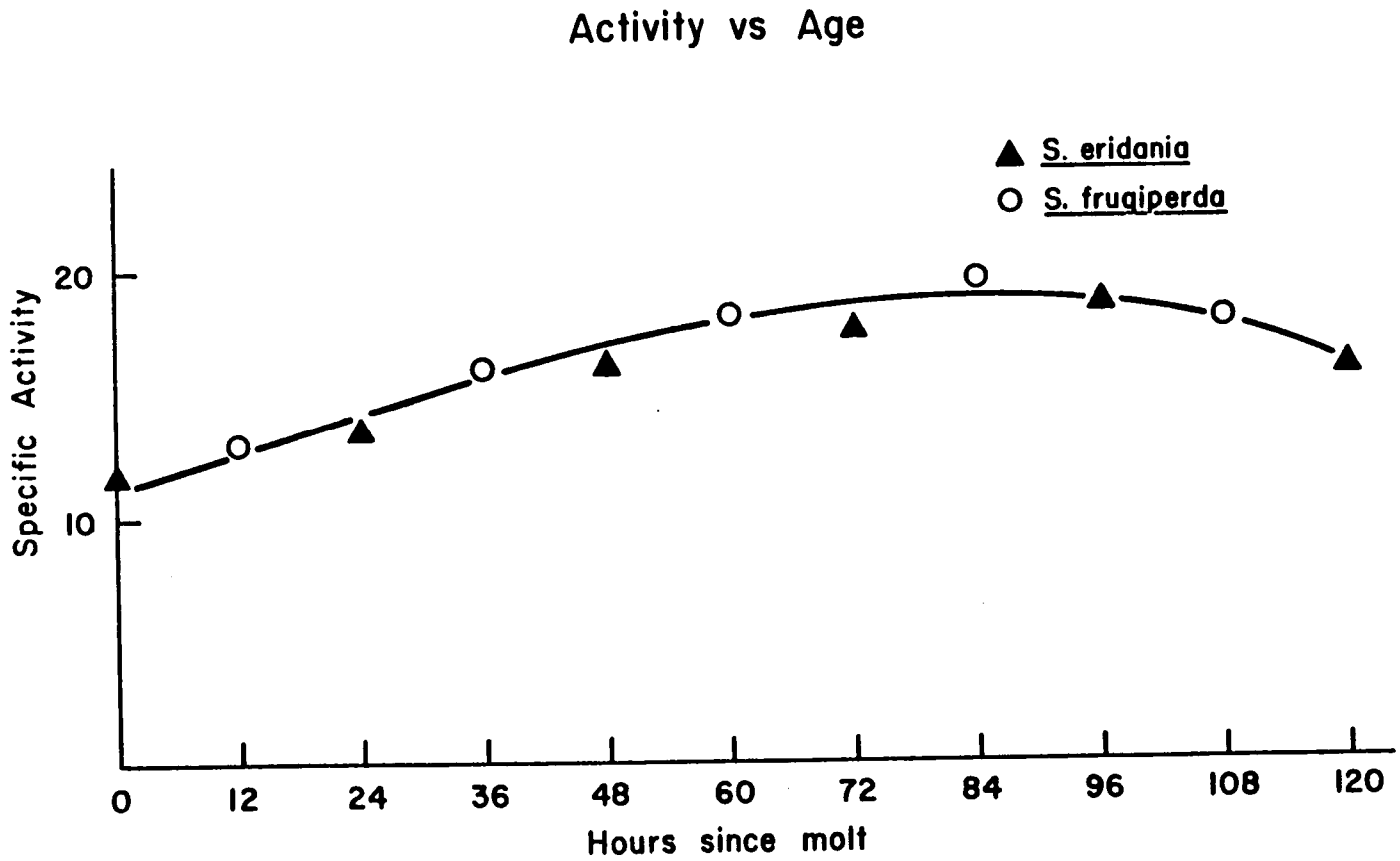


Figure 9. GSH S-transferase activity in the sixth instar, nmoles of conjugate/min/mg protein.

larvae (in gut tissue), pupae (using whole pupae), in adult moths (abdomens), and in eggs.

Tissue distribution. Endogenous activity levels for DCNB conjugation in each of four tissues were determined for both species (Figure 10). Transferase activities in the gut and testes were very similar in the two species. The specific activities in the fatbody and malpighian tubules, however, were much lower in the fall armyworm--less than half of those in the southern armyworm.

Enzyme induction in gut tissue. The effects of several dietary chemicals on GSH S-transferases were investigated in both species. Figure 11 shows the effects of 0.2% pentamethylbenzene (PMB), α-pinene, and coumarin on specific activities in each species. PMB and α-pinene, which both induce microsomal PSMO activities (Brattsten and Wilkinson, 1973; Brattsten et al., 1977), were highly effective in inducing the GSH S-transferases in the southern armyworm, but much less so in the fall armyworm. Coumarin, however, which has little effect on the PSMO system (Gunderson et al., 1983), induced GSH S-transferases approximately 7-fold in both species.

The inducing effects of five other monoterpenes on the GSH S-transferases in southern armyworm gut tissues are shown in Table 3. Activity toward DCNB was increased by 1.6- to 2.6-fold in all cases.

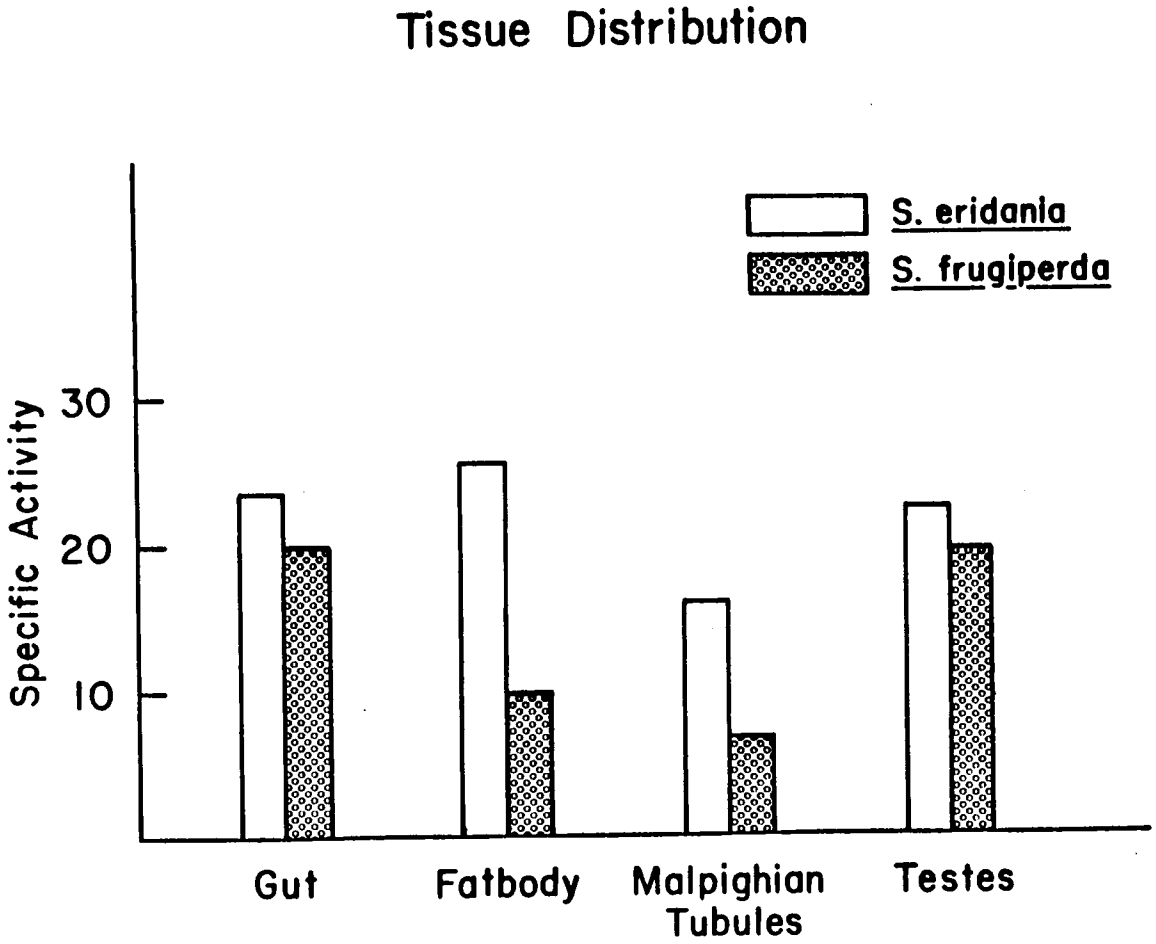


Figure 10. Tissue distribution of GSH S-transferase activity in sixth instar larvae, nmoles/min/mg protein.

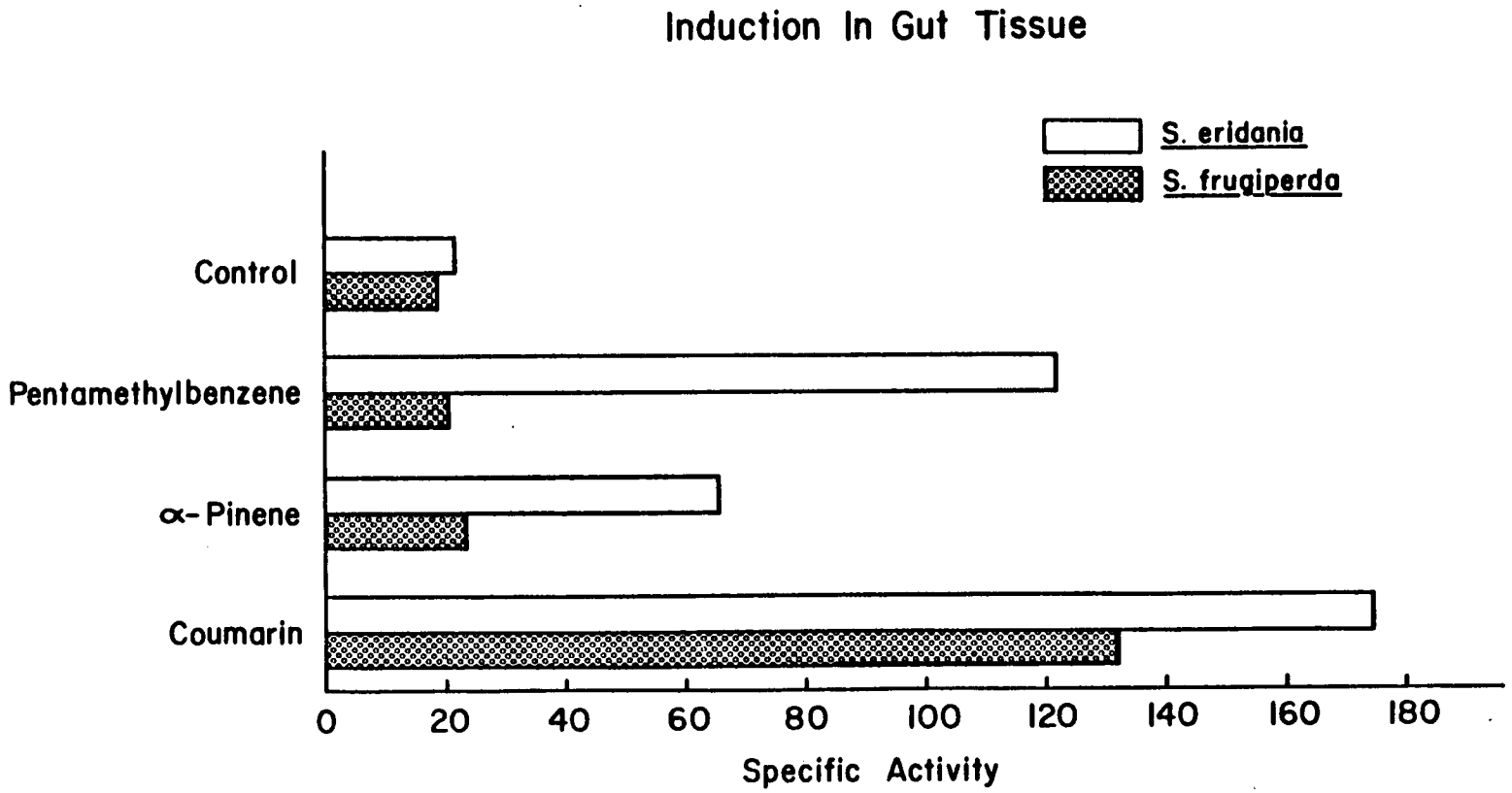


Figure 11. Induction of GSH S-transferase activity in gut tissue of sixth instar larvae, nmoles of product/min/mg protein.

Table 3. Induction in S. eridania.

Compound*	Specific Activity**	% of Control
β-pinene	47.47 ± 6.58	204
α-terpinene	38.76 ± 3.04	166
γ-terpinene	59.68 ± 0.55	256
(+)-limonene	48.74 ± 4.21	209
(-)-limonene	61.76 ± 2.02	265

*0.2% in diet for 3 days.

**nmoles of conjugate per minute per mg protein, mean ± its standard error.

Table 4 shows the induction of transferase activity in the gut of both species by pulegone and menthofuran in the diet at 0.05% for 3 days.

Discussion

Assays. Optimum assay conditions for GSH S-transferase activity with DCNB were fairly consistent with those reported in the literature for lepidopterous larvae (Gould and Hodgson, 1980; Yu, 1982b). The broad peak of the pH-activity profile between approximately pH 7.5 and 8.5 is also fairly typical of those reported for transferases in other invertebrates (Chang et al., 1981; Tate and Herf, 1978; Yu, 1982b).

Developmental stage versus activity. The changes in GSH S-transferase activity levels during the last larval instar (Figure 9) are insignificant when compared to the changes in PSMO activity levels reported by Brattsten and Wilkinson (1973) and Gould and Hodgson (1980), who found 8- and 28-fold increases in PSMO activity in lepidopterous larvae during the same period. The small changes in GSH-conjugating activity are consistent, however, with the small age-related changes in GSH S-transferase reported in tobacco budworm and fall armyworm larvae and in adult house flies (Gould and Hodgson, 1980; Hayaoka and Dauterman, 1981; Yu, 1982b). Evidently

Table 4. Effects of 0.05% Dietary Pulegone and Menthofuran on Glutathione S-Transferase Activity.

Species	Activity*		
	Control	Pulegone	Menthofuran
S. eridania	19.35 ± 1.25	22.72 ± 1.72	17.23 ± 0.32
S. frugiperda	19.02 ± 1.09	24.84 ± 1.84	22.41 ± 1.40

*nmoles of conjugate produced per minute, per mg protein, means ± their standard errors.

the variation in GSH S-transferase activity during larval development is not as significant in determining changing detoxification potential as the variation in microsomal enzyme activity, although in the adult mosquito a decrease in GSH S-transferase activity (and GSH levels) is correlated with an increase in acetaminophen toxicity (Richie and Lang, 1982). No conclusions can be drawn in the present study regarding changing activity in other developmental stages due to the high variability of results. This variability is partially the result of technical problems in dissecting out the smaller guts of early larval instars and adults and the probable presence of inhibiting substances in the cruder preparations (whole pupae and eggs, abdomens of adults). A similar variation was observed in house fly abdominal preparations and was attributed to inhibition by quinones produced in vitro by tyrosinase (Kulkarni et al., 1978; Motoyama et al., 1978). In this study as in those, lowered activity was usually associated with the darkening of the soluble fraction by tyrosinase (polyphenol oxidase).

Tissue distribution. It is interesting to note the tissue distribution of GSH S-transferase activity in these two species (Figure 10). Specific activity is highest in gut, testes, and, in S. eridania, the fatbody. Because of the relative contribution of each tissue to

body mass, however, it is obvious that the transferases of the testes and malpighian tubules play a smaller role in overall metabolism, regardless of specific activity. These transferases may, of course, perform a specific protective function, e.g., in the reproductive tissues (Hales et al., 1980; Mukhtar et al., 1978).

Since any ingested compound will first be acted upon in the gut, enzyme activity in that tissue is obviously important. The fatbody, however, has approximately twice the mass of the gut tissue in these larvae, and for that reason may be expected to play an important part in xenobiotic metabolism (Brattsten et al., 1980). Since endogenous fatbody GSH S-transferases are higher in the southern armyworm than the fall armyworm, it is quite possible that these enzymes could be involved in a metabolic difference in susceptibility, e.g., to pulegone.

Induction. The response of the glutathione S-transferases of the southern armyworm to many common inducers is much greater than the response of the fall armyworm (Figure 11). This enzyme induction by dietary chemicals could significantly increase the detoxifying capacity of this species. Many of the monoterpenes do induce transferase activity in the southern armyworm at 0.2% in the diet (Table 3). The response of both

species to 0.05% pulegone and menthofuran (Table 4) is naturally less pronounced because the concentration is lower, though this was necessary to insure acceptance of the food by the fall armyworm. Any induction of this family of enzymes will increase the insect's ability to detoxify ingested foreign compounds such as pulegone, menthofuran, or their primary metabolites.

CHAPTER IV

GENERAL CONCLUSIONS

The deleterious effects of long exposure to pulegone and menthofuran are more pronounced in Spodoptera frugiperda than in S. eridania. Growth and development are significantly suppressed in the fall armyworm, and higher concentrations in the diet are lethal. The biochemical differences in the detoxifying potentials of these two closely related species very likely contribute to the observed differences in susceptibility.

Microsomal oxidation is not a likely candidate to account for this difference, since S. eridania has higher levels of PSMO activity, and since menthofuran is actually more chronically toxic than pulegone itself. In addition, inducers of this system, pentamethylbenzene and α-pinene, for example, increase the acute toxicity of pulegone (Gunderson et al., 1983).

Glutathione S-transferases are far more likely to contribute to the differential detoxification of pulegone in these two species. Endogenous GSH S-transferase activities are higher in several tissues of the southern armyworm. Perhaps most importantly, southern armyworm gut transferases are induced more readily, and to much higher levels, by dietary exposure to xenobiotic compounds,

including plant allelochemicals. Coumarin, which is the most effective inducer of GSH S-transferase tested, does not substantially induce the PSMO system, yet it significantly reduces the acute toxicity of pulegone (Gunderson et al., 1983).

Thus, while no direct evidence has been presented for the metabolism of pulegone by GSH S-transferase, the indirect evidence strongly suggests such a role. It may be, in fact, that neither pulegone nor menthofuran serves directly as a substrate for this enzyme. Ongoing work in this laboratory has found no evidence for the conjugation of GSH to either of these compounds using the DTNB assay for the disappearnace of GSH (Ellman, 1959). This assay is less sensitive, however, than the spectrophotometric assay with DCNB used here; it may not be sensitive enough to detect conjugation with these monoterpenes if they are not strong substrates.

The most likely explanation, however, is that GSH S-transferase may not act directly on these terpenes but rather on one of their microsomal oxidation products. None of these primary metabolites are currently available as pure compounds to use in an assay as possible substrates for GSH conjugation.

Further study is indicated, as always, to more completely answer the questions at hand. Future work with thin layer chromatography of the aqueous fraction

of an incubation of pulegone with cytosolic enzymes is planned, as are further assays for the disappearance of GSH in the presence of pulegone plus microsomal and cytosolic fractions from induced insects.

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