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UNIVERSITY OF OTTAWA

In memory of my

father

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Abstract

The toxicity of alpha-terthienyl [2,2':5',2"-terthienyl], a secondary metabolite of various Asteraceae, was studied in three phytophagous insect species. In topical applications, alpha-terthienyl was highly toxic to *Manduca sexta* (LD_{50}=10 μg/g), moderately toxic to *Heliothis virescens* (LD_{50}=474 μg/g) and relatively non toxic to *Ostrinia nubilalis* (LD_{50}=698 μg/g). A similar diverse response was observed in feeding studies.

To investigate the reasons for the considerable variation in the insecticidal activity of alpha-T, the opacity of the cuticle, toxicokinetics and metabolism of alpha-T were studied in the three species. The absence of significant differences in the absorbance of the cuticle at the λmax of α-T in the three species suggested that cuticular properties may not play an important role in the diverse response indicated. However, toxicokinetics studies with ^{3}H-α-T demonstrated that following either oral or topical administration, larvae of *M. sexta* were unable to excrete this allelochemical rapidly. However, *H. virescens* and *O. nubilalis* were able to clear this chemical rapidly from the body via the feces preventing lethal concentrations from reaching the cuticle where light mediated toxic reactions may occur. For example, after 48 hrs. of feeding on ^{3}H-α-T treated diet, the ratio of the label in the body to that in the feces was 58:42 for *M.sexta*, 32:68 for *H.virescens* and 16:84 for *O.nubilalis*. Elimination of ^{3}H after topical application is much more rapid in *O.nubilalis* ($t_{1/2}$=8.5 hrs) as compared to *H.virescens* ($t_{1/2}$=22 hrs.) or *M.sexta* ($t_{1/2}$=48 hrs.).

Subsequently, comparative metabolism, *in vivo*, was studied. Analysis of the body (minus gut contents) of the larvae of the three species showed slightly (but not significantly) higher levels of the α-T:metabolite ratio in *M.sexta* as compared to *H.virescens* or *O.nubilalis*. On the other hand, significantly increased levels of α-T:metabolite in the frass, was observed for *M.sexta* as opposed to *H.virescens* or

O.nubilalis. A significant amount (> 40 per cent) of activity excreted was unchanged α-T in all the three species.

Striking differences in the capacity of the midgut microsomes to generate metabolites was observed in an *in vitro* study. Alpha-T was metabolised 16 and 30 times faster by *H.virescens* and *O.nubilalis* microsomal fractions respectively as compared to *Manduca sexta* microsomal fractions. Evidence gained with the use of piperonyl butoxide indicated that mixed function oxidases are major enzymes involved in metabolism. Examination of cytochrome P-450 levels in the three species showed increasing levels in the order: *M. sexta*, *O. nubilalis* and *H. virescens* with the latter two species having 3 and 4 times more cytochrome P-450 respectively than *M. sexta*. Alpha-T, at 10 and 30 μg/g in the diet, had no significant effect on cytochrome P-450 levels in *M. sexta* and *O. nubilalis*. However, for *H. virescens*, slight but significant inhibition of cytochrome P-450 levels were noticed at these two concentrations.

The results suggest that increased metabolism, mediated by mixed function oxidases, leading to rapid clearance of this phototoxic thiophene is one method by which phytophagous insect species deal with this type of allelochemical in host plants. The significance of these results with respect to the host range of the insects is discussed.

Résumé

La toxicité de l'α–terthienyl (2,2':5',2"–terthienyl) (α–T), un métabolite secondaire de diverses espèces d'Astéraceae, a été étudiée pour trois espèces d'insectes phytophages. L'α–T est très toxique pour *Manduca sexta* (LD_{50}=10 µg/g), moyennement toxique pour *Heliothis virescens* (LD_{50}=474 µg/g), et relativement peu toxique pour *Ostrinia nubilalis* (LD_{50}=698 µg/g). Des résultats semblables ont été obtenus dans des études de nutrition.

Afin de déterminer quels sont les facteurs responsables des variations considérables de l'activité insecticide de l'α–T, l'opacité de la cuticule, la toxicocinèse et le métabolisne de l'α–T ont été étudiés pour les trois espèces.

L'absence de différences significatives dans l'absorbance de la cuticule au lmax de l'α–T pour les trois espèces suggère que les propriétés de la cuticule ne sont pas responsables des variations e la toxicité de l'α–T.

Les études de toxicocinèse utilisant ^{3}H–α–T démontrent que les larves de *M. sexta* ne peuvent pas excréter cette substance allélochimique rapidement suite à l'administration orale ou topique. Cependant, *H. virescens* et *O. nubilalis* peuvent rapidement éliminer l'α–T par excrétion dans les fèces, évitant l'accumulation du composé à des concentrations léthales dans la cuticule, où les réactions toxiques peuvent prendre place sous l'effet de la lumière. Par exemple, suite à une période de 48 hr pendant laquelle les larves se nourrissent d'une diète contenant de l'^{3}H–α–T, la proportion de l'élément radioactif retrouvé dans le corps de l'organisme comparé aux fèces est de 58 : 42 pour *M. sexta*, de 32 : 68 pour *H. virescens* et de 16 : 84 pour *O. nubilalis*. L'élimination de ^{3}H après application topique est beaucoup plus rapide pour *O. nubilalis* ($t_{1/2}$=8.5 hr) que pour *H. virescens* ($t_{1/2}$=22 hr) ou *M. sexta* ($t_{1/2}$=48 hr).

Le métabolisme de l'α–T fut comparé par des études *in vivo*. Les analyses du corps (après nettoyage du tube digestif) des larves démontrent un plus grand rapport (quoique non significatif) de α–T : métabolite chez *M. sexta* que chez *H.*

virescens ou *O. nubilalis*. Par contre, cette proportion est plus grande pour les fèces de *M. sexta* que pour celle de *H. virescens* ou *O. nubilalis*. Pour les trois espèces, plus de 40% des composés radioactifs récupérés dans les fèces est de l'α–T non métabolisé.

Une étude *in vitro* a démontré des différences marquées de métabolisme de l'α–T par les microsomes de l'intestin moyen. L'α–T est métabolisé 16 et 30 fois plus rapidement par les fractions microsomales de *H. virescens* et *O. nubilalis*, respectivement, comparé à *M. sexta*. L'utilisation de pipéronyl butoxide démontra que les oxidases à fonctions mixte sont les principaux enzymes participant au métabolisme de l'α–T.

La teneur en cytochrome P–450 chez *O. nubilalis* et *H. virescens* est respectivement 3 et 4 fois plus élevée que chez *M. sexta*. L'α–T incorporé dans la nourriture à des taux de 10 et 30 µg/g n'a aucun effet significatif sur le niveau de cytochrome P–450 chez *M. sexta* et *O. nubilalis*. Par contre, pour *H. virescens*, une légère inhibition du cytochrome P–450 fut observée pour les deux concentrations d'α–T.

Les résultats indiquent qu'un métabolisme accru, dépendant des oxidases à fonctions mixtes, peut mener à l'élimination rapide de ce thiophène phototoxique; ceci semble être une façon par laquelle les insectes herbivores font face à ce genre de produit allélochimique présent dans les plantes hôtes. La signification de ces résultats est discutée, la gamme des plantes hôtes utilisées par les insectes.

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INTRODUCTION

Green plants and phytophagous insects account for half of the living species on our planet (Strong et al., 1984). The encounter between the two groups is perhaps the most frequent and important interaction in terrestrial ecosystems. In recent years, the study of plant-insect interactions has increased dramatically. It is now being generally accepted that plants and insects coevolve and the continuous struggle for existence is being reflected in the biosynthesis of defence strategies by the plant and the development of counter strategy by the insects (Ehrlich and Raven, 1964; Berenbaum, 1983). However, the coevolution hypothesis has not gone unchallenged (Jermy, 1976, 1984).

Plants are subject to attack by pathogens, nematodes, and large and small herbivores at all stages of their life cycle. Plants lack the ability to move, which is usually the first method of defence in animals. Still, they have restricted the enormous destructive potential of herbivores with the use of physical (morphological) and/or chemical barriers. Physical defences include thick epicuticular waxes, thorns, spines and trichomes (Swain, 1977 and references therein). Less obvious but more important, plants synthesize a diverse group of chemicals ranging from simple structures such as amino acids to very complex molecules such as tannins, tetraterpenoids or extended quinones. Swain (1977) lists the various classes to which the chemicals belong and gives a few examples for each class. Numerous papers have appeared in the literature suggesting that these chemicals act as attractants or repellents, ovipositional or feeding stimulants, acute or chronic toxicants (Berenbaum,1986; Frazier,1986).

More recently, an increasing number of plant secondary compounds have been found to be more toxic to insects when light activated. These phototoxins are produced by a number of biosynthetic pathways and operate by various modes of action,

suggesting that this property may have evolved several times in plants. The selective advantage of phototoxicity is that it provides access to excited state chemistry and affords protection to plants using a relatively free commodity, light.

To feed successfully on plants possessing physical barriers or containing toxic chemicals which are active in their 'ground state' or 'excited state', insects have developed behavioral (see review by Tallamy, 1986) or physiological countermeasures. Behavioral adaptations include avoidance or feeding restricted to plant parts that are non-toxic. Physiologically, insects have evolved tissue or components of tissue which are insensitive to toxic compounds and/or a battery of detoxifying enzymes which enable them to cope with many of the harmful chemicals in their host plants (Dowd et al., 1983).

Although there are numerous reports on insect adaptations to a number of non-phototoxic chemicals in plants (see review by Dowd et al., 1983), the furanocoumarins are the only group of phototoxic compounds for which insect adaptation has been investigated in reasonable depth (Berenbaum, 1978; Ivie et al., 1983; 1986). Behavioral adaptation includes concealed feeding such as leaf miners and leaf rollers (Berenbaum, 1978) which feed successfully on plants containing high concentrations of furanocoumarins. Biochemical adaptations in tolerant species include rapid conversion of the parent molecule to non-phototoxic metabolites prior to excretion (Ivie et al., 1983; 1986).

The role of another group of phototoxins, the polyacetylenes and thiophenes in plant-insect interaction has recently drawn considerable interest. In an initial screening test for the biological activity of these groups of chemicals, the thiophene, alpha-terthienyl (α-T), was shown to have outstanding activity and a novel mode of action towards a wide variety of organisms (see review Downum , 1986). From previous studies (Champagne, 1985; Downum et al., 1984) and part of the present research, it is

evident that α-T shows considerable variation in its insecticidal activity towards herbivorous lepidopterans: the tobacco hornworm, *Manduca sexta*; the tobacco budworm, *Heliothis virescens*; and the European corn borer, *Ostrinia nubilalis*. To investigate the reasons for this, the following studies were undertaken :

1) To determine if there is any selective absorption by the cuticle of the 3 above mentioned lepidopteran species to light, in the near UV (320 -400 nm) and visible region;

2) To determine the distribution of α-T in the different tissue compartments of the 3 different species after a fixed time of ingestion;

3) To investigate the rate of excretion of the compound after topical applications and feeding studies;

4) To study the *in vitro* and *in vivo* metabolism rates of the allelochemical in the three insect species.

5) To quantify the cytochrome P-450 titres in the gut of the three lepidopterans.

These studies were designed not only to provide additional information on how 'tolerant' and 'sensitive' insect species handle this allelochemical in their food, but were also necessary to evaluate the effective use of this chemical as a potential botanical insecticide for an integrated pest management program.

LITERATURE REVIEW

Studies of phytophagous insects have long been prominent in evolutionary literature. The involvement of chemicals as protective agents in plants to deter herbivory was first suggested by a German botanist, Stahl, a hundred years ago (Stahl, 1888). In developing a preliminary hypothesis concerning how insects identify their host plants, researchers drew from prevailing models in ethology and vertebrate neurobiology. The initial discovery that glucosinolates (mustard oil glycosides) in cruciferous plants acted as strong feeding stimulants for certain insects (Verschaffelt, 1910) stimulated research into the effect of secondary plant compounds on insect behavior. A major controversy, which still remains unresolved, is the extent to which individual insects and plant taxa have coevolved (engaged in adaptation and counteradaptation through evolutionary time).

Interest grew after publications by Fraenkel (1959, 1969) and Ehrlich and Raven (1964) which related host specificity and pattern of insect evolution to the defensive properties of secondary plant compounds. Summarizing the early theories, Fraenkel (1959) concluded that these secondary plant compounds acted as a class of 'token stimuli' (Tinbergen, 1951) to insects. Failure to extend the token stimuli concept far beyond the plants of the family Cruciferae and their associated insects (Dethier, 1982) may have led in part to the hypothesis that secondary plant compounds defined host ranges by signalling to the insects what not to eat (Jermy, 1958; 1961). The idea was consistent with the growing evidence that many secondary plant compounds such as nicotine, strychnine and pyrethrins were toxic to insects. However, mechanisms regulating host selection in insects are considerably more complex than were first suspected.

Traditional concepts stressing single compound control of host choice have failed to account for the accumulated evidence, and new theories are gaining

acceptance which emphasize the integration of total sensory input and the interaction of pre- and post ingestive mechanisms (Dethier, 1982; see review by Miller and Strickler, 1984). Dethier (1937) stressed the importance of perception of total plant stimuli. He reemphasized this integrative approach of the perception of 'Gestalt' (Dethier, 1982), i.e., sensory neurons in insects are not narrowly tuned to individual stimuli (labelled lines), but have a broad and overlapping spectra (across-fibre pattern) like that in vertebrates (Pfaffman, 1941).

2.1 Assessing toxicity

The protocol followed to determine whether a plant secondary compound plays a defensive role involves assessing its toxicity towards different insect species. A detailed critical account of the different biological assays involved was provided by Lewis and Emden (1986), Berenbaum (1986) and Kogan (1986).

In general there are three basic methods used in toxicity testing:

a) Topical application of the compound in question and monitoring mortality over a period at 24h intervals and calculating the LD_{50} or LC_{50} values (lethal concentration required to kill 50% of the test population) by probit analysis (Finney, 1971). Although this method is not ecologically relevant, it is still useful for screening the activity of a large number of chemicals in a relatively short period of time. Moreover, the data obtained from such studies can be used in quantitative structure activity relationships (QSAR), and helps draw comparisons of the efficacy of the compound to that of other natural or synthetic insecticides. A major disadvantage is that behavioral responses of the insects are overlooked, for example, feeding deterrency.

b) Feeding the compound to the insect and studying the acute and chronic effects (Berenbaum, 1986). In such studies the developmental parameters such as larval weight,

pupal weight, days to pupation, per cent adult emergence, sex ratios, fertility and fecundity are measured. The ED_{50}, i.e., the dose required to reduce growth by 50%, is also measured. Thus, by recording relative consumption and growth rates, one can distinguish antifeedant effects from toxicity. Measurement of nutritional indices (approximate digestibility, efficiency of conversion of ingested and digested food) (Waldbauer, 1968) gives additional information which pertains to the mode of action of the compound.

c) Direct injection of the chemical into the body cavity of the insect has been used in classical insect physiological studies, for example, for screening antihormonal activity of plant compounds. This method is, however, inappropiate from an ecological or evolutionary standpoint and is not recommended if the purpose of the study is to assess testing the defensive nature of allelochemicals from plants.

2.2 Adaptations by insects

There are very few reports on behavioral adaptations of insects to physical and chemical barriers imposed by the plants. An example of avoidance of physical defence is exhibited by the caterpillars of an ithomiid butterfly, *Mechanistis isthmia* , which spin silk over the leaves, using this to crawl to the spineless edges where they feed successfully (Rathcke and Poole, 1975). In another example, the green peach aphid, *Myzus persicae*, feeds on the leaves of tobacco which contains the toxic alkaloid nicotine, by restricting its feeding to the phloem of the plant which is devoid of nicotine (Guthrie et al., 1962). In an interesting study, Carroll and Hoffman (1980) provided evidence for a behavioral counterresponse by a coccinelid beetle, *Epilachna tridecimnotata*, to induced resistance mechanisms in *Cucurbita moschata*. The authors suggest that trenching behavior observed in the coccinelid beetle is probably an adaptive response to the plant's rapid mobilization of chemical substances, like cucurbitacins, to the site of insect feeding.

Such behavior was not observed in a chrysomelid beetle, *Acalymma vittata*, where cucurbitacins serve as feeding stimulants.

Physiologically, insects employ three strategies to overcome chemical barriers in plants (Duffey, 1980; Blum, 1983) :

a) they store the chemical in an unaltered form where it will not harm the basic metabolic process (sequestration);

b) the rate of excretion increases with the chemical remaining in unaltered form;

c) there is biochemical alteration which is followed by rapid excretion or storage.

Before discussing the physiological processes involved in detoxification, some classical studies of insect response to specific plant allelochemicals are presented.

2.2.1 Insect response to specific chemicals

2.2.1.1. L-canavanine

L-canavanine, a non-protein amino acid, is found in seeds of plants belonging to the family Leguminoseae and can account for upto 13% of the dry weight of some seeds. At this level, when added to the larval diet of the seed beetle *Callosobruchus maculatus* and the tobacco hornworm *Manduca sexta*, it is highly toxic (Dahlman and Rosenthal, 1975). The mode of action involves incorporation of L-canavanine in place of L-arginine, since L-canavanine is an analogue of arginine, leading to the formation of aberrant proteins. However, the seed beetle *Carydes brasiliensis*, is known to feed on seeds containing L-canavanine without any detrimental effects. This is explained by the ability of arginyl t-RNA synthetase of this bruchid beetle to discriminate between L-arginine and L-canavanine (Rosenthal et al., 1976). Furthermore, the unusually high titres of arginase and urease activity in *C.brasiliensis* transform the guanidinoxy group of

L-canavanine into carbon dioxide and ammonia (Rosenthal et al., 1977); the latter is utilised for primary metabolism (Rosenthal and Janzen, 1985). Moreover it is reported that when ^{14}C-L-canavanine was fed to the bruchid beetle or tobacco hornworm larvae, the hornworm proteins contained significant amounts of the label whereas the beetle proteins have only had traces of label (Rosenthal, 1986).

2.2.1.2. Nicotine

Nicotine, an alkaloid present in the tobacco plant, *Nicotiana tabacum*, is a cholinergic toxin i.e., it binds to acetylcholine receptors evoking massive discharges of neural activity leading to blockage of synaptic transmission (Flattum and Sternburg, 1970). Although nicotine has been used as an insecticide and is highly toxic to several insect species (Flattum and Sternburg, 1970; Sattelle, 1978), the tobacco plant containing this neurotoxin is a host plant for a number of insect species. Rapid excretion of intact nicotine has been demonstrated in *M. sexta*, *Trichoplusia ni* and *Heliothis virescens* (Self et al., 1964 a) whereas in the tobacco wireworm, *Conoderus vespertinus*, the cigarette beetle, *Lasioderma serricorne* and the grasshopper *Melanopus spp.*, it is effectively metabolised into cotenine (Self et al., 1964b). Additional mechanisms for insensitivity in *M. sexta* have been shown to be local metabolism of nicotine by the central nervous system preventing nicotine from reaching vulnerable sites of the neuropile (Morris, 1983 a,b), as well as impermeability to nicotine ("blood brain barrier"), tissue insensitivity and regulation of nicotine entry by the CNS (Morris, 1984 a,b). Other modes of avoiding toxicity has been attributed to a carrier system in the hemolymph capable of concentrating nicotine in the Malpighian tubules, thereby regulating nicotine entry into sensitive areas or forming barrier complexes around the nervous system. This may involve protein binding and could also protect the nerve cord against nicotine effects (Maddrell and Gardiner, 1976).

2.2.1.3 Chromenes

Chromenes present in the common bedding plant *Ageratum sp.* and avocado *Persea amaericana* (Slama, 1979; Bowers et al., 1976), were shown to possess antijuvenile hormone effects leading to precocious metamorphosis in hemimetabolous insects, thus their name precocenes. Interruption of normal nymphal development to produce precocious adults and the sterilization of adult females has been reported mostly in hemimetabolous insects (Bowers, 1985). The mode of action of precocenes involves conversion of the precocene to its reactive epoxide in the corpora allata which binds to cellular proteins and which, in turn, leads to failure of juvenile hormone synthesis (Pratt, 1983). The differential sensitivity in hemi- and holometabolous insects has been attributed to the excretion rates. Ohta et al. (1977) studying the rates of metabolism of precocene in nine different species, demonstrated a 37 fold variation *in vivo* . The rates were low in precocene sensitive insects (for example, *Oncopeltus*), and high in precocene insensitive insects (examples in *Ostrinia nubilalis*, and in *Trichoplusia ni*). Recently Haunderland and Bowers (1985) compared the pharmacokinetics of precocene II in *O. fasciatus* (sensitive) and *Heliothis zea* (insensitive) species. In *H. zea*, precocene II was transported to the gut, and metabolised quickly and excreted (90% of the applied dose), whereas metabolism was significantly slower in *O. fasciatus*. Rapid metabolism in the gut or fat body preventing toxic concentrations from reaching the corpora allata was suggested to be the mechanism involved.

2.2.1.4 Cardenolides

Cardenolides are sterol derivatives which inhibit the Na^+-K^+- ATPase system. The monarch butterfly, *Danaus plexiplus*, which forages on plants of the family Apocynaceae and Asclepiadaceae containing high concentrations of cardenoloides, has been shown to have a Na^+-K^+-ATPase located in the neural tissues that is approximately

300 times less sensitive than the same enzyme from non-adapted species (Vaughan and Jungries, 1977). Impermeability of the neural sheath to cardenolides is the result of the presence of high titres of K^+ in the insect hemolymph which antagonizes the binding of the chemical to the active site of the enzyme. Lack of conventional K^+, stimulated ATPase and sequestration of the compounds in dorsolateral abdominal spaces, as in *O. fasciatus* (Scudder et al., 1986), have been suggested as adaptive methods in insects feeding on cardenolides.

2.2.1.5 Pyrollizidine alkaloids

Pyrollizidine alkaloids are found in the groundsell, *Senecio vulgaris* and the tansy ragwort, *Senecio jacobaea.* The mechanism of action of pyrollizidine alkaloids involves conversion of the alkaloid *in vivo* to a metabolite (pyrolle 1) which is cytotoxic. The tiger moth, *Arctia caja* , and the cinnabar moth, *Tyria jacobaeae*, store the chemical unchanged, thereby avoiding toxicity (Harborne, 1982).

2.2.1.6 Cyanogenic glycosides

Cyanogenic compounds are present mainly as glycosides in plants such as Cycads and at least 60 flowering plant families. On ingestion by an insect, the cyanogenic glycoside undergo disassociation with the release of HCN which interferes with the electron transport system and leads to death. Studies by Teas (1967) showed that larvae of the moth, *Seirarctic echo* , that feed on the seeds of many Cycads, can tolerate high levels of methylazoxy-methanol, commonly known as cycasin, because of their rapid ability to reconvert the toxic aglycone formed as a result of beta-glucosidase activity quickly back to the glucoside form (cycasin) and store it. The enzyme rhodanase, found in a few insect species, is capable of converting the toxic cyanide to thiocyanate, a relatively nontoxic form (Beesley et al., 1985).

2.3 Detoxification

The term detoxification refers to the chemical alteration of an ingested compound such that the altered product is non-toxic to the organism. Transformation of the compound is achieved in two steps, primary and secondary, also referred to as phase I and phase II (Brattsten, 1979). The two phases, are carried out by complexes of relatively non-specific enzymes. In phase I, a polar group is added to the substrate. The different chemical reactions that are involved include oxidation, reduction, hydrolysis or group transfer. The resulting molecule, if sufficiently hydrophillic, is excreted at this stage; otherwise it acts as a substrate for the phase II reaction which involves addition of sugars, amino acids, sulphates or phosphate groups to achieve a still more polar compound to facilitate excretion (Brattsten, 1979). The phase I reactions are catalysed by a group of non-specific enzymes, the catalytic centre of which is occupied by cytochrome P-450; the conjugating or phase II reactions are catalysed by the enzyme glutathione-S-transferase (Yu, 1982). The polysubstrate monooxygenase system has attracted considerable attention from both the ecological and the applied standpoints and is discussed below.

2.3.1 Polysubstrate monooxygenase system

In insects, the polysubstrate monooxygenase system (PSMO) is localised in the midgut, fatbody and Malpighian tubules. The midgut is generally, but not always, the site of greatest activity (Hodgson, 1983). Within a cell, the enzyme is firmly attached to the smooth and rough endoplasmic reticulum, the specific activity in the latter is usually higher (Hodgson, 1985). The system is composed of three basic components: two enzymes and a lipid. The catalytic centre, as previously mentioned, is occupied by cytochrome P-450. The enzyme NADPH cytochrome P-450 reductase transports reducing equivalents from NADPH to cyt P-450. Reconstitution studies have shown the

neccessity of phosphatidyl choline, a lipid, for normal operation of the system (Lu and Coon, 1981; Mayer and Durant, 1979; Naquira et al., 1980).

Cytochrome P-450 is a b type cytochrome with protophyrin IX as the prosthetic group and iron in the native state. It has a molecular weight of 40,000-60,000 daltons. The reduced cytochrome-CO complex shows a difference spectrum with an absorption peak at 450 nm (Omura and Sato, 1964). This characteristic absorption maximum can be modified by numerous ligands to yield Type I, II and III spectra (Brattsten, 1979). The molar extinction coefficient of cytochrome P-450 from rabbit liver has been estimated to be 91 $mM^{-1}cm^{-1}$ (Omura and Sato, 1964). Levels of cytochrome P-450 vary considerably within and between insect species, strain, age and sex (Hodgson, 1985 and references within). A variety of direct and indirect studies have demonstrated that cytochrome P-450 exists in a number of related forms which can co-exist in the same cell type (Naquira et al., 1980). The details of the catalytic events are however not known. The generally recognized steps are summarized in Fig. 1.

From induction (*de novo* protein synthesis), toxicity and metabolism studies, the primary function of polysubstrate monooxygenase system (PSMO) in insects is assumed to be the detoxification of allelochemicals present in host plants (Kreiger et al., 1971; Brattsten, 1983; Yu, 1987), although evidence leading to this assumption has been questioned (Gould, 1984). Other roles for MO in insects include lipid peroxidation, sterol biosynthesis, juvenile hormone and ecdysone biosynthesis (Hodgson, 1985 and references within).

An ecological role for PSMO in host plant selection has not been properly established. Kreiger et al. (1971) tested *in vitro* activity of PSMO from the larval gut of 35 lepidopteran species and found that the microsomal fraction of polyphagous insects showed 15 times more activity than that of monophagous insects. This led Whittaker and

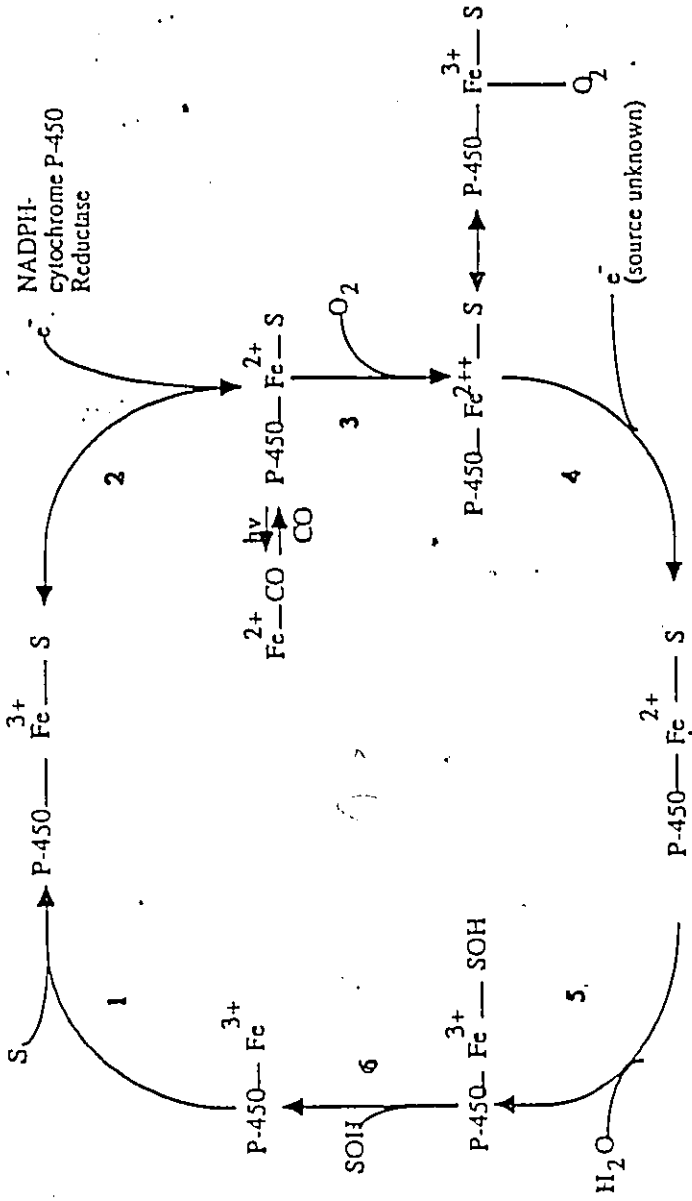


Fig. 1 : Recognised events of cytochrome P-450 operation. S=substrate
(Adapted from Hodgson, 1985)

Feeny (1971) to postulate that specialist herbivores carry a lower "metabolic load" than polyphagous insects, which expend more energy to maintain higher titres of PSMO in order to cope with the diverse group of chemicals they encounter. However, Rose (1985) found no correlation between the degree of polyphagy and MO activity for a number of lepidopteran larvae, suggesting that polyphagous insects do not necessarily carry a heavier "metabolic load". Experimental evidence lending support to this idea has been derived from the work of Neal (1987) who showed that there is no difference in food utilization or growth rate in larvae of *H.zea*, with a seven fold greater cytochrome P-450 level and a 9 fold O-demethylase activity, to controls in which the enzyme was not induced.

Gould (1984) and Dowd et al. (1983) argue that although allelochemicals have been shown to induce PSMO, there is no evidence to suggest that the same chemicals are metabolised by PSMO. However, recent experiments have shown that plant allelochemicals such as coumarins, ß-pinene and many others, induce PSMO's and are metabolised by PSMO's (Yu, 1987).

2.4 Photosensitizers

In addition to compounds present in plants which are allelochemicals in their 'ground state', there is a unique group of secondary plant substances that require light (320 nm-700 nm) for the full expression of their toxicity. These are known as photosensitizers and occur in a large number of plant families (Towers 1984, Knox and Dodge, 1985; Downum, 1986). Some examples of natural photosensitizers are iron free porphyrins, chlorophyll, flavins, benzofurans, furanocoumarins, ß-carboline alkaloids, polyacetylenes, thiophenes and quinones. A photosensitizer is defined as a chemical which can be excited to a higher electronic state on absorption of a photon of light, which is then capable of reacting with some other suitable molecule in a given system (Towers,

1984). These sensitized reactions in biological systems usually create some form of damage.

Most of these reactions involve the participation of molecular oxygen, i.e., they are photodynamic. There are two mechanisms of photodynamic action: Type I and Type II (Gollnick, 1968). In both Type I and type II the ground state sensitizer (S_0) is excited to its singlet state (^1S). This short lived ^1S species readily forms the triplet excited state (^3S) of the sensitizer by undergoing intersystem crossing.

In a type I process, the triplet state of the sensitizer reacts with the substrate via an electron transfer. The final product is a superoxide radical or hydrogen peroxide which may oxidize biomolecules (e.g., anthraquinone dyes and flavins). In a type II process, the triplet state interacts with ground state oxygen ($^3O_2$, which is a triplet state) yielding ground state sensitizer and singlet oxygen (e.g., isoquinoline alkaloids and thiophenes). In this energy transfer mechanism, the sensitizer is not consumed or destroyed, but is used repeatedly in a catalytic fashion. The activated species of oxygen from Type I and Type II processes cause biological damage by oxidizing molecules such as amino acids, proteins, lipids and nucleic acids (see review Spikes and Straight, 1987). Lipid peroxidation is one of the most serious consequences and leads to membrane damage and lysis.

In contrast, there are a few sensitizers that do not require molecular oxygen for their action (non-photodynamic sensitizers). The excited triplet state of these molecules interacts with DNA forming mono or difunctional adducts with pyrimidine bases (usually thymine) to form irreversible cycloadducts which then interfere with template formation and transcription (Scott et al.,1976). Unlike photodynamic reactions, the sensitizer is consumed in the process (e.g., furanocoumarins and furoquinolines).

The first documented study in which light was understood to cause an

enhancement of a chemically induced toxic effect was that of Maracci (1888 - cited in Heitz, 1987). He reported that alkaloids were more effective against seeds, plants, and amphibian eggs in sunlight than in darkness. By 1904, Jodlbauer and van Tappeiner (1904) had demonstrated the requirement of oxygen, and had coined the term "photodynamic action". Raab (1900) following systematic quantitative studies, showed that the toxicity of the dyes acridine and eosin to the protozoa was several orders of magnitude higher in the presence of daylight. The first reported use of photodynamic action against insect targets was that of Berbieri (1928 - cited in Heitz, 1987) in which *Anopheles* and *Culex* mosquito larvae were shown to be susceptible to solutions of dyes in direct sunlight. There was no mortality in the absence of light, suggesting a phototoxic mode of action. Since then, the phototoxicity and the biological activity of a large number of synthetic dyes (see review by Heitz, 1987) and natural products (see reviews by Arnason et al., 1983a; Towers, 1984; Downum and Rodriguez, 1986), have been studied. The biological activity of the thiophene α-T has drawn considerable attention in the past few years and is discussed at length below.

2.4.1 Alpha-terthienyl

Alpha-terthienyl (Fig. 2) is a sulphur derivative of polyacetylenes consisting of a series of three thiophene (sulphur heterocycles) units bonded at the alpha position. This compound was first prepared by Steinkoph in 1941 even before its occurrence in plants was reported. Zechmeister and Sease (1947) isolated milligram quantities of the blue fluorescing compound from the petals of the "African" marigold *Tagetes erecta*. The concentrations reported in the petals was approximately 15-21 mg/kg. There were two statements made in this seminal paper which were rather interesting. First the authors say 'its role in marigolds remains problematical' and second it "is void of antibiotic potency against microorganisms like *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, or *Pseudomonas ovalis*" (Zechmeister and Sease, 1947). Modern

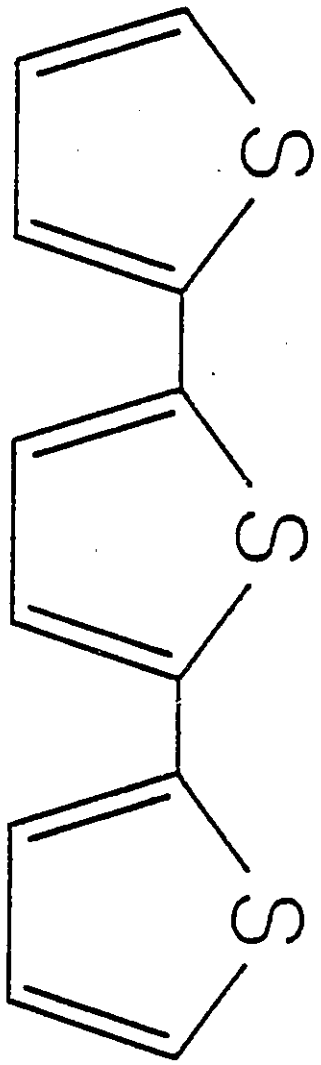


Fig. 2 : Structure of 2, 2'5', 2"-Terthienyl

chemical ecology has much to say about these points.

Natural sources of this compound are restricted to advanced tribes of the family Asteraceae including Helianthae, Inuleae, Vernoneae, Senecioneae, Anthemidae, Cynareae and Tagetae (Bohlman et al., 1973). In the Helianthae, the genera *Adenophyllum*, *Chrysactinia*, *Dyssodia*, *Hymenatherum*, *Nicolletia* and *Porophyllum* contain α-T, the concentration in these plants ranging from 20-440 μg/g dry weight (Downum et al., 1985).

Using labelling studies, Bohlman et al. (1967) showed that the precursor of thiophene biosynthesis in plants is oleic acid. Polyacetylenic compounds, which are long chain molecules with conjugated triple bonds, are intermediates formed by ß-oxidation of the C_{18} fatty acid. Decarboxylation of specific polyacetylenes has been proposed to lead to C13 intermediates, the penta-yne-ene. Following this, cyclisation of two adjacent acetylenic bonds and the incorporation of a sulphur atom leads to thiophene production (Bohlman et al., 1973). The sulphur source in plants is speculated to be methionine or sulphate (Schuetz et al., 1965). A detailed description of the different schemes proposed for α-T synthesis in plants has been given by Bohlman and Zdero (1985).

Research related to the enzymes that may be involved in the biosynthesis of α-T has received little attention. Sütfield and Towers (1982) and Pensl and Sütfield (1985) suggest that the bithiophene, 3-hydroxy-4-acetoxy butinyl bithiophene found in *Tagetes* seedlings, is formed from a highly specific enzyme 3,4-diacetoxy butinyl bithiophene:4 acetate esterase. On the other hand, an organic synthesis has been developed for large quantities of α-T (50-100g) using the one pot Grignard Wurtz coupling reaction (Tamao et al., 1982). In this reaction, 2-bromothiophene and 2,5-dibromothiophene react, in the presence of the catalyst nickel, to give α-T. The crude α-T

formed is then purified by continuous Soxhlet extraction of a crude α-T/silica gel mixture (1:1) with hexane as the solvent (Mac Eachern 1987). Purity of >99% has been obtained. Yields in the order of 75% have been reported (Philogène et al., 1985).

2.4.1 Biological activity of α-T

The observation made by Slootweg (1956) that *Tagetes* was resistant to nematode induced root lesions in the field, promoted a study by Uhlenbroek and Bijloo (1958, 1959), which showed that α-T and BBT (5-(but-3-en-1-ynyl)-2-2`-bithenyl) were the active nematicidal principles present in the marigold *Tagetes erecta*. In addition, the study involved screening a number of analogues of α-T for activity, which indicated that a bithienyl molecule was a minimal requirement for bioactivity. Daniels (1965), working with crude extracts of marigolds and other members of the family Asteraceae, showed that light enhanced the toxicity of the extracts towards the yeast, *Candida albicans*. Following up previous studies, Gommers (1972) and Gommers and Gerrlings (1973), showed that sunlight enhanced α-T toxicity towards nematodes, and that the spectral region responsible for the increment was near UV (300-400 nm) which corresponds to the absorption maxima of α-T. Later these findings were also reported by Chan et al. (1975) who showed that UV enhanced the toxicity of α-T. In subsequent experiments, Gommers et al. (1980), using scavengers of singlet oxygen such as sodium azide, methionine or histidine, demonstrated that in the presence of the quenchers there was no inactivation of the enzymes glucose-6-phosphate dehydrogenase, malate dehydrogenase or acetylcholine esterase. A significant decrease in activity occurred in the absence of these quenchers. Additionally, loss in enzyme activity was not protected when superoxide dismutase, catalase or mannitol, scavengers of superoxide ion, hydroxyl radical and peroxide, were used.

These studies clearly demonstrated that α-T operated as a Type II

photosensitizer, with singlet oxygen being required for toxicity. The studies also indicated that Type I mechanisms were not involved. Conversely, in the first *in vivo* study, Kagan et al. (1980) showed α-T to be non-photodynamic towards *E.coli*, as it was toxic to the organism in the absence of oxygen. Furthermore, in these studies using *C.utilis*, no protective effect was reported when singlet oxygen quenchers were used, inferring the absence of Type II mechanisms. Binding of ^{14}C labelled α-T to calf thymus DNA suggested a possible photogenotoxic effect. Since then there have been a number of studies which contradict Kagan et al. (1980) findings and confirm the neccessity of oxygen involvement in α-T toxicity, ruling out the possibility of α-T binding to DNA. Wat et al. (1980) showed chemical evidence of singlet oxygen production by using cholesterol, which yields 5, α-hydroperoxide cholesterol, and 7, ß-hydroperoxy cholesterol in the presence of $^1O_2$ and a free radical, respectively. In the presence of UV light and α-T, the 5, α-hydroperoxide was formed, showing the production of $^1O_2$ and absence of radical formation.

In the same study, Wat et al. (1980) examined the effects of α-T on human erythrocytes and found hemolysis, K^+ leakage and acetylcholine esterase (AChE) inhibition. Furthermore, $^1O_2$ was shown to enhance AChE inactivation. It was suggested that membranes may be the target site for toxicity and that $^1O_2$ is required. Further evidence for $^1O_2$ involvement was provided by Arnason et al. (1981a) who showed that the bioactivity of α-T against *E.coli* and *S.cerviseae* was enhanced by about 5 orders of magnitude in the presence of $^1O_2$, and $^1O_2$ involvement confirmed, with cell survival enhanced in the presence of $^1O_2$ quenchers.

MacRae et al. (1980) examined α-T for ability to induce cytogenetic damage and using Syrian hamster cell cultures and sister chromatid exchange protocol, demonstrated no chromosomal aberration. The work of Downum et al. (1982), who reported DNA repair deficient *E.coli* showed the same sensitivity as wild type, and

provided further evidence that there is no DNA involvement in the mode of action of α-T.

The larvicidal activity of α-T has drawn considerable attention. Wat et al. (1981) found the compound to be highly toxic to the mosquito *Aedes aegypti* and the blackfly *Simulium vittatum* in the presence of near UV light at 0.5 mg/ml α-T. Subsequent studies by Arnason et al. (1981b) showed α-T to have unique phototoxic effects on *A. aegypti*. The LC_{50} value for 4th instar larvae was 19 ppb in a UV regimen indicating α-T to be more toxic than DDT (LC_{50}, 70ppb). The action spectrum for photosensitization resembled the absorption spectrum, indicating the photoreceptor molecule to be α-T and not a complex with another molecule or a photosensitizing metabolic product (Arnason et al., 1981b). Alpha-T has also been been shown to be highly phototoxic to the blackfly larvae *S. verecundum* with an LC_{50} of 28 ppb and an LC_{90} of 70 ppb under controlled laboratory conditions (Philogène et al., 1985).

Field trials with α-T and sunlight on 3rd and 4th instar larvae of *A. intrudens* have indicated an EC_{50} of 0.02 kg/ha and an EC_{90} of 0.05 kg/ha. Studies on non-target species have been inconclusive. Arnason et al. (1987) showed that α-T was nontoxic to the trout *Salmo gairdneri* and the snail *Physa sp.* at an application rate of 10 kg/ha. The EC_{50} and EC_{90} for caddisfly, midge and damsel fly were several fold higher than for mosquitos but for *Daphnia* the LC_{50} was similar to that of mosquitoes. Studies by Kagan et al. (1984, 1986) report LC_{50} values of 18-111 ppb and 50-650 ppb for tadpoles and fathead minnows respectively, but the use of dimethyl sulphoxide as a carrier in these studies has been questioned (Arnason et al., 1987).

Photoovicidal activity of α-T towards *Drosophila melanogaster* has been demonstrated by Kagan and Chan (1983). In the presence of UV light, α-T was ovicidal at a concentration of 0.0006 g/L whereas in the dark the LD_{50} value was 1.26 g/L. Recently Kagan et al. (1987) have shown phototoxicity of α-T towards mosquito pupae,

the LC_{50} being 0.06 ppm.

The first reports on the toxicity of α-T to herbivorous insects were published in separate studies by Champagne et al. (1985) and Downum et al. (1984). When fifth instar larvae of the tobacco hornworm were administered the chemical topically or by direct injection into the body cavity at 50 µg/g body weight, and irradiated with near UV light, reduced larval weight, delayed pupation and pupal deformities resulted. In topical applications, pronounced tissue necrosis was noticed (Downum et al.,1984). Champagne et al. (1985, 1986) have shown that α-T, at a concentration of 100 µg/g in the diet, leads to reduced feeding, a slower growth rate, and reduced efficiency of conversion of digested food to insect biomass in *M. sexta* and *Euxoa messoria*. On the other hand, α-T toxicity towards *O. nubilalis* was minimal.

At the third trophic level, McDougall (1986) found that when *O. nubilalis* larvae were reared on a diet containing 100 µg/g α-T and parasitized by their natural parasitoid *Diadegma terebrans*, no females of the parasitoid were produced. The weights of the male pupae and adults were significantly lower than the controls. Per cent parasitization was reduced in dual and no choice tests.

Although the full expression of toxicity of α-T requires UV light, light independent toxicity referred to as 'dark toxicity', has also been shown in mosquitoes (Arnason et al., 1983; Kagan, 1983); fruitfly eggs (Kagan, 1983) and herbivorous insects (Champagne et al., 1985, 1986; Mc Dougall, 1986).

The cell membrane may be the primary site of action of α-T. Wat et al. (1980) showed that cytoplasmic enzymes of erythrocytes were deactivated following hemolysis, and that the enzyme AChE, a membrane bound enzyme, was deactivated by cytoplasmic enzymes. Crosslinking of membrane proteins of *E.coli* in the presence of UV light and α-T was demonstrated by Downum et al. (1982). Recently Hudson et al. (in

press) have found that membrane bound viruses are more sensitive to α-T treatment than viruses that are not membrane bound.

2.5 Experimental insects

2.5.1 *Manduca sexta* (L.) (Lepidoptera:Sphingidae)

The tobacco hornworm, *Manduca sexta*, is an oligophagous insect pest feeding on a wide range of Solanaceous plants including tobacco, potato, tomato, pepper, egg plant, Jerusalem cherry, ground cherry, etc. (Teitz, 1972). The most common occurrences have been in Canada, the United States, Mexico, West Indies and in the South American continent. In Canada, southern Ontario and Quebec are infested with the insect (Madden and Chamberlain, 1945). The adults emerge in early summer from the pupae which overwinter in the soil. The females deposit mature eggs singly on the undersurface of leaves. The incubation period is usually 3-8 days, depending on the temperature. There are five instars, occasionally six. The most damaging stage is the 5th instar, since 80% or more of the feeding is done at this stage. The larvae reach 7-11 cm in length and attain a weight of 8-10 gm. The larval period lasts about 20 days. On maturing the dorsal aorta becomes distinctly visible along the mid dorsal line. At this stage the larvae stop feeding, go below the soil and form a tunnel to pupate. Between the larval and pupal stage, the larva enters a prepupa stage. There are 1-4 generations/year depending on the latitude (Davidson and Lyon, 1979). In the absence of diapause and in insects entering diapause, the complete life cycle takes around 60 days and 130 days, respectively (Bell et al., 1975). Because of its size, the tobacco hornworm has become the "laboratory rat" for experiments in insect physiolgy and insect toxicology.

2.5.2 *Heliothis virescens* (Fab.) (Lepidoptera:Noctuidae)

The tobacco budworm, *Heliothis virescens* , is found throughout most of

the Western Hemisphere, more specifically in the Southern United States, Canada, West Indies and South America as far south as Argentina. The host range of this insect pest includes cotton, tobacco, pigeon pea, corn, clover, alfalfa, soybean, snapdragon, tomatoes, garden and sweet peas (Teitz, 1972).

Females lay eggs on either upper or lower leaves. About 500 fertile eggs are laid by a female. Color variation in the larvae is common and can range from light green to pink or reddish brown. The larval development period is usually 18-31 days, depending on the temperature and diet. The larvae overwinter as pupa about 1-2" below the soil. In the field, there are one to two generations per year, with every generation spanning between 33 and 46 days.

2.5.3 *Ostrinia nubilalis* (Hübner) (Lepidoptera:Pyralidae)

The European corn borer (ECB), *Ostrinia nubilalis*, is a notoriously polyphagous insect which feeds on a large number of plant families. Although the major host is corn with sweet corn being preferred, the host range also includes pepper, beet, chrysanthemum, dahlia, gladiolus, egg plant, bean, potato and many weeds. An extensive host range has been published by Caffrey and Worthley (1927). The European corn borer (ECB) is known to occur in Europe, Asia, USSR (West Belt), North Africa and North America. It is beleived that the pest was introduced into the USA through shipments of broomcorn from Europe around the begining of the century and hence its common name. In Canada, sporadic outbreaks occur in the Maritimes. In Ontario and Quebec, the borer populations are higher than in other provinces. Borer damage is slight in Saskatchewan and Manitoba. Recent incidence of corn borer outbreaks have been reported in Alberta. The adult moths emerge and escape through the burrows dug in the plants during May and June. After mating, the females lay eggs on the under surface of the lower leaves on stalks, flag leaves or ear husks. The eggs are found in masses of 15-

30. Each female lays approximately 500 eggs during her lifespan. The incubation period lasts for 4-7 days. On hatching the larvae feed on the leaves for a few days causing a pin-hole like appearance. Later, in corn they tunnel into all parts of the stalks and ears. This leads to broken stalks and tassels, poor ear development and dropped ears. In other hosts, damage is done primarily by tunneling of the stalks or stems by the larvae. The larvae go through five instars. The larval period lasts about 35 days. The larvae pupate in tunnels formed inside the stem. There are 1-4 generations/year depending on the latitude; the number increasing with decreasing latitude. In Canada there are two strains of the species known; a single generation strain and a two generation strain. Borers overwinter as larvae in diapause concealed in parts of plants in which they have been feeding. Pupation takes place in late spring.

MATERIALS AND METHODS

3.1 Insects

3.1.1 *Manduca sexta*

The culture of tobacco hornworm, *M. sexta* was originally obtained from Carolina Biological Supply Co., N.Carolina, as pupae that were reared through many generations according to the following procedure. The pupae were sexed and approximately 20-25 males and 20-25 females were placed together in a screened plexiglass cage (60 x 60 x 48 cm) in an incubator at 25±1^{0}C and with a 18:6, L:D photoperiod after adult emergence. At the onset of mating, a tobacco plant was placed in the cage for oviposition and the adults were provided with 20% sucrose solution. Eggs were collected and placed in plastic petri dishes lined with a moistened Whatman #1 filter paper.

Larvae were reared according to the methods outlined by Bell and Joachim (1976) and Stewart (1984) with a few modifications. The meridic diet (Appendix I) described by Stewart (1981) was used without dried tobacco leaves and addition of 0.01% benomyl (a fungicide) and 0.01% chlorotetracycline (an antibiotic). Diet cubes were placed in the petri dishes with the eggs to make sure that food was readily available to the hatching neonates. Three to four day old larvae were transferred to 24 cell Linbro or Falcon culture plates. Tiny holes were drilled in the lid to prevent moisture build up. Early to mid 4th stadium larvae were transferred to 50 cell trays model plastic (Bioserv). Larvae going into the 5th instar with their head capsule or first day 5th instar were transferred to plexiglass containers (50 x 32.5x 5 cm) with about 50 individual compartments. The lid and floor of each compartment was drilled to prevent water condensation. Prepupae were transferred to polystyrene *Drosophila* culture vials

(10 x 3.3 cm hxd) with vermiculite inside and allowed to pupate in the dark. The larvae were maintained in an incubator at a temperature of 25±1^{0}C and a 16:8 L:D cycle to avert diapause.

3.1.2 *Heliothis virescens*

Tobacco budworm pupae were obtained from Dr. Peter Krell, Department of Microbiology, University of Guelph, Ont., Canada. On arrival, they were placed in a plastic cage with a brown paper towel (to serve as oviposition site) lining the inside of the cage. On adult emergence, two liquid scintillation vials containing 20% sucrose solution were placed in the cage. The paper towel with the egg masses was removed and placed in a 1% formaldehyde solution for 5 minutes and then rinsed several times with deionised water following which they were allowed to air dry. Small pieces of paper with 20-25 eggs were then placed in 1 oz. plastic cups with a diet cube. Halifax diet (Appendix II) was used. After about a week larvae were tranferred into new cups, this time 5 larvae/vial. Fifth and sixth instar larvae were raised individually in either the cups or 50 well trays, to avoid cannabilism. The colony was maintained in a Environator incubator, the temperature was 25±1^{0}C and a L:D period of 18:6 h maintained.

3.1.3 *Ostrinia nubilalis*

Eggs of *O. nubilalis* were initially supplied by M. Hudon, (Agriculture Canada, St. Jean, P.Q.) and subsequently reared in the laboratory. Egg masses were laid on wax paper suspended in cages of adults. These were cut out and pinned to a meridic diet (Appendix III). The protocol outlined by Guthrie et al. (1971) was followed to rear the larvae except that corn grits were added to the diet. Larvae were kept in round plastic containers with a screened black lid (185 x 85 cm, dxh). They were kept in an incubator (Conviron model E7) at 26.5^{0}C during the 16h light period and 19^{0}C for the 8h dark period. Relative humidity was maintained at 80%. When the larvae were in their last

instar, a corrugated cardboard ring was placed in the plastic chamber to serve as a pupation chamber. The rings were placed in a cage (45 x 45 x 61 cm, lxwxh) for adults made of plexiglass and aluminium screen, with temperature and light conditions similar to that described above. Water was sprayed several times daily with a hand held plant mister. This was necessary for optimal mating and egg laying. The relative humidity was maintained at a high level with a humidifier placed adjacent to the cage. Wax paper was hung inside the cage and served as the oviposition site.

3.2 Chemicals

Alpha-T was synthesised in a one pot Grignard reaction as described previously (Philogéne et al.,1985) with purity greater than 99%. Radiolabelled ^{3}H-α-T was prepared by a high temperature dilute acid procedure (Werstiuk and Timmins, 1981) which permits prediction of the order of exchange of aromatic protons. The method was optimised with D_2O before final work up in THO. Specific activity was 1.3 mCi/mmole and the compound was found to be more than 99% radiochemically pure by NMR and TLC in several solvent systems. For sample oxidation and subsequent liquid scintillation counting Monophase 40 Plus and Permafluor were purchased from Packard Instruments Company, Downers Grove, Illinois. Scintiverse and Scintilene were purchased from Fisher Scientific Company. Glucose-6-phosphate, Glucose-6-phosphate dehydrogenase, NADP and NADPH were purchased from Boehringer Mannheim, Montreal.

3.3 Toxicity Studies

Alpha-T was topically applied in 10 µL of acetone to the dorsum of early last instar larvae of *M. sexta*, *O. nubilalis*, and *H.virescence*, using a 10 µL Hamilton syringe. Control larvae were treated with acetone only. Six to eight concentrations were used with 3 replicates of 10 larvae at each concentration. Mortality was recorded 24 h post treatment. LD_{50} values were obtained from probit analysis of the data (Finney,

1971).

3.4 Growth and development studies

3.4.1 Conditions of test

In addition to the solar simulating 48 in Vita-light fluorescent lamps (Duro test No 48T12), the growth chamber was equipped with four 24 in, 20 watt Westinghouse black light blue fluorescent lamps (FT 20T12/BLB) and three 24 in Westinghouse 20W DLX warm white (F20T12/WWX) lamps placed 65 cm above the experimental animals. The black light blue lamps emitted 320-400 nm wavelength light and had an emission maximum at 350nm, the λmax of alpha-terthienyl. The RH was 80% and the temperature was maintained at 25±1^{0}C. The additional lamps in the chamber (i.e., UV and warm white lamps) were on a 12:12 h L:D photoperiod whereas the Vita-light fluorescent lamps were on a 16:8 L:D photoperiod.

3.4.2 Survivorship studies

Alpha-T dissolved in acetone was incorporated into freshly prepared meridic diet. To ensure uniform distribution, the diet was mixed vigorously with α-T. The amount of acetone was 0.5% for all concentrations prepared. The experiment consisted of four treatment groups: a) Control: -UV, -α-T; b) +UV, -α-T; c) -UV, +α-T; d) +UV, +α-T. For -UV treatment, a Kodak Wratten filter (400nm cut off) was placed on top of the experimental group to exclude UV light. For each concentration of α-T (0, 10, 31, 56 and 100 μg/g), 20 neonate larvae of *M. sexta*, were placed individually in liquid scintillation vials plugged with cotton and presented with an appropriately treated diet cube. The diet cubes were changed every two days until the larvae had reached 5th instar at which point they were fed daily. Fifth instar larvae were transferred to plexiglass trays covered with a very thin UV transparent glass. Twenty larvae of *H.*

virescens, in each of five treatment sets (0, 10, 31, 56 and 100 µg/g) were reared from egg in plastic cups containing a test diet cube. The cups were covered with a polyethylene cover. The diet cube was changed on day seven and every second day subsequently. Larvae were raised individually after day 10 to prevent cannibalism. In these experiments larval survivorship was assessed.

3.5 Distribution of ^{3}H-α-T after 24 h

Early last instar larvae of all three species were provided a cube of artificial diet containing 60,000 dpm of ^{3}H-α-T (2.5 µg/g diet). Larvae of *M. sexta* completely consumed the diet cube within 24 h, however *H. virescens* and *O. nubilalis* larvae had consumed only a part of the diet cube at 24 h. For each experiment, 5 larvae of *M. sexta*, 10 larvae of *H. virescens*, and 20 larvae of *O. nubilalis* were dissected. The experiment was repeated twice. The larvae were kept in individual compartments, under photosensitizing conditions with a combination of four cool white fluorescent lights (20 w/m^2) and four black light blue lamps (20 w Westinghouse, # F20T12) providing 2.5 w/m^2 of near UV. At harvest, hemolymph from larvae of *M. sexta* was collected by cutting off the horn and from *H. virescens* larvae by an incision in the proleg. The larvae were then placed in a dissection tray on a filter paper over wax paper. The remaining hemolymph was collected upon opening the animal along the mid dorsal line and gently absorbing the fluid onto ashless filter paper, without the fat body adhering to the paper. At this point, an appropiate amount of saline (0.15M NaCl) was applied along the gut and the saline wash absorbed onto filter paper. All the filter papers were collected including the one placed below the larva constituted a part of the hemolymph fraction. For *O. nubilalis*, all the hemolymph was collected as described above after slitting open the body. Subsequently, the tissues were separated as far as possible into gut with contents, fat body and carcass. The different tissues were taken onto ashless filter papers. Fecal pellets if found in the hindgut were removed. The fecal material produced by each larva

was also collected. With *O. nubilalis* tissue, samples from two larvae were pooled to obtain sufficient counts.

3.6 Continuous label feeding

Early last instar larvae of *M. sexta*, *H. virescens*, and *O. nubilalis*, were reared on their respective diets containing 2.5 μg ^{3}H-α-T (1 μg approx 10,000 dpm). After 24, 48, 72 and 96 h on the α-T diet, larvae were collected and immediately frozen at -20^{0}C. Feces produced by each larva for that particular time period were also collected. The amount of ^{3}H in the body and feces was determined as outlined below.

3.7 Pulse label feeding

In another set of experiments, insects were fed radiolabelled diet as above but after 24 h were provided fresh diet cubes lacking ^{3}H-α-T. The amount of ^{3}H in the body and feces was determined at 24, 48, 72, and 96 h post treatment.

3.8 Topical application

Early fifth instar larvae of the three insect species were treated on the dorsum with 50,000 dpm of ^{3}H-α-T with a 10 μL Hamilton syringe. Because of the differences in size of the larvae of the three species, α-T was applied in 5 μL of acetone for *M. sexta*, 3 μL for *H.virescence* and 2 μL for *O. nubilalis*. Treated larvae were held individually for time intervals of 0.5, 1, 2, 3, 6, 12, 24, and 48 h. Three or four larvae of each species were analysed at each time interval. At the end of the designated exposure time, each larva was rinsed in 3 mL of acetone. The acetone rinse, plus the amount remaining in the scintillation vial in which the larvae were kept, constituted the external radioactivity. Following this, the hemolymph, gut, fat body, carcass and feces were separated at the 6 h time period and thereafter.

3.9 Quantification of ^{3}H

Hemolymph collected directly from *M. sexta* and *H.virescence* was dispensed into Scintiverse cocktail and counted. The hemolymph collected on filter paper and the tissue samples with the filter paper were burnt in a Packard Tricarb 306 sample oxidizer. Fecal samples were placed in cellulose thimbles prior to oxidation. The time of burning was between 1-3 min depending on the nature and size of sample. Each *M. sexta* larva was divided into three parts to ensure complete oxidation. Samples were placed in Combusto-cones (Packard) and ashless cellulose powder to ensure proper combustion. Prior to and after running the samples, the efficiency of the machine was determined by running known amounts of ^{3}H. Controls were run on every tissue. Unlabelled samples for each tissue type were spiked with known amounts of radiolabel and recovery determined. Overall recovery was between 87-93%. After trapping the ^{3}H in Monophase, Permafluor and water, the radioactivity was measured on a Beckman LS 3133P Liquid Scintillation counter. Samples were repeatedly counted until consistent dpm were noted. This ensured that artificial photo and chemiluminescence (occurring especially in feces and gut samples) did not contribute to the final results. Quench corrections were made using quench curves from external standards to correct for chemical and color quench.

3.10 Cuticle absorbance

Fifth instar larvae of the three species under study were dissected under cold 0.15M NaCl. A longitudinal cut was made along the the mid ventral side. The gut, fat body, Malpighian tubules, trachea and muscles were removed. A small piece of cuticle was stapled onto an opaque plastic sheet (55x8.5 mm, lxw) with a mini stapler and staple #10. The plastic sheet had an opening 8x4 mm (lxw), 4.5 mm from the lower end. The plastic sheet with the cuticle was placed in a 3 mL quartz cuvette and the

absorbance read between 700 and 250 nm on a Beckman 50 spectrophotometer.

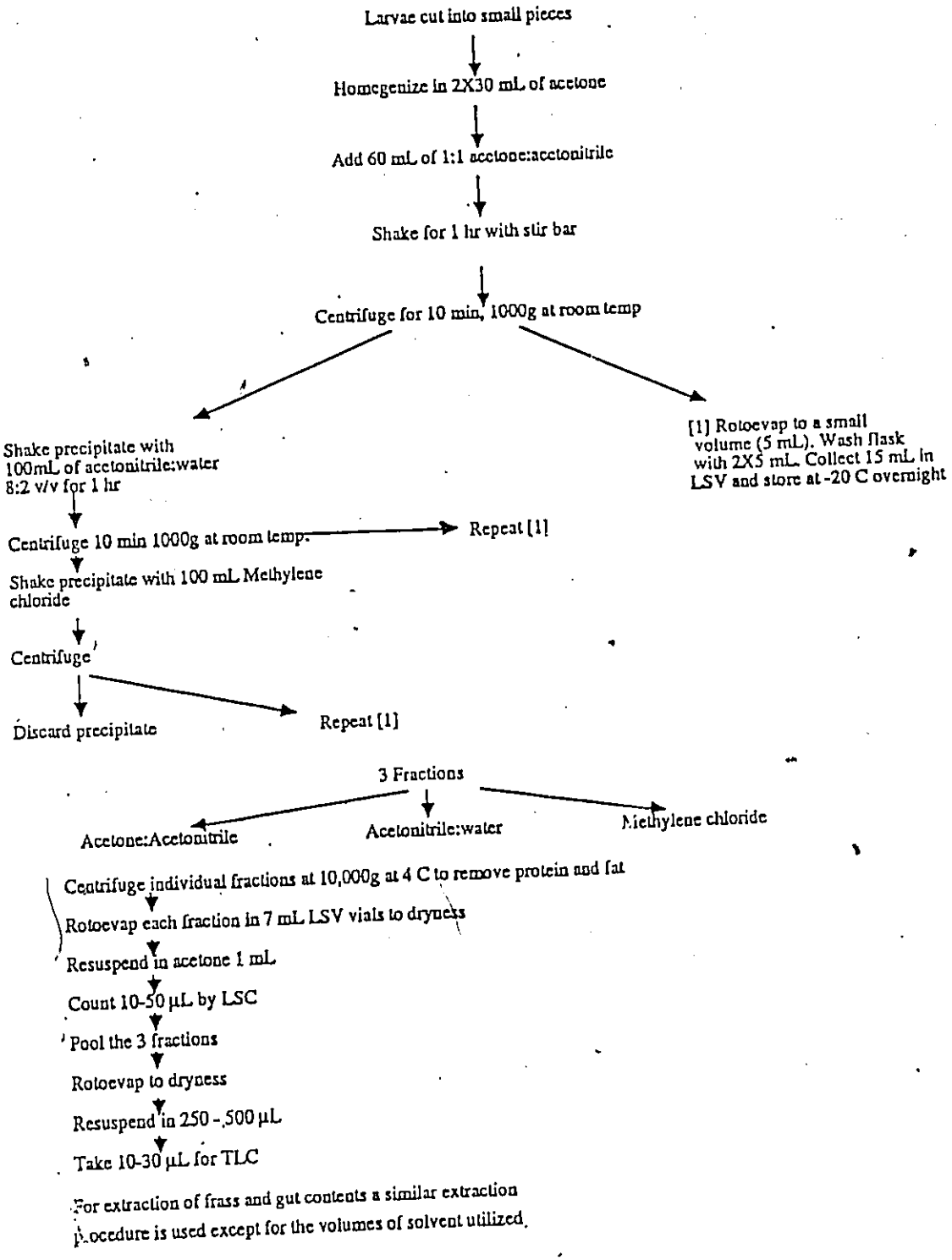
3.11 *In vivo* metabolism

To study the *in vivo* metabolism of α-T, early last instar larvae of *M. sexta* (2), *H. virescens* (10) and *O. nubilalis* (20), were fed α-T at a concentration of 20 μg/g in the diet. The diet of *M. sexta* and *H. virescens*, consisted of both hot and cold α-T. The amount of α-T present in the diet was 91,000 dpm/g diet for *M. sexta*, 28,000 dpm/g diet for *H. virescens* and 150,000 dpm/g diet for *O. nubilalis*. The treated larvae were held individually in controlled environmental growth chambers as described previously. After 24 and 72 h, the larvae were dissected and gut contents removed. The extraction procedure for α-T and its metabolites from larvae (minus gut contents) and frass is presented as a flow diagram in Fig 3. In a parallel experiment, larvae and fecal samples of the three species were spiked with 50,000 dpm of ^{3}H-α-T and recoveries calculated using the extraction procedure described. Controls were run to investigate if α-T breaks down to metabolites in spiked feces which were left in the incubator under the same experimental conditions for 24 h.

Thin layer chromatography was performed on Kodak chromagram sheets (#13181, silica gel with fluorescent indicator) 100 micron thick. The samples were delivered to the chromagram sheet with the help of a 5 μl pipette as spots 30 mm across, forming a band at the origin. The solvent system was ethyl acetate : methanol (70:30 v/v). An α-T standard was run on each sheet. On completion of the run, the sheet was dried and the bands visualized with a UV lamp. Strips (30 x 8 mm) were cut from each lane i.e., from origin to solvent front. The strips were placed in a 7 mL scintillation vial, to which cocktail added and the amount of radioactivity in each strip estimated by LSC.

3.12 *In vitro* metabolism

Fig. 3 : Flow diagram of extraction procedure used in *in vivo* studies



For extraction of frass and gut contents a similar extraction procedure is used except for the volumes of solvent utilized.

3.12.1 Preparation of microsomes

Last instar larvae of *M. sexta* (9 larvae, 5.4±0.3g), *H. virescens* (15 larvae, 363±16 mg) and *O. nubilalis* (30 larvae, 98±6 mg) were used for the experiment. Actively feeding larvae were selected and dissected in chilled (4^0C) 0.15M NaCl. The midguts were removed, the gut contents were emptied and the Malpighian tubules were carefully discarded. The guts were rinsed in chilled 0.15M NaCl and then transferred to cold sodium phosphate buffer (0.1M, pH 7.5). Guts were homogenised in cold buffer using a Wheaton glass homogeniser with a teflon pestle. The crude homogenate was then filtered through cheesecloth and centrifuged at 10,800 g at 4^0C for 10 minutes in a Sorvall RC2-B centrifuge. The supernatant from the first spin was centrifuged at 105,000 g at 4^0C for 1 h to sediment the microsomes. The supernatant was discarded and the microsomal pellet washed with 3x30 µl buffer, then resuspended in buffer with gentle stirring with a smooth round end glass rod. The resuspended microsomes were homogenised very briefly to ensure uniform suspension. Aliquots (2x100 µl) were removed for protein assay and frozen at -20^0C. Protein was determined by the method of Lowry et al. (1951), using a bovine serum albumin standard. The standard curve consisted of seven concentrations, 0, 5, 10, 15, 20, 25 and 30 µg protein, and the absorbance was read at 750 nm.

3.12.2 Microsomal assay

Incubation mixtures were contained in 25 mL Erylenmeyer flasks placed on a shaker. Each incubation mixture contained 4, 3 and 2 ml of microsomal preparation for *M. sexta*, *H. virescens*, and *O. nubilalis*, respectively. An NADPH generating system consisting of 1.8 µmol NADP, 18 µmol of glucose-6-phophate and 1 unit of glucose-6-phosphate dehydrogenase or 10^{-3} M NADPH was added directly to the system. I opted to test the metabolic capabilities of the microsomes under both conditions for two reasons.

First, the NADPH generating system has been more widely used in the recent literature, and second, α-T has been shown to inhibit glucose-6-phoshate dehydrogenase activity in the presence of UV light and oxygen. Although my experiment was conducted under-UV conditions, I ran a parallel experiment in which NADPH was directly added to the system to circumvent any potential problems with the generating system.

An appropiate amount of phosphate buffer was added to give a final volume of 5 ml to each incubation mixture for *M. sexta* and *H. virescens*, and 3 ml for *O. nubilalis* ^{3}H-α-T (151,000±224 dpm, 20 μg) in acetone was added to each flask first and the acetone allowed to evaporate before addition of tissue preparation. Controls were incubation mixtures consisting of all components less microsomes. In a separate experiment, controls were also run on boiled microsomes with the incubation mixture described above. The incubation was carried out at 30^{0}C for 30 min in a GCA Precision Scientific shaker at 80 oscillations/min in an aerobic atmosphere. The reaction was stopped using 2 mL of petroleum ether. Extraction was carried out with 3x2 mL petroleum ether, by vortexing for 2 minutes. This was followed by centrifugation at 1000 g at room temperature in a IEC HN-II centrifuge to ensure clear partitioning. The petroleum ether fraction was then dried by rotoevaporation and then resuspended in 1 mL, 0.7 ml and 0.3 ml of acetone for *M. sexta*, *H.virescence*, and *O. nubilalis*, respectively. The aqueous fraction was filtered through cotton contained in a pasteur pipette. Aliquots 0.5-1.0 mL of the aqueous fraction and 100 μl of the petether fraction were counted by LSC. The petroleum ether fraction was subjected to Thin Layer Chromatography (70:30 v/v, ethyl acetate:methanol). Following this the aqueous fraction was rotoevaporated to dryness, resuspended in methanol (0.5 ml), and 100 μl of the methanol fraction was chromatographed (ethyl acetate : methanol, 70:30 v/v). Both fractions were subjected to scanning between 200-500 nm on a Cary Model 2200 spectrophotometer.

3.13 Piperonyl butoxide studies

Early last instar larvae of *H. virescens* (10 larvae/treatment) and *O. nubilalis* (6 larvae/treatment) were provided a cube of artificial diet containing 60,000 dpm of ^{3}H-α-T (2.5 μg/g diet). When piperonyl butoxide was used, it was applied on the dorsum at a dose of 75 μg per larva of *H. virescens* and 25 μg per larva of *O. nubilalis*. Acetone was used as a carrier for *O. nubilalis* (2.5 μL) and *H. virescens* (7.5 μL) respectively. After 24 h of feeding, the amount of ^{3}H in the body and frass was quantified by sample oxidation followed by LSC (details described in Section 3.9).

3.14 Cytochrome P-450 concentrations

Early fifth instar larvae of *M. sexta* (1.8±0.3 g, 6 per treatment), *H. virescens* (370±14 mg; 10 per treatment) and *O. nubilalis* (102±11 mg; 20 per treatment) were exposed to 0, 10, and 30 μg/g of α-T in their diet for 48 hours. Microsomes were prepared from the guts of the three species as described previously (Section 3.11.1) and resuspended in 2 mL of cold sodium phosphate buffer (0.1M pH 7.5). Two x 100 μl samples were withdrawn for protein assay. Cytochrome P-450 difference spectra were recorded on a Varian Model 2200 spectrophotometer according to the method described by Omura and Sato (1964). Two x 900 μl aliquots of microsomal fraction were dispensed in 1 mL quartz cuvettes. A baseline was recorded between 500-400 nm. Carbon monoxide was bubbled through the sample cuvette for 1 min, sodium dithionate (a few mg at the end of a pasteur pipette) was added to both cuvettes and a CO difference spectra recorded. The concentration of cytochrome P-450 were calculated using the equation cited in Guengerich (1982).

3.15 Statistical Analysis

Whenever required, data collected from experiments were subjected to an

ANOVA followed by a Tukeys test ($p < 0.05$). On some data an arcsine transformation was done to normalize the distribution and an analysis of variance performed on the tranformed data. Differences were determined by a Tukey's test at the 0.05 level (Tukey, 1949).

RESULTS

4.1 Effect of α-T on larval survivorship

The effect of dietary α-T on larval survivorship of the three species of insect herbivores is shown in Table 1. With *M. sexta*, treatments as low as 10 μg/g, α-T resulted in only a 55 % survivorship in the +UV groups and 68 per cent in the -UV groups. At or above 31 μg/g α-T, all the larvae died before reaching the pupal stage either in the presence or absence of near UV light. For *H. virescens*, no marked effect was noticed at 10 μg/g. However, at a concentration of 31 μg/g and above, there was a decrease in survivorship with increasing concentrations of α-T. Sensitivity to the presence of UV was also made evident at these concentrations by a lower survivorship; for example, 69.4 % as opposed to 82.3 % at 31 μg/g. With both *M. sexta* and *H. virescens*, appropriate controls indicated that near UV alone had no effect on survivorship. *O. nubilalis* was the least sensitive of the three species with reduction of survivorship observed only at 100 μg/g. Phototoxicity could not be assessed in this group because the larvae burrow into the diet and thus avoid exposure to light.

4.2 Topical studies

Topical studies were conducted with the three species of insects for the following reasons. First, thiophenes in some plants, (e.g., *Porophyllum*) are stored in large glands on the leaf margins at high concentration. When lepidopterans feed on this type of leaf, feeding damage may lead to exudation of the compounds and subsequent cuticular contact with the insect. Second, in topical studies, excretion can be unambiguosly quantified without having to account for dietary α-T which may be unabsorbed. In contact toxicity tests under photosensitizing conditions, *M. sexta* larvae were 47 times more susceptible to the α-T than *H. virescens* larvae and nearly 70 times

Table 1 : Effect of dietary α-T on survivorship of three insect herbivores

Concentration of α-T in diet (µg/g)	Larval survivorship of the three species (% control)					
	M.sexta		*H.virescens*		*O.nubilalis* *	
	-UV	+UV	-UV	+UV	-UV	+UV
10	67.7	55.5	100	96.6	100	-
31	0	0	82.3	69.4	97	-
56	0	0	56.5	35.5	-	-
100	0	0	33.6	17.7	75	-

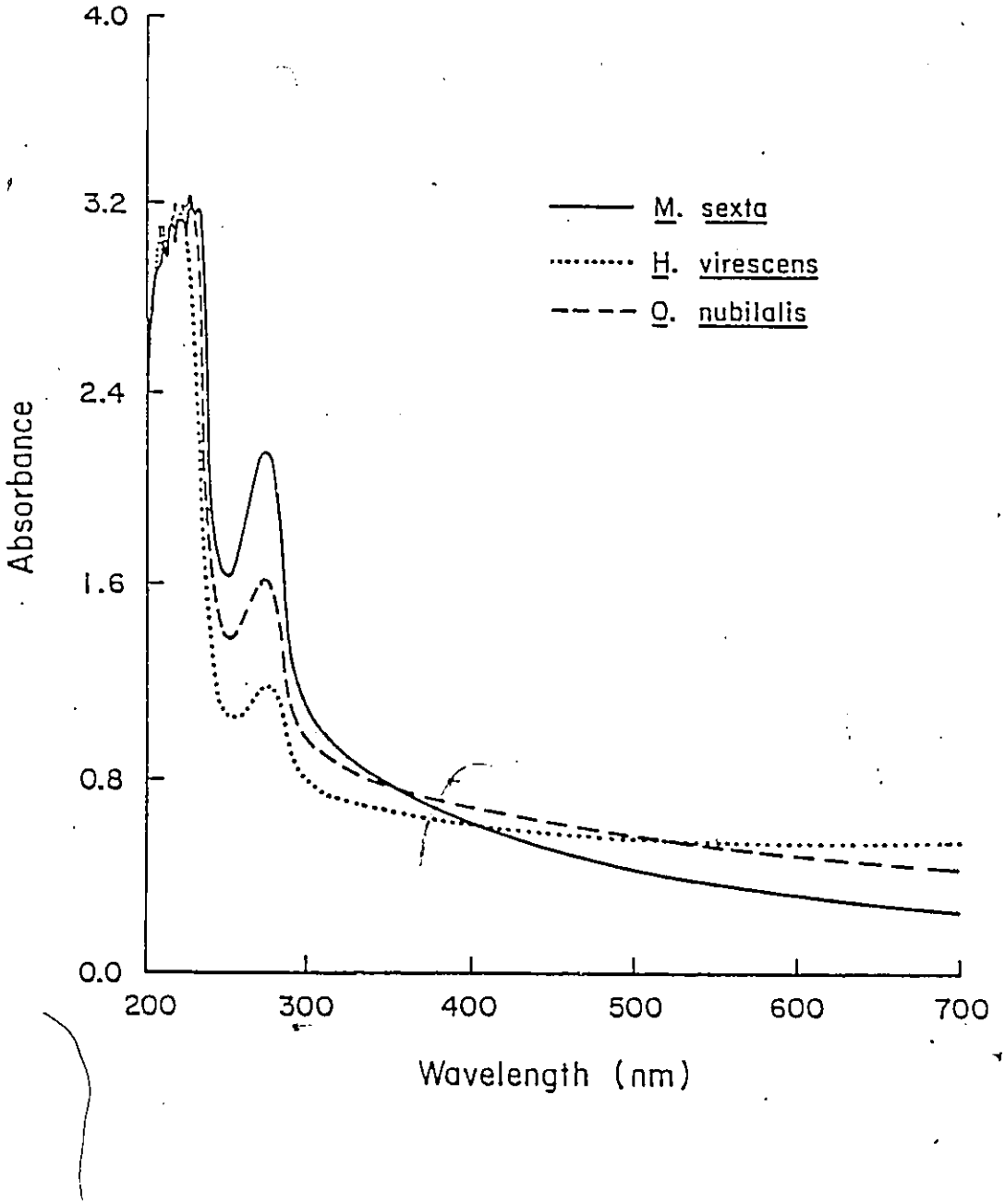
* Data obtained from Mc Dougall (1986)
N=2 (20 larvae/treatment)
Near UV, 2.5 w/m^2 used
Control sets consisted of -α-T,+UV and -α-T,-UV treatments

Table 2 : Acute toxicity of α-T after topical application under photosensitizing conditions*

Species	24h LD_{50} (µg/g)
Manduca sexta	10
Heliothis virescens	474
Ostrinia nubilalis	698

* 2.5 w/m^2 near UV light provided by 4 black light blue lamps

Fig 4. Absorption spectra of the cuticles of the last instar of the three lepidopteran species studied.


4.0
3.2
2.4
1.6
0.8
0.0
Absorbance
200
300
400
500
600
700
Wavelength (nm)
M. sexta
H. virescens
O. nubilalis

more susceptible than *O. nubilalis* larvae (Table 2). No mortality was observed in the acetone treated controls over the period of the experiment.

4.3 Absorbance of insect cuticle

To determine if the differential absorbance of near UV by the insect cuticle was a contributing factor to the diverse sensitivities observed, the absorption spectra of the insect cuticle was determined (Fig.4). No significant selective absorption at the λmax of α-T at 350 nm was recorded. However, from the spectra at 280 nm, differential absorption is evident, the characteristic absorption maxima for cuticular proteins.

4.4 Distribution and clearance studies

Possible sites of accumulation of α-T and its metabolites in the three insects were determined following administration of ^{3}H-α-T. Twenty four hours after oral treatment with ^{3}H-α-T, the distribution of the label in the different tissue compartments was determined. The results are expressed as a percentage of the total recovered activity in disintegrations per minute (dpm) (Fig. 5). Almost 25% of the recovered label remained in the gut (tissue and contents) in *H. virescens* as compared to 18% in *M. sexta* and 17% in *O. nubilalis*. The amounts of ^{3}H detected in the hemolymph and the fat body differed significantly ($p<0.05$) between all three species. In the hemolymph, the highest levels were recorded in *M. sexta* (9.5%) and the lowest in *O. nubilalis* (4.7%), with intermediate levels in *H. virescens* (6.5%). In contrast, for the fat body, maximum levels were found in *H. virescens* (8.0%), intermediate levels in *M. sexta* (4.7%), and lowest levels in *O. nubilalis* (2.3%). However, higher levels of tritium were detected in the dermal and subdermal tissues (carcass + fatbody + hemolymph) in *M. sexta* (28.2%), compared to *H. virescens* (20.7%) and *O. nubilalis* (11.2%). A significant amount of the ingested dose had been excreted in the feces by the larvae of all three species. *M. sexta* and *H. virescens* excreted 53 and 55% label respectively, and *O.*

Fig 5: Distribution of ^{3}H-α-T in the different components of last instar larvae of *M,sexta*, *H.virescens* and *O.nubilalis* after 24 h. of feeding with ^{3}H-α-T . G=gut, H=hemolymph, FB=fat body, C=carcass, F=feces. Error bars = 1SD.

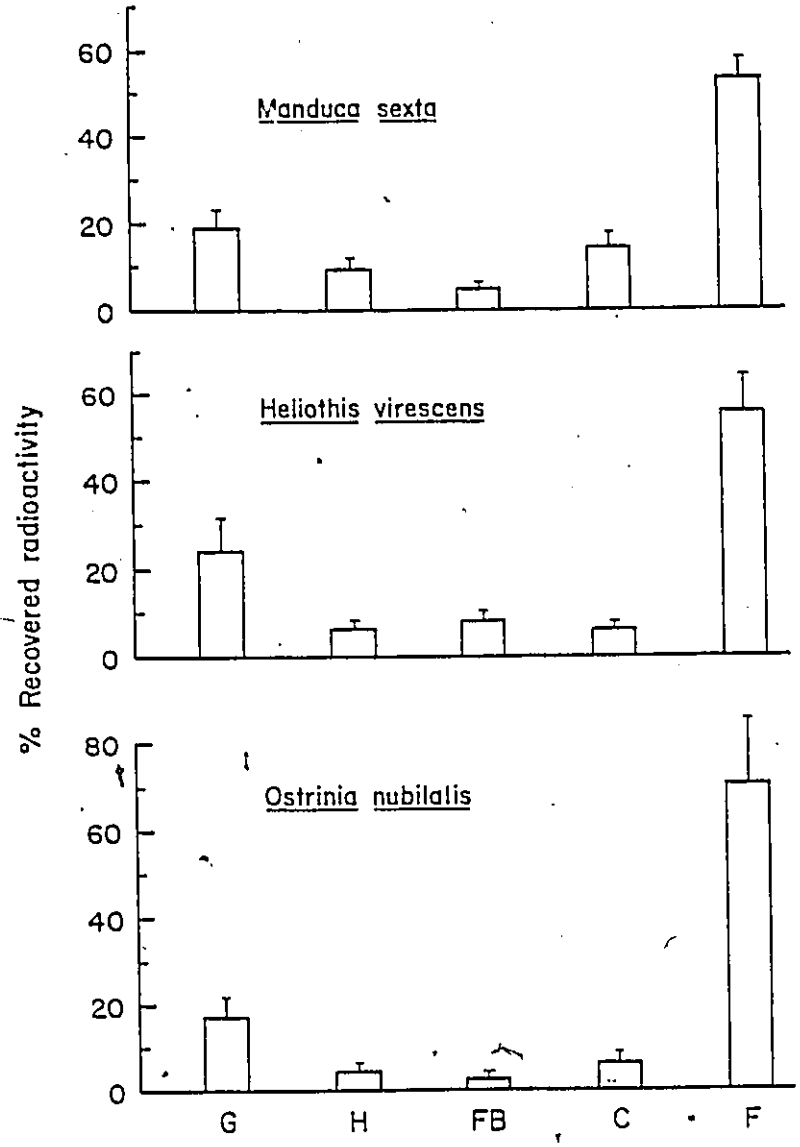

% Recovered radioactivity
Manduca sexta
Heliothis virescens
Ostrinia nubilalis
0
20
40
60
80
G
H
FB
C
F

Fig 6: Patterns of ^{3}H-α-T excretion in last instar larva of *M.sexta*, *H.virescens* and *O.nubilalis*, after ^{3}H-α-T was continuously available in diet. B/F ratio=amount in body/amount in feces. Error bars= 1SD.

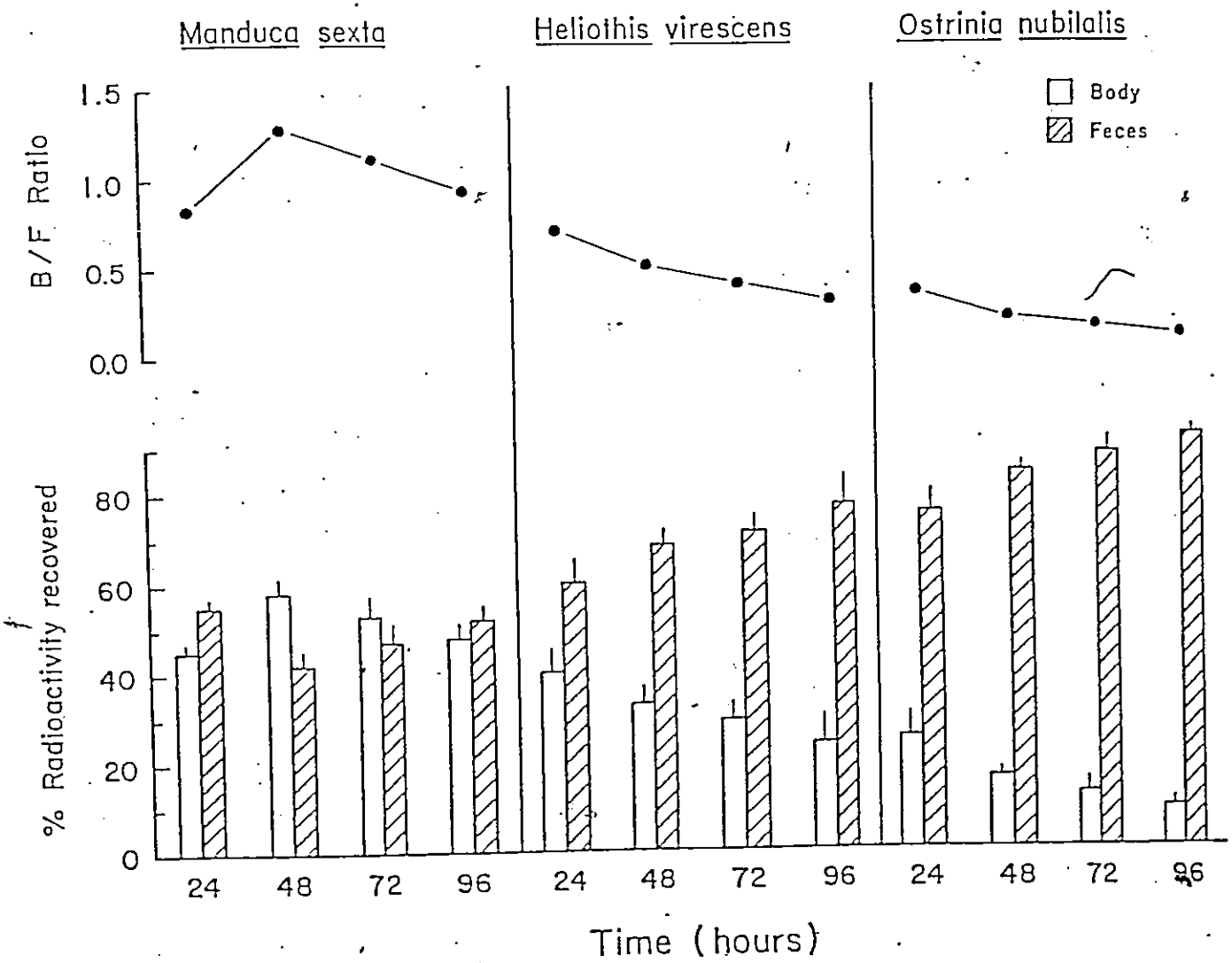
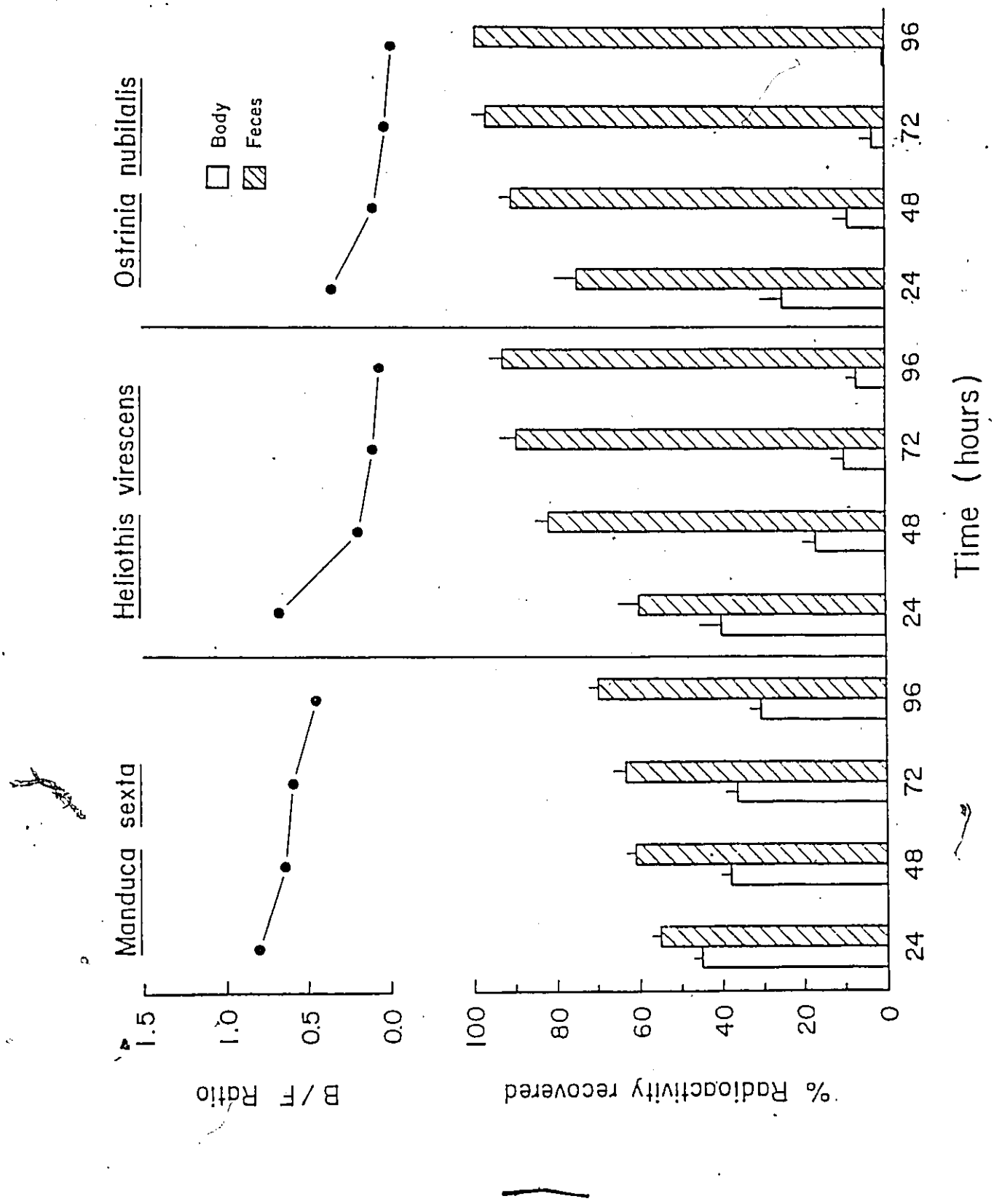

Manduca sexta
Heliothis virescens
Ostrinia nubilalis
Body
Feces
B/F Ratio
1.5
1.0
0.5
0.0
% Radioactivity recovered
80
60
40
20
0
24
48
72
96
Time (hours)

Fig 7: Patterns of ^{3}H-α-T excretion in last instar larvae of *M.sexta*, *H.virescens* and *O.nubilalis*. Larvae were fed ^{3}H-α-T for 24 h after which they they were transferred to diet without α-T. Error bars are 1SD.


Manduca sexta
Heliothis virescens
Ostrinia nubilalis
Body
Feces
B / F Ratio
1.5
1.0
0.5
0.0
% Radioactivity recovered
100
80
60
40
20
0
24
48
72
96
Time (hours)

nubilalis eliminated 70% in the feces. The clearance pattern of label in the three insect species during a four day continuous feeding experiment is shown in Fig. 6. After 48 h of treatment, 42, 67, and 87% of the recovered label were in the feces of *M. sexta*, *H. virescens* and *O. nubilalis* respectively. At 96 h, 48% of the label remained in the body of *M. sexta* , then declined gradually, whereas in *H. virescens* and *O. nubilalis*, the amounts were lower and declined from 24 to 48 h.

In a parallel pulse experiment, the larva of the three species were fed ^{3}H-α-T for only 24 h and were then transferred to diet containing no α-T. This study was undertaken to ascertain whether complete clearance of the label is possible. *O. nubilalis* was the only species that had eliminated all the label in the excreta 96 h after treatment (Fig. 7). Small amounts of radiolabel (7%) were detected in the body of *H. virescens* whereas in *M. sexta* 30% of the ^{3}H remained. Furthermore, in *M. sexta*, the B/F ratio remained relatively high upto 96 h but declined rapidly in *H. virescens* and *O. nubilalis*.

Distribution of radioactivity after topical application of ^{3}H-α-T is presented in Figs 8, 9 and 10. A comparison of the penetration of radiolabel through the cuticle, measured either as the amount of label present in the internal tissues within a few hours after application, or the cuticle + rinse fraction, shows that there is greatest penetration into *M. sexta* (Fig. 8) followed by *O. nubilalis* (Fig. 10) and then *H. virescens* (Fig. 9). At 2h post treatment, 48, 29 and 37% of the α-T were found in the internal tissues, i.e. fat body + hemolymph + gut, and 52, 71 and 63% of the label was observed in the cuticle+ rinse fractions for *M. sexta*, *H. virescens* and *O. nubilalis* respectively. By 48 h most of the radiolabel was eliminated in the feces by *H. virescens* and *O. nubilalis* after an initial increase in fat body, hemolymph and gut. In *H. virescens*, the label recovered, reached a maximum in the fat body at 6 h and in the hemolymph at 3 h and subsequently declined rapidly. In *O. nubilalis* , ^{3}H in the hemolymph, gut and fat

Fig 8: Distribution of ^{3}H-α-T in early last instar larvae of *M.sexta* after topical application with 5 μg (50,000 dpm) of ^{3}H-α-T. Cuticle and rinse (○), feces (●), fat body (□), hemolymph (△) and gut (▲). Points represent means of three or four larvae per sample time, error bars, the 95% confidence interval about the mean. The error was less than 10% of the mean wherever not indicated.

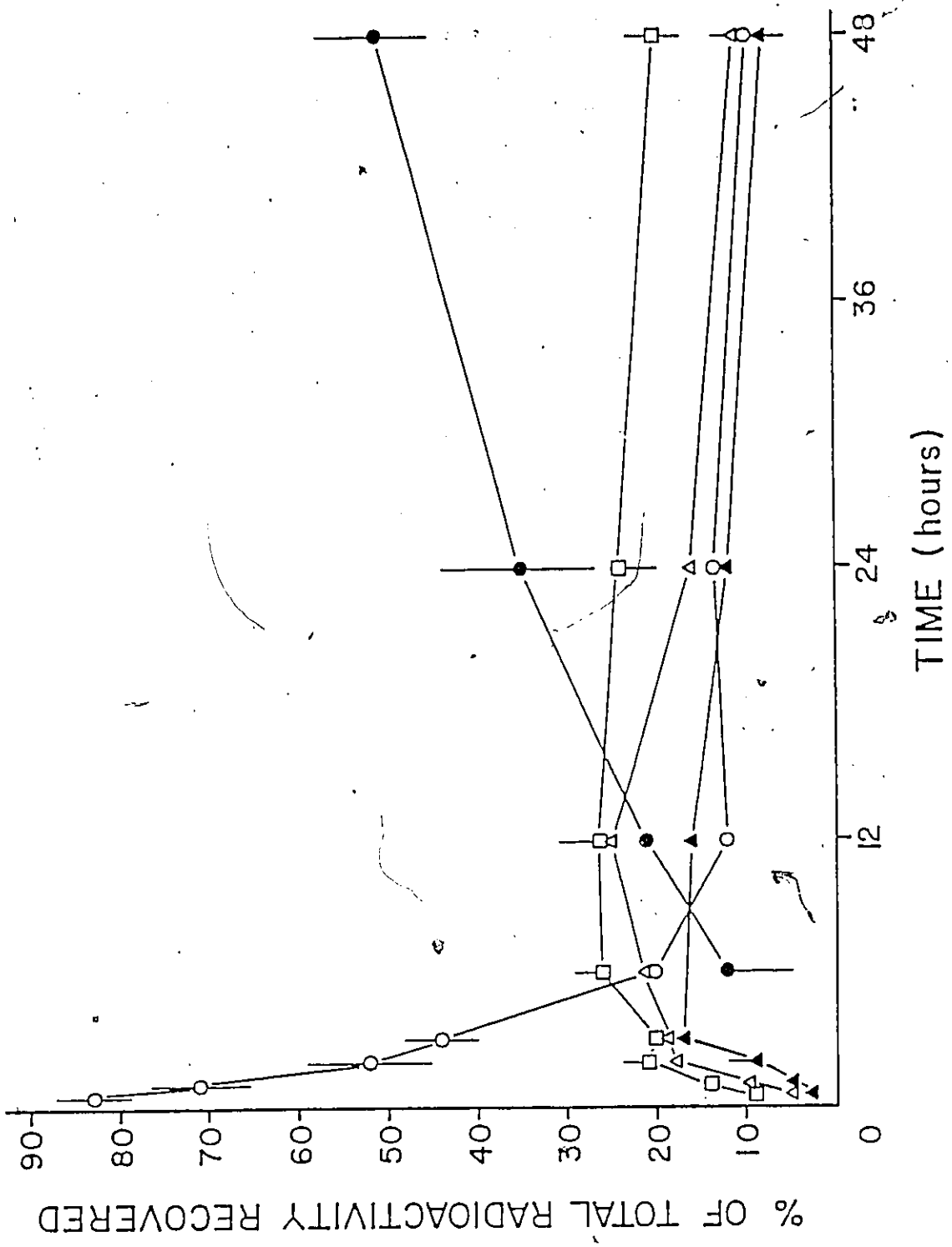

% OF TOTAL RADIOACTIVITY RECOVERED
90
80
70
60
50
40
30
20
10
0
12
24
36
48
TIME (hours)

Fig 9: Distribution of ^{3}H-α-T in early last instar larvae of *H.virescens*, after topical treatment with 5 µg (50,000dpm) of ^{3}H-α-T. Cuticle and rinse (○), feces (●), fat body (□), hemolymph (△) and gut (▲). Points represent means of three to four larvae per sample time; error bars, the 95% confidence interval about the mean. The error was less than 10% of the mean wherever not indicated.

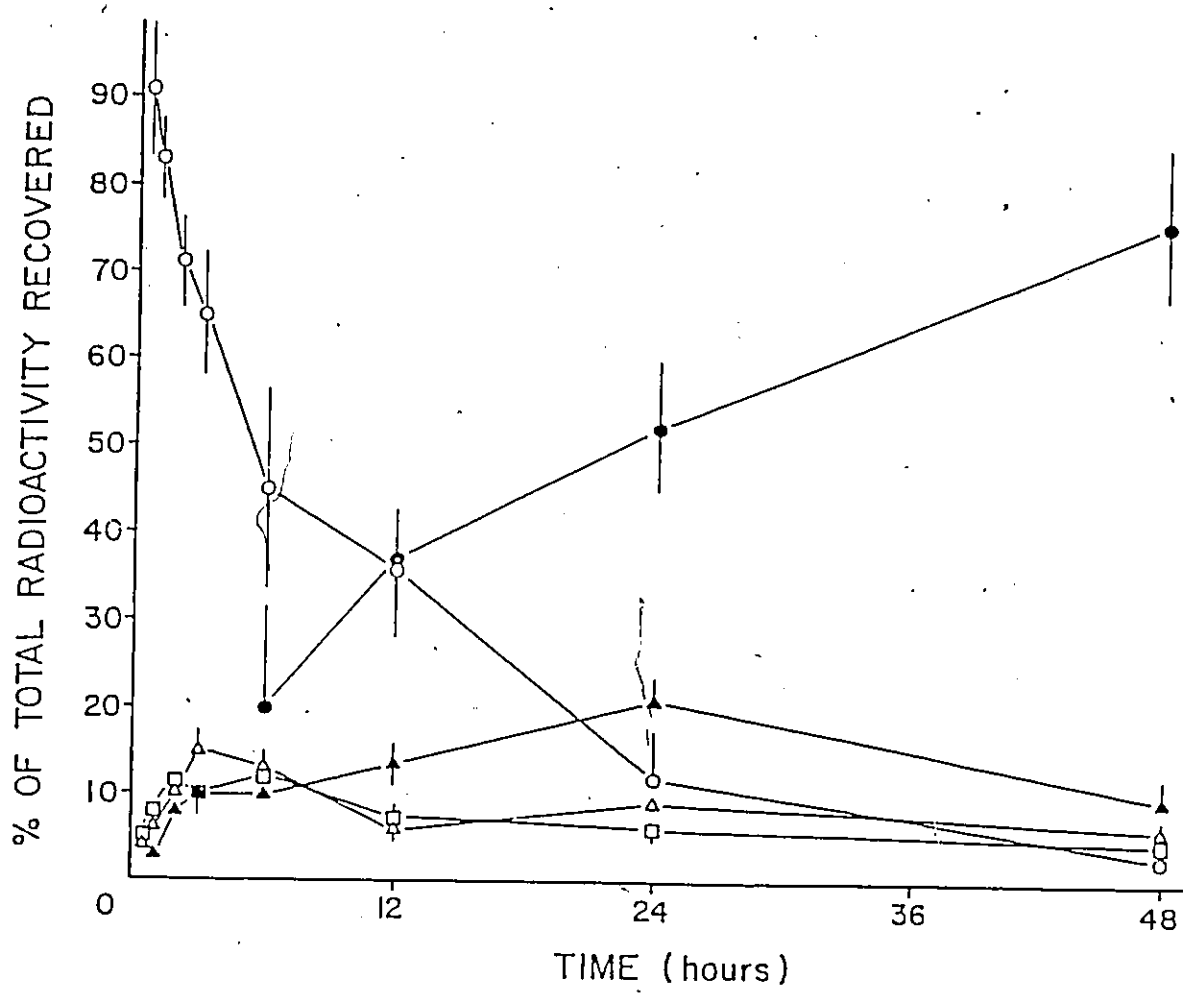
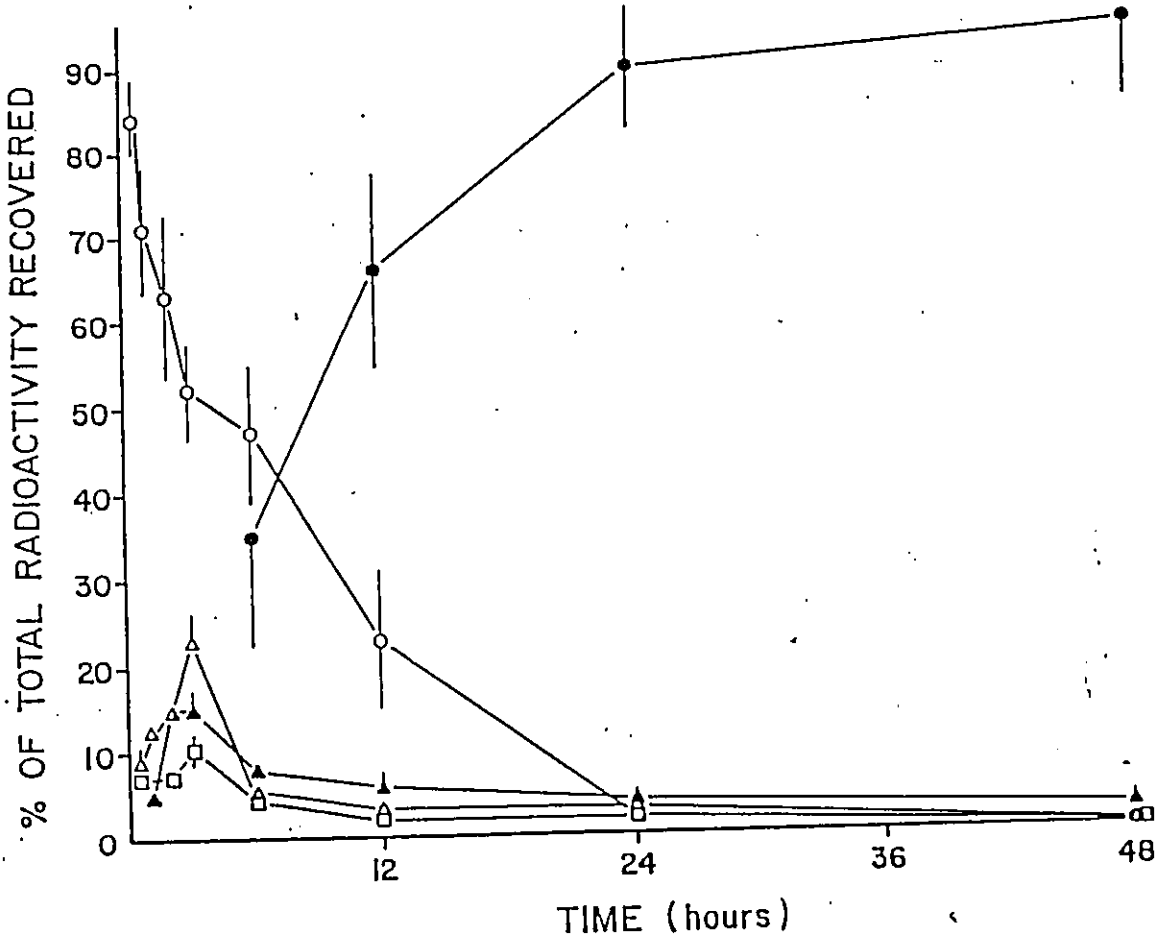

% OF TOTAL RADIOACTIVITY RECOVERED
90
80
70
60
50
40
30
20
10
0
12
24
36
48
TIME (hours)

Fig 10 : Distribution of ^{3}H-α-T in early last instar larva of *O.nubilalis*, after topical appplication with 5 µg (50,000 dpm) of ^{3}H-α-T. Cuticle and rinse (○), feces (●), fat body (□), hemolympn (△) and gut (▲). Points represent means of four larvae per sample time; error bars, the 95% confidence interval about the mean. The error was less than 10% of the mean wherever not indicated.


% OF TOTAL RADIOACTIVITY RECOVERED
90
80
70
60
50
40
30
20
10
0
12
24
36
48
TIME (hours)

body peaked at 3 h, then declined rapidly. In *M. sexta* however, the α-T concentration increased in the fat body, hemolymph and gut over a relatively long period; label recovered in the gut and fat body peaked at 6 h and in hemolymph at 12 h. Slower elimination resulted in almost 40% of the tritium remaining in the tissues at 48 h post treatment. Thus, elimination of ^3H after topical application is much more rapid in *O. nubilalis* (time for 50% elimination, $t_{1/2}$ =8.5 h) and *H. virescens* ($t_{1/2}$=22 h) than in *M. sexta* ($t_{1/2}$=48 h).

4.5 *In vivo* studies

It was observed that about 50% or more of the radioactivity recovered from the body after 24 h and 72 h of feeding was the parent compound α-T in all the three species (Table 3). However, both at 24 and 72 h, a greater amount of the radioactivity isolated was present as parent α-T in *M. sexta* than in *H. virescens* or *O. nubilalis*. In the feces a larger proportion of the radioactivity recovered was the parent compound. At 24 and 72 h, a similar trend in the results obtained for the body was seen i.e., the ratio of the amount of α-T to metabolite being greater in *M. sexta* as compared to the other two species.

The efficiency of extraction of α-T from body and feces components for *M. sexta*, *H. virescens*, and *O. nubilalis* was 61-73%, 73-76% and 72-81% respectively of dietary administered dose. On the other hand, when body or the feces was spiked with known amounts of α-T, the extraction efficiency was higher than in the *in vivo* situation, being 82-89%. A TLC run from this check showed the presence of only α-T, suggesting that no breakdown or transformation of the compound had occurred during the extraction procedure.

From the chromatograms obtained (Table 4) it can be seen that there are possibly 3 metabolites present in the body fractions of the three species and that the R_f

Table 3 : *In vivo* metabolism of ^{3}H-α-T in *M.sexta*, *H.virescens* and *O.nubilalis* expressed as % of parent:non-parent compound

Species	% in body		% in feces	
	24 h	72 h	24 h	72 h
M.sexta	72:28[a]	56:44[b]	67:33[c]	65:35[e]
	(8.3)	(9.4)	(4.5)	(3.8)
H.virescens	53:47[a]	34:66[b]	49:51[d]	42:58[f]
	(10.2)	(10.9)	(5.6)	(6)
O.nubilalis	61:39[a]	47:53[b]	53:47[d]	45:55[f]
	(9.5)	(9.5)	(4.0)	(5.1)

Larvae of each species were fed alpha-T cold + hot, to give a final concentration of 20 µg/g in the diet
For a given time period (column), a Tukey's test was done comparing the values between the three species. Values with the same letter are not significantly different ($p<0.05$)
Values in parentheses indicate ± 1 S.D of both parent and non parent values

Table 4 : Summary of the three chromatograms of whole body and feces extracts for the three species investigated

	Rf at which radioactivity detected			
	Body		Frass	
	24 h	72 h	24 h	72 h
		M.sexta		
	0.82* (72)	0.83 (56)	0.83 (68)	0.83 (64)
	0.67 (11.3)	0.67 (11.2)	0.24 (14)	0.63 (3.4)*
	0.60 (4.5)	0.60 (8.5)	0.14 (6.8)	0.24 (12.4)
	0.24 (7.8)	0.24 (14.0)		0.14 (7.1)
	origin (4.5)	origin (10.2)	origin (11.1)	origin (13.0)
Total counts spotted	10,360	8791	13,242	17,055
Counts recovered	8761	7364	11,255	14,497
		H.virescens		
	0.83 (53.9)	0.83 (34.9)	0.83 (51.8)	0.82(41.8)
	0.67 (6.6)	0.64 (13.3)	0.24 (12.5)	0.24 (7.8)
	0.60 (13.4)	0.24 (37.6)	0.14 (10.9)	0.14 (19.2)
	0.24 (19.0)			
	origin (7.2)	origin (13.7)	origin (24.6)	origin (31.1)
Total counts spotted	9223	9564	8763	10,132
Counts recovered	8612	9012	7981	9073
		O.nubilalis		
	0.82 (61)	0.81 (47)	0.83 (53)	0.81 (45.4)
	0.63 (12.9)	0.63 (18.7)	0.21 (18)	0.42 (1.3)
	0.25 (21.4)	0.25 (21.5)		0.23 (11.8)
		0.16 (9.5)		0.19 (17.2)
	origin (4.5)	origin (13.2)	origin (29.0)	origin (23.7)
Total counts spotted	11,356	10,461	8056	13,333
Counts recovered	10,534	9841	7126	11,534

*= Rf of ^{3}H-α-T

Solvent system used: ethyl acetate:methanol (70:30, v/v)

Values in parentheses: per cent of total activity recovered

Table 5 : In vitro metabolism of ^{3}H-α-T by the gut microsomal fraction of*M.sexta*, *H.virescens* and *O.nubilalis*

Species	Alpha-T metabolised (p moles/min/mg protein) NADPH	NADPH generating system	p moles/min/n mole* cytochrome P-450
M.sexta	41.1 ± 2.4 a	34.5 ± 5.5 a	304
H.virescens	672.0 ± 102 b	564.0 ± 19 b	1538
O.nubilalis	1271 ± 51 c	1373 ± 95.5 c	3984

Values followed by the same letter are not significantly different at ($p<0.05$ level) (Tukey's test)
Total volume of incubation mixture for *M.sexta* and *H.virescens* was 5 ml and 3 ml for *O.nubilalis*. It consisted of sodium phosphate buffer (0.05M, pH 7.5), 151000 ± 224 dpm = 20 µg of ^{3}H-α-T. When NADPH was used alone, the final concentration was 10^{-3} M. The generating system consisted of 1.8 µM NADP, 18 µM glucose-6-phosphate and one unit of glucose-6-phosphate dehydrogenase.
* Calculated from cytochrome P-450 data shown in Table 7

values (0.67, 0.60, 0.24) of these metabolites are similar among the species. In the feces, there were 3 to 4 metabolites were detected with a large proportion (11-13%) of the activity staying at the origin.

4.6 *In vitro* studies

The results of *in vitro* studies, presented in Table 5, reveal significant differences ($p < 0.05$) in the metabolic capabilities of the isolated microsomes of the three species. *H. virescens* gut microsomes were capable of metabolising α-T 16 times faster than *M. sexta,* whereas the microsomes of *O. nubilalis* converted α-T to a metabolite 31 fold more rapidly than *M. sexta* and twice as faster than *H. virescens.*

There were no significant differences in the results when NADPH was directly incorporated into the incubation mixture or when the NADPH generating system was used, i.e., glucose-6-phosphate dehydrogenase activity was not affected under the experimental conditions used.

Extraction efficiency of α-T calculated for the controls (minus microsomes) was 89-94%. When boiled microsomes were used, the extraction efficiency was slightly lower, 81-85%. Absence of metabolite formation using boiled microsomes was confirmed by TLC analysis of the petroleum ether and aqueous fraction. With viable microsome preparations, the petroleum ether fraction showed the presence of only α-T while the water fraction showed no α-T, but a band containing radioactivity with an R_f of 0.69 when subjected to TLC. Furthermore, an absorption spectrum of the aqueous fraction showed no α-T ($\lambda max = 350$) but the presence of a metabolite with a λmax of 250.

4.7 Studies with Piperonyl butoxide

The amount of ^3H recorded in the body of the last instar larvae fed ^3H-α-

Table 6 : Distribution of label in the body and frass of *H.virescens* and *O.nubilalis* in the presence and absence of piperonyl butoxide

Species	N	^{3}H-αT administered		^{3}H-αT + piperonyl butoxide administered	
		% label in body	% label in feces	% label in body	% label in feces
H.virescens	6	42.7 (4.1)	57.2 (4.1)	67.7 (5.6)	32.3 (5.6)
O.nubilalis	10	28.3 (4.5)	71.6 (4.5)	46.5 (6.1)	53.5 (6.1)

Values in parentheses represent ± 1 S.D.

Table 7 : Cytochrome P-450 + cytochrome P-420 levels in larvae of three lepidopteran species after feeding with control or α-T treated diets

Species	Dietary α-T (µg/g)	Cyt-P-450 + Cyt-P-420 (n moles / mg protein)	
M.sexta			
	0	0.137±0.017	a 1
	10	0.125±0.016	1
	30	0.129±0.016	1
H.virescens			
	0	0.429±0.013	b 1
	10	0.374±0.031	2
	30	0.340±0.016	3
O.nubilalis			
	0	0.320±0.037	c 1
	10	0.318±0.006	1
	30	0.321±0.081	1

Values followed by the same letter or numbers are not significantly different at $p<0.05$ (Tukey's test)
The test was performed among the three species on controls (letters) and within a given species for α-T treatments (numbers).

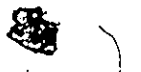
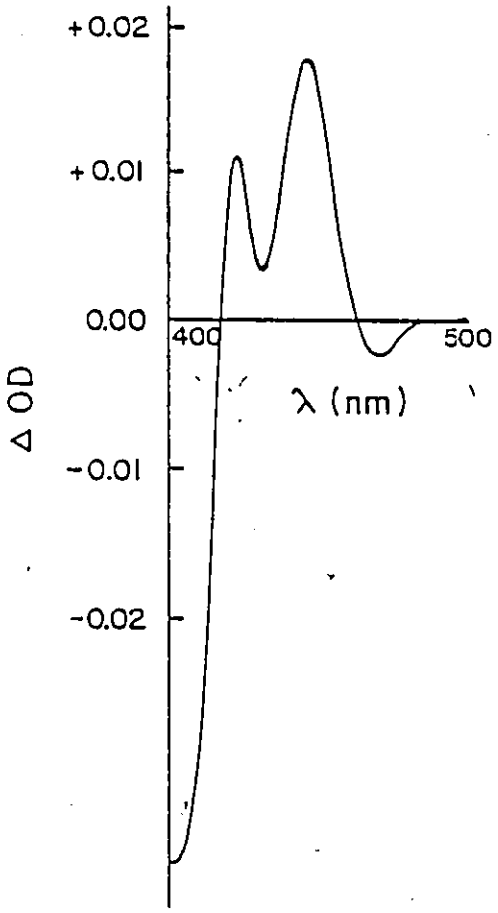


Fig 11: A typical CO difference spectrum of the midgut microsomes of *H.virescens*.


+0.02
+0.01
0.00
-0.01
-0.02
400
500
λ (nm)
Δ OD

T, 4 h after topically applying piperonyl butoxide, showed 25 per cent more label than larvae fed α-T alone, 24 h post feeding (Table 6). Similar results were found for *O. nubilalis*, an 18% increase in label when piperonyl butoxide was added to the treatment (Table 6). Recovery of label from body and frass was between 82-89 per cent.

4.8 - Cytochrome-P-450

A typical CO difference spectrum is presented in Fig 11. Two peaks at 450 and 420 nm were seen. The peak at 420 nm represents a denatured form of cytochrome P-450 that is present in almost all reported preparations. On average, cytochrome P-420 levels were 29.8%, 23.1% and 9.4% of the total cytochrome P-450 + 420 levels recorded in *M. sexta*, *H. virescens* and *O. nubilalis* respectively. Table 7 summarises results of the cytochrome P-450 determinations in the guts of the three species studied and the effects of α-T. Cytochrome P-450 + cytochrome P-420 are presented together. It can be seen (Table 7) that levels of Cyt P-450 + Cyt P-420 were about three fold higher in *H. virescens* and *O. nubilalis* as compared to *M. sexta*. An analysis of the data revealed significantly higher ($p<0.05$) levels of cyt P-450+P-420 as compared to *O. nubilalis*. Alpha-T, at concentrations of 10 and 30 μg/g in the diet, did not markedly increase or decrease the amount of cyt P-450 +P-420 in *M. sexta* and *O. nubilalis*, whereas a slight but significant decrease was observed for *H. virescens*.

DISCUSSION

The results obtained in the present study demonstrate that differential toxicity to phototoxins is exhibited by phytophagous lepidoptera having different host ranges. Toxicokinetics, as expressed by excretion patterns and metabolism mediated by mixed function oxidases, may explain this differential sensitivity. Differential light absorption through the cuticle does not account for the observed differences in toxicity.

5.1 Survivorship studies

From the results presented in Table 1, three main inferences can be drawn. First, α-T is toxic to the three species; secondly, toxicity is enhanced in the presence of near UV light at some concentrations; and third, differential responses is exhibited by larvae of *Manduca sexta*, *Heliothis virescens* and *Ostrinia nubilalis*. The results for *O. nubilalis* from McDougall (1986) have been included in this table for comparative purposes.

The concentration of α-T in most plants of the family Asteraceae has been shown to be between 1-40 μg/g fresh weight of plant (recalculated from Downum et al. (1985) - assuming the fresh weight to dry weight ratio is 10:1), although higher values in *Flaveria* (60 μg/g) after induction with fungi have been reported (Arnason et al., 1983b).

The concentrations at which α-T was effective towards *M. sexta* and *H. virescens* in reducing the growth and survivorship was an order of magnitude lower than most other allelochemicals studied, for example, sesquiterpene lactones (Isman and Rodriguez, 1983), methylated flavonoids and various phenolic compounds (Isman and Duffey, 1982) and alkaloids (Devitt et al., 1980). These results suggest that α-T may may play an important role in the plant's defence against certain phytophagous insects.

Studies by Champagne et al. (1985) suggested there are two components leading to the toxicity of α-T in lepidopteran larvae: feeding deterrency and inhibition of nutrient utilization. At 10 μg/g in the diet, feeding was reduced by 15 % in *M. sexta* larvae whereas at 100 μg/g, α-T suppressed feeding of neonates. A 35 % reduction in feeding was reported when *O. nubilalis* larvae were presented with a diet containing 100 μg/g α-T. Their study also showed that there is a decrease in efficiency of conversion of the ingested and digested food, but no reduction in approximate digestibility when habituated larvae of the dark sided cutworm, *Euxoa messoria*, were fed 10 μg/g α-T. This result suggested that α-T interfered with the conversion of ingested food into insect biomass without affecting digestibility. These results may explain the reduced larval survivorship observed in *M. sexta* and *H. virescens* in the present study and in *O. nubilalis* by McDougall (1986). Recently we have shown by scanning electron microscopy (results not shown in the present dissertation) that perforations appear in the gut of *M. sexta* larvae exposed to 50 μg/g α-T in the diet which may explain the dramatic effects noticed at concentrations at and above 31 μg/g for this species.

Addition of near UV light (2.5 w/m^2), a fraction of what is present in sunlight (50 - 100 w/m^2), increased the toxicity of α-T to *M. sexta* and *H. virescens* larvae. Near UV treatments were not included in the study of McDougall (1986) as *O. nubilalis* larvae bore into their food or spin silk thus avoiding UV light.

Alpha-T in the presence of UV light produces singlet oxygen ($^1O_2$). This reactive species of oxygen has been shown to oxidize essential biomolecules such as amino acids, proteins, lipids and nucleic acids (see review by Spikes and Straight, 1987), which may partly explain the increased toxicity observed under near UV conditions. Phototoxicity of α-T has been demonstrated in *M. sexta* and *E. messoria* (Champagne, 1985; Downum et al., 1984). Champagne et al. (1986) reported a small but significant reduction in larval growth and survivorship when neonates were fed 10 μg/g in the diet

and exposed to near UV light, these results reflecting closely the findings of the present study. Furthermore, Champagne et al. (1986) reported photosensitized lesions in the dorsal cuticle particularly along the dorsal blood vessel, leading to failure of molting and eventually death. The authors suggested that α-T may be transported from gut through the hemolymph to tissues adjacent to the hemocoel. This hypothesis was tested and is discussed in a later section. Although lesions were not noticed in larvae of *M. sexta* fed 10 μg/g α-T and exposed to UV light in the present study, concentrations of 50 μg/g in the diet fed to early fifth instar larvae caused severe "burns" (dark lesions) throughout the cuticle (Appendix IV). Black lesions were noticed even below the cuticle, probably in the fat body, suggesting the presence of α-T and/or a metabolite in the cuticle and fat body. The present study also suggested that near UV light can pass through the cuticle in sufficient amounts to cause photomediated reactions. In a separate study, Downum et al. (1984) reported tissue necrosis in the cuticle of 5th instar larva after topical application of 50 μg/g α-T. No necrotic lesions were seen in the cuticle of *H. virescens* even at the highest concentration (100 μg/g) suggesting photosensitized targets at the subcuticular level, as has been previously reported for *Euxoa messoria* (Champagne et al., 1986) and for *Spodoptera eridania* (Berenbaum, 1981).

The UV enhanced toxicity could be the result of oxidation of biomolecules such as proteins and lipids in the fat body or the hemolymph which is unlikely to occur in -UV groups. This hypothesis warrants further testing at the molecular level. In summary, three components have been indicated leading to toxicity of α-T in lepidoptera larvae: feeding deterrency, reduction in nutritional indices and tissue necrosis.

The most noteworthy aspect of the data was that α-T was highly toxic to *M. sexta* and moderately toxic to *H. virescens* under both photosensitizing and non-photosensitizing conditions. Similar trends were observed in the 24 h topical studies under photosensitizing conditions (Table 2) confirming the results of the feeding studies

without having to account for feeding deterrency as a contributing factor.

Major differences in optical density of the cuticle, penetration, distribution, metabolism and excretion are hypothesised as the primary reason for the differential response of α-T towards the three insect species studied.

5.2 Cuticle absorbance

The absence of a significant difference in the selective absorption of near UV light at the λmax of α-T in the three species investigated suggests that cuticular properties are not an important component in the differential response to α-T observed in the three species (Fig. 4).

5.3 Distribution studies

The distribution studies clearly indicate that the ^{3}H label (representing α-T or a metabolite of α-T) does not accumulate in any specific tissue, but is absorbed through the gut and reaches the fat body and the cuticle in significant amounts via the hemolymph . The high lipophilicity of α-T (log K_{ow}=5.6, McLachlan, 1984) may be a factor enabling it to pass through the gut and possibly bind to lipid-rich tissues within the insect. The higher levels of label found in dermal and subdermal tissues (cuticle+fat body+hemolymph) in *M. sexta* larvae (28%) as compared to *H. virescens* (20%) or *O. nubilalis* (11%) may explain why *M. sexta* larvae were more susceptible to phototoxic effects, demonstrated by dark necrotic lesions in the larvae seen in the present study and earlier reports (Champagne et al., 1986; Downum et al., 1984). Furthermore, the percent of radiolabel recovered from the cuticle was also highest in *M. sexta* (14 %) compared to *H. virescens* (6 %) and *O. nubilalis* (6 %). The cuticle is one tissue where photosensitizing reactions are clearly evident (Appendix IV). Differential permeability of the gut to α-T, although not considered in the present study, would be a factor worth

investigating to see if the molecule reaches the cuticle and the fat body faster in susceptible species.

There were only small but nevertheless significant differences in label found in the various components within the bodies of the three insect species, but striking differences were observed in the frass. Maximum recovery of ^{3}H in the frass was observed in the most resistant species *O. nubilalis* (70%), as compared to 60 % in *H. virescens* and 54 % in *M. sexta* suggesting that faster elimination may contribute to the differential sensitivity noticed. Further support is provided by continuous and pulse label feeding experiments which showed that rate of elimination contributes to the differential sensitivities observed.

B/F ratios (the amount of label present in the body relative to that in the frass) showed that the most susceptible species, *M. sexta*, retained higher levels in the body than did the two more resistant species, *H. virescens* and *O. nubilalis*. Whether faster metabolism followed by rapid excretion or simply rapid excretion explains the higher rates of elimination observed in *H. virescens* and *O. nubilalis* remains to be determined.

Pulse labelling studies gave results very similar to the continuous feeding studies. *O. nubilalis* completely eliminated all the ingested α-T in the frass.*H. virescens* completely eliminated α-T whereas, in contrast, almost 30 percent of the ingested dose remained unexcreted in the body of *M. sexta*. These results support our hypothesis that faster elimination is one possible way by which certain lepidopteran larvae may successfully feed on α-T containing plants, without having to contend with adverse effects.

The presence of efficient excretory mechanisms in insects to cope with toxic plant allelochemicals has been demonstrated in a number of studies. Rapid

elimination of nicotine in *M. sexta* larvae (Self et al., 1964 a,b), canavanine by the bruchid beetle (Rosenthal et al., 1977), or phenolics by *Heliothis zea* (Isman and Duffey, 1983) has enabled these species to succesfully feed on plants containing such chemicals. Ivie et al. (1983) compared the pharmacokinetics of labelled ^{14}C xanthotoxin, a phototoxic furanocoumarin, in the larva of the black swallowtail butterfly, *Papillio polyxenes*, and the armyworm, *Spodoptera frugiperda*. They showed that elimination of label after oral treatment was much more rapid in *P.polyxenes* than in *S.frugiperda*. Within 1.5 h, 50% of all the administered ^{14}C was recovered in the excreta of the adapted species whereas only 1% of the label was eliminated by the armyworm. The authors suggested that fast clearance of the phototoxin is the prime adaptation leading to the successful foraging of the black swallowtail on furanocoumarin containing plants.

Further evidence for a differential response is drawn from the topical studies. The penetration rate of α-T was fairly rapid in all three species. This result is surprising since compounds with a high hydrophobicity often have a low penetration rate (Olson and Brian, 1963). Penetration rates alone cannot account for the differential response observed between *O. nubilalis* and *H. virescens* as at any given time, it was higher in *O. nubilalis* as compared to than *H. virescens*; however in the contact toxicity tests the larvae of *H. virescens* were more susceptible than *O. nubilalis*. This suggests that if increased penetration rate is followed up by rapid detoxification and excretion, the insect can circumvent the problem of toxicity. In *M. sexta*, slower elimination rates appear to explain the susceptibility of this insect to this phototoxin.

The fate of α-T after topical application could follow two paths: i) the chemical passes through the cuticle into the hemolymph, reaches the Malpighian tubule and is excreted through the hindgut and/or ii) α-T moves from the cuticle through the hemolymph to the gut and is detoxified and excreted with the ingested food. Evidence lending support to the latter comes from the results which showed label increasing in the

gut of all the three insect species at the initial time periods. Since gut contents were not separated from the gut in the present study, it was not possible to determine whether the α-T was metabolised in the gut and passed back to the hemolymph for excretion in Malpighian tubules or moved into the gut cavity and cleared with the undigested food.

At the end of the continuous and pulse label feeding studies, the following three questions remained unanswered: 1) What is the percent label representing α-T, or its metabolite in the body and frass of the three insect species? 2) Is faster excretion a result of increased metabolism? and 3) Are higher levels of mixed function oxidase activity responsible for faster detoxification leading to faster elimination?

5.4 Metabolism

5.4.1 *In vivo* studies

If an insect efficiently metabolises α-T as a means of detoxification we would expect less α-T excreted than if it were unable to do so. Apparentiy from *in vivo* studies, it is clear that not all of the α-T was metabolised before excretion as α-T was detected in significant amounts in the frass of all three species, suggesting that fast excretion rather than metabolism could protect insects from toxicity.

The results of the *in vivo* study showed that there were no significant differences in the parent:metabolite ratios in the body (without the gut contents) among the three species, however, significant differences were apparent in the frass between the most susceptible species and the two more resistant species.

From the results of the clearance studies, one would expect to find higher α-T to metabolite ratio in *M. sexta* compared to *H. virescens* and *O. nubilalis* if metabolism prior to excretion was essential. Significant differences were not noticed probably due to the low extraction efficiencies obtained in these studies for the body

component (63 % for *M. sexta*, 71 % for *H. virescens* and 72 % for *O. nubilalis*) which left 37, 29 and 28 % of the label unaccounted. Evidence supporting higher levels of parent:metabolite ratio in susceptible species is derived from the work of Ivie et al. (1983). These authors incorporated ^{14}C xanthotoxin (5 µg/g) into twigs of parsley and fed them to *P. polyxenes*, an adapted species and *S. frugiperda*, a polyphagous but non-adapted species. Larvae of both species consumed the entire dose in 5-15 min. After 1.5 and 3 h the percent of unmetabolised xanthotoxin was 41.6 and 20.4 % in *Spodoptera frugiperda* and only 0.7 and 0.1 per cent in *Papilio polyxenes*. The study showed that within 3 h sufficient amount of unmetabolised xanthotoxin had reached the dermal and subdermal tissues to bring about phototoxicity in *S. frugiperda*. The absence of xanthtoxin in the body of both the species after 12 h suggested that the rate of metabolism determines levels of toxicity.

Lower levels of unchanged α-T were noticed 72 h post feeding in all three species. Increases in the amounts of mixed function oxidases in the gut and fat body, with larval age shown previously (Kreiger and Wilkinson, 1969; Berry et al., 1980; Tate et al., 1982) may account for increased metabolism and lower levels of α-T, assuming that the mixed function oxidases are involved in the detoxification process.

Gut content analysis from *M. sexta*, *H. virescens* and *O. nubilalis* shows almost all the α-T in unchanged form in all three species suggesting that the gut microflora or the digestive enzymes secreted into the gut lumen do not play a major role in α-T metabolism. The minute quantities of metabolite detected may be from the contents of the hindgut, which receives metabolites from the Malpighian tubules.

Analysing the parent:metabolite ratio in the excreta of the three species showed significant differences between *M. sexta* and *H. virescens* or *O. nubilalis* but no differences between *H. virescens* and *O. nubilalis*. Higher levels of the ingested dose

was excreted as α-T in *M. sexta*, suggesting lower metabolising capabilities in this insect species as compared to *H. virescens* or *O. nubilalis*. Alpha-T could follow two routes before being voided in the feces. The possibilities are α-T going directly through the gut, unabsorbed and/or being absorbed through the gut, into the hemolymph and ending up in the hindgut through the Malpighian tubules. Quantification of α-T : metabolite distribution in the different components (gut, fat body, hemolymph cuticle) in all three species is an important next step.

The excretion of allelochemicals without metabolism has been shown by Isman and Duffey (1983). They reported that the phenolics, chlorogenic acid and rutin, are not metabolised but can be recovered unchanged in the frass of *Heliothis zea*, suggesting that these compounds pass straight through the gut. The high polarity of these phenolics was suggested as a contributing factor. In a earlier study, Self et al. (1964a) showed excretion of intact nicotine, an amphipathic neurotoxin, by *M. sexta* and suggested that *M. sexta* avoids toxicity from nicotine by fast excretion without having to metabolise the compound. The method of rapid excretion without metabolism may hold true for compounds which are hydrophilic but certainly not for molecules that are highly lipophilic like α-T. This was indirectly indicated in the present study which showed that metabolism prior to excretion does play an important role in the excretion of α-T.

There were 4 metabolites detected in the three species by TLC suggesting that larvae of the three species break down α-T to similar metabolites, probably using the same detoxification enzymes. However, the percent of metabolite with a higher polarity was appreciably higher for the resistant species, *H. virescens* and *O. nubilalis* as compared to *M. sexta*, clearly suggesting that conversion of α-T to a more polar metabolite results in faster clearance into the frass. A significant amount of label was detected at the origin indicating a highly polar metabolite, most probably a conjugate, in all the three species. Preliminary studies (Appendix V,VI and VII) indicate that α-T is

converted to metabolites which no longer absorb in the near UV region suggesting that this may be a method to avoid phototoxic effects.

5.4.2 *In vitro* studies

The low extraction efficiency obtained in the *in vivo* studies for the body component (63 % for *M. sexta*, 71% for *H. virescens* and 72 % for *O. nubilalis*) left 37, 29 and 28 % of the label unaccounted. The ratio of α-T : metabolite being unknown in a significant proportion cautions one from making conclusive statements. This and the question whether MFO's are involved in the detoxification promoted the *in vitro* studies.

Studies on the comparative metabolism of α-T by the microsomal fraction isolated from the midguts of the three species showed striking differences. The rate at which α-T was converted to a metabolite was 16 times faster in *H. virescens* and 30 times faster in *O. nubilalis* as compared to *M. sexta*. The results followed the same trend when expressed as pico-mole α-T metabolised/min/mg protein or pico-mole α-T metabolised/nano-mole cytochrome P-450 suggesting differences in specific activity of the MFO in the three species. These results explain clearly the fast excretion rates observed in *O. nubilalis* and *H. virescens* as compared to *M. sexta* and also indicate that mixed function oxidases are involved in the detoxification process. In similar *in vitro* assays, Bull et al. (1986) demonstrated that the gut microsomal fraction of *P.polyxenes* converted xanthotoxin 6 to 6.5 times faster than *S.frugiperda*, implying that the activity of MFO alone can explain the differential toxicity noticed in these insects.

In the present study, disappearance of substrate rather than product formation was used since no attempt was made to identify the metabolites formed. Judging from the structure of α-T, one might expect epoxidation, hydroxylation, sulphoxidation or desulphuration as possible mechanisms of detoxification, although epoxidation or sulphoxidation could lead to products more toxic than the parent

compound.

5.5 Piperonyl butoxide experiments

It has been argued that the demonstration of metabolism of an allelochemical by mixed function oxidases in an *in vitro* system may not necessarily reflect the involvement of the enzyme in an *in vivo* situation (Gould, 1984). To demonstrate the involvement of PSMO in *in vivo* , the method generally followed is application of piperonyl butoxide, a methylene dioxyphenol (MDP), with the chemical in question and monitoring LD_{50} values. If the LD_{50} is lower with the mixture as opposed to the insecticide alone, it indicates PSMO involvement (Brattsten et al., 1977; Gould et al., 1980)). In the present study, a different approach was taken. Body burdens of label when α-T was presented alone as opposed to α-T+ piperonyl butoxide were monitored. The study clearly indicated that PSMO's play an important role in the detoxification of α-T. Higher levels of label detected in larva of both *H. virescens* and *O. nubilalis* given piperonyl butoxide and α-T suggests that inhibition of PSMO by piperonyl butoxide led to lower metabolic rates of α-T in turn resulting in slower excretion. This explains higher levels of radioactivity in the body. Again, in the frass the levels of label detected was lower when α-T+piperonyl butoxide was used. This supports the earlier suggestion that PSMO's are involved in the detoxification process.

It has been demonstrated that the mode of action of piperonyl butoxide is an inhibition of the inactivation of the xenobiotic in question. Piperonyl butoxide is a known competitive inhibitor of certain microsomal oxidations. It is now generally accepted that most MDP's exert their inhibitory effect on PSMO activity through the formation of active metabolite intermediates possibly carbenes (Mansuy et al., 1979), that generate relatively stable complexes with cytochrome P-450 (Hodgson and Philpot, 1974; Wilkinson et al., 1984). It has been recognised for some time that MDP's elicit a

biphasic response with respect to MFO activity by eliciting a transient inhibitory effect followed by a more prolonged inductive effect leading to actually enhanced cytochrome P-450 levels and several oxidase activities (Thongsinthusak and Kreiger, 1974). Marcus et al. (1986) showed that after 72 h, *S.eridania* fed 0.05 % MDP's showed a 30 fold increase in aryl hydrocarbon hydroxylase activity in midgut microsomes. Unfortunately, piperonyl butoxide was not included in their study. In the present study sampling of larvae might have occurred during the transient inhibitory phase of piperonyl butoxide which would explain the observed results.

5.6 Cytochrome P-450

Quantitative studies on cytochrome P-450 levels showed that the amount present in *M. sexta* is about a third lower than levels in *H. virescens* or *O. nubilalis*. There is still uncertainty regarding the most satisfactory unit to express cytochrome P-450 levels. Expressing cytochrome P-450 on a larval basis showed higher levels in *M. sexta* compared to *H. virescens* or *O. nubilalis*. Expression of the levels on a larval basis is inappropriate because of the large variation in weight of the three species studied. In the present study, expressing cytochrome P-450 on a g animal, mg protein or mg protein / animal basis consistently showed *M. sexta* having lower levels than *H. virescens* or *O. nubilalis* (Appendix VIII). Reporting activity on a mg protein basis was preferred since comparisons can be made to results obtained in different laboratories and also among species.

Threefold greater levels of cytochrome P-450 were found in *H. virescens* when it was compared with *M. sexta* on a mg protein basis, and a 5.7 fold difference was observed when the levels were reported on a gram animal basis suggesting that the ratio for the weight of the individual to midgut protein is not constant between these species. Similar results were noticed when *M. sexta* and *O. nubilalis* were compared.

However, results expressed in the two forms showed no differences when *H. virescens* and *O. nubilalis* were compared indicating that mg protein in midgut to weight ratio is relatively constant between these species.

Significantly higher levels of cytochrome P-450 were detected in *H. virescens* compared to *O. nubilalis* although one might expect higher amounts in the latter from the results of the *in vitro* metabolism studies. In this study, cytochrome P-450 levels were measured quantitatively but no attempt was made to measure qualitative differences which could explain the higher specific activity of *O. nubilalis* gut microsomes in the *in vitro* studies inspite of lower cytochrome P-450 titres, as compared to *H. virescens*. Moreover, the cytochrome P-450 in *O. nubilalis* may be more specific for α-T than in *H. virescens*. Further research regarding the multiple forms of cytochrome P-450 which may co-exist in the same cell type is warranted.

Although cytochrome P-450 is present in the midgut, fat body and Malpighian tubules, no attempts were made to quantify the amounts in the latter two tissues, as the literature shows that the midgut is usually the site where maximum amounts of cytochrome P-450 is found in lepidopteran insects (Hodgson, 1983). For example, the reported values for cytochrome P-450 in midgut is 0.032 nmoles/mg protein as compared to 0.009 nmole/mg protein in the fat body (Tate et al., 1982). Cytochrome P-450 levels measured for *M. sexta* and *H. virescens* are in close agreement to literature values (Kulkarni et al., 1976, cited in Agosin, 1983). To the best of my knowledge, this is the first time cytochrome P-450 levels are being reported in *O. nubilalis* although indirect epoxidation activity has been studied for this species (Williamson and Schechter, 1970).

In brief, these studies substantiate the conclusion that higher levels of cytochrome P-450 in *H. virescens* and *O. nubilalis* as compared to *M. sexta* explain the

observed faster metabolism and excretion rates of α-T.

Cytochrome P-450 levels were not significantly affected when *M. sexta* and *O. nubilalis* larvae were fed 10 μg/g and 30 μg/g α-T in the diet, suggesting that lower levels or specific activity in *M. sexta* may be the prime reason for the inability of fast clearance of α-T in this species. Slightly lower (significant) dose-dependent levels were noticed in *H. virescens*. These results indicated that the measured decrease in levels of cytochrome P-450 may not be sufficient to adversely affect metabolism of α-T in this insect, or affect growth, as larval weights taken before sacrificing the larvae for analysis in treated and control were very similar. Significant decreases in larval weights were recorded for *M. sexta* only.

Induction of cytochrome P-450 was not seen. This was not surprising as levels of α-T used in this study were an order of magnitude lower than other allelochemicals which have been shown to induce cytochrome P-450 levels. The result also suggest that induction is not necessary in *H. virescens* and *O. nubilalis*, natural levels being sufficient for rapid excretion of the chemical.

5.7 An Ecological viewpoint

From the results of previous studies (Ivie et al.,1983; Berenbaum,1983) and the present work, it may be inferred that the differences in host range and evolutionary history of the lepidopteran species are significant elements of differential toxicity of the phototoxins tested. *M. sexta* is an oligophagous insect feeding principally on solanaceous plants. As a result, the larvae of this species never, or only rarely encounter α-T or related thiophenes, which explains the lack of rapid clearance capabilities in this insect of this phototoxin. Champagne et al. (1986) suggested that the ability of the larva to detect and avoid α-T is probably an adaptation in *M. sexta*. However, in the same study at 10 μg/g α-T in the diet, little feeding deterrency was

observed while toxicity was clearly indicated.

On the other hand, the polyphagous nature of *H. virescens*, which occassionally feeds on Asteraceae, and the host range of *O. nubilalis*, including 30 species of phototoxic Asteraceae, may have led to preadaptation in these species to thiophenes. In addition to physiological adaptation in *O. nubilalis*, concealed feeding and spinning profuse amounts of silk represent behavioral adaptations to avoid phototoxic effects (Champagne et al.,1986).

A relationship between feeding range (monophagy, oligophagy and polyphagy) and detoxification capabilities in insects was first demonstrated by Kreiger et al. (1971). Measurement of epoxidation activity of gut microsomes from 35 species of Lepidoptera he showed that polyphagous insects have 3 and 35 times more activity than oligophagous and monophagous insects respectively. The higher levels of activity in polyphagous insects was considered to be a countermeasure to the wider range of potential toxins these insects may encounter (Kreiger et al., 1971). My results though limited to only three species, lend support to this hypothesis. One may question the study of Kreiger et al. (1971) since it was based on MFO activity recorded from the guts of insects. MFO's are present in the fat bodies and Malpighian tubules as well. Optimization of measuring MFO activity in fat body and Malpighian tubule, followed by quantifying epoxidation, hydroxylation, O and N demethylation, desulphuration, sulphoxidation and the actual amounts of cytochrome P-450, should give more reliable data for or against the hypothesis. Furthermore, the hypothesis by Kreiger et al. (1971) has been questioned by Rose (1985) who found no significant differences in mean epoxidation activities in 58 species of monophagous to polyphagous Lepidoptera. Finally, Gould (1984) has also argued that evidence in support of the role of MFO's as an effective biochemical defence against diverse allelochemicals is not strong.

SUMMARY AND CONCLUSIONS

Alpha-T is toxic to *M. sexta* and *H. virescens* when fed to larvae at field relevant concentrations. Toxicity increases in the presence of near UV light, i.e., α-T is phototoxic to these phytophagous insects. *M. sexta* is extremely sensitive, whereas *H. virescens* is moderately susceptible to this allelochemical. Differential sensitivity of α-T towards *M. sexta*, *H. virescens* and *O. nubilalis* is seen in both feeding (results for *O. nubilalis* adapted from McDougall, 1986) and topical studies. Toxicity increases in the order *O. nubilalis*, *H. virescens* and *M. sexta*.

Alpha-T does not accumulate in any specific tissue within the three insect species, but passes through the gut and reaches the hemolymph, fat body and the cuticle, and is finally eliminated.

Larvae of *M. sexta* are unable to clear this compound rapidly enough to avoid toxicity. On the other hand, moderate to extremely high clearance rates seen in *H. virescens* and *O. nubilalis* indicate the mechanism by which tolerant insect herbivores may effectively deal with this allelochemical in host plants.

Rapid metabolism preceding excretion plays a role leading to faster excretion patterns. This was substantiated in *in vitro* studies, but did not correlate well with the *in vivo* studies on metabolism. Poor extraction efficiencies of α-T or its metabolite, especially in the fat body of the three species may have led to small but significant differences in the rate of metabolism of α-T noticed.

Alpha-T is metabolised by the mixed function oxidase enzyme system. This was clearly demonstrated in both *in vivo* and *in vitro* studies. Increased cytochrome P-450 levels in tolerant insects correlates well when these results are compared to the *in vitro* metabolism study of *M. sexta* and *H. virescens*,but not between *H. virescens* and

O. nubilalis. Further investigation of qualitative differences in the cytochrome P-450 enzyme between these insects is suggested.

This study in conjunction with a similar study on the phototoxic furanocoumarins (Berenbaum, 1978; Ivie et al., 1983; Bull et al., 1986) has shown that rapid metabolism followed by fast clearance is one method by which phytophagous insects feeding on plants containing these phototoxins avoid the adverse physiological effects of these allelochemicals.

6.1 Suggestions for future research

a) It is important that an attempt be made at elucidating definitive structures of the alpha-terthienyl metabolites formed in the lepidoptera larvae after ingesting α-T in the diet.

b) It would be worthwhile to study the levels of antioxidants (for example, vitamin C, K^+, ß-carotene) in larvae of the three species, after removing their gut contents to relate this to their potential for protection from phototoxins.

c) It would be interesting to study further the mode of action of α-T by investigating *in vivo* oxidation of biomolecules such as amino acids and proteins in the hemolymph, fat body and the gut of the insects studied. Using ^{3}H-α-T, an attempt could be made to find out if α-T is moved in the hemolymph by a carrier protein.

d) Further studies should include investigation of the effect of α-T on the guts of the three insect species. Enzyme activity in the gut, structural deformity and effect on gut microflora are some of the parameters worth considering.

e) Estimating cytochrome P-450 levels in the fat body and Malpighian tubules would provide more meaningful data to enable one to accurately correlate cyt P-

450 levels and α-T clearance patterns.

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APPENDIX I : Meridic diet used to rear laboratory colony of tobacco hornworm larvae

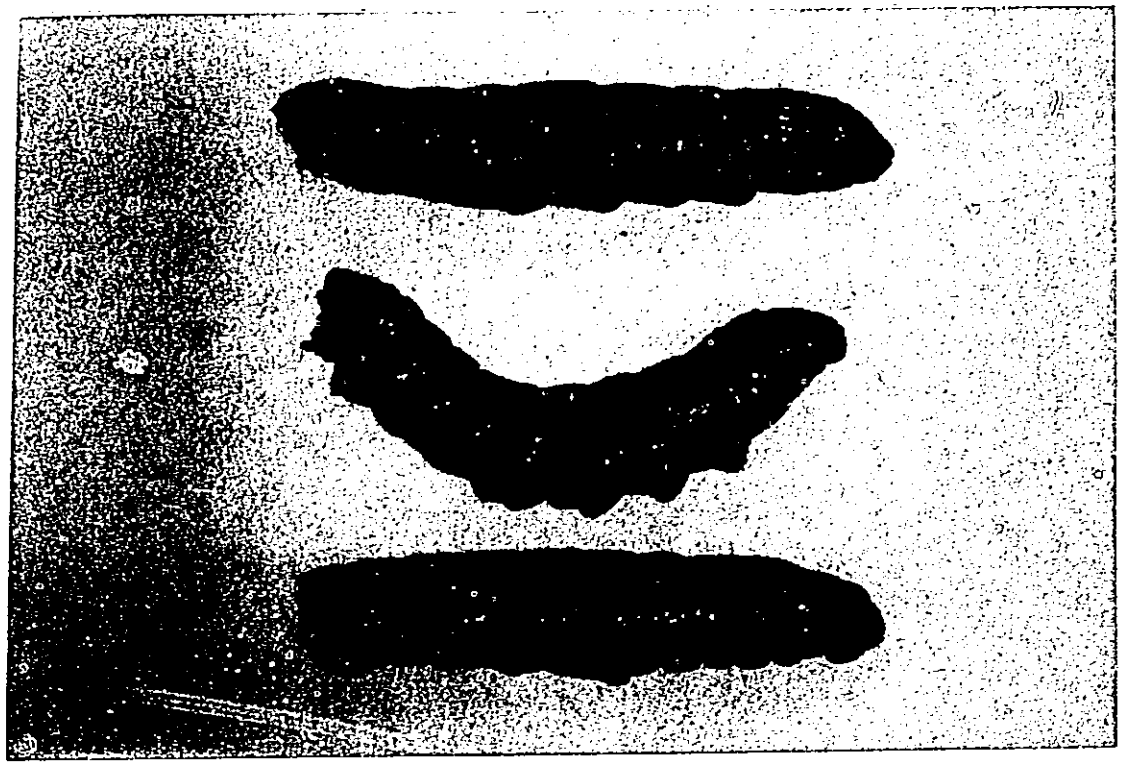
Ingredients	Quantities
Premix (g)	
Wheat germ	50.00
Casein	22.50
Sucrose	20.00
Brewers yeast	15.00
Wesson's salt mixture	7.50
Ascorbic acid	2.00
Sorbic acid	0.75
Methyl-p-hydroxy benzoate	0.60
Cholesterol	0.25
Benomyl	0.10
Vitamins (mg)	
Nicotinic acid	5.00
Calcium pantothenate	5.00
Riboflavin	2.50
Thiamine HCl	1.65
Pyridoxine HCl	1.65
Folic acid	0.10
Biotin	0.10
Others	
Agar (g)	10.00
Formalin (mL)	10.00
Raw linseed oil (mL)	2.00
Distilled water (mL)	750.00

APPENDIX II : Meridic diet (Halifax diet) used to rear *H. virescens* larvae in the laboratory

Ingredients	Quantity
A. Wet Mix (Stir till neeeded)	
Water (deionized)	100 ml
Sorbic acid	2.0 g
Ascorbic acid	2.5 g
Choline chloride	1.0 g
Inositol	0.2 g
Tocopherol	0.16 g
Chlortetracyline	0.15 g
Vitamin mix	10.0 g
B. Dry mix	
Wheat germ	120 g
Casein	25 g
Salt mix W	10 g
Methyl paraben	1 g
C. Agar mix	
Water (deionized)	750 ml
Agar	20 g

Appendix III : Meridic diet used to rear *O. nubilalis* larvae in the laboratory

Ingredients	**Quantity**
Distilled water	433 ml
Agar	14.9 g
Distilled water	172+87 mls
Dry mix	98.3 g
Methyl paraben	4 g
Propionic acid	4.6 ml
Formaldehyde	0.4 ml
Corn cob grits	21.3 g
Fumidil B	0.37 g



Appendix IV : Acute toxicity of α-T to *Manduca sexta* fed 50 µg/g α-T in the diet and exposed to 12 h of near UV light. Note lesion on subdermal and dermal tissues. Control larvae are bluish green in color.

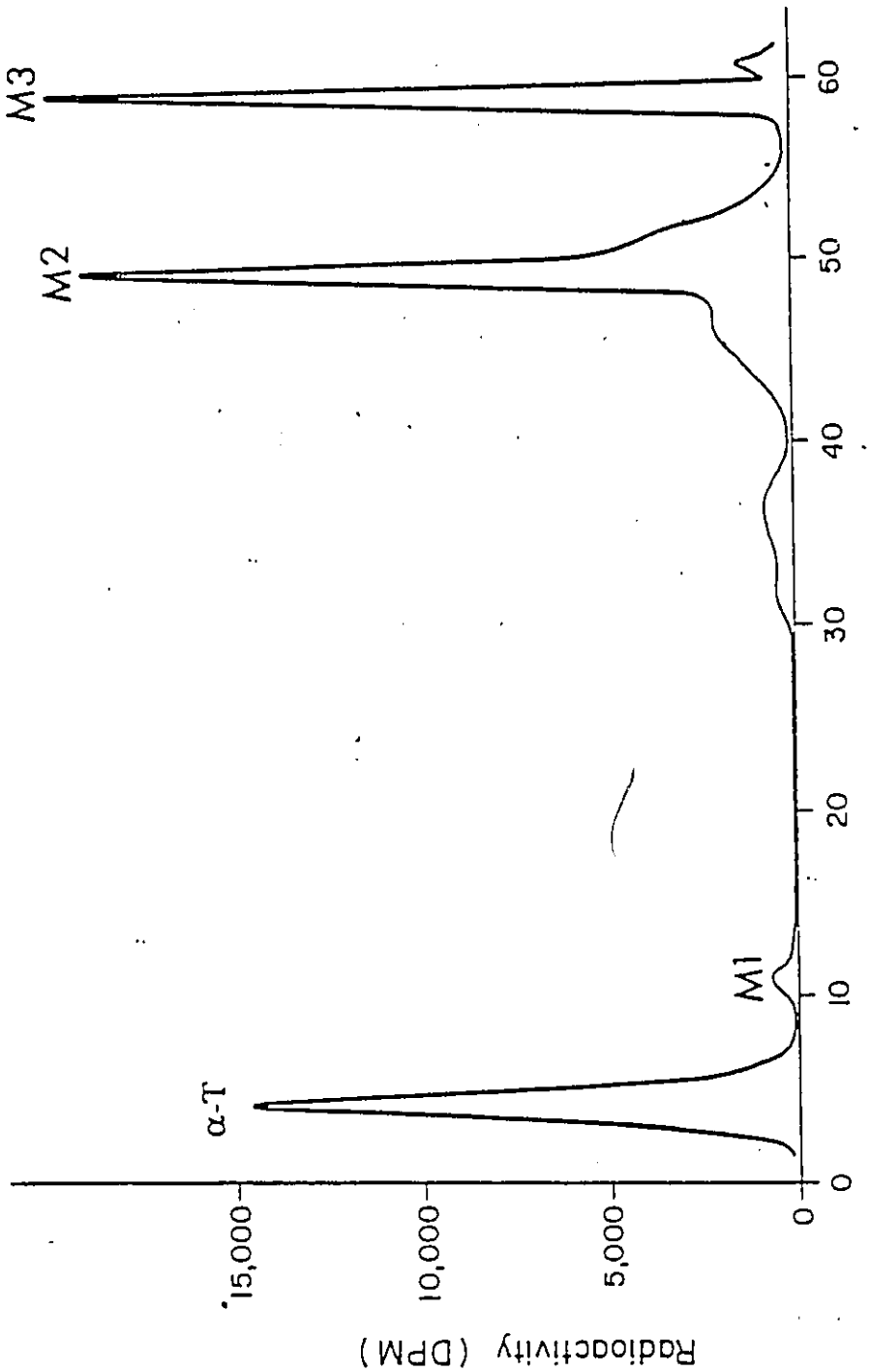
Appendix V: Preliminary studies on metabolite identification

Twenty five early fifth instar larvae of *M. sexta* were fed radiolabelled ^{3}H-α-T (0.532 μg/g) and cold α-T (20μg/g) to give a final concentration of 20.5 μg/g in the diet. Feces were collected every 24 h and pooled together at the end of 72 h. Extraction for α-T and metabolite was done as described previously (Section 3.11). The final volume was 3 mL. α-T and metabolites was separated using a Chromatotron (model 7924 T, Harrison Research Inc.). The extract was loaded onto a silica gel PF plate and eluted using a gradient elution as follows:

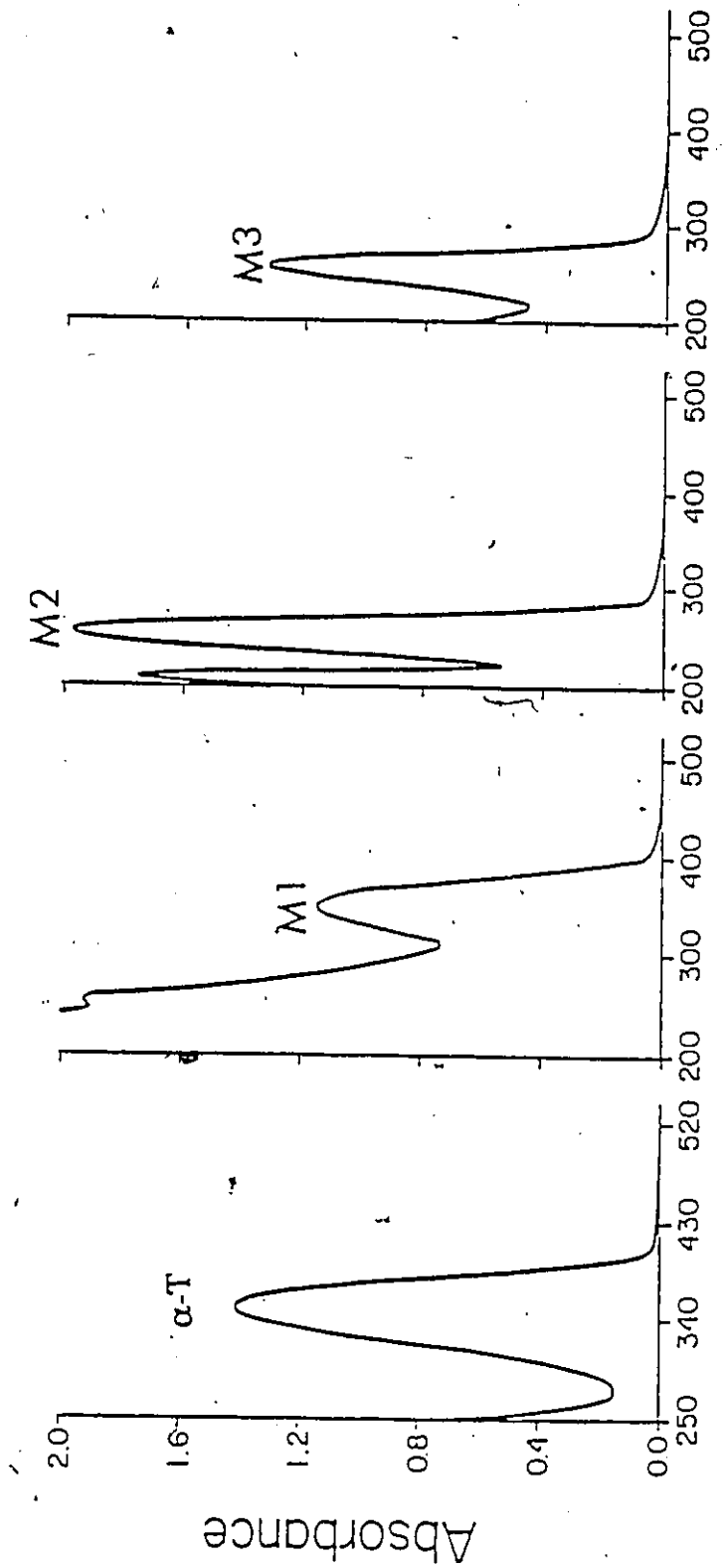
40 mL	100 % hexane
80 mL	5 % ethyl acetate in hexane
120 mL	10 % ethyl acetate in hexane
160 mL	15 % ethyl acetate in hexane
200 mL	20 % ethyl acetate in hexane
240 mL	25 % ethyl acetate in hexane
280 mL	30 % ethyl acetate in hexane
320 mL	35 % ethyl acetate in hexane
360 mL	40 % ethyl acetate in hexane
400 mL	50 % ethyl acetate in hexane
440 mL	75 % ethyl acetate in hexane
480 mL	100 % ethyl acetate in hexane
520 mL	100 % methanol
560 mL	1% acetic acid in methanol
600 mL	3 % acetic acid in methanol
640 mL	5 % acetic acid in methanol

Eluted fractions (7 mL) were collected for gradients 0% ethyl acetate to 100 % ethyl acetate. A single 200 mL methanol fraction was collected. Acetic acid in methanol was collected as a single fraction. The fractions were evaporated to dryness and resuspended in 300 μL methanol. A 100 μL aliquot was taken for LSC counting.

There were four separate peaks with radiolabel (Appendix VI). A spectrum of peak 1 indicated alpha-terthienyl. Spectra of α-T and three peaks are shown in Appendix VII. Peak M2 indicated that the α-T chromophore was still intact whereas peaks M3 and M4 showed spectra with a λmax of 250 indicating a metabolite of α-T which no longer absorbs in the near UV region. A disc test bioassay using yeast on peaks M3 and M4 showed that they were not phototoxic. Structural elucidation of peaks M2, M3 and M4 are being pursued.



Appendix VI : Elution profile of the frass sample of *M.sexta* on a chromatotron. Early last instar larvae were fed ^{3}-H-α-T and frass sample analysed for α-T and metabolite. M1-M3 represents metabolites



Appendix VII: Absorption spectra of α-T and metabolites 1, 2 and 3 (M1, M2, M3) from frass sample of *M.sexta*

Appendix VIII : Cytochrome P-450 levels expressed in different units

Species	n mol/mg protein	n mol/animal	n mol/g animal	nmol/mg protein/g animal
M.sexta	0.136	0.255	0.043	0.147
H.virescens	0.437	0.089	0.247	0.495
O.nubilalis	0.319	0.014	0.142	0.302