NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

ISBN 0-315-75063-4

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Many secondary plant compounds are capable of photoactivation resulting in the production of toxic species of oxygen. One mechanism of defense for insects feeding on phototoxic plants may be the presence of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOX), and glutathione reductase (GR). The activities of these enzymes were examined in larvae of three lepidoptera: *Ostrinia nubilalis*, *Manduca sexta*, and *Anaitis plagiata*. Highest levels of antioxidant enzyme activity were found in *A. plagiata*, a specialist feeder on *Hypericum perforatum*, which contains high levels of the phototoxin hypericin. Larvae of *A. plagiata* fed leaf discs treated with hypericin exhibited a short-term, concentration-dependent decline in enzyme activity. Longer term studies with *A. plagiata* fed either the phototoxic *H. perforatum*, or the closely related but non phototoxic *H. calycinum*, resulted in increased CAT and GR activity in larvae fed the phototoxic plant whereas superoxide dismutase activity was not significantly different. These results suggest that CAT and GR may be inducible defenses against phototoxins.

Other insect defenses against phototoxins include specific biochemical defenses such as antioxidants. These antioxidant defenses eliminate or quench the deleterious singlet oxygen and free radicals formed by these phototoxins. We examined the role of dietary antioxidants in protecting the phototoxin-sensitive insect herbivore *M. sexta*. Elevated dietary levels of the lipid-soluble antioxidants beta-carotene and vitamin E resulted in a

concentration-dependentreduction in the mortality associated with treatment of larvae with the phototoxic thiophene α-T. Elevated levels of dietary ascorbic acid had no effect whereas reduced levels greatly increased the toxicity of α-T. Tissue levels of antioxidants were shown to increase substantially in larvae fed antioxidant-supplemented diets.The results suggest that the ability to absorb and utilize plant-derived antioxidants could be an important defenseagainst photo-activated plant secondarycompoundsand may have allowed some insects to exploit phototoxic plants.

The effects of oxidative stress induced by α-T at the biochemical level and the protective effect of antioxidants and antioxidant enzymes were also examined. The phototoxin α-T strongly induced lipid peroxidation (LPO) in midgut tissues of the phototoxin-sensitive *M. sexta* in a UV-dependent manner, however this LPO was prevented when the compound was administered to larvae raised on high vitamin E diets. In the absence of UV, α-T caused a significant increase in GPOX, GR, and non-GSH-dependent PER activity over 72 h. However in the presence of UV, α-T strongly inhibited GPOX and GR and prevented the increase in PER. α-T also affected cellular thiol status with approximately a 50% increase in total and GSH content in midgut tissue, although this was not UV-dependent. The effectiveness of antioxidant enzymes and the antioxidant GSH in providing protection against phototoxins were also examined. Neither the SOD inhibitor DEDC nor the CAT inhibitor 3AT affected the acute toxicity of topically applied α-T to *M. sexta* larvae. The GSH-depleting agent BSO also had no effect on acute toxicity. In contrast, GSH depletion strongly enhanced the chronic (72 h) toxicity of α-T when the phototoxin was incorporated into diets. GSH depletion also enhanced LPO in midgut tissue

of α-T-treated larvae.

Implications of the results are discussed in terms of the role antioxidants and antioxidant enzymes may have played in the successful adaptation of some insect species to phototoxin-containing plants. The interrelationships of biochemical, physiological, physical, and behavioural mechanisms of defense are considered.

RESUME

Plusieurs composés secondaires des plantes peuvent être photo-activés et produire ainsi des formes toxiques d'oxygène. Un des mécanismes de défense pour les insectes qui se nourrissent sur les plantes phototoxiques pourrait être la présence d'enzymes anti-oxydantes tels que la superoxyde dismutase (SOD), la catalase (CAT), la glutathione peroxydase (GPOX) et la glutathione réductase (GR). L'activité de ces enzymes a été examinée pour les larves de trois insectes lépidoptères: *Ostrinia nubilalis*, *Manduca sexta* et *Anaitis plagiata*. Les niveaux les plus élevés d'activité enzymatique ont été trouvés pour *A. plagiata* qui est un insecte spécialiste se nourrissant sur *Hypericum perforatum*, une plante ayant une teneur élevée en hypéricine, une phototoxine. Les larves de *A. plagiata*, nourries sur des rondelles de feuilles traitées avec de l'hypéricine, ont présenté une diminution à court terme de l'activité enzymatique en fonction de la concentration utilisée. Les études à long terme sur *A. plagiata* nourri soit sur la plante phototoxique *H. perforatum* ou sur la plante apparentée de près mais non phototoxique *H. calycinum*, ont démontré une augmentation de l'activité de CAT et GR pour les larves nourries sur la plante phototoxique, alors que l'activité de SOD n'a pas été significativement différente. Ces résultats suggèrent que les enzymes CAT et GR pourraient être un moyen de protection contre les phototoxines.

Parmi d'autres défenses des insectes contre les phototoxines, il y a des mécanismes comportementaux tel que la protection contre le rayonnement solaire, ainsi que des défenses biochimiques tels que les antioxydants. Ces défenses antioxydantes éliminent ou réduisent les effets délétères de l'oxygène singulet et des radicaux libres formés par ces phototoxines.

Nous avons examiné le rôle d'antioxydants alimentaires dans la protection de l'insecte herbivore *M. sexta*, insecte sensible aux phototoxines. Lorsque des teneurs élevées des antioxydants liposolubles beta-carotène et vitamine E ont été ajoutées au regime, une diminution de la mortalité a été observée en fonction des concentrations utilisées pour les larves de *M. sexta* en présence du thiophène phototoxique α-terthienyl. Des teneurs élevées d'acide ascorbique dans le regime n'ont eu aucun effet, alors que des teneurs faibles ont résulté en une forte augmentation de la toxicité d'α-terthienyl. Les niveaux tissulaires d'antioxydants ont augmenté substantiellement pour les larves nourries sur une diète contenant un supplément d'antioxydants. Les résultats suggèrent que la capacité d'absorber et d'utiliser des antioxydants dérivés de plantes, pourrait être un moyen de protection important contre les composés secondaires photosensibles des plantes et pourrait permettre à certains insectes d'exploiter les plantes phototoxiques.

Les effets stressants de l'oxydation induite par l'α-T au niveau biochimique et l'effet de protection des antioxydants et des enzymes antioxydants ont aussi été examinés. La phototoxine α-T a fortement induit la peroxydation des lipides (LPO) pour *M. sexta*, un insecte sensible à la phototoxine, et ce, en fonction des rayons ultra-violets (UV), alors que la LPO était évitée lorsque le produit a été administré aux larves élevées avec un regime ayant une teneur élevée en vitamine E. En absence d'UV, l'α-T a causé une augmentation significative de l'activité de GPOX, de GR et de peroxydase (PER) à 72 heures. Cependant, en présence d'UV, l'α-T a fortement inhibé l'activité de GPOX et GR et a empêché l'augmentation de PER. L'alpha-T a aussi affecté l'état des thiols cellulaires avec une augmentation approximative de 50% du contenu en GSH dans les tissus de

l'estomac moyen, mais sans relation avec les UV. L'efficacité des enzymes antioxydants et de l'antioxydant GSH à fournir une protection contre les phototoxines a aussi été examinée. Ni l'inhibiteur du superoxyde dismutase DEDC, ni l'inhibiteur du catalase 3AT, n'ont affecté la toxicité aigüe d'α-T appliqué localement pour les larves de *M. sexta*. Le buthionine sulfoxymine (BSO), un inhibiteur de la synthèse de GSH, n'a eu aucun effet non plus sur la toxicité aigüe. A l'opposé, l'épuisement en GSH a fortement augmenté la toxicité chronique (72 heures) de l'α-T lorsque la phototoxine a été incorporée à la diète. L'épuisement en GSH a aussi augmenté la LPO dans les tissus de l'estomac moyen des larves traitées à l'α-T.

Les résultats et leurs implications sont discutés en fonction du rôle que les antioxydants et les enzymes antioxydants peuvent avoir joué dans le succès d'adaptation de quelques espèces d'insectes sur les plantes contenant des phototoxines. Les inter-relations entre les mécanismes de défenses biochimiques, physiologiques, physiques et comportementales sont considérées.

ACKNOWLEDGEMENTS

Many friends, relatives and co-workers are responsible for encouraging me to enter graduate school and complete a Masters degree and now a Doctorate. To all of these people I am sincerely grateful. In particular I would like to thank the following:

My supervisors Thor Arnason and Bernard Philogène for encouragement, support, and optimism, and for teaching me the importance of biology and the need for excellence in science.

My lab and cell-mates, Yong-shou Xie, Paul Fields, Mark Lewis, Diane Gagnon, Dave Bergvinson, Jane-Anne Dugas, Claude Bernard, M.Chantal Bertrand, Bernard C.de Beyssac, France Duval, Janet Gail, Danna Leaman, Christine Murray, Rafik Assabagui, Norman Thie, Ru-Ying Feng, Robin Marles, Cliff Beninger, and Mamdouh Abou Zaid have all given me help and friendship, and made this project an enjoyable learning experience.

The Natural Sciences and Engineering Research Council of Canada Scholarship Program, The Ontario Graduate Scholarship Program, and The University of Ottawa for financial support without which this project would not have been possible.

Last but not least my wife and friend Pauline without whose love, encouragement, and support this thesis would not have evolved.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS USED

α-T	alpha-terthienyl
LPO	lipid peroxidation
8-MOP	8-methoxypsoralen
PHT	phenylheptatryne
PSMO	polysubstrate mono-oxygenases
SOD	superoxide dismutase
CAT	catalase
GPOX	glutathione peroxidase
GR	glutathione reductase
PER	non-glutathione dependent peroxidase
GST	glutathione-S-transferase
GSH	reduced glutathione
GSSG	oxidized glutathione
SH	sulfhydryl
UV	ultraviolet light
TSH	total sulfhydryl
PBSH	protein-bound sulfhydryl
NPSH	non protein bound sulfhydryl
DTNB	dinitrobenzoic acid
BSO	buthionine sulfoximine
DEDC	diethyldithiocarbamate
3AT	3-amino-triazole

Chapter 1: Literature Review: Insect Defenses against Photo-activated Plant Secondary Compounds

1.0 Allelochemicals

Plants have evolved a variety of defense mechanisms which deter insects and other herbivores. These include physical defenses such as spines, hairs, trichomes, and tough leaves. As well, plants produce a diverse array of phytochemicals known as allelochemicals (Whittaker and Feeny, 1971). These are non-nutritional chemicals whose function is usually to repel, poison, or interfere with the assimilation of food by herbivores (Reese, 1979; Rhoades, 1986). They may normally be present in various plant tissues or may be induced in response to attack by herbivores or plant pathogens (Rhoades, 1979). The approximately 20,000 allelochemicals identified to date exhibit an enormous diversity with respect to both their physical and chemical properties as well as in the ways in which they exert their often toxic effects on biological systems (McKey, 1979; Brattsten and Ahmad, 1986).

Many allelochemicals act as toxins and directly interfere with essential metabolic processes. For example, some non-protein amino acids (e.g canavanine) can be incorporated into proteins where they may disrupt both structure and function (Rosenthal, 1977). Cyanogenic glycosides are enzymatically altered to release hydrogen cyanide, a potent inhibitor of cytochrome oxidase and hence respiration (Conn, 1979). Other allelochemicals may act in a more indirect fashion by acting as digestibility reducers. These compounds may reduce the nutritional value of ingested

plant materials, often by complexing with important digestive enzymes and restricting the insects ability to assimilate nutrients. For example, protease and amylase inhibitors may reduce an insects ability to use polypeptides or starch (Ryan, 1979) and hence slow or prevent normal growth and development.

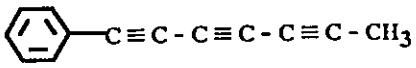
2.0 Phototoxins

Phototoxins are a novel class of allelochemicals that have been isolated from over thirty flowering plant families (Downum, 1986; Berenbaum, 1987), with members of the Asteraceae and Rutaceae synthesizing the widest range of phototoxic compounds (see Appendix 1). They are produced by numerous biosynthetic pathways and include polyine compounds (e.g. alpha-terthienyl (α-T)), alkaloids (e.g. berberine), quinones (e.g.hypericin) and furanocoumarins (e.g. psoralens) (see Figure 1). Within the plant these phototoxins may or may not be localized to specific tissues. In many members of the genus *Hypericum* for example, the phototoxic extended quinone hypericin is located in prominent, marginal leaf glands where it is ideally located to provide a defense against herbivory (see Appendix 2).

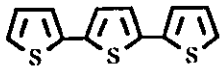
2.1 Mode of Action of Phototoxins

Phototoxins are unique in that they exhibit greatly enhanced toxicity in the 8presence of specific wavelengths of light (often in the near UV range, 300-400 nm).

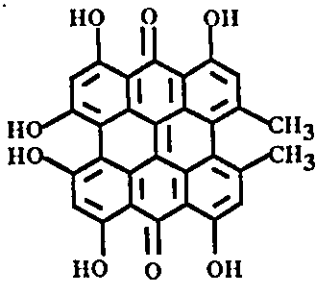
Figure 1: Chemical structures of some common photo-activated plant secondary compounds.



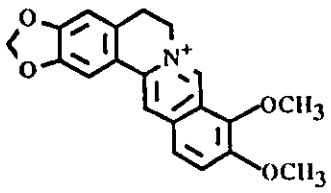
Phenylhepatriyne



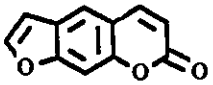
α-Terthienyl



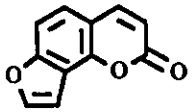
Hypericin



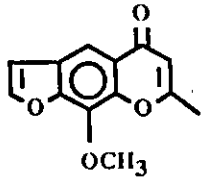
Berberine



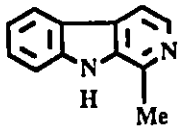
Psoralen



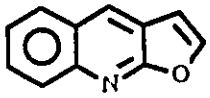
Angelicin



Khellin



Harmane



Dictamnine

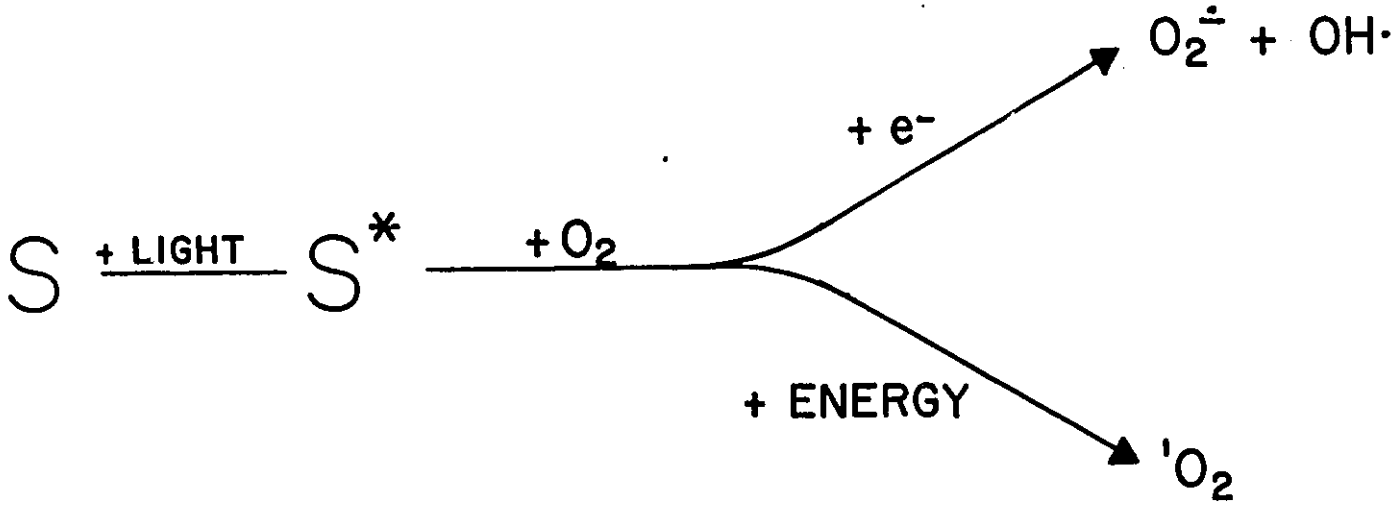
They have been shown to have broad spectrum biocidal properties against a wide variety of organisms including herbivorous and non-herbivorous insects (Wat *et al*, 1981; Arnason *et al*, 1981a) and may be classified according to their mode of action, although some overlap may occur. There are two major groups of phototoxins, the photo-oxidants and the photogenotoxins.

2.1.1 Photo-oxidants

The majority of phototoxins examined to date are active only in the presence of oxygen and hence have been traditionally described as "photodynamic" compounds but are more accurately described as photo-oxidants. These include the polyines, their thiophene derivatives, and the quinones. Light activation of these compounds results in a short-lived, electronically excited state. These 'sensitizer' molecules may then react with oxygen and other substrates to produce highly reactive and highly toxic species of oxygen including singlet oxygen ($^1O_2$) and free radicals such as superoxide anions (O_2-) and hydroxyl radicals (OH)(see Figure 2). All of these activated species of oxygen are capable of causing serious cytological and genetic 2damage (Mead, 1979; Halliwell and Gutteridge, 1985).

The most common reaction of the photo-oxidants is an energy transfer (type II) mechanism (Foote, 1986) in which the photosensitizer molecule in its ground electron state (S_0) is activated by a photon to its first singlet excited state (S_1) and

Figure 2: Light activation of a sensitizer molecule (S) to an excited state (S^*) and the transfer of energy or electrons to produce free radicals or singlet oxygen.


S
+ LIGHT
S*
+ O_2
+ e^-
$O_2^{\cdot-}$ + OH·
+ ENERGY
$^1O_2$

then undergoes intersystem crossing (ISC) to a relatively long-lived, triplet state (T_1). This triplet state sensitizer can then undergo energy transfer with ground state oxygen ($^3O_2$) to produce $^1O_2$ and regenerate the ground state sensitizer S_0. Under continuous irradiation, this production of $^1O_2$ is catalytic, allowing a few sensitizer molecules to produce millions of molecules of $^1O_2$. Singlet oxygen thus produced may further react with other molecules such as membrane components where it may lead to lipid peroxidation (LPO) and protein modifications (Valenzeno, 1987).

An alternative mechamism involves electron transfer processes (type I) leading to the formation of free radicals. These type I and type II mechanisms are normally in competition with each other with the products formed dependent on factors such as O_2 concentration and the reactivity of the sensitizer and substrates available (Foote, 1987). The phototoxin α-T for example, has been shown to react primarily by a type II mechanism (singlet oxygen generation) with less than 1% of the activated O_2 produced by electron transfer (Scaiano *et al*, 1989).

2.1.2. Photogenotoxins

Some phototoxins (known as photogenotoxins) have been found to bind and cause damage at the level of DNA without a requirement for oxygen. These include the furanocoumarins and furanoquinoline alkaloids which appear to act primarily by intercalating with pyrimidine bases in DNA and RNA in a dark reaction. Subsequent absorption of photons leads to formation of photoadducts which can be monoadducts

or give rise to interstrand crosslinking of the DNA (Ashwood-Smith *et al*, 1980). These mutagenic products are frequently lethal to target organisms (Song and Tapley, 1979) resulting in gross chromosomal aberrations (Towers and Abramowski, 1983; Averbeck, 1989).

Although photogenotoxins such as the furanocoumarins act primarily through non-photodynamic, genotoxic mechanisms, and the photo-oxidants such as α-T act through oxidative processes, some overlap can occur. The furanocoumarins for example are capable of producing $^1O_2$ and O_2- (Joshi and Pathak, 1983). Both the isoquinoline alkaloids (e.g.berberine) and harmane alkaloids generate $^1O_2$ and O_2- yet are also mildly photomutagenic perhaps because they intercalate DNA and generate $^1O_2$ at this site (Towers and Abramowski, 1983; Philogène *et al*, 1984; Hudson *et al*, 1986).

2.2 Toxicology of Photo-activated Compounds and Their Physiological Effects on Insects

Much of the original research on the mode of action of phototoxins was carried out using photo-oxidative dye sensitizers such as methylene blue and rose bengal on mostly non-herbivorous insects (reviewed in Robinson, 1983; Weaver, 1987). These agents were found to be highly toxic and, like the plant-derived photosensitizers, are known to generate activated oxygen species. Phototoxins have been known for some time to be capable of killing or inactivating a wide range of organisms including viruses, bacteria, fungi, and nematodes (reviewed in Towers,

1984) however it is only during the last decade or so that the susceptibility of h2erbivorous insects to these compounds has been explored. The polyines, quinones, alkaloids, and furanocoumarins are the major classes of phototoxins which have been shown to be lethal or to interfere with insect growth and development.

2.2.1 Polyines

A number of polyines and thiophene derivatives have been shown to be phototoxic to a wide variety of organisms including insects (Wat *et al*, 1981; Arnason *et al*, 1981a) The thiophene α-T, for example, has exceptional phototoxic activity towards mosquito and blackfly larvae, with LC_{50}'s of 19 ppb and 28 ppb respectively (Arnason *et al*, 1981a; Philogène *et al*, 1985). This high toxicity of α-t has led to its evaluation as a commercial mosquito larvicide in laboratory and field trials (Philogène *et al*, 1985; Arnason *et al*, 1989).

In herbivorous insects a number of polyines have been tested for both feeding deterrency and toxicity. Phenylheptatryne (PHT) has strong antifeedant effects on *Euxoa messoria* (McLachlin *et al*, 1982) and both PHT and α-T have been shown to strongly deter feeding of neonate larvae of *Manduca sexta*, and to a lesser extent larvae of *E. messoria* and *Ostrinia nubilalis* (Champagne *et al*, 1986). Both compounds had detrimental effects on growth and development and negative effects on nutrient utilization as evidenced by changes in nutritional indices. In this same study, both light-dependent and light-independent effects were seen. α-T and PHT also caused

black necrotic lesions on the dorsal surfaces of larvae of *M. sexta* fed the compounds in the presence of UV but not in its absence (see also Downum *et al*, 1984). As with most phototoxins examined to date however, there are a number of insects which appear to be very tolerant of phototoxins. α-T for example shows considerable variation in its insecticidal activity. Topical studies with α-T have indicated LD_{50}'s of 10, 474, and 698 ug/g for *M. sexta, Heliothis virescens,* and *O. nubilalis* respectively (Iyengar *et al*, 1987). The highly polyphagous *O. nubilalis* is also very tolerant of ingested α-T, an attribute which may be primarily explained by its ability to rapidly metabolize and excrete the compound (Iyengar *et al*, 1987; 1990).

Because most polyines are such strong photo-oxidants, generating highly toxic species of oxygen, their toxicity to insects might be expected to be a result of protein oxidation and lipid peroxidation in sensitive tissues. *In vitro* evidence has shown that α-T induces potassium ion leakage and hemolysis in erythrocytes (Wat *et al*, 1981) and initiates glucose leakage and lipid peroxidation in egg lecithin liposomes (McRae *et al*, 1985). *In vivo*, ingested α-T severely damages midgut epithelial cells in *M.sexta* (Sen *et al*, 1990). In mosquito larvae, α-T treatment in the presence of UV light causes a rapid increase in tissue lipid peroxidation (Hasspieler *et al*., 1990).

2.2.2 Extended Quinones

The extended quinone pigment hypericin is a strong photo-oxidant found in many members of the genus *Hypericum* where it may make up 0.05% of the plant weight (Giese, 1980). Unlike α-T, hypericin is primarily activated by visible light

(450-600 nm). However, as with α-T, it generates a high yield of singlet oxygen (Knox and Dodge, 1985; Duran and Song, 1986).

The insecticidal properties of ingested hypericin have been investigated in *M.sexta* where an LD_{50} of purified hypericin to third instar larvae was found to be 16 ug/g larval weight under constant light conditions (Samuels and Knox, 1989). At sub-lethal doses, hypericin caused a reduction in growth and development. The phototoxic effects of hypericin diminished rapidly when treated larvae were subsequently maintained in darkness for periods prior to irradiation. For example, mortality was reduced to only 6% if irradiation was delayed for 8 h after treatment (compared to about 80% if irradiation immediately followed treatment). In a separate study, Sandberg and Berenbaum (1989) have shown that hypericin is phototoxic to the generalists *H. zea* and *Platynota flavedana* forced to feed on *Hypericum perforatum* under daylight conditions; survivorship was reduced and development times increased significantly.

2.2.3 Alkaloids

Little information is available on the effects of phototoxic alkaloids on insects although many have been identified as capable of causing mortality including dictamnine, khellin, berberine, and beta-carbolines such as harmane (reviewed in Arnason *et al*, 1991). The isoquinoline alkaloid berberine is very phototoxic to mosquito larvae with an acute 24 h LC_{50} of 8 ppm compared to 250 ppm in the dark

(Philogène *et al*, 1984). Chronic effects of berberine included a reduction in larval, pupal, and adult survival. In herbivorous insects, Larson *et al* (1988) have tested a number of beta-carboline alkaloids in artificial diets fed to the cabbage looper *Trichoplusia ni*, however only harmane had significant negative effects on relative growth rate and consumption. Harmine reduces relative growth rates and consumption without affecting the conversion of ingested food to biomass (Lee and Berenbaum, 1989).

The phototoxic alkaloid sanguinarine has also been tested against *T. ni* where mortality increased with increasing concentration of the compound in the diet. However, no significant effects of light on mortality were observed (Tuveson *et al*, 1989). Arnason *et al*.,(1991) have shown that sanguinarine is clearly phototoxic to mosquito larvae with an LC_{50} of 0.096 mg/ml in the presence of UV and 23 mg/ml in the absence of UV.

2.2.4. Furanocoumarins

Much of the research on the effects of photogenotoxins on insects has been carried out with xanthotoxin (8-methoxypsoralen), primarily because of its commercial availability. Berenbaum (1978) was the first to demonstrate the phototoxic effects of xanthotoxin on herbivorous insects. She reported that when larvae of the southern armyworm *Spodoptera eridania* are fed on diets containing xanthotoxin (but not its biosynthetic precursor umbelliferone) under simulated

daylight conditions, all of the larvae failed to develop past the second instar. However when the activating wavelengths (UVA) were excluded, nearly 40% of the larvae survived through to pupation, even though the time to pupation was significantly increased. In contrast, the black swallowtail *Papilio polyxenes* (an umbellifer feeding specialist) is not intoxicated by linear furanocoumarins such as xanthotoxin but is adversely affected by angelicin, an angular furanocoumarin found in only a few advanced tribes of the Umbelliferae. Larvae fed leaf discs treated with angelicin exhibited reduced growth rates and greater than three-fold poorer fecundity than controls (Berenbaum and Feeny, 1981). Although derived from the common precursor umbelliferone, linear and angular furanocoumarins differ in their toxicological properties to insects. Linear furanocoumarins such as xanthotoxin are capable of forming both DNA monadducts and DNA-DNA crosslinking whereas angular furanocoumarins normally produce only DNA monadducts (Ashwood-Smith *et al*, 1984).

The differential toxicity of xanthotoxin to *S. eridania* and *P. polyxenes* has been ascribed to the ability of the latter insect to more rapidly and efficiently detoxify the compounds (Ivie *et al*, 1983). *P. polyxenes* excretes 50% of administered [^{14}C]-xanthotoxin within 1.5 h after dosing, whereas *S. frugiperda* only eliminates about 1% (Bull *et al*, 1984). Metabolic detoxification also appears to be the reason for furanocoumarin tolerance in the leafminer *Phytomyza spondylii* (Diptera: Agromyzidae) feeding on *Heracleum lanatum* (Ashwood-Smith *et al*, 1984) and the relative insensitivity of the parsnip webworm *Depressaria pastinacella* (Lepidoptera:

Oecophoridae), a specialist on furanocoumarin-rich plants (Nitao, 1989; Lee and Berenbaum, 1990). In the phototoxin sensitive *T. ni*, Ahmad *et al*(1987) have established an LC_{50} for xanthotoxin of 0.0004% under UV light and 0.0013% in the absence of UV. In a separate report, Lee and Berenbaum (1989) have shown that xanthotoxin has a negative effect on relative consumption rates and relative growth rates of *T. ni*. However, xanthotoxin has no effect on the food utilization indices in the furanocoumarin-tolerant *D. pastinacella* (Lee and Berenbaum, 1990) except in the presence of the cytochrome P-450 inhibitor, piperonyl butoxide which results in significantly lower growth and consumption rates and reduced efficiencies of conversion of ingested food. These results are consistent with both the antifeedant effects of furanocoumarins as well as indicative of their post-digestive toxicity. They also point to the central role of P-450 based detoxification mechanisms in determining sensitivity and tolerance to furanocoumarins (see Section 3.0).

3.0 Insect Defenses and Adaptations to Allelochemicals

3.1 General Defenses

Many insect herbivores have evolved mechanisms which have allowed them to overcome the defenses of their host plants. These include behavioural, biochemical, and physiological adaptations. Much of the research conducted to date on the behavioural adaptations of insects relates to host plant selection and

specificity, with perhaps less attention paid to specific behavioural mechanisms with which insects overcome plant defenses. Insects have an array of sensory structures with which to detect plant chemicals including contact chemoreceptors usually located on the maxillae, labium, and tarsi (Schoonhoven, 1968) and olfactory chemoreceptors usually located on the antennae. These olfactory receptors detect volatile chemicals which may act as repellents or attractants and hence may aid in the location of suitable (or avoidance of unsuitable) host plants (Schoonhoven, 1982). One example of a specific behavioural adaptation to overcoming plant defensive chemistry is found in beetles of the genus *Epilachna* which specialize on plants of the family Curcubitaceae. Carroll and Hoffman (1980) have shown that, prior to feeding, larvae and adults cut a circular trench through the upper layers of leaf tissue. This apparently isolates a section of tissue from curcubitacins which are bitter-tasting, triterpenoid allelochemicals mobilized in response to insect feeding.

Another way insects may deal with allelochemicals is to actively sequester them and store them in "less sensitive" tissues (Duffey, 1980). This is the case with the oleander aphid *Aphis nerii* for example, which sequesters highly toxic cardiac glycosides from oleander and the more familiar example of sequestration of cardenolides from milkweed by monarch butterflies (Harborne, 1978; 1989). In this latter example, the sequestered toxins may act as a defense against insect predation.

The major line of defense insects have against plant allelochemicals (and synthetic pesticides) is that they have developed numerous enzyme systems whose function is to detoxify xenobiotics. These enzymes metabolically degrade foreign

substances to products which are more water soluble than the parent compound and hence are more readily excreted. The primary products of this metabolic degradation may be polar enough to be excreted directly but often must first be converted by secondary enzymes to easily excretable, conjugated materials (Brattsten, 1979). The microsomal, cytochrome P-450 dependent polysubstrate monooxygenases (PSMOs) are by far the most important enzymes involved in the primary metabolism of lipophilic foreign compounds in insects (Brattsten, 1979; Ahmad *et al.*, 1986). These enzymes catalyze diverse oxidative reactions including hydroxylations, epoxidations, and oxidative dealkylations and are often induced in insects in response to ingestion of host allelochemicals (Yu, 1983). High activities of these enzymes have been found in the midguts of insects, particularly polyphagous species which would be expected to encounter many types of allelochemicals (Krieger *et al.*, 1971). Other enzymes known to be important detoxification enzymes include the epoxide hydrolases (Mullin and Wilkinson, 1980), carboxylesterases (Kapin and Ahmad, 1980), and the glutathione transferases (Yu, 1982).

3.2 Adaptations to Phototoxins

It has become apparent that many insects must face the double challenge of dealing with allelochemicals as potentially lethal poisons as well as the added possibility that some of these chemicals are also phototoxic and capable of causing severe oxidative stress. Even though most insects are very sensitive to

phototoxins, there appears to be a guild of insect herbivores which are tolerant of or even specialize on phototoxic plants. These insects have likely developed mechanisms to deal with this light-dependent aspect of toxicity which have allowed them to exploit these plants as food sources.

3.2.1 Physical and Behavioural Adaptations to Phototoxins

In dealing with a phototoxin the first line of defense used by some insects might be avoidance of light in general or specific sensitizing wavelengths. Many insects may be pre-adapted to feeding on phototoxin-containing plants by means of physical factors such as opaque cuticles or highly reflective body surfaces. This may be the case with adult leaf beetles of the genus *Chrysolina* which are characteristically metallic blue-black and feed with impunity on phototoxic plants of the genus *Hypericum* (Fields *et al.*,1991).

Numerous tortricid and oecophorid caterpillars, which feed on a range of phototoxin-containing plants, are known to feed in a concealed manner (e.g. in leaf rolls, leaf mines, or webbed flowerheads) and this may serve as a behavioural resistance mechanism (Berenbaum, 1983,1987; Sandberg and Berenbaum, 1988). Fields *et al.*,(1989) have shown that late instar larvae of *C. quadragemina*, a specialist feeder on *Hypericum* sp., avoid potential phototoxicity by feeding very early or late in the day when light intensity is low. In contrast, larvae of the geometrid *Anaitis plagiata*, another specialist on *Hypericum*, feed throughout the day. If larvae of *C.*

quadragemina are forced to feed in the light they suffer an intensity-dependent mortality whereas larvae of *A. plagiata* do not. This suggests that *A. plagiata* may have other defensive mechanisms to deal with phototoxins.

3.2.2 Biochemical Adaptations to Phototoxins

3.2.2.1 Metabolic Detoxification

One of the most effective biochemical adaptations insects may have against the ingestion of phototoxins is metabolic detoxification. As with any xenobiotic, high elimination/ metabolism rates may reduce the possibility of adverse effects and, in the case of phototoxins, rapid elimination of the compounds or degradation to non-phototoxic metabolites, would likely be an effective defense. In mosquito larvae, the toxicity, localization, and elimination rates of [^{3}H]-αT have been examined (Hasspieler *et al.*, 1988) and the toxicity of the compound was directly correlated to rates of elimination.

In only a few cases has the metabolism of phototoxic compounds been examined in herbivorous insects. Both the metabolism of [^{3}H]α-T and [^{14}C]-xanthotoxin have been examined in lepidopteran larvae, particularly with respect to the toxicokinetics of these compounds (Ivie *et al.*, 1983; Iyengar *et al.*, 1987) and their comparative metabolism in phototoxin-tolerant and phototoxin-sensitive species (Bull *et al.*, 1984; Iyengar *et al.*, 1990). Iyengar *et al* (1987) have shown that elimination of

^{3}H after topical application to the phototoxin-tolerant *O. nubilalis* is much more rapid ($t_{1/2}$=8.5h) as compared to *H. virescens* ($t_{1/2}$=22h) or the phototoxin sensitive *M. sexta* ($t_{1/2}$=48h). This suggests that rapid clearance of the toxin is one method by which tolerant insects can deal with this type of allelochemical. Further studies with midgut microsomal preparations (Iyengar *et al.*, 1990) have confirmed that PSMOs are involved in its elimination and that the compound is degraded to at least four non-phototoxic metabolites. Similarly, elimination of [^{14}C]-xanthotoxin after oral treatment is much more rapid in *P. polyxenes* (a specialist feeder on psoralen containing plants) than in *S. frugiperda*, a psoralen-sensitive species. Within 1.5 h, 50% of the administered ^{14}C is recovered in the frass of *P. polyxenes*, whereas only about 1% is eliminated by *S. frugiperda* (Ivie *et al.*, 1983). As well, it appears that metabolism involves extensive modifications of the parent nucleus (cleavage of the furan ring) producing metabolites that are non-phototoxic.

The parsnip webworm *D. pastinacella* also appears to be enzymatically adapted to feeding on xanthotoxin-containing plants. Larvae metabolize about 95% of ingested labelled phototoxin (Nitao, 1989) primarily through midgut PSMO activity which has a high specificity for and can be induced by xanthotoxin (Berenbaum and Lee, 1989). In contrast, PSMO activity towards xanthotoxin is very low in the cabbage looper *T. ni*, a generalist feeder whose host range includes a number of furanocoumarin-containing plants (Lee and Berenbaum, 1989). Induction of PSMO metabolism has also been observed in the black swallowtail *P. polyxenes* (Cohen *et al*, 1989). When xanthotoxin was added to diets, a dose-dependent

induction (up to 4.7 fold) of P-450 activity in midgut microsomes was recorded. Total P-450 content did not change significantly however, suggesting that multiple forms of P-450 may exist in the black swallowtail. In contrast, *P. zelcaon* feeding on parsley (which contains low levels of furanocoumarins) had no detectable xanthotoxin-metabolizing activity (Ashwood-Smith *et al*, 1984).

3.2.2.2 Antioxidant Enzymes

A number of enzymatic defense mechanisms are known to exist which may offer some degree of protection to insects feeding on plants containing phototoxins; particularly those phototoxins which result in the production of toxic oxygen species (see Figure 3).

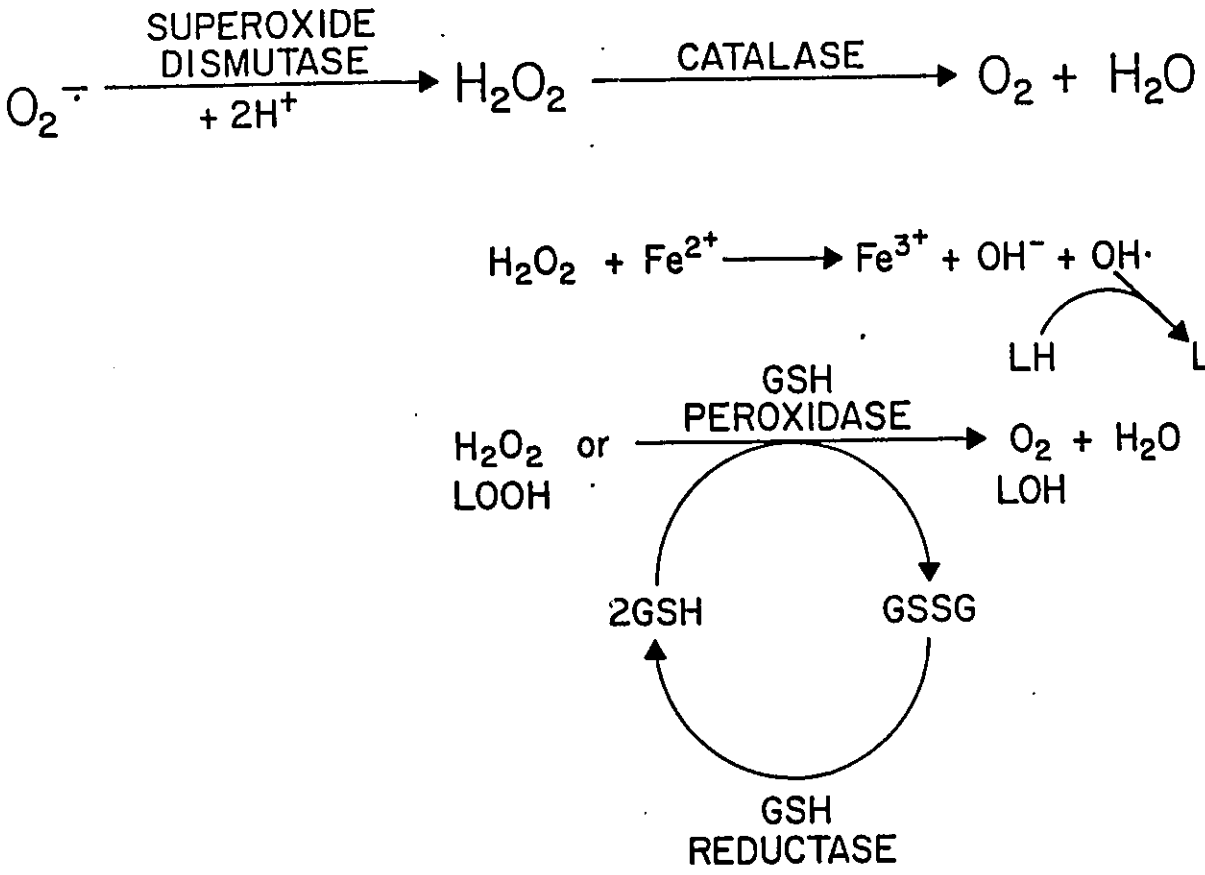
Superoxide dismutase (SOD) (E.C.1.15.1.1) (McCord and Fridovich, 1969) catalyzes the dismutation reaction of superoxide anions (1):

$$O_2^- + O_2^- + 2H^+ \text{---} > H_2O_2 + O_2 \qquad (1)$$

This reaction results in the removal of superoxide radicals but also results in the formation of hydrogen peroxide. Hydrogen peroxide can penetrate cell membranes even more readily than superoxide anions and once inside the cell may react with iron or copper ions to form hydroxyl radicals (Halliwell and Gutteridge, 1986). This may be the origin of many toxic effects of these radicals.

Catalases (CAT) (E.C.1.11.1.6) catalyze the following reaction (2):

Figure 3: Enzymatic defenses against activated oxygen species.


$O_2^{\cdot-}$
SUPEROXIDE DISMUTASE
+ $2H^+$
H_2O_2
CATALASE
O_2 + H_2O
H_2O_2 + Fe^{2+} → Fe^{3+} + OH^- + $OH\cdot$
LH
LOOH
GSH PEROXIDASE
H_2O_2 or LOOH
O_2 + H_2O or LOH
2GSH
GSSG
GSH REDUCTASE

$$2H_2O_2 \text{ ---> } 2H_2O + O_2 \qquad (2)$$

and hence, to some degree, control the accumulation of hydrogen peroxide and hydroxyl radicals.

SOD and CAT have been studied in insects (e.g.Allen *et al.*, 1983; Bird *et al.*, 1986; Ahmad *et al.*, 1987, 1988a, 1988b; Pritsos *et al.*, 1988a, 1988b) although it is not yet clear whether they have a protective role solely in terms of the normal metabolic processes which result in free radical formation, or whether they confer any special advantage or defense to adapted insects feeding on phototoxins. Allen *et al.*(1983) have examined SOD and CAT activities in the housefly and found, with the use of SOD and CAT inhibitors, that longevity is not affected by altering the levels of these enzymes. Neto *et al.*, (1986) have shown that SOD and CAT activities are 5 to 15 fold higher in luminescent as opposed to non-luminescent elaterid larvae. This, they suggested, may be related to protection against deleterious oxygen species arising from the storage of oxygen which is necessary for bioluminescent reactions. Pardini *et al.*(1988) have examined antioxidant enzymes in a group of insects composed of three Lepidoptera which normally encounter phototoxins in low, moderate, and high levels as follows: *Trichoplusia ni* << *Spodoptera eridania* << *Papilio polyxenes*. All three species in this model system have been found to contain high levels of antioxidant enzymes, though no correlation appears to exist between enzyme levels and level of exposure to pro-oxidant phototoxins. Pritsos *et al.* (1988a) have however, found that in response to the allelochemical quercetin, SOD activity in whole body homogenates of *P. polyxenes* nearly doubles.

In mammals, a Se-dependent glutathione peroxidase (E.C.1.11.1.9) (GPOX) has been well characterized (Flohe, 1982) and catalyzes the removal of H_2O_2 and a wide range of lipid and organic hydroperoxides (ROOH) according to the following processes:

$H_2O_2 + 2GSH \text{ ---> } GSSG + H_2O$ and (3)

$2GSH + ROOH \text{ ---> } GSSG + H_2O + ROH.$ (4)

In insects, Se-dependent GPOX activity has not been observed (Pardini *et al.*, 1988) and attempts to demonstrate GPOX activity towards H2O2 have not been successful (see Pardini *et al*, 1988). Peroxidase activity has ,however recently been shown to exist in the cabbage looper *T. ni* against the organic hydroperoxide cumene hydroperoxide (Ahmad and Pardini, 1988).This activity was ascribed not to a Se-dependent GPOX but to a non Se-dependent glutathione transferase. In the absence of GPOX, the peroxidase activity of glutathione transferase may be important as an antioxidant defense.

In insects, GSSG is recycled to GSH in the presence of NADPH by a glutathione reductase (G.R.) (E.C.1.6.4.2). In this way a high GSH:GSSG ratio can be maintained which is important since GSH is itself a scavenger of singlet oxygen and free radicals (Halliwell and Gutteridge, 1986). Significant levels of GR have been measured in a number of herbivorous insects (e.g. Pritsos *et al*, 1988a).

3.2.2.3 Antioxidants

A toxic oxygen species such as singlet oxygen can interact with other molecules

in two ways: it can either combine chemically with them, or else it can transfer its excitation energy to them and return to its ground state. This latter process is known as chemical quenching. Larson (1986) has suggested that many insects produce or actively sequester chemicals which can act to quench excited state molecules (see Table 1). The presence of these chemical quenchers may be particularly important as an antioxidant defense against singlet oxygen since no enzymatic mechanisms to remove this toxic species are yet known to exist. As well, it appears that most phototoxins examined to date produce singlet oxygen. Some of the more common antioxidants which may be important in insects include ascorbic acid (vitamin C), uric acid, reduced glutathione (GSH), α-tocopherol (vitamin E), and beta-carotene (see Figure 4).

Ascorbic acid is a hydrophilic antioxidant which may be a particularly important extracellular defense against free radicals and singlet oxygen since the antioxidant enzymes are almost exclusively intracellular (Levine and Kidd, 1986). It reacts rapidly with O_2 and HO_2 and even more rapidly with $OH^{\cdot}$ to give relatively innocuous

semidehydroascorbate. It also scavenges singlet oxygen and is a necessary substrate in the removal of lipid peroxides by ascorbate peroxidases (Halliwell and Gutteridge, 1986). Ascorbic acid is also capable of reacting with and thereby reducing the tocopherol quinone radical (see Figure 5), and hence regenerates reduced tocopherol as a membrane interceptor of activated oxygen species. In insects, no protective role has been ascribed to ascorbic acid although it appears to be ubiquitous in insect

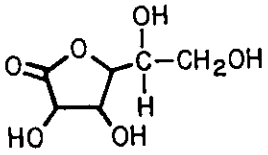
Table 1: Potential defensive antioxidants in insects*.

1. Quenchers of Excited-State Molecules
   - Beta-carotene
   - Amines
   - Furans
   - Some anions (I^-, N^-)

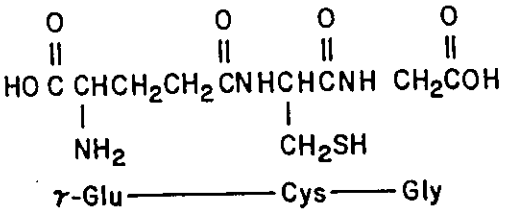
2. Quenchers of Reactive Oxygen Species
   - Amines
   - Carotenoids
   - Furans
   - Some O and S Derivatives
   - Some Plant Alkaloids i.e. Nicotine
   - Vitamin E
   - Vitamin C
   - Uric Acid
   - Glutathione

*References: Larson, 1986; Levine and Kidd, 1986; Larson and Berenbaum, 1989.

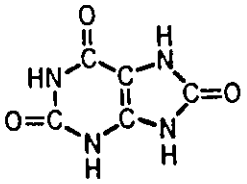
Figure 4: Chemical structures of some common antioxidants which may be important cellular defenses against oxidative stress in insects.



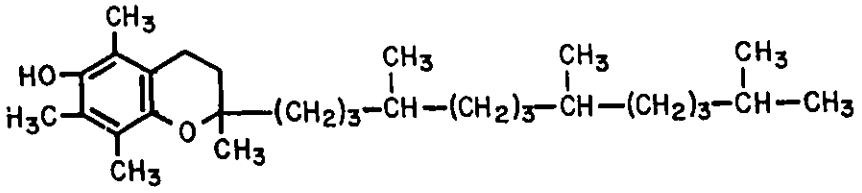
Ascorbic acid



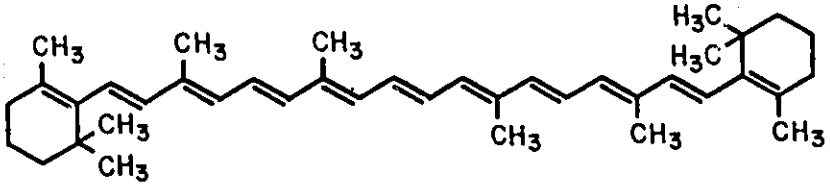
Reduced glutathione



Uric acid



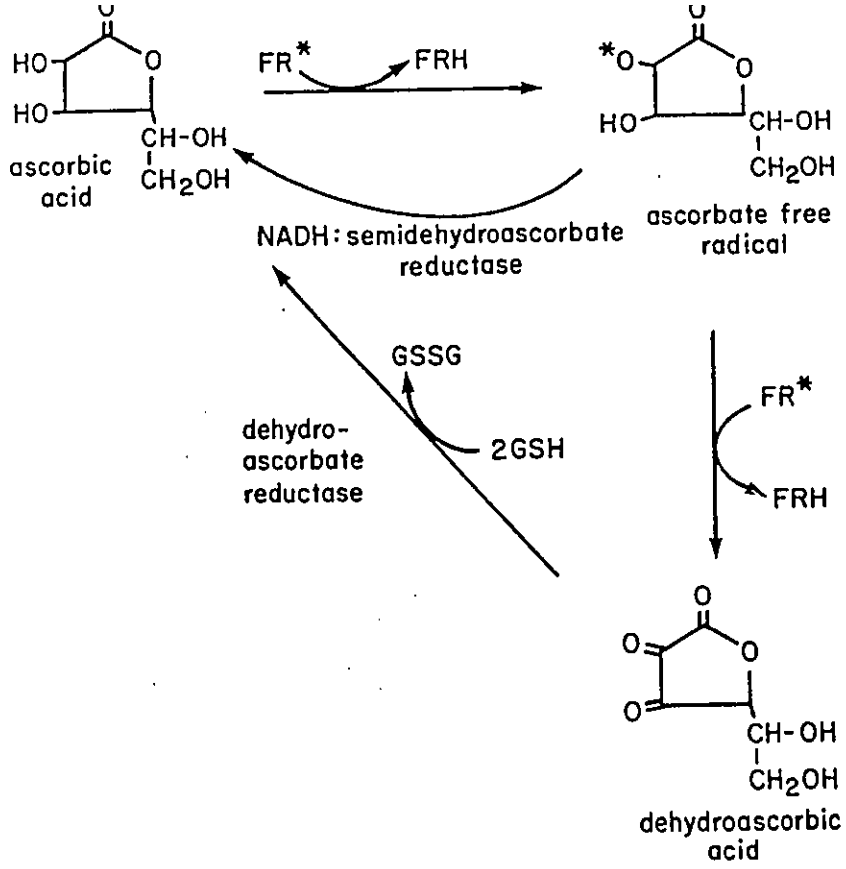
α-Tocopherol

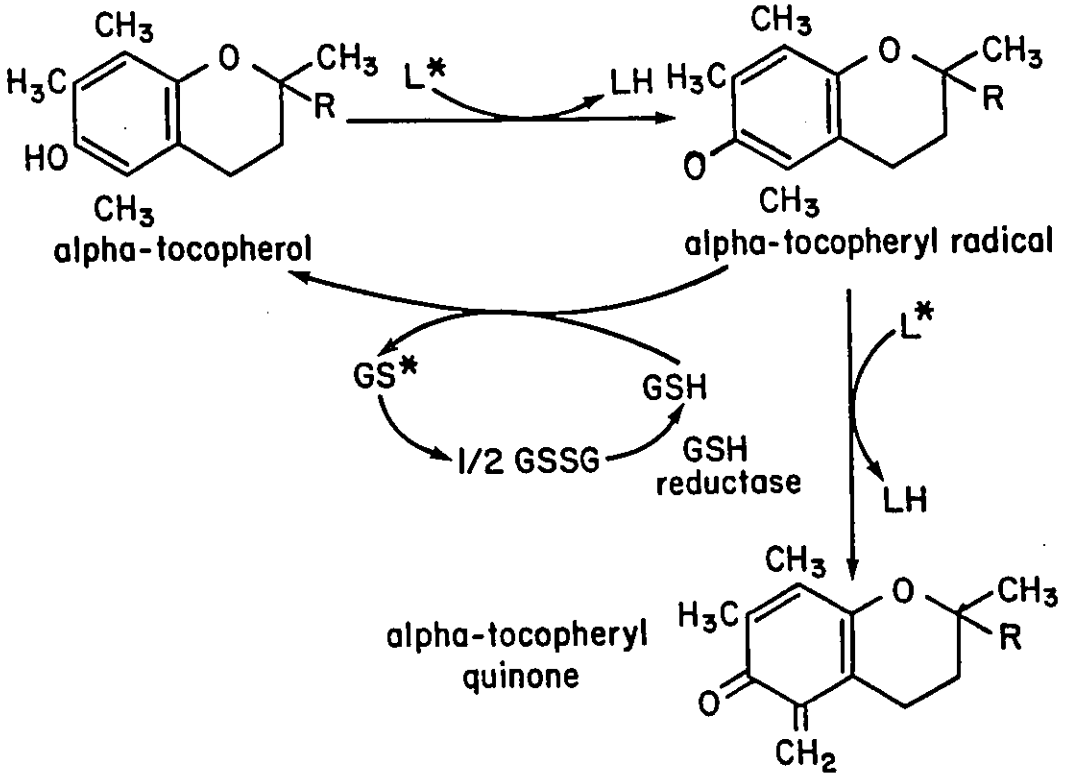


Provitamin A (β-Carotene)

Figure 5: Upper: Quenching of free radicals by ascorbate forming ascorbate free radicals and dehydroascorbate and the regeneration of ascorbate by semidehydroascorbate and dehydroascorbate reductases.

Lower: Quenching of free radicals by alpha-tocopherol (vitamin E) to form alpha-tocopherol radicals and alpha-tocopherol quinone and the GSH-dependent regeneration of vitamin E.


ascorbic acid
FR*
FRH
ascorbate free radical
NADH: semidehydroascorbate reductase
GSSG
2GSH
dehydro-ascorbate reductase
FR*
FRH
dehydroascorbic acid


alpha-tocopherol
L*
LH
alpha-tocopheryl radical
GS*
GSH
1/2 GSSG
GSH reductase
L*
LH
alpha-tocopheryl quinone

tissues and fluids (Kramer *et al.*, 1981).

Uric acid is another water soluble antioxidant which may be an important defense since it is a powerful scavenger of singlet oxygen and inhibits lipid peroxidation (Ames *et al.*, 1981; Hochstein *et al.*, 1984). Because the extracellular fluids contain low levels of SOD and CAT activity and yet have high uric acid levels, it may, like ascorbate, provide an antioxidant defense. In insects, uric acid is often found in high concentrations in body fluids and tissues, particularly fat body where it may be used as a form of storage excretion (Wigglesworth, 1974).

Carotenoid pigments are the most effective quenchers of singlet oxygen known and can eliminate other oxygen-centered radicals as well (Foote and Denny, 1968). In insects, carotenoids have been examined from a wide variety of tissues (see, for example, Feltwell, 1978; Goodwin, 1980). They are lipophilic pigments, as demonstrated by their preferred accumulation in oil droplets of fat body and eggs (Kayser, 1982). Some evidence has accumulated which suggests that carotenoids may have a photoprotective role in insects. Poff (1976) for example, observed that cryptic *Leptinotarsa* species had lower concentrations of carotenoids and a relatively low carotene to xanthophyll ratio than in their host plants and conversely that aposematic species had higher concentrations of carotenoids and a higher ratio of carotenoids to xanthophylls. Poff (1976) suggested this high level of carotenoids might have a protective role against incoming radiation.

Reduced glutathione (GSH) is an antioxidant tripeptide composed of glutamate, cysteine, and glycine. As well as being an essential co-substrate for the GSH-

peroxidase reaction, it is also a strong nucleophile and thus can react with O_2-radicals, hydroxy radicals, and $^1O_2$ to neutralize these species (Levine and Kidd, 1986). GSH is also a versatile conjugating agent and can protect -SH containing enzymes from inactivation. GSH is present in high concentrations in the mammalian liver where it can conjugate with a variety of metabolites to increase water solubility and hence subsequent excretion. In insects, a similar function is accorded to GSH transferase, an important detoxification enzyme. Thus GSH is important in both the detoxification of xenobiotics and preventing oxidative stress.

The tocopherols, like the the carotenoids, are lipid soluble compounds and are excellent quenchers of singlet oxygen and free radicals (Foote *et al.*, 1978). They are believed to be the major lipid soluble antioxidants in blood plasma where they provide antioxidant protection for circulating lipids and the hydrophobic segments of blood proteins (Levine and Kidd, 1986). Thus, they can dramatically inhibit lipid peroxidation, primarily by interrupting free radical-initiated chain reactions. It has been hypothesized (Yang and Desai, 1978) that Vitamin E acts from within membranes to prevent lipid peroxidation by directly neutralizing free radicals at their site of formation. GSH peroxidases act intracellularly to decompose lipid peroxides from within.

4.0 Coevolution

Janzen (1980) has defined coevolution as reciprocal evolutionary changes between interacting populations. In the original hypotheses of Ehrlich and Raven

(1964) the chemical defenses of plants against insects (including phototoxins) evolved as a series of chance mutations and recombinations which produced more complex allelochemicals derived from simpler phytochemicals. The success of insects in overcoming some of these plant defenses would then leave these plants open to attack by herbivores; natural selection would then favor the elaboration of new allelochemicals which would in turn select for more resistant insects and more complex defense strategies. The result of these interactions has been described as coevolutionary "arms race" (Gilbert and Raven, 1975, Berenbaum, 1978).

Very good evidence for such an "arms race" has apparently occurred between insects and various furanocoumarin-containing plants. The phototoxic linear furanocoumarins are found in only eight plant families, mainly in the Rutaceae and Apiaceae, and these are more toxic to generalist insects than their precursor umbelliferone which occurs in over thirty families (Berenbaum, 1978). Linear furanocoumarins can be tolerated by a limited insect fauna which includes the specialist *P. polyxenes* (Ivie *et al*, 1983)(see Section 2.2.4). However *P. polyxenes* cannot tolerate angular furanocoumarins such as angelicin which occur in only a few families (Berenbaum and Feeny, 1983). Thus the evolutionary sequence from umbelliferone to linear furanocoumarins to angular furanocoumarins may represent successive escalations in the coevolution of these plants and the herbivorous insects they interact with. This is supported by furthur work (Berenbaum, 1981) in which the increasing specialization of the insect fauna on these plants is observed to be closely correlated with plant chemistry. The strong selective pressures of phototoxic plants

appears to have resulted in some complex defensive strategies in insects.

5.0 Rationale

5.1 Hypothesis

Many insect herbivores have specific biochemical and physiological mechanisms (e.g. inducible PSMOs) which allow them to exploit plants containing potentially toxic chemicals. Many of these chemicals are known to be photo-activated and their toxicity due to the generation of free radicals and/or singlet oxygen. In addition to general detoxification enzymes, I propose that some insects make use of both endogenous antioxidants (e.g. ascorbate) and antioxidant enzymes (e.g. SOD, CAT) to protect against phototoxicity. These antioxidants and antioxidant enzymes are known to be present in insects and, as in mammals likely have an important role in controlling the levels of potentially toxic free radicals and peroxides which are produced during normal metabolism and respiration. In insects adapted to feeding on phototoxins, the antioxidant defenses may be particularly important in reducing the toxicity of these chemicals and may in some cases account for their ability to use certain plants as a food source. Species of insects which are tolerant of phototoxins may have well developed antioxidant defenses.

5.2 Objectives

1) To determine the role of constitutive and inducible antioxidant

enzymes as biochemical defenses against phototoxin ingestion in phototoxin-tolerant and phototoxin-sensitive insects.

2) To determine if a) the sensitivity to phototoxins in some insects can be explained by differences in tissue antioxidant levels and if b) levels of dietary antioxidants can alter this sensitivity to phototoxins.

3) To investigate furthur the mode of action of the phototoxin α-T. This aspect will provide insight into the effectiveness of antioxidants and antioxidant enzymes in protecting against oxidative stress and lipid peroxidation at the biochemical level.

4) To examine the effect of manipulation of antioxidant enzyme activity inhibitors and a GSH depleting agent on the short and long-term toxicity of α-T.

CHAPTER 2: ANTIOXIDANT ENZYMES AS BIOCHEMICAL DEFENSES AGAINST PHOTOTOXIN-INDUCED OXIDATIVE STRESS IN THREE SPECIES OF HERBIVOROUS LEPIDOPTERA

2.1 INTRODUCTION

The recent literature in chemical ecology provides many examples of defensive plant substances with unusual modes of action against phytophagous insects. Among these are the so-called phototoxins, phytochemicals that are activated by the absorption of light. They can mediate a variety of toxic processes that are initiated photochemically in susceptible species but are nonetheless tolerated by a guild of insects successfully exploiting phototoxic plants.

Our recent work has concentrated on one group of phototoxins, the plant-derived photo-oxidants which include biosynthetic classes such as the thiophenes, extended quinones, and isoquinoline alkaloids. After excitation, these photo-oxidants can generate activated species of oxygen such as singlet oxygen ($^1O_2$) by energy transfer or superoxide (O_2-) or hydroxyl (OH) radicals by electron transfer (Spikes, 1977). All of these activated species are highly reactive and highly toxic (Mead, 1976; Halliwell and Gutteridge, 1985). After ingestion, photo-oxidants can produce symptoms such as black lesions of the cuticle leading to high rates of mortality, or at subacute doses cause a reduction in growth and efficiency of conversion of ingested food (Downum, 1986; Downum *.et al*, 1986; Iyengar

et al., 1987).

Insect species that are relatively tolerant of phototoxins in their diet or which exploit plants containing phototoxic compounds have also been studied. Numerous tortricid and oecophorid caterpillars, which feed on a range of phototoxin-containing plants, are known to feed in a concealed manner (e.g. in leaf rolls) and hence may be able to avoid potential phototoxicity (Sandberg and Berenbaum, 1989). Leaf beetles of the genus *Chrysolina* (Coleoptera: Chrysomelidae) successfully exploit *Hypericum perforatum* (St. John's Wort) which contains high levels of the extended quinone hypericin which is highly phototoxic (Giese, 1980). *Chrysolina* spp. larvae have been shown to be highly susceptible to photo-oxidative damage and suffer an intensity-dependent mortality when exposed to light while feeding on this plant (Fields *et al.*, 1990). To avoid the phototoxicity of hypericin larvae feed just before dawn and then retreat to hide in soil during the day. Adult *Chrysolina*, in contrast, are able to feed in full sunlight, possibly as a result of their irridescent elytra which transmit almost no light (Fields *et al.*, 1991). The European corn-borer *Ostrinia nubilalis* (Lepidoptera: Pyralidae), whose host range includes many phototoxin-bearing plants of the Asteraceae, has been shown to tolerate a 40-fold higher concentration of the phototoxic thiophene alpha-terthienyl in meridic diets than the susceptible tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) (Iyengar *et al.*, 1990). Tracer studies with labelled α-T suggest that tolerance to the phototoxin is due to a much higher rate of clearance of the phototoxin in *O. nubilalis*. Excretion is associated with metabolism of the compound to several non-phototoxic metabolites by the

polysubstrate monooxygenases of the gut. *O. nubilalis* is also very tolerant of dietary hypericin (Aucoin, unpublished observations) whereas *M. sexta* is very sensitive (Samuels and Knox, 1989). Thus behavioral, physical, and metabolic factors may account for the successful adaptation of some species of insects to phototoxic plants. Recently, biochemical adaptations of insects to photo-oxidants have also been examined. The antioxidant enzymes SOD (E.C.1.15.1.1), CAT (E.C.11.1.6), GR (E.C.1.6.4.2), and GPOX (E.C.1.11.1.9) are well known, in mammalian systems, to provide an important line of defense against deleterious species of oxygen (Halliwell and Gutteridge, 1985). Fridovich (1983) has proposed that these enzymes together provide an essential cellular defense against free radicals. SOD is known to catalyze the dismutation of superoxide anions (McCord and Fridovich, 1969). However one of the byproducts of this reaction is the potentially toxic H_2O_2 molecule. This in turn is acted upon by either CAT or GPOX; GPOX may also act on a wide variety of lipid and organic hydroperoxides formed through interactions of membranes with hydroxyl radicals (Flohe, 1982). The enzyme GR is an NADPH-dependent enzyme which catalyzes the reduction of GSSG and hence maintains high levels of GSH for the GSH-dependent GPOX. These same enzymes have recently been examined in a number of herbivorous insects (Ahmad *et al.*, 1988a; 1988b; Lee and Berenbaum, 1989) and may provide some degree of protection to insects feeding on plants containing phototoxins, particularly those phototoxins which generate toxic oxyradicals.

The present investigation was initiated to determine whether there was any evidence of biochemical antioxidant adaptation in herbivorous insects exposed to phototoxic plants.

In particular, we examined *Anaitis plagiata* (Lepidoptera: Geometridae) which as larvae are specialist feeders on phototoxic *H. perforatum*. Larvae of this species feed in full sunlight and yet have cuticles which transmit a significant amount of light (Fields *et al.*,1991). Levels of the antioxidant enzymes SOD, CAT, GR, and GPOX in this insect were compared to the generalist *O. nubilalis*, and to *M. sexta* which is highly sensitive to phototoxins (Samuels and Knox, 1989; Champagne *et al.*, 1986). We also examined the short and long term effects of hypericin ingestion on antioxidant enzyme activity in *A. plagiata* in order to determine whether antioxidant enzymes may be inducible defenses in specialists on phototoxic plants.

2.2 METHODS AND MATERIALS

2.2.1 Insects

Laboratory colonies of *O. nubilalis* and *M. sexta* were raised on artificial diets as described by Guthrie (1971) and Bell and Joachim (1976). A colony of the geometrid *A. plagiata*, originally from British Columbia, was maintained at a 20^0C with a 16:8, L:D photoperiod. The lighting system consisted of a bank of 10 X 60 W vitalites (Durotest, Toronto, Ontario, Canada) suspended 60 cm above the cages. Cages used (60 X 40 X 40 cm) were constructed of wood covered with a cloth mesh to provide good ventilation. Larvae were fed potted or fresh cut *H. perforatum*. Last instar larvae were allowed to pupate in trays of vermiculite and then newly emerging adults transferred to oviposition

cages containing potted *H. perforatum* and cotton-plugged vials containing a 15% sucrose solution. Under these conditions, *A. plagiata* was observed to have five larval instars spanning about 21 days. The fifth instar lasted 7-8 days.

2.2.2 Chemicals

All purified enzymes, substrates, and co-factors used in the enzyme assays were from Sigma Chemical Co., St. Louis, Missouri. Hypericin (95% pure) was obtained from ICN Pharmaceuticals, Plainsview, N.Y.

2.2.3 Sample Preparation and Enzyme Assays

For *A. plagiata* and *O. nubilalis*, 8 last instar larvae or 30 to 40 third instar larvae were homogenized in 4 ml of 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA. For *M. sexta*, 5 last instar larvae were homogenized for 30 s in 200 ml of cold buffer using a motor-driven homogenizer. An aliquot of 4 ml was taken for centrifugation. After centrifugation in the cold for 20 min at 14000g, 3-ml aliquots of the supernatants were desalted on Sephadex G-25 columns (Pharmacia, 20 X 1.5cm i.d.) to remove low molecular weight and potentially interfering compounds such as glutathione. The final eluant (6 ml) was kept on ice and examined for antioxidant enzyme activity within 5 h. Initially, we examined whole body homogenates of insects in which the gut contents had

been left intact. Preliminary experiments (results not shown) indicated that the presence of gut contents only contributed about 5-6 % of total sample protein thus ruling out the possibility of gut contents significantly diluting enzyme activity, which was expressed on a per mg protein basis. As well, the presence of gut contents had no effect on total sample enzyme activity with the exception of CAT activity in *A. plagiata*. CAT activity in this species was found to be inhibited 10-15% by the presence of plant material (*H. perforatum*). No correction factor was applied, however, since the variation between samples was also in this range. Protein determinations were carried out according to the method of Bramhall *et al.*(1969) using bovine serum albumin as standard.

SOD assays were carried out according to the procedure of Flohe and Otting (1984) which uses a ferricytochrome c reduction assay. Briefly, the reduction rate of cytochrome c by superoxide radicals is monitored at 550 nm using a xanthine-xanthine oxidase system as the source of superoxide anions. The inhibition of the rate of reduction of cytochrome c by superoxide anions can thus be measured by using either pure SOD or insect samples. In this assay, 1 unit of SOD activity is defined as that amount which inhibits by 50% the rate of reduction of cytochrome c at 25^0C (pH 7.8) and is expressed in units/mg protein. A typical reaction mixture in a 1- ml cuvette contained 950 ul potassium phosphate buffer (50 mM, pH 7.8) with 2 uM cytochrome c, 5 uM xanthine, and 0.1 mM EDTA; 20 uL xanthine oxidase (0.2 U/ml) in 0.1 mM EDTA, and 20 ul of buffer, insect sample, or pure SOD.

CAT assays were carried out according to the method of Aebi (1984) which monitors

the breakdown of H_2O_2 at 240 nm. One unit of CAT activity is defined as that which decomposes 1 umol H_2O_2/ mg protein/ min at pH 7.0 and 25^0C. A typical reaction mixture contained 1 ml of H_2O_2 solution (50 mM) and 1 to 5 ul of insect sample.

GR activity was determined according to the method of Racker (1955) which is based on the reduction of GSSG by GR to GSH with concomitant oxidation of NADPH to NADP. One unit of GR activity is expressed as the change in absorbance due to the disappearance of NADPH at 340 nm of 0.001A/ mg protein/ min at 25^0C. A typical reaction mixture contained 600 ul of potassium phosphate buffer (0.1 M, pH 7.6), 100 ul NADPH (1 mM), 100 ul BSA (1% (w/v) solution in buffer), 100 ul GSSG (2% solution), and 100 ul of sample. Endogenous oxidation of NADPH was measured in the absence of GSSG and used as a correction factor.

GPOX was measured using the method of Strauss *et al* (1980). The reaction mixture contained 900 ul potassium phosphate buffer (50 mM, pH 7.8) containing 1 mM EDTA, 5 ul of a 1 M solution of NaN_3, 30 ul of 150 mM GSH, 30 ul of 8 mM NADPH, and 2 ul of GR. After addition of 30 ul 2 mM H_2O_2, the reaction rate was monitored at 340 nm until a linear rate was established (10 min.). The insect sample (50 ul) was then added and the difference between the initial and final rates recorded. This corresponded to the GPOX activity which is defined as the oxidation of 1 nmol NADPH/ min/ 100 ug protein.

All enzyme assays were carried out at 25^0C using either a Varian DMS 300 or a Varian CARY 2200 spectrophotometer.

2.2.4 Induction of Antioxidant Enzymes

To examine the short-term effects of hypericin ingestion on the antioxidant enzymes in *A. plagiata*, mid-last instar larvae were starved for 16 h and then fed hypericin treated leaf discs of *H. calycinum* leaves for 1 h. Starvation insured that leaf discs were fully consumed within 1 h. Groups of 10 larvae were placed individually in plastic cups containing a single leaf disc (6 mm dia., 4.5±0.4 mg fresh weight) treated with either 2 ul of 90% acetone (controls) or 0.01, 0.1, or 1.0 ug of hypericin in 2 ul 90% acetone. Only leaf discs of *H. calycinum*, rather than *H. perforatum*, were used because this species does not contain hypericin (Mathis and Ourisson, 1963). Since hypericin is strongly bioactivated under visible light (550-600 nm) (Giese, 1980), larvae were then placed in an incubator for 1 h (25°C) equipped with 4 x 60 W vitalite fluorescent lights. Larvae which had not consumed the entire leaf disc (usually 2-3 per group) were discarded and the remaining larvae used in the enzyme assays.

To examine the longer-term influence of hypericin ingestion on antioxidant enzyme activity, a separate experiment was designed. This was felt to be necessary because treated leaf discs typically dried out after 1-2 h and were then not eaten by larvae. Groups of 20 last instar larvae were starved for 16 h as before, and then placed in cages containing either the phototoxic *H. perforatum* or non-phototoxic *H. calycinum*, and allowed to feed *ad libitum* for up to 72 h. Larvae were provided with fresh plant material daily and cages

were placed in an incubator (25°C, 16:8 L:D photoperiod). After 24, 48, or 72 h, groups of 10 larvae were removed and assayed for antioxidant enzyme activity after removal of digestive tract contents by dissection and aspiration using a pasteur pipette.

2.3 RESULTS

2.3.1 Antioxidant Enzyme Activity

The SOD activity of the three species is compared in Table 2. *A. plagiata* was found to have the highest specific activity, particularly during the mid-third and mid-fifth instars. Mid-third instars had nearly four-fold higher activity than the mid-fifth instars. In late last instar larvae, all species had relatively low levels of SOD activity. In the case of *A. plagiata*, this low level represented nearly a 20-fold decrease from the levels observed in mid-third instars (23.9 units versus 1.3 units).

When CAT activity (Table 3) is compared, *A. plagiata* also had the highest activity of the three species examined. During the third instar it had three-fold greater activity than *M. sexta* and more than twenty-fold greater activity than *O. nubilalis* on a per milligram protein basis. In both *A. plagiata* and *M. sexta*, CAT declined from third to fifth instar however, unlike SOD activity, did not decline furthur during the latter part of the fifth instar. *O. nubilalis* exhibited the lowest overall CAT activity.

There were only slight differences in GR activity (Table 4) between the three species with relatively low overall activity. *O. nubilalis* again was observed to have the

Table 2: SOD activity of Third and Fifth Instar Larvae of *A. plagiata*, *O. nubilalis*, and *M. sexta**.

	Larval Instar**		
Species	M3	M5	L5
A. plagiata	23.9 ± 0.3a	5.5 ± 0.3a	1.3 ± 0.3a
O. nubilalis	1.5 ± 0.3c	1.4 ± 0.1b	1.9 ± 0.2a
M. sexta	12.4 ± 1.4b	1.5 ± 0.2b	1.6 ± 0.2a

** Values represent the means ± S.D. of at least three samples and are expressed as units per mg protein. Values in each vertical column followed by the same letter are not statistically different (Tukeys test).*

*** M, mid instar; L, late instar.*

Table 3: CAT activity of Third and Fifth Instar Larvae of *A. plagiata*, *O. nubilalis*, and *M. sexta*[*].

	Larval Instar[**]		
Species	M3	M5	L5
A. plagiata	592.0 ± 121.0a	171.1 ± 14.0a	189.9 ± 13.9a
O. nubilalis	23.1 ± 2.3c	33.6 ± 3.7b	43.7 ± 2.8c
M. sexta	182.0 ± 8.4b	153.9 ± 16.7a	163.0 ± 11.5b

** Values represent the means ± S.D of at least three samples and are expressed as units/ mg protein. Values in each vertical column followed by the same letter are not statistically different (Tukeys test).*

*** M, mid instar; L, late instar.*

Table 4: GR activity of Third and Fifth Instar Larvae of *A. plagiata*, *O. nubilalis*, and *M. sexta*[*].

	Larval Instar[**]		
Species	M3	M5	L5
A. plagiata	3.0 ± 0.2a	4.3 ± 1.7a	2.2 ± 0.3a
O. nubilalis	1.6 ± 0.4a	2.1 ± 0.5a	0.9 ± 0.2b
M. sexta	2.8 ± 0.9a	1.6 ± 0.3a	2.5 ± 2.5a

** Values represent the means ± S.D of at least three samples and are expressed as units/ mg protein. Values in each vertical column followed by the same letter are not statistically different (Tukeys test).*
*** M, mid instar; L, late instar.*

lowest overall GR activity, particularly during the third instar.

GPOX activity using H_2O_2 as substrate was observed at extremely low levels which were not substantially above the level of expected instrument error. As well, this activity could not be reduced or eliminated using heat-treated (boiled for 1 h) samples.

2.3.2 Effect of Hypericin on Antioxidant Enzyme Activity

To examine the short-term effects of hypericin ingestion on the antioxidant enzyme defenses of the *Hypericum* specialist *A. plagiata*, larvae were fed leaf discs treated with hypericin and then assayed for antioxidant enzyme activity 1 h after ingestion. Starved controls were found to have antioxidant enzyme levels that were considerably depressed relative to *H. perforatum*-fed larvae (Table 5). GR activity, for example, was nearly 9-fold higher in fed larvae as compared to starved larvae. Larvae fed leaf discs containing 0.01 ug hypericin had SOD and CAT activity that was significantly higher than fed controls. At a higher dose of hypericin, 0.1 ug/disc, SOD, CAT, and GR all declined significantly. Further inhibition of enzyme activity was observed at the highest dose of hypericin used, 1.0 ug/disc.

To examine the longer-term effects of hypericin ingestion on the antioxidant enzyme activity of *A. plagiata*, larvae were fed either phototoxic *H. perforatum* or non-phototoxic *H. calycinum* (Figure 6). Larvae appeared to feed equally well on both plants. However

TABLE 5: The Short-term Effect of Hypericin Ingestion on Antioxidant Enzyme Levels in *A. plagiata.*

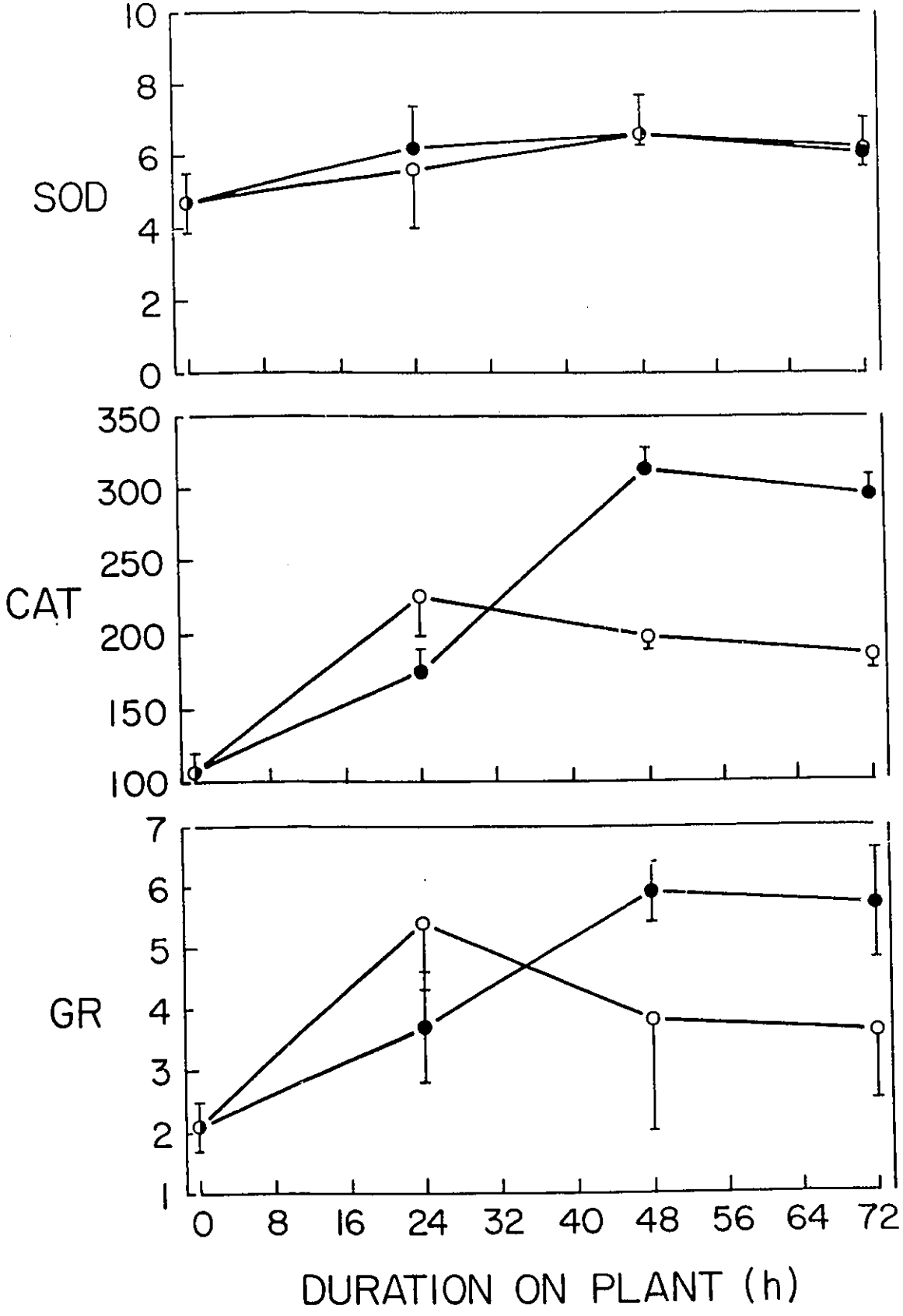
	Hypericin Consumption[b]		Enzymes[c]		
Treatment[a]	ug/disc	% fr.wt.	SOD	CAT	GR
Starved	0	0	4.7 ± 0.8c	107.7 ± 12.6c	2.1 ± 0.4d
Fed	0	0	7.9 ± 0.2a,b	450.6 ± 33.7a	18.0 ± 1.4a
Fed	0.01	0.002	8.9 ± 0.3a	483.6 ± 40.8a	18.2 ± 0.4a
Fed	0.1	0.02	7.0 ± 0.3b	345.0 ± 32.4b	12.0 ± 1.2b
Fed	1.0	0.2	6.6 ± 0.6b	344.8 ± 26.9b	7.8 ± 1.1c

[a] *Starved larvae consisted of larvae starved for 17 h. Fed larvae were starved for 16 h followed by 1 h of feeding.*

[b] *Consumption is expressed as ug hypericin applied per leaf disc as well as a percent of fresh leaf tissue weight.*

[c] *Values represent means ± S.D. of three experiments each with three determinations and are units per mg protein. Values in each vertical column followed by the same letter are not statistically different (Tukeys test).*

Figure 6: Effect of feeding fifth instar larvae of *A. plagiata* either non-phototoxic *H. calycinum* (open circles) or phototoxic *H. perforatum* (filled circles) on the antioxidant enzymes SOD (upper), CAT (middle), and GR (lower). Values represent means $\pm$ S.D. of three experiments (n=3). All enzyme activities are expressed as units per milligram protein.


SOD
10
8
6
4
2
0
CAT
350
300
250
200
150
100
GR
7
6
5
4
3
2
1
0
8
16
24
32
40
48
56
64
72
DURATION ON PLANT (h)

larvae fed on *H. calycinum* for 72 h typically had a weight gain of 10-15% less than those fed on *H. perforatum* (results not shown). Although we believe the only major difference between the two species to be the presence of hypericin, and that this accounts for the effects observed, we cannot rule out the possibility that other differences (due to different compounds) exist as well (e.g. other pro-oxidant allelochemicals may be present). During the first 24 h, larvae fed on *H. calycinum* had significantly higher (t-test, $P < .05$) levels of both CAT and GR than groups fed on *H. perforatum*. After 48 h this trend was reversed, with larvae fed on *H. perforatum* showing much higher enzyme activity. During the 24- to 48- h period, larvae fed *H. perforatum* showed significant increases in both CAT and GR activity while larvae fed *H. calycinum* exhibited a decline in the activity of both of these enzymes. Both CAT and GR remained at a relatively high level for the 48- to 72- h period in groups fed *H. perforatum*, whereas groups fed *H. calycinum* maintained a much lower level of activity. SOD activity, although increasing over the length of the experiment, was not significantly different between the two groups.

2.4 DISCUSSION

2.4.1 Constitutive Antioxidant Enzymes

We have examined the possibility that antioxidant enzymes such as SOD, CAT, and GR are necessary and logical defenses for insect herbivores feeding on phototoxin-containing plants. Strict specialist feeders such as *A. plagiata* might require a high basal level of these enzymes as a defense against the potentially lethal effects of hypericin ingestion. The SOD activities reported in Table 1 for *A. plagiata* are substantially higher than that of either *M. sexta* or *O. nubilalis*, and are greater than the SOD activity reported for a number of other lepidoptera including *Trichoplusia ni, Spodoptera eridania, and P. polyxenes* (Pritsos *et al.*, 1988; Pardini *et al.*, 1989). In the specialist feeder *P. polyxenes*, SOD activity was observed to be generally higher (particularly during the third instar) when compared to the two generalist feeders, *T. ni and S. eridania*. These results are consistent with the hypothesis that specialist feeders on phototoxic plants may require higher levels of protective antioxidant enzymes than other insects.

It is interesting to note that in the present study, third instar larvae of *A. plagiata* had nearly four-fold greater levels of SOD compared to fifth-instar larvae (Table 1). Fields *et al.*(1990) have observed that third instar larvae of this species

tend to remain on their food plants (*H. perforatum*) at all times, feeding both day and night. Last instar larvae however, tend to be more cryptic, with a distinct preference for feeding later in the day and at night. The high levels of SOD in third instar larvae might allow them greater protection from oxidative stress caused by hypericin and hence the ability to be freely exposed to sunlight without detrimental effects. The lower levels of SOD in late fifth instars may be related to the cessation of daylight feeding at this stage.

The CAT activity of the three species examined in this study followed the same trends as SOD activity. *A. plagiata* exhibited the highest activity, particularly in the third instar. This high CAT activity might be expected given the high SOD activity which would produce high levels of hydrogen peroxide. The low levels of CAT found in *O. nubilalis* are similar to levels observed in *S. eridania* and *Musca domestica* (Allen *et al.*, 1983; Pritsos *et al.*, 1988).

We examined GR levels in the three species and found that GR levels in *A. plagiata* were only slightly higher than in the other two species. Levels of GR tended to decline from third to late fifth instar, much in the same way as SOD levels declined. Similar levels of GR have been observed in *P. polyxenes* (Pritsos *et al.*, 1988). It has been proposed that GR provides the necessary recycling of GSSG in order, among other functions, to supply a Se-dependent, GSH-dependent GPOX which is an essential defense against the accumulation of lipid peroxides (Flohe, 1982). Even though we observed GR activity in all three species examined, we did

not measure significant levels of GPOX using H_2O_2 as substrate. This result is consistent with the original findings of other researchers (Ahmad *et al.*, 1987; 1988a; Pardini *et al.*, 1989) who also observed only negligible GPOX activity against this substrate. It has been demonstrated, however, that there exists high levels of GSH-dependent peroxidase activity in the cabbage looper *T. ni* when assayed using the substrate cumene hydroperoxide (cumOOH) instead of H_2O_2 (Ahmad and Pardini, 1988; 1989). This peroxidase activity was ascribed to a non Se-dependent, GSH-transferase (E.C.2.5.1.18) which accepts a wide variety of lipid hydroperoxides as substrates but not H_2O_2 (Flohe, 1982; Ahmad and Pardini, 1988; 1989). In the absence of an effective GPOX against H_2O_2 in insects, it has been suggested that high CAT levels could limit the need for this enzyme (Pardini *et al.*, 1989). An additional possibility might be the presence of active, non GSH-dependent peroxidases as has been examined in *Drosophila melanogaster* (Allen *et al.*, 1983; 1984, Ahmad and Pardini, 1988) and in the granary weevil *Sitophilus granarius* (Boulter and Chefurka, 1990). More recently, GPOX activity in insects has been reassessed and extremely low levels of GPOX-like activity using H_2O_2 observed in *T. ni, S. eridania*, and *P. polyxenes* (Ahmad *et al.*, 1989). However, over 95% of this activity was retained in enzyme preparations which had been heat-treated. This was not the case with samples of *M. domestica* however (also examined in in this report) where heat treatment destroyed all enzyme activity. In mammalian erythrocytes, the selenium-dependent GPOX is rapidly and completely destroyed by heat treatment

(Paglia and Valentine, 1967). This GPOX-like activity against H_2O_2 in insects (2-12 units; (Ahmad *et al.*, 1989)) is relatively low compared to mammalian tissues where levels may reach 300 units per mg protein (Levine and Kidd, 1986). It appears that cellular defenses in insects against lipid peroxides, which are a major consequence of photo-oxidative damage to membranes, may take the form of both GSH-dependent (e.g. GSH-transferase) and non GSH-dependent peroxidases with perhaps only a minor (if any) role for the Se-dependent GPOX. It has been suggested that the low Se content of plants, and the fact that plants do not have a Se requirement, may have led phytophagous insects not to rely on a Se-dependent GPOX (Ahmad *et al.*,1989). As well, the high CAT activity in insects and its broad subcellular distribution could protect against the accumulation of H_2O_2 (Ahmad *et al.*,1988a). We are investigating further the nature of the peroxidases in lepidopterous larvae and their ability to provide protection against lipid peroxidation.

The differences in antioxidant enzyme activities we observed could be related, in part, to differences in diets. For example, *A. plagiata* is reared on fresh plant material whereas *M. sexta* and *O. nubilalis* are reared on artificial diets. However to determine if pre-conditioning has a major effect on these species we have compared (results not shown) SOD and CAT activity in *M. sexta* larvae raised on either artificial diets or fresh tobacco and found that in the latter case larvae had only 10 to 20% higher levels. This pre-conditioning might affect induced enzyme activity however.

Our results support the view that specialist feeders (e.g. *A. plagiata, P. polyxenes*) which regularly encounter high levels of phototoxins may require high constitutive levels of antioxidant enzymes. Generalist feeders (e.g. *T. ni, O. nubilalis*) which intermittently ingest phototoxins may have lower levels of these enzymes and this, in part, may make them potentially more susceptible to photo-oxidative damage.

2.4.2 Response of Antioxidant Enzymes to Hypericin

If some insects use antioxidant enzymes as a defense against photo-activated secondary plant compounds, it might be expected that antioxidant enzyme activity in these species is inducible, much in the same way as other xenobiotic detoxification enzymes in insects are inducible. As well, it could be argued that for antioxidant enzymes to be an effective defense against free radicals and singlet oxygen, either high constitutive levels of these enzymes must be maintained and/or that enzyme activity should be rapidly inducible. This might be necessary given the rapidity with which many phototoxins can generate toxic oxyradicals.

Only a few studies have examined the short-term effects of plant photo-oxidants on antioxidant enzyme activity in insects. We initially examined the short-term effects (within one hour) of ingestion of hypericin by *A. plagiata*. At the lowest level of hypericin given, 0.01 ug/disc, only a slight increase in enzyme activity occurred. At higher doses however, a strong inhibition of SOD, CAT, and GR

activity was observed. This inhibition was significant at the 0.1 ug/disc level (0.02% fresh weight), a level of hypericin which is normally found in *A. plagiata*'s host plant, *H. perforatum*. In this species, hypericin may reach 0.04% of fresh weight (Pace and McKinney, 1941). In *P. polyxenes*, SOD levels have been shown to increase significantly when this species was fed for 1 to 12 h on diets containing quercetin, a flavonoid known to produce activated oxygen species (Pritsos *et al.*,1988; Joshi and Pathak, 1983). However both CAT and GR activity were both strongly inhibited by this compound. Very similar short-term results have also been obtained with the furanocoumarin xanthotoxin when used in diets fed *T. ni* (Ahmad *et al.*, 1988b). Both quercetin and xanthotoxin have been shown to exhibit a time and concentration-dependent inhibition of the GPOX activity observed in the southern armyworm *S. eridania* (Ahmad *et al.*,1987) although this could reflect an inhibition of GSH transferase.

The nature of the short term inhibition of antioxidant enzymes by these phototoxins is unknown but it is most likely caused by the generation of free radicals or singlet oxygen leading to photo-oxidation, although direct inhibition cannot be ruled out. Most phototoxins examined to date display a dark toxicity and we cannot rule out the possibility that the inhibition seen is not strictly light-dependent. The fact that phototoxins can strongly inhibit antioxidant enzymes such as CAT and GR suggests that many phytophagous insects, particularly those specializing on phototoxic plants, may need high constitutive levels of these enzymes if they are to act as an

effective antioxidant defense. At present, we are unable to properly assess and quantify the amount of oxidative stress caused by plant phototoxins. Xanthotoxin, even though known to produce toxic oxygen radicals (Joshi and Pathak, 1983), also acts through a genotoxic mechanism (Ashwood-Smith *et al.*, 1980) whereas hypericin is believed to operate primarily through the generation of singlet oxygen (Knox and Dodge, 1985). The fact that both hypericin and quercetin can inhibit CAT and GR whereas xanthotoxin has relatively little effect suggests that they may be more likely to cause oxidative damage.

Very little work has been carried out on the longer term (e.g. greater than 12 h) consequences of photo-oxidant ingestion on antioxidant enzyme activity. It has recently been shown that larvae of *T. ni* , when fed for 36 h on diets containing xanthotoxin have elevated levels of SOD, CAT, and GPOX activity (Lee and Berenbaum, 1989). In *S. granarius*, use of the fumigant phosphine, which produces toxic oxygen species, has shown that treated insects have elevated levels of SOD yet have greatly depressed levels of peroxidase activity (Allen *et al.*, 1983; Boveris *et al.*, 1972). In our study, larvae fed either the hypericin-containing *H. perforatum*, or the hypericin-free *H. calycinum* maintained relatively similar SOD levels, however CAT and GR were, after 48 h, maintained at a significantly higher level of activity in the groups ingesting hypericin. The fact that no furthur changes in enzyme activity were seen after 48 h may be related in part to the fact that some larvae may be approaching late instar when levels of activity have been shown to decline (Tables

2-4). This response of *A. plagiata* to hypericin ingestion suggests that the antioxidant enzymes CAT and GR may be important inducible defenses against the accumulation of free radicals and singlet oxygen. High constitutive levels of SOD may preclude the need for induction of this enzyme and may be sufficient to rapidly remove superoxide anions. CAT, together with the presence of peroxidases, would likely minimize the risk of peroxidative damage. GR could maintain high levels of GSH for use as both a general antioxidant and for GSH-dependent peroxidase activity.

As yet, we do not know the relative contribution of cytochrome P450-based detoxification or other mechanisms such as sequestration to deal with hypericin in *A. plagiata*. It is likely that they play an important role in reducing the amount of unmetabolized phototoxin capable of generating toxic oxyradicals, and this area requires investigation. However it is likely that the detoxification enzyme system and the antioxidant enzyme system work in concert to prevent oxidative damage. Our results suggest that high constitutive levels may be an important defense mechanism and that photo-oxidants are capable of inhibiting these enzymes. Phytophagous insects which have highly developed antioxidant enzyme systems may be preadapted to exploit phototoxic plants.

CHAPTER 3: ANTIOXIDANTS AS A DEFENSE AGAINST PHOTOTOXIN INGESTION

3.1 INTRODUCTION

Many plant secondary compounds are capable of acting as photosensitizers and causing biological damage to insects (Towers, 1984; Knox *et al.*, 1987). Upon absorption of light the photo-oxidants, which are the sensitizers of interest in this study, generate, either directly or indirectly, highly toxic species of oxygen including singlet oxygen and free radicals such as superoxide and hydroxy anions. Biological damage is caused primarily through the oxidation of proteins, lipids, and nucleic acids (Spikes, 1977).

A large degree of variation in the sensitivity of insect herbivores to these plant phototoxins has been demonstrated (Champagne *et al.*, 1986; Iyengar *et al.*, 1987; Berenbaum and Lee, 1991) and it has been postulated that some insects may have specific behavioural and biochemical defense mechanisms to deal with either the accidental or necessary ingestion of phototoxic plant chemicals (Larson, 1986; Larson and Berenbaum, 1988; Pardini *et al.*, 1988; Sandberg and Berenbaum, 1989). Behavioural mechanisms of defense include the leaf-tying behaviour of tortricid caterpillars feeding on phototoxic *Hypericum perforatum* (Sandberg and Berenbaum, 1989) and the light-avoidance behaviour of *Chrysolina sp.* larvae (Fields *et al.*, 1990). Recently, much work has focussed on the presence of antioxidant enzymes such as

superoxide dismutase, catalase, glutathione reductase, and peroxidases in lepidopteran larvae (e.g.Pritsos *et al.*, 1988; Lee and Berenbaum, 1989). In mammalian systems these enzymes are well known to be a primary line of defense against free radical damage (Halliwell and Gutteridge, 1985). In some insects, the levels of these antioxidant enzymes have been shown to increase in response to the ingestion of phototoxins such as the flavonoid quercitin (Pritsos *et al.*, 1988) and the furanocoumarin 8-methoxypsoralen (8-MOP) (Lee and Berenbaum, 1989). These enzymes could provide an important defense mechanism which is complementary to the P450-based detoxification enzyme system of insects.

There are also a number of small molecular weight molecules which are capable of preventing the deleterious effects of free radical and singlet oxygen formation. Beta-carotene, ascorbic acid (vitamin C), and alpha-tocopherol (vitamin E) are all known to be either effective quenchers of toxic oxygen species or to be capable of preventing lipid peroxidation (Halliwell and Gutteridge, 1985). Larson (1986) has reviewed the occurrence of potential inhibitors and quenchers of free radicals and singlet oxygen in insects. These include carotenoids, vitamin E, ascorbic acid, and some amines and sulphur compounds. Uric acid has been postulated to be an important antioxidant in mammals (Ames *et al.*, 1981) and it could have an antioxidant role in insects as well. Little experimental work has been carried out however, to determine whether these molecules are an effective defense against plant phototoxins. In housefly larvae, Robinson and Beatson (1985) have shown that

dietary beta-carotene exerts a protective effect in larvae treated with the photosensitizing dye erythrosine B. Ascorbate, tocopherol, and commercial antioxidants such as butylated hydroxytoluene (BHT) actually enhanced the toxic effect of the dye. In herbivorous insects it is unknown whether dietary antioxidants play a role in protecting them against photo-oxidative damage.

We have been examining the phototoxin alpha-terthienyl (α-T), which is a thiophene found in many species of the family Asteraceae and which shows a wide degree of variation in its toxicity to insect herbivores. Larvae of the tobacco hornworm, *Manduca sexta*, are particularly sensitive to this compound whereas the polyphagous *O. nubilalis* and the specialist feeder *A. plagiata* are not sensitive to phototoxins (Downum *et al.*, 1984; Downum, 1986; Iyengar *et al.*, 1987; see also Appendix III). We have investigated firstly, whether the differences in sensitivities of these larvae can be explained in part by differences in tissue antioxidant levels and secondly, whether any degree of protection can be afforded to the phototoxin-sensitive M. sexta by the addition of dietary antioxidants such as ascorbate, beta-carotene, and vitamin E.

3.2 METHODS AND MATERIALS

3.2.1 Insects and Antioxidants

Insects were raised as previously described (Chapter 2). For *M. sexta*, newly hatched larvae were transferred to trays of diet cubes which contained varying

amounts of antioxidants. For groups of larvae raised on diets supplemented with beta-carotene or vitamin E (0.001, 0.01, 1.0% of diet), the antioxidant was first mixed with 2 mls of linseed oil and then added to 100 g of diet. Control diets also received 2 mls of linseed oil. For diets in which the level of ascorbic acid was altered (0.0024, 0.024, 0.24, 1.2% of diet) the antioxidant was added as a dry ingredient to cooled diet.

3.2.2 Mortality Assessments

Four to seven groups of ten, third-instar *M. sexta* larvae (50-80 mg mean weight) which had been raised on diets containing antioxidants were placed into glass petri dishes and then treated with α-T using a Potters spray tower. After treatment the larvae, together with diets, were placed under a bank of near-UV lamps (300-400 nm, Westinghouse BLB, 5 W/m^2) for eight hours and then returned to the incubator. Mortality assessments were made at 24 and 48 hours after spraying the insects. Five mls of a 1.5 mg/ml solution of α-T in acetone containing 1% propylene glycol was found to result in approximately a 50% mortality in control groups and so this level of treatment was used in all trials. Three round glass coverslips (22 mm dia.) were also included in each petri dish in order to measure actual application rates. The α-T on the coverslips was dissolved in 1 ml of methanol and its concentration measured using a spectrophotometer (Varian 2200). The application rate was 1.31 ± 0.37 ug/cm^2.

3.2.3 Tissue Analysis

3.2.3.1 Vitamin E

Vitamin E content was examined in midgut, fat body, and carcass (tissue remaining after removal of digestive tract) tissues using a modification of the procedures of Zaspel and Csallany (1983) and Miller and Yang (1985). Fifty to one hundred mg of tissue was dissected into cold 0.15 M NaCl containing 5% ascorbic acid (BDH Chemicals) and then homogenized on ice in 1 ml of 1:1 methanol: 5% ascorbic acid in water. Samples were then transferred to 1.5 ml Eppendorf plastic tubes and 0.5 mls hexane added. After vortexing for 10 seconds the tubes were centrifuged in the cold for two minutes in a Beckman microfuge (13000g), the hexane layer removed to a glass vial and the extraction procedure repeated twice. The combined hexane extracts were dried under nitrogen in dim light, reconstituted in 300 ul methanol, and then 20 ul injections used in the HPLC. Unused portions of samples were frozen (-70°C) for subsequent beta-carotene analysis.

Vitamin E was analyzed by HPLC with a Perkin-Elmer LC-250 binary pump and an LS-40 fluorescence detector. Vitamin E was detected using an excitation wavelength of 295 nm and emission wavelength of 340 nm. The column used was a Beckman Ultrasphere C18 (4.6 mm x 25 cm) with a solvent system consisting of methanol:water (98:2) delivered at 2.0 ml/min. The elution time of vitamin E was 7.9 min. Extraction efficiencies using vitamin E-dosed samples averaged 87%.

Standards were prepared from fresh stock solutions of alpha-tocopherol (Sigma) in methanol.

3.2.2.2 Beta-carotene

Beta-carotene was analyzed using a Waters Model 441 Absorption detector containing a mercury lamp and 436 nm filter. The column was eluted isocratically with methanol:acetonitrile:chloroform (25:60:15) at a flow rate of 1.5 ml/min (Miller and Yang, 1985). Standards were prepared using a stock solution (2mgs/ml) of beta-carotene (Sigma) in chloroform. Extraction efficiencies averaged 84% with dosed samples while the retention time of beta-carotene was 15.1 min.

3.2.2.3 Vitamin C

For vitamin C determinations, 50 to 100 mg tissue was dissected in to cold 0.15 M NaCl. After weighing, the tissue was homogenized on ice in a glass homogenizer in 3 mls 6% metaphosphoric acid, and then samples centrifuged in the cold for 10 min at 6000 rpm and the supernatants filtered (0.45 um, Millipore).

The HPLC system used to detect vitamin C in tissue samples consisted of a Beckman Model 110 pump with Model 420 Controller and Model 165 variable wavelength detector set at 242 nm. The buffer used was 70 mM sodium acetate (pH 4.2) containing 0.15% metaphosphoric acid delivered at 1.0 ml/min. The column used was a Beckman Ultrasphere C-18 (4.6 mm x 25 cm). Ascorbic acid was identified by

comparison with the retention time (2.8 min) and UV spectra of pure ascorbic acid. Extraction efficiencies averaged 92%.

3.2.2.4 Bound and Free Sulfhydryls

Total (TSH), protein bound (PBSH), and non-protein bound (NPSH) sulfhydryls were determined by the method of Sedlak and Lindsay (1968) and Habeeb (1972). Tissues (50-100 mg) were homogenized on ice in 4.0 mls of 0.02 M EDTA (pH 4.7). For TSH, 250 ul of tissue homogenate was combined with 750 ul TRIS buffer (0.2 M, pH 8.2), 4.0 mls methanol, and 50 ul dithiobisnitrobenzoic acid (DTNB) (4.0 mg/ml in methanol). Standards were prepared using 950 ul TRIS, 4.0 mls methanol, 50 ul DTNB, and 50 ul of a stock solution of cysteine in H_2O. A reagent blank (no sample) and TSH sample blanks for each tissue sample (no DTNB) were also prepared. After color development (30 min), solutions were vortexed for 5 s, centrifuged for 4 min at 6000 rpm, and the absorbance read on a Cary 2200 spectrophotometer. The extinction coefficient of 13,600 $M^{-1}cm^{-1}$ was used for calculations. For NPSH, 2.5 mls of homogenate was combined with 2.0 mls distilled water and 0.5 ml 50% trichloroacetic acid. After vortexing for 1 min, the solutions were centrifuged for 4 min as above. After filtering (0.45 um, Millipore) 1.0 ml was added to 2.0 ml TRIS buffer and 50 ul DTNB and the samples read after 15 min. PBSH was calculated by subtracting NPSH from TSH.

3.3 RESULTS

3.3.1 Tissue Analysis for Antioxidants and Sulfhydryls

The tissue levels of antioxidants found in the three species are presented in Table 6. Vitamin E levels are clearly highest in *A. plagiata*, the specialist feeder on phototoxic plants, although *O. nubilalis* also had high fat body levels. In contrast, vitamin C levels were found to be highest in all tissues of *M. sexta*. Beta-carotene levels were also found to be highest in *A. plagiata* and not detectable in any tissue of *O. nubilalis* nor in the fat body of *M. sexta*.

Bound and free sulfhydryls were also examined (Table 7) in these three species since thiol status (e.g. cysteine, reduced glutathione) may potentially be an indicator of sensitivity to phototoxins. NPSH levels were found to be quite high in the fat body of *M. sexta* and *A. plagiata*. PBSH levels were highest in all tissues of *A. plagiata*, with *M. sexta* exhibiting the lowest overall levels. This resulted in overall highest TSH in *A. plagiata*, particularly midgut tissues.

3.3.2 The Protective Effect of Dietary Antioxidants

The 24 hour mortality of α-T treated (1.37 ug/cm^2) groups of larvae fed control diets ranged from 32 to 56% whereas the 48 hour mortalities of these groups ranged from 54 to 84% (Figure 7-9). Because of the high level of ascorbic acid in control diets (Figure 7), we primarily examined the effect of reducing the concentration of this vitamin in the diet on the sensitivity of *M. sexta* to α-T. As the

Table 6: Antioxidant levels in tissues of third instar *M. sexta*, and fifth instar *A. plagiata* and *O.nubilalis.*[*]

a) Vitamin E

Species	Midgut	Fatbody	Carcass
O. nubilalis	2.39 ± 0.14[b]	9.01 ± 1.61[a]	2.56 ± 0.30[a]
A. plagiata	5.77 ± 0.17[a]	6.74 ± 1.28[b]	2.40 ± 0.72[a]
M. sexta	3.34 ± 0.39[b]	6.98 ± 1.35[b]	1.97 ± 0.21[a]

b) Vitamin C

Species	Midgut	Fatbody	Carcass
O. nubilalis	574.4 ± 76.2[b]	367.1 ± 7.7[b]	475.1 ± 110.6[b]
A. plagiata	342.2 ± 44.2[c]	232.2 ± 23.7[c]	288.9 ± 35.0[c]
M. sexta	1009.1 ± 37.0[a]	709.1 ± 79.7[a]	1048.2 ± 55.0[a]

c) Beta-carotene

Species	Midgut	Fatbody	Carcass
O.nubilalis	N.D.	N.D.	N.D.
A. plagiata	1.1 ± 0.2	N.D.	0.4 ± 0.1
M. sexta	0.2 ± 0.1	N.D.	0.3 ± 0.1

[*]Values are μg/g tissue and are means ± S.D. of four samples. Using a simple one-way ANOVA, means in each vertical column were compared using a Tukeys test. Values in each vertical column of each section followed by the same letter are not significantly different.

Table 7: Bound and Free Sulfhydryls in tissues of third instar *M. sexta* and fifth instar *A.plagiata* and *O.nubilalis.*

a) Non-Protein Bound

Species	Midgut	Fatbody	Carcass
O. nubilalis	0.60 ± 0.15^{a}	0.84 ± 0.41^{b}	0.52 ± 0.13^{a}
A. plagiata	0.64 ± 0.05^{a}	2.01 ± 0.41^{a}	0.32 ± 0.02^{a}
M. sexta	0.53 ± 0.12^{a}	2.59 ± 0.52^{a}	0.63 ± 0.03^{a}

b) Protein Bound

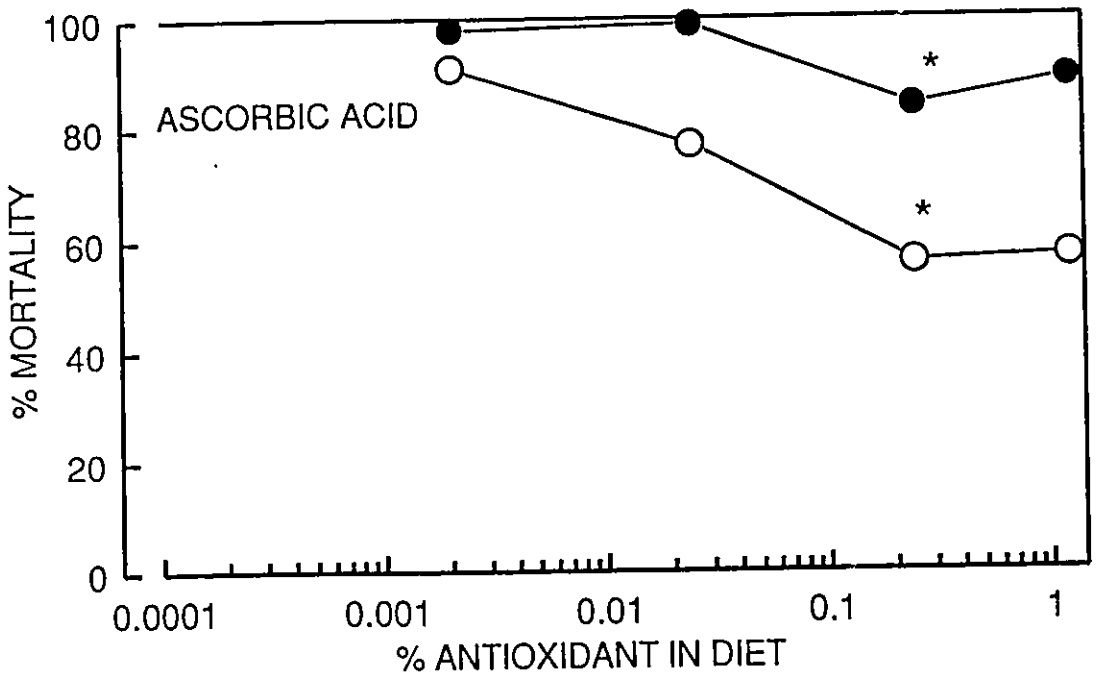
Species	Midgut	Fatbody	Carcass
O. nubilalis	5.07 ± 1.30^{a}	1.88 ± 0.80^{b}	4.08 ± 0.15^{a}
A. plagiata	6.20 ± 0.32^{a}	4.05 ± 0.37^{a}	4.15 ± 0.21^{a}
M. sexta	3.46 ± 0.14^{b}	1.61 ± 0.69^{b}	2.13 ± 0.42^{b}

c) Total

Species	Midgut	Fatbody	Carcass
O.nubilalis	5.67 ± 1.45^{a}	2.72 ± 0.66^{c}	4.60 ± 0.23^{a}
A. plagiata	6.85 ± 0.38^{a}	6.05 ± 0.57^{a}	4.47 ± 0.22^{a}
M. sexta	4.01 ± 0.10^{b}	4.20 ± 0.35^{b}	2.77 ± 0.41^{b}

*Values are umoles SH/ g tissue and are means $\pm$ S.D. of four samples. Using a simple one-way ANOVA, means in each vertical column were compared using a Tukeys test. Values in each vertical column of each section followed by the same letter are not significantly different. Non-protein bound = total - protein-bound.

Figure 7: Toxicity of α-T to third instar larvae of *Manduca sexta* raised on diets containing various levels of ascorbic acid. Open circles: 24 h mortality; closed circles: 48 h mortality. Asterisks indicate levels of antioxidant in control diets.


100
80
60
40
20
0
% MORTALITY
ASCORBIC ACID
*
*
0.0001
0.001
0.01
0.1
1
% ANTIOXIDANT IN DIET

level of the vitamin was reduced (from 0.24% to 0.024%), greater mortality was observed. In contrast, a five-fold increase in the vitamin, from 0.24 to 1.2%, afforded no degree of protection against the phototoxin.

The lipid soluble antioxidants, beta-carotene and vitamin E, were both found to provide excellent protection against the toxicity of α-T. Beta-carotene, at a concentration of 0.1%, reduced the 24 hour mortality of *M. sexta* to only 3% (Figure 8). Substantial reduction in mortality (to 24%) was also seen at a ten-fold lower level of this antioxidant (0.01%) whereas ten-fold greater levels (1.0%) provided no further protection. The 48 hour mortality values closely reflected the 24 hour trends with only slightly greater mortality at each level of antioxidant.

The results with vitamin E (Figure 9) resembled those of beta-carotene. A significant level of protection (mortality dropped to 25%) was observed at the 0.01% level while near maximal protection (mortality only 5%) was obtained using 0.1% vitamin E. The 48 hour mortalities again only showed slight increases at each level of antioxidant.

3.3.3 Tissue Antioxidants in Larvae Fed Control and Antioxidant-Supplemented Diets

In order to confirm that the lipid soluble antioxidants which had provided protection against α-T were actually increasing in *M. sexta* tissues, we examined levels

Figure 8: Toxicity of α-T to third instar larvae of *Manduca sexta* raised on diets containing various levels of beta-carotene. Open circles: 24 h mortality; closed circles: 48 h mortality. Asterisks indicate levels of antioxidant in control diets.

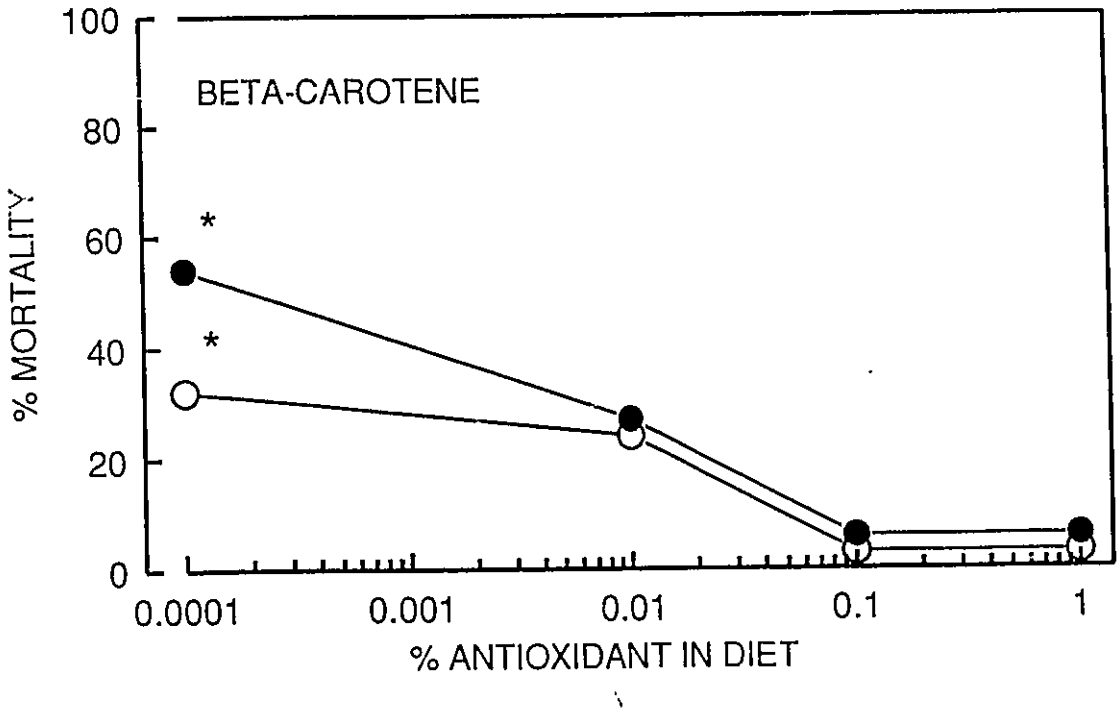
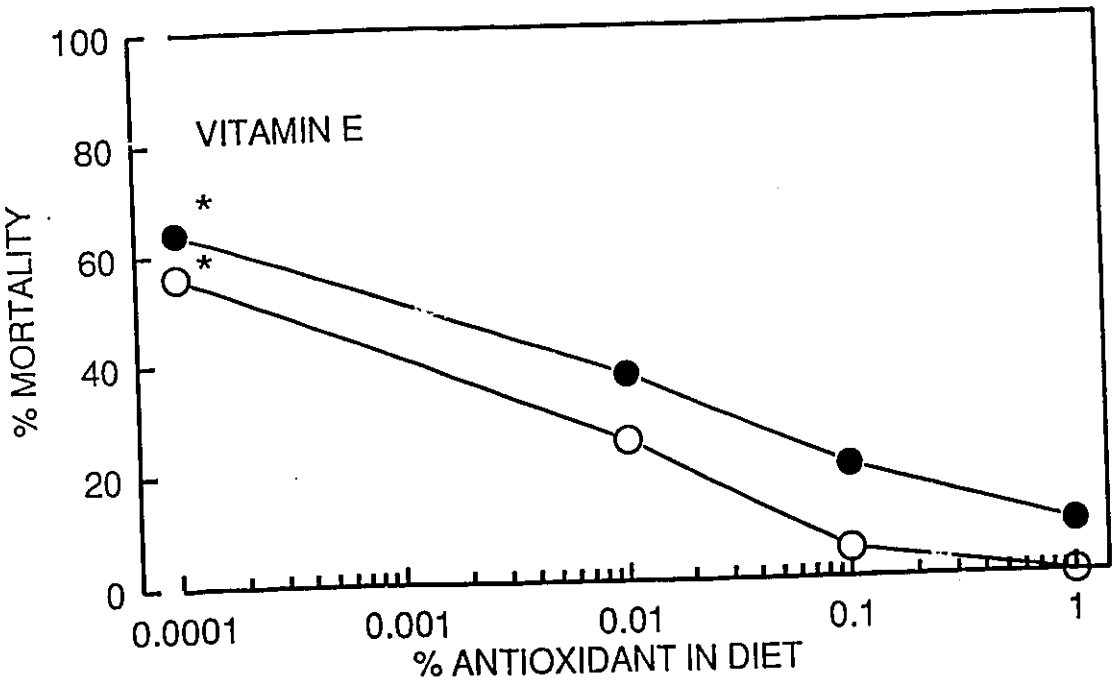

BETA-CAROTENE
% MORTALITY
100
80
60
40
20
0
*
*
0.0001
0.001
0.01
0.1
1
% ANTIOXIDANT IN DIET

Figure 9: Toxicity of α-T to third instar larvae of *Manduca sexta* raised on diets containing various levels of vitamin E. Open circles: 24 h mortality; closed circles: 48 h mortality. Asterisks indicate levels of antioxidant in control diets.


100
80
60
40
20
0
VITAMIN E
*
*
% MORTALITY
0.0001
0.001
0.01
0.1
1
% ANTIOXIDANT IN DIET

in midgut, fat body, and carcass of larvae fed on control diets and on diets supplemented with 0.1% antioxidant (Table 8). In the case of both beta-carotene and vitamin E, the increased dietary antioxidant level resulted in a corresponding increase in tissue levels. In the standard *M. sexta* diet the level of beta-carotene and vitamin E is extremely low (<0.0001%) and this is close to the levels observed in the tissues of control groups. For vitamin E, increasing the dietary concentration by 1000-fold resulted in 445 to 2644-fold increases in tissue levels while increases in dietary beta-carotene resulted in tissue beta-carotene levels which were only 48-57 fold higher.

3.4 DISCUSSION

We observed the highest level of vitamin E in tissues of *A. plagiata*. This is perhaps not surprising, however, since this species was raised on fresh plant material whereas the other two species were raised on artificial diets. Similarly, beta-carotene levels might be expected to be higher in tissues of larvae fed fresh material. *A. plagiata* had relatively high levels of beta-carotene in midgut tissues as compared to the other two species. In contrast to the lipid soluble antioxidants, vitamin C levels were found to be highest in *M. sexta*. This likely reflects the high level of vitamin C in this species diet and suggests that vitamin C is not very effective as a protective antioxidant, at least with this species - photosensitizer combination.

An examination of the thiol status in tissues of these larvae showed only slight differences in sulfhydryl levels with *A. plagiata* exhibiting an overall higher level of

Table 8: Tissue levels of Antioxidants in *M. sexta* larvae fed control or antioxidant-supplemented diets.

	Vitamin E[a]			Beta-carotene[a]		
	Midgut	Fatbody	Carcass	Midgut	Fatbody	Carcass
Control[b]	3.3 ± 0.4	7.0 ± 1.4	2.0 ± 0.2	0.2 ± 0.1	N.D.	0.3 ± 0.1
0.1% antioxidant in diet	4980 ± 1259	3103 ± 97	5209 ± 60	5.7 ± 1.4	1.0 ± 0.3	18.9 ± 3.2
Fold Increase	1491	445	2644	48	-	57

a: ug/g fresh weight tissue (means ± S.D.) of 3 samples.
b: fresh diet was analyzed and found to contain approximately 1 ug/g vitamin E and 0.1 - 0.5 ug/ g beta-carotene.
N.D.: not detected.

both PSH and TSH. This suggests that the basal level of sulfhydryls could contribute to the variation in sensitivity to phototoxins although this status could be affected differentially in the three species (see also Chapter 4). Many sulfhydryls and sulfhydryl-containing proteins (e.g. enzymes) are susceptible to oxidation such as might be expected by photosensitization with α-T.

The lipid soluble antioxidants beta-carotene and vitamin E, when administered in artificial diets, substantially reduced the mortality associated with treatment of *M. sexta* larvae with the phototoxin α-T. Ascorbic acid provided no additional protection when dietary levels were increased whereas a reduction in the control levels resulted in much higher mortality. This latter result may however be due in part to a decreased fitness of the larvae. Ascorbic acid is an essential nutrient for most herbivorous insects (Dadd,1985) and in *M. sexta*, suboptimal dietary ascorbic acid has been shown to cause abnormal growth and development (Kramer *et al.*, 1978). Ascorbic acid is capable of scavenging activated oxygen species such as superoxide anions although its efficiency is orders of magnitude lower than superoxide dismutase (Levine and Kidd, 1986). Ascorbic acid can also react with and reduce the tocopherol quinone radical resulting from the oxidation of tocopherol and in this way regenerate reduced tocopherol which is an interceptor of free radicals and singlet oxygen (Witting, 1976). The fact that increased dietary ascorbic acid provided no additional protection against α-T might be related to its hydrophilic character (in contrast to the lipophilicity of α-T (log P=5.7)). In the housefly, *Musca domestica*, ascorbate

actually enhanced slightly the phototoxic effect of erythrosine B treatment of larvae (Robinson and Beatson, 1985).

Unlike ascorbic acid, vitamin E appears to be an essential dietary requirement in far fewer species (Dadd, 1985). Vitamin E has been shown to be required for normal adult reproductive function in *Spodoptera exempta* (David and Ellaby, 1975) and in crickets (McFarlane, 1972). An important role for vitamin E in insects is likely as a general antioxidant since it is an excellent scavenger of activated oxygen species (Foote *et al.*, 1978). It may also have a role in the conservation of tissue polyunsaturated fatty acids as has been suggested for *Pieris brassicae* (Turunen, 1976). In our study, vitamin E was found to be an extremely effective antioxidant capable of protecting *M. sexta* larvae against lethal oxidative damage from α-T. The toxicity of α-T to insects is believed to be primarily due to its strong production of singlet oxygen and subsequent damage to essential membranes (Bakker *et al.*, 1979; Arnason *et al.*, 1981a,b; Scaiano *et al.*, 1989). McRae *et al.*(1985) have shown that antioxidants can markedly reduce α-T induced lipid peroxidation in egg lecithin liposomes. In the case of *M. sexta*, treatment of larvae with sublethal levels of α-T has been shown to cause black lesions of the cuticle (Downum, 1986; Champagne *et al.*, 1986) and to cause rapid destruction of midgut epithelial cells (Sen *et al.*, 1990). The lipid-soluble nature of vitamin E and the ability of *M. sexta* tissues to rapidly accumulate large amounts of this antioxidant from their diet (Table 8) may account for its protective effect.

Carotenoid pigments can also function as effective quenchers of singlet oxygen and other oxygen-centered radicals (Foote and Denny, 1968; Krinsky and Deneke, 1982). Beta-carotene has the highest quenching rate known for any quencher of singlet oxygen (2-3 x $10^{10}M^{-1}sec^{-1}$)(Foote, 1976). Carotenoids may react directly with peroxy and alkoxy radicals and hence prevent lipid peroxidation (Halliwell and Gutteridge, 1985). Beta-carotene has been shown to protect against phototoxicity induced by 8-MOP in mammals (Giles *et al.*, 1984). In insects, carotenoids have been examined from a variety of tissues (see, for example, Feltwell, 1978; Goodwin, 1980) and the dominant carotenoids found are lutein and beta-carotene (Feltwell and Rothschild, 1974). Large variations exist in the ability of some insects to absorb these carotenoids (Kayser, 1985); variations which are also found in insects reared on identical host plants (Feltwell and Rothschild, 1974). In terms of their function in insects, it is known that deprivation of either vitamin A or suitable carotene precursors, results in an impairment of the visual and light responses in several species (Veerman, 1980). In addition to their role in insect warning coloration (Rothschild, 1975), it has been suggested that they may also have a photoprotective role (Feltwell and Rothschild, 1974). In a short report, Poff (1976) observed that cryptic *Leptinotarsa* species had low concentrations of carotenoids and a lower carotenoid to xanthophyll ratio than in their host plants and conversely that aposematic species had higher concentrations of carotenoids and a higher ratio of carotenoids to xanthophylls. Poff (1976) suggested this high level of carotenoids might

have a protective role against incoming radiation.

In our study, beta-carotene effectively protected *M. sexta* at a dietary concentration of 0.1%, a concentration of beta-carotene frequently found in plants in nature (Bureau and Bushway, 1986). It is not known whether *M. sexta* might be able to attain this level of beta-carotene and/or protection by feeding on its typical host plant, tobacco. Nevertheless, we have demonstrated that dietary antioxidants such as beta-carotene can dramatically alter the sensitivity of this insect herbivore to a plant phototoxin.

Our results with beta-carotene and vitamin E are consistent with the view that the lethal damage caused by α-T is primarily due to $^1O_2$ formation as both antioxidants are excellent quenchers of this species of O_2. However, Type I (electron transfer) and Type II (energy transfer) processes are always in competition (Foote, 1987) hence we cannot rule out that the phototoxicity of α-T is due in part to a free-radical mechanism. Vitamin E is also a good radical scavenger. α-T is capable of O_2^- production (Kagan *et al.*, 1989) although laser flash photolysis studies (Scaiano *et al.*, 1989) indicate that electron transfer accounts for only 1% of the activated O_2. Perhaps specific scavengers and quenchers of different activated species of O_2 could be used to resolve this question.

We have also been investigating antioxidant defenses in phototoxin-tolerant insects. Larvae of the chrysomelid beetle *Chrysolina hyperici*, for example, are specialist feeders on *H. perforatum* which contains hypericin, a strong generator of

singlet oxygen (Knox and Dodge, 1985). These larvae, though primarily using light-avoidance as a defense mechanism against phototoxicity (see Fields *et al.*, 1990), have been observed to have brightly colored (orange-red) fat body. HPLC analysis of last-instar larvae (results not shown) indicated levels of beta-carotene of 336.2 $\pm$ 45.7 ug/g(x $\pm$ S.E.M., n=3); levels which are two to five fold higher than have been observed in many coleopteran and lepidopteran larvae (Feltwell and Rothschild, 1974). The protection provided by beta-carotene to *M. sexta* suggests that high levels of beta-carotene in *C. hyperici* may also protect these insects from host-plant phototoxins.

A number of factors may be involved in explaining the extreme sensitivity of *M. sexta* larvae to phototoxins, not the least of which might be the low level of lipid-soluble antioxidants in its artificial diet. *M. sexta* has also been recently shown to have a relatively low complement of the antioxidant enzymes superoxide dismutase, catalase, and glutathione reductase (Aucoin *et al.*,1991) and this may contribute to its sensitivity. Physical factors such as the amount of light penetration through the cuticle may also play an important role. Berenbaum (1987) has observed that a black mutant strain of this species is less sensitive to phototoxins than normally pigmented larvae. As well, *M. sexta* is an oligophagous herbivore and may not have had sufficient exposure to phototoxins in its natural diet breadth to develop an adaptive response in the form of specific defenses.

The fact that *M. sexta* can be protected from the toxicity of α-T suggests that

the sensitivity of other insect herbivores to phototoxins may be dependent in part on the nature and level of plant chemicals in their diet which can act as antioxidants. This would also depend on the ability of insects to absorb and utilize these chemicals. Antioxidants may have played an important role in the ability of some insects to develop successfully on phototoxic plants.

CHAPTER 4: Biochemical Effects of Photo-oxidative Stress Induced by α-T and the Protective Effect of Antioxidants and Antioxidant Enzymes.

4.1 Introduction

Relatively little definitive evidence regarding the mode of action of the photo-oxidant α-T in herbivorous insects is available and much of what is known has been elucidated using photo-oxidative dye sensitizers on non-herbivorous insects (e.g. Pimprikar, 1986). Biochemical sites of action of photo-oxidants include membrane lipids, proteins, and nucleic acids. In mosquitoes, one critical target of α-T appears to be the anal gill membranes, where the toxin has been shown to accumulate and cause structural damage (Arnason *et al*, 1987) as well as increase lipid peroxidation (LPO) (Hasspieler *et al*, 1990). Other targets in the mosquito are the midgut epithelium and malpighian tubules where the toxin also localizes (Hasspieler *et al.*, 1988). In the tobacco hornworm *M. sexta*, topically applied α-T has been shown to rapidly induce cuticular lesions (Downum, 1984; Champagne *et al.*, 1984,1986), while at subacute doses, ingested phototoxin can damage midgut epithelial cells (Sen *et al.*, 1990), and severely impair larval growth and the efficiency of conversion of ingested food (Samuels and Knox, 1989). One of the likely consequences of oxidative stress caused by photo-oxidant chemicals such as α-T in insect midgut tissue is an increase in LPO which in turn could be responsible for impaired digestion. Cell membrane phospholipids are susceptible to peroxidation which may lead to altered membrane

permeability and cell lysis (Mead, 1976). *In vitro*, photosensitization by α-T has been shown to induce potassium ion leakage and hemolysis in erythrocytes (Wat *et al.*, 1981), and to initiate both glucose leakage and LPO in egg lecithin liposomes (MacRae *et al.*, 1985). *In vivo*, it initiates leakage of halide from mosquito larvae (Arnason *et al.*, 1987). These results strongly suggest that LPO may be an important factor in the toxicity of this compound to insect herbivores.

A second possible biochemical target of photo-oxidation is proteins. α-T is known to photo-oxidize glucose-6-phosphatase *in vitro* (Bakker *et al.*, 1979) and acetylcholine esterase in human erythrocytes (Yamamoto *et al.*, 1979) and in mosquito larvae (Kagan *et al.*,1982).

Nucleic acids are also a possible target of photo-oxidants and ample evidence of mutagenesis is available in the case of DNA intercalating substances such as berberine or psoralens. With α-T, there is little evidence of mutagenesis in several studies including Ames test, sister chromatid exchange, etc.(e.g. MacRae *et al.*, 1980) and little evidence of DNA intercalation.

In this report we examine the potential for α-T to cause LPO in the phototoxin-sensitive herbivore, *M. sexta* and the potential for vitamin E to prevent damage. As well, we examine the effect of photo-oxidative stress on several antioxidant enzymes which may play an important role as cellular defenses. GPOX and GR are well known, in mammalian systems, to be an important line of defense against deleterious oxygen species (Mead, 1976). GPOX removes a variety of

potentially harmful peroxides while GR catalyzes the reduction of oxidized glutathione (GSSG) and hence maintains high levels of GSH for GPOX. GSH itself has long been considered an antioxidant and an important component of insect detoxification systems, such as the GST (E.C.2.5.1.18) -mediated conjugation of xenobiotics. GST itself catalyzes, via peroxidase activity, the removal of a wide variety of lipid peroxides but not H_2O_2 (Mannervik, 1985). Other, non-glutathione dependent peroxidases (e.g. PER, E.C.1.11.1.7) may also exist to prevent the build-up of harmful peroxides (Mannervik, 1985). Because of the dependence of some of these antioxidant enzymes on GSH, its potential role as an endogenous antioxidant, and its role in detoxification, we also investigated whether α-T could affect cellular thiol status.

4.2 Methods and Materials

4.2.1 Chemicals

All purified enzymes, substrates, and co-factors used in the enzyme assays were from Sigma, St.Louis, Missouri. 5-sulfosalicylic acid, 2-vinylpyridine, and triethanolamine were from Aldrich Chemicals, Milwaukee, Wis. Hydrogen peroxide (30%) was from BDH Chemicals, Toronto, Ont.

4.2.2 Lipid Peroxidation

To examine the effect of α-T on lipid peroxidation in midgut tissue of *M.*

sexta, the method of Dillard and Tappel (1984) was used. This assay is based on the fluorescent Schiff-base products formed from the reaction of endogenous amines with the decomposition products of lipid peroxides (e.g.malondialdehyde). These bases have characteristic excitation maxima of 315 to 400 nm and emission maxima of 420 to 470 nm and therefore the extent of LPO can be quantified (on a relative but not absolute basis) by fluorescence spectrophotometry.

Early third instar *M. sexta* larvae were raised according to the methods of Bell and Joachim (1976) until early fifth instar on either control diets (<1 ug/g vitamin E) or diets supplemented with vitamin E (1 mg/g). Controls (-α-T, +UV), dark treated (+α-T, -UV), and light treated groups (+α-T, +UV) were then fed control diets containing 0 or 50 ug/g α-T for 24 h. Light treated groups received 4 h UV from a bank of blacklight-blue lamps (Westinghouse 4 X 40 W). Groups not receiving UV were shielded using a Kodak CP2B UV filter.

Two hundred mg of midgut tissue was dissected from larvae, rinsed 3 times in cold 0.1 M NaCl, and then homogenized for 1 min in 3 mls cold 2:1 chloroform: methanol. After addition of 3 mls water the samples were vortexed for 10 s, centrifuged for 2 min at 1500g and the top layer removed to a glass vial. Methanol was then added (0.1 ml/ml sample) and the samples irradiated under UV for 30 s to remove retinol, a potentially interfering flourescent compound. Samples were then transferred to quartz cuvettes and the fluorescence spectra recorded using a Perkin-Elmer LS-50 spectrophotofluorimeter. Excitation and emission slit widths were set

at 2.5 nm.

4.2.3 Antioxidant Enzymes

To investigate the effect of α-T on antioxidant enzyme activity, fifth instar larvae were raised in UV-transparent petri dishes for up to 72 h on control diets or diets containing 50 ug/g α-T. Groups receiving light treatment were placed under UV lights as above for 8 h per day. After dissection, 150 mg of rinsed midgut tissue was homogenized for 1 min in 1.5 mls cold sodium phosphate buffer (50 mM, pH 7.0). After centrifugation for 5 min at 14000g, the supernatants were transferred to glass vials and used as the enzyme source.

GPOX was measured according to the method of Strauss *et al.* (1980) as previously described (Aucoin *et al.*, 1991). Activity is expressed as nmol NADPH oxidized per min per mg protein.

GR was determined using the method of Racker (Racker, 1955) as previously described (Aucoin *et al*, 1991). One unit of activity is defined as the change in absorbance due to the disappearance of NADPH at 340 nm of 0.001A per min per mg protein.

Non-GSH-dependent peroxidase activity (PER) was determined according to the method of Armstrong *et al* (1978). Phenylenediamine is used as a hydrogen donor cosubstrate that is oxidized to purpurogallin which absorbs at 412 nm. A typical

reaction mixture in a 1 ml cuvette contained 20 mM H_2O_2 in phosphate buffer (0.15 M, pH 7.5), 18 mM phenylenediamine, and an aliquot of insect sample. Absorbance was followed for 3 min and enzyme activity defined as units per mg protein where 1 unit is the change in absorbance per minute.

All assays were conducted at 25^{0}C using a programmable Varian DMS 300 spectrophotometer. Protein determinations were carried out using the Lowry method (Lowry *et al.*, 1951).

4.2.4 Cellular Thiol Status

Total, oxidized, and reduced glutathione levels were determined according to the procedure of Griffith (1980). Larvae were treated as with the enzyme studies for a total of 72 h. One hundred mg of tissue was then dissected, rinsed in cold NaCl, and homogenized for 1 min in 1.0 ml cold phosphate buffer (125 mM, pH 7.4, with 6.3 mM EDTA) containing 5% 5-sulfosalicylic acid. Samples were then centrifuged in the cold for 5 min at 13500g in a Beckman microfuge. A 0.1 ml aliquot of the supernatant was then diluted 50-fold with distilled water and kept on ice for analysis for total glutathione content. Another 0.5 ml aliquot of supernatant was combined with 10 ul 2-vinylpyridine and 30 ul 50% aqueous triethanolamine. After vortexing for 30 s, the preparation was allowed to stand at room temperature for at least 30 min prior to analysis for GSSG.

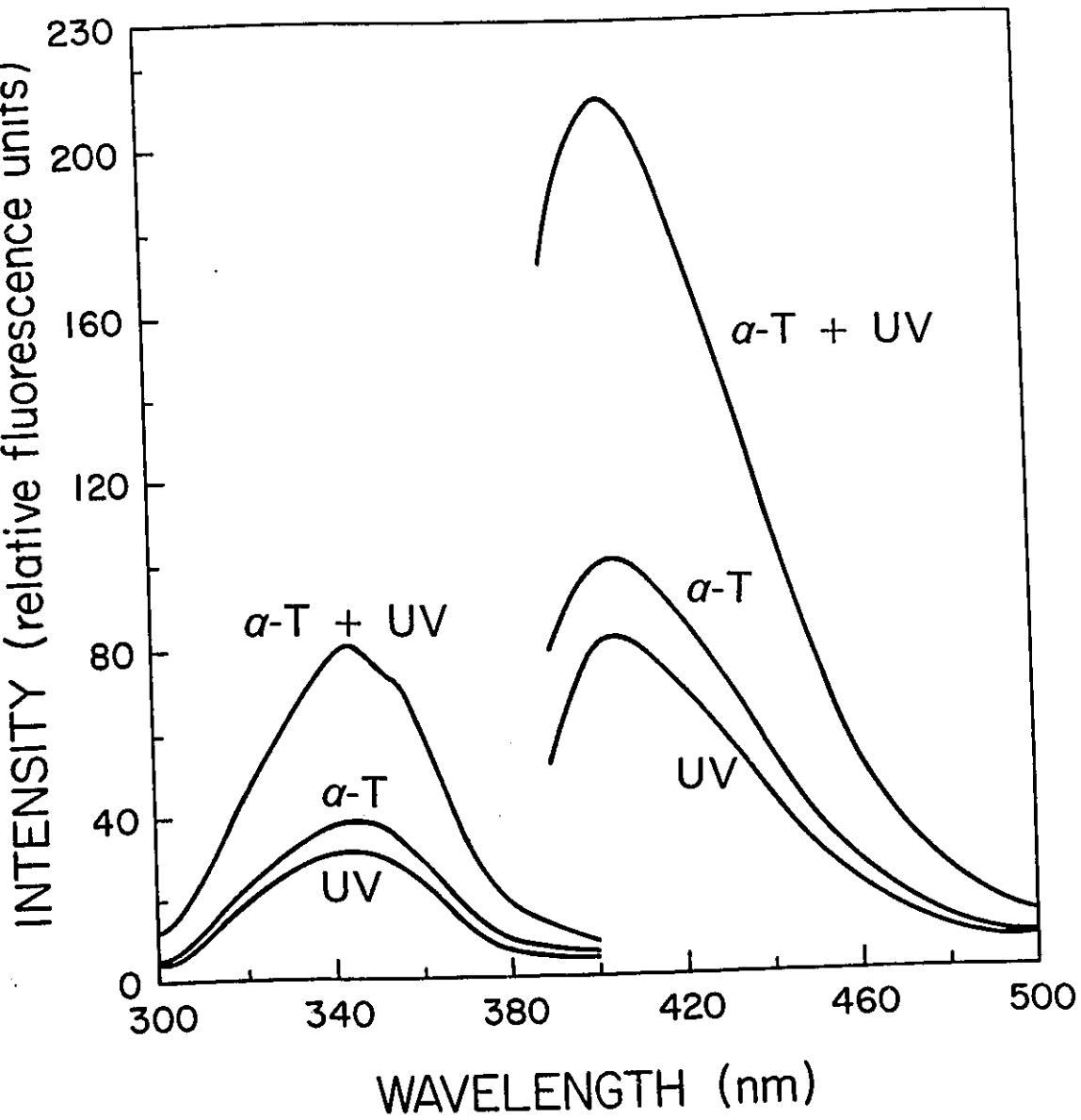
Quantification of oxidized and total glutathione was accomplished using an enzymatic cycling assay using pure GR. A working buffer solution consisted of phosphate buffer as above containing 0.3 mM NADPH. To assay for total glutathione, 0.1 ml of diluted supernatant was combined with 0.75 ml working buffer and 0.1 ml 6 mM 5,5'-dithiobis(2-nitrobenzoic acid) in working buffer. After equilibration for 1 min at 25^0C, 50 ul GR (20 units/ml) was added and the absorbance at 412 nm followed for 4 min. To assay for GSSG, the 2-vinylpyridine treated supernatant was assayed in a similar manner except that 50 ul sample and 50 ul water was used. Standards containing known amounts of GSH or GSSG and an appropriate amount of 5-sulfosalycylic acid were assayed concurrently with supernatant samples to establish standard curves. The glutathione concentration was calculated using the rate of change in absorbance and by comparing samples with the standard curve.

4.3 Results

4.3.1 Lipid Peroxidation

The fluorescence spectra (Figure 10) illustrate that all tissue extracts from insects on low vitamin E diets, including controls, exhibit some fluorescent damage products of LPO. Characteristic broad emission peaks of Schiff bases were observed

Figure 10: Fluoresence spectra of midgut tissue extracts from larvae of *Manduca sexta* receiving either control (UV), α-T, or α-T + UV treatments. Left, excitation spectra; right, emission spectra.


INTENSITY (relative fluorescence units)
230
200
160
120
80
40
0
300
340
380
420
460
500
WAVELENGTH (nm)
α-T + UV
α-T
UV
α-T + UV
α-T
UV

at 410-460 nm as a result of excitation in the range 330-370 nm. In preliminary experiments (data not shown) there was no difference between fluorescence in +UV or -UV controls. α-T did not enhance fluorescence very much compared to UV controls however a large, UV-potentiated increase in fluorescence was observed in tissues treated with α-T +UV. Qualitatively similar peaks have been observed in mosquito tissue extracts (Hasspieler *et al.*, 1990) where rigorous controls have demonstrated that the increase in fluorescence due to α-T treatment is not due to the inherent fluorescent properties of α-T itself. As well, we have observed (results not shown) similar increases in fluorescence caused by hypericin, a chemically very different phototoxin.

On low vitamin E diets (Table 9), α-T significantly increased the formation of fluorescent damage products, particularly in the presence of UV where levels increased nearly two-fold. In the high vitamin E diets however, the increase in flourescence was not observed, which is evidence that the protective effects of this molecule operate in herbivorous insects in a similar manner to other systems.

4.3.2 Effect of α-T on Antioxidant Enzymes

Figures 11-13 illustrate the effect of the phototoxin α-T on midgut antioxidant enzyme activity. GPOX activity was observed to increase after 24 to 48 h of ingestion of α-T in the absence of UV. In contrast, the GPOX activity of α-T +UV groups

Table 9: Effect of α-T and vitamin E on the formation of fluorescent products of lipid peroxidation in midgut tissue of *Manduca sexta*.*

a) Low vitamin E diet (< 1 ug/g)		
	-UV	+UV
Controls	417.8 ± 102.3^{b}	462.5 ± 84.3^{b}
α-T	561.3 ± 70.6^{a}	881.8 ± 155.6^{a}
b) High vitamin E diet (1 mg/g)		
	-UV	+UV
Controls	326.5 ± 46.0^{bc}	317.2 ± 16.7^{c}
α-T	266.4 ± 31.2^{c}	118.5 ± 35.9^{d}

*units are relative fluorescence units per 100 mg tissue and represent means ± S.D. of 3-5 samples. Using a simple one-way ANOVA, means in each vertical column were compared using a Tukeys test. Values followed by the same letter are not significantly different. A standard solution of quinine sulfate (1 ug/ml) in 0.1 N H_2SO_4 gave a fluorescence intensity reading of 94.0.

Figure 11: Time dependent effects of the phototoxin α-T on GPOX activity in midgut tissue of *Manduca sexta*. Values are means $\pm$ S.D. for 4-5 samples.

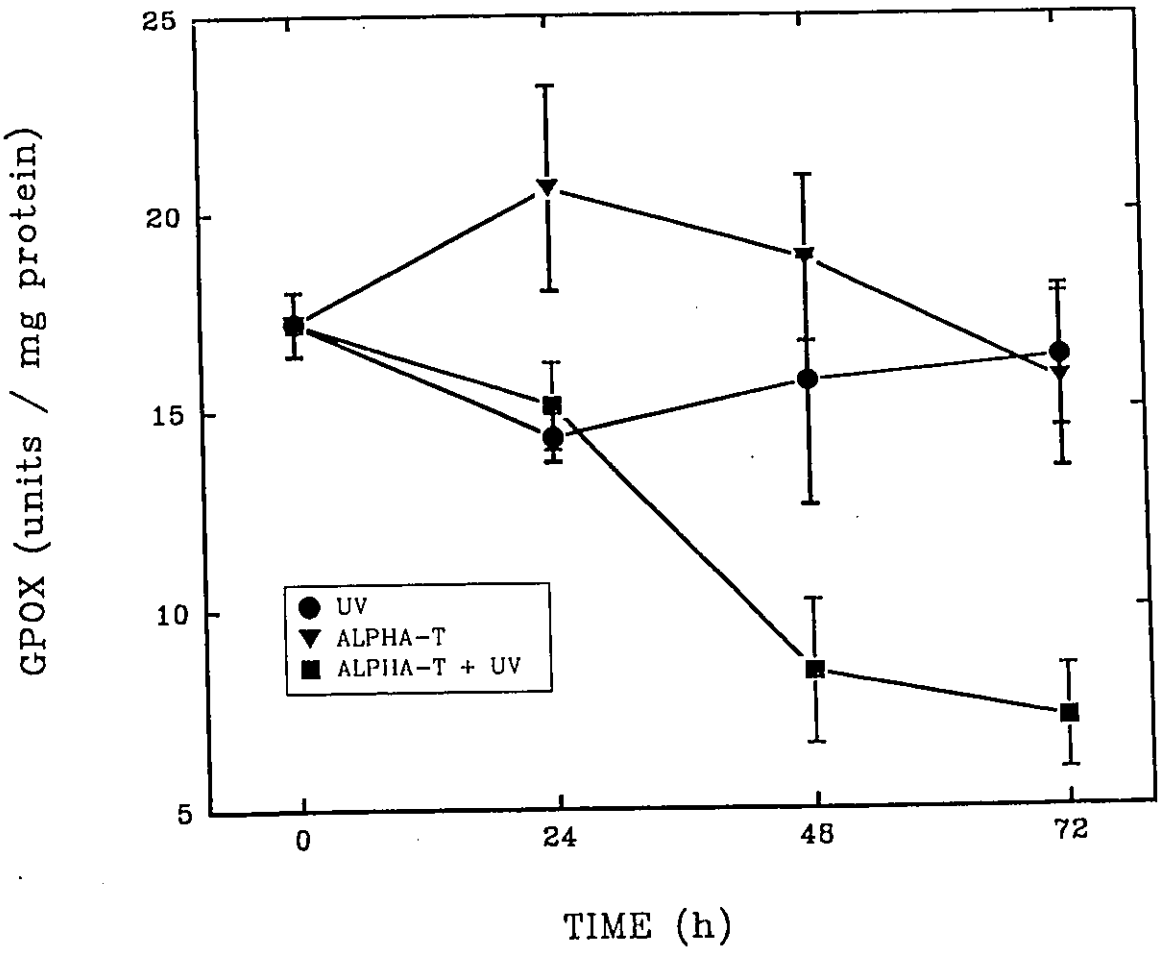
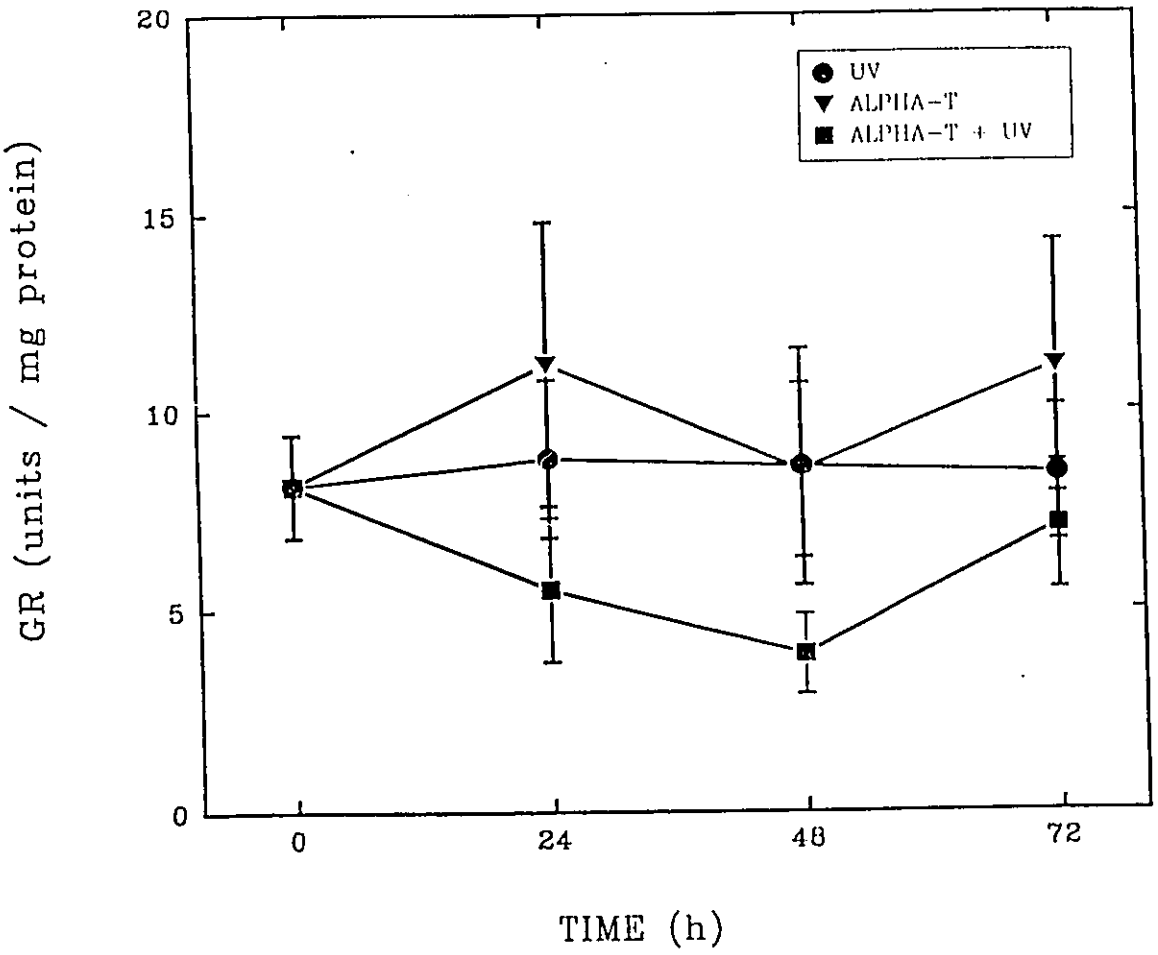

GPOX (units / mg protein)
25
20
15
10
5
0
24
48
72
TIME (h)
UV
ALPHA-T
ALPHA-T + UV

Figure 12: Time dependent effects of the phtotoxin α-T on GR activity in midgut tissue of *Manduca sexta*. Values are means $\pm$ S.D. of 4-5 samples.


20
15
10
5
0
GR (units / mg protein)
UV
ALPHA-T
ALPHA-T + UV
0
24
48
72
TIME (h)

gradually declined and by 72 h was less than half that of controls (7.2 vs. 16.3 units)(Figure 11).

GR activity over the 72 h ingestion period showed a similar pattern (Figure 12). α-T +UV groups had the lowest activity which tended to decline with time while the α-T -UV groups were not significantly different from controls.

PER activity was observed to increase greater than 3-fold (0.46 to 1.57 units) over the 24 to 72 h period in the α-T -UV groups (Figure 13). However both controls and the α-T +UV groups had comparable levels of enzyme activity (0.29 to 0.41 units) throughout this period.

4.3.3 Effect of α-T on Thiol Status

Control groups (+UV) were found to have levels of total glutathione of 53.2 $\pm$ 9.4 ug equivalents GSH per 100 mg midgut tissue (Table 10). GSSG in these groups represented about 1.6% of the total. In groups fed α-T ($\pm$UV), both total and GSH levels were observed to nearly double while GSSG levels declined. This increase in total glutathione produced by increased GSH levels resulted in strong increases in the GSH:GSSG ratio.

Figure 13: Time dependent effects of the phototoxin α-T on PER actiovity in midgut tissue of *Manduca sexta*. Values are means $\pm$ S.D. of 4-6 samples.

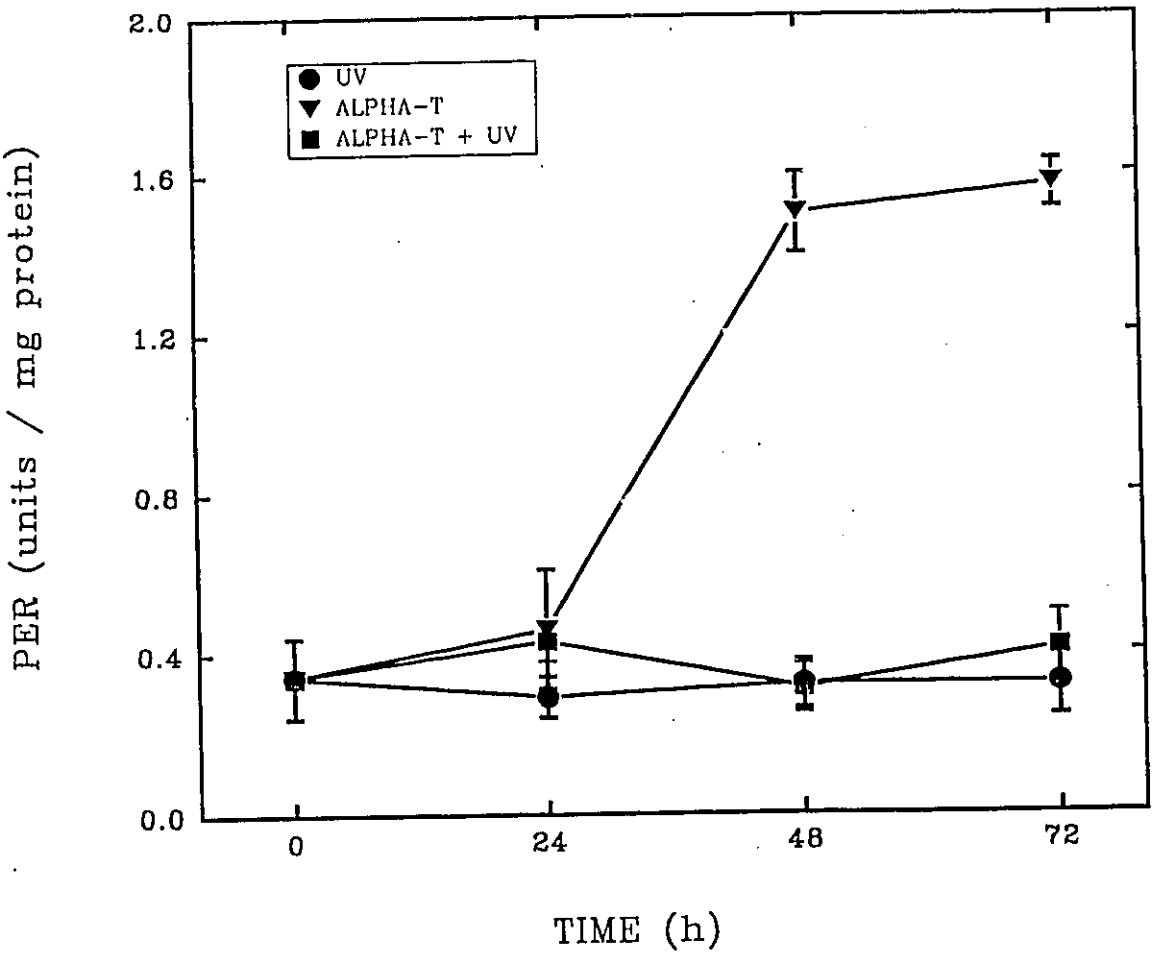

UV
ALPHA-T
ALPHA-T + UV
2.0
1.6
1.2
0.8
0.4
0.0
0
24
48
72
PER (units / mg protein)
TIME (h)

Table 10: Effect of the phototoxin α-T on levels of reduced and oxidized glutathione* in midgut tissues of *M. sexta.**

Treatment	Total	GSH	GSSG	GSSG as % Total	GSH:GSSG
UV	53.2 $\pm$ 9.4[a]	52.4 $\pm$ 9.6[a]	0.84 $\pm$ 0.26[a]	1.6	62:1
α-T	113.9 $\pm$ 19.3[b]	113.3 $\pm$ 19.2[b]	0.57 $\pm$ 0.28[a]	0.5	198:1
α-T + UV	100.6 $\pm$ 24.9[b]	100.2 $\pm$ 22.9[b]	0.44 $\pm$ 0.17[a]	0.4	228:1

*units are ug equivalents GSH per 100 mg tissue and values represent the means $\pm$ S.D. of 8 samples. Using a simple one-way ANOVA, means in each vertical column were compared using a Tukeys test. Values followed by the same letter are not significantly different.

4.4 Discussion

We have demonstrated that ingestion of α-T results in a strong, UV-enhanced increase in fluorescent damage products of LPO in midgut tissue of *M. sexta*. This large increase in LPO (nearly 50% in α-T +UV groups on low vitamin E diets) is likely an important factor in the toxicity of this compound to this species. Sen *et al* (1990) observed that α-T caused a swelling and eventual rupture of midgut epithelium cells in this species. Cell lysis might be caused by an LPO-induced disruption of membrane integrity, resulting in altered ion permeability. This is consistent with the observed *in vitro* effect of potassium leakage from α-T-treated erythrocytes (Wat *et al.*, 1981) and the *in vivo* observation of halide leakage from mosquito larvae (Arnason *et al.*, 1987).

Although LPO is an early symptom of phototoxicity occuring in stressed but still living cells, it may not be the primary toxic event. Oxidation of important membrane proteins and critical -SH groups, may lead to cell damage and subsequent loss from the cell of protective antioxidants and transition metal ions that can stimulate LPO (Gutteridge and Halliwell, 1990). LPO could therefore accompany rather than initiate cell lysis. Our results with vitamin E demonstrate the effectiveness of this lipid-soluble antioxidant in preventing LPO in insect midgut tissue for the first time in a herbivorous insect. Vitamin E is a well known quencher of singlet oxygen and inhibitor of LPO (Halliwell and Gutteridge, 1985) and is believed to prevent LPO mainly by reacting with lipid peroxy radicals and

interrupting the chain reaction process of lipid peroxidation. We have shown recently that dietary lipid soluble antioxidants such as vitamin E and beta-carotene can dramatically alter the acute toxicity of α-T to *M. sexta* (Aucoin *et al.*, 1990). Larvae raised on antioxidant-supplemented diets exhibited a concentration-dependent decline in the mortality associated with topical treatment with α-T. We hypothesized that this protective effect was because vitamin E and beta-carotene are excellent quenchers of singlet oxygen and/or the lipid peroxy radicals formed during propagation of LPO. The present experiments provide biochemical evidence consistent with this model. The sensitivity of insect herbivores to phototoxins may therefore depend to a large degree on the levels of protective antioxidants in their food sources.

In mammals, the primary line of defense against oxidative stress caused by singlet oxygen and free radicals is thought to be antioxidant enzymes such as SOD, CAT, and peroxidases such as GPOX (Halliwell and Gutteridge, 1985). In lepidopteran larvae, a number of these antioxidant enzymes have already been shown to be inducible in response to pro-oxidant or photo-oxidant phytochemicals such as xanthotoxin (Lee and Berenbaum, 1989), quercetin (Pritsos *et al.*, 1988), and hypericin (Aucoin *et al.*, 1990). In a previous study (Aucoin *et al.*, 1990) we demonstrated that phototoxin-tolerant insect herbivores such as specialist feeders on phototoxic plants may have higher constitutive levels of antioxidant enzymes to deal with these compounds. LPO in the present study is likely an important manifestation

of α-T toxicity, and so defensive antioxidant enzymes such as GPOX and GR may be necessary to prevent its progression.

As well, our results show that α-T, in the presence of UV, causes a time-dependent decline in GPOX activity relative to (+UV) controls and this could be a factor in explaining the sensitivity of this species to phototoxins. Also, the control activity of GPOX in this species appears to be somewhat low as compared to a number of more phototoxin-tolerant species such as the black swallowtail *P. polyxenes* (Lee and Berenbaum, 1989). The decline in GPOX activity is most probably a result of direct photo-oxidation of the enzyme since α-T (-UV) had no negative effect on activity and α-T has been shown to photo-inactivate a number of erythrocyte enzymes (e.g Yamamoto *et al.*, 1979). The significant increase in GPOX activity after 24 h (α-T-UV) could represent an induction of GPOX activity however it could also represent induction of GST activity since GPOX in insects is thought to be associated with this same protein (Ahmad *et al.*, 1988). In mammals, GPOX and GST may in fact compete for lipid hydroperoxides (Flohe, 1982).

Our results with the effect of α-T on GR show a similar pattern of activity as with GPOX although this enzyme appears to be less sensitive to photo-oxidation. This could be due to the absence of more readily oxidized -SH groups or perhaps the location of the enzyme itself. In mammals both GR and GPOX are primarily cytosolic enzymes whereas in insects it appears that most activity is found in the non-cytosolic fractions. In *Spodoptera eridania* for example, Ahmad *et al* (1988a) found

that the highest levels of GR activity (5.49 $\pm$ 0.90 units/mg protein) were in the mitochondrial fraction whereas no GR activity was detected in the cytosol. In *Trichoplusia ni*, GPOX activity was found to be highest in the nuclear and microsomal fractions while being absent in mitochondria (Ahmad *et al.*,1988b). The presence of high GR activity (and absence of GPOX) in mitochondria may be necessary to prevent GSSG accumulation since thiol status is apparently critical to mitochondrial integrity and proper function (Ahmad *et al.*, 1988a). The relative insensitivity of GR to photo-oxidation may therefore be beneficial.

We also examined the effect of α-T on a general, non-GSH dependent peroxidase (PER) which has previously been examined in the housefly (Armstrong *et al.*, 1978) and the granary weevil (Boulter *et al.*, 1990). Its ability to remove a wide range of peroxides including hydrogen peroxide may be important given that the insect GPOX appears to have little or no activity against this substrate (Ahmad *et al*, 1989). As well, we have observed (results not shown) that α-T rapidly inhibits CAT activity in *M. sexta*. Our results indicate that α-T -UV can induce a large (greater than 3-fold) increase in PER activity. This increase was not observed however in the α-T +UV groups, suggesting that the enzyme is very sensitive to photo-oxidation.

One of the manifestations of oxidative stress may be a perturbation of cellular thiol status (Halliwell and Gutteridge, 1985). With strong photo-oxidants such as α-T, we might expect thiol oxidation to occur resulting in increased GSSG levels and a concomitant decrease in GSH. These changes would affect the operation of GPOX

(and perhaps GST) and likely increase the susceptibility of tissues to peroxidative damage. In our experiments however, we observed the opposite i.e. that α-T ($\pm$ UV) induced an increase in both total and GSH levels while GSSG declined slightly. Similar results have been obtained in houseflies treated with paraquat (Allen *et al*, 1984), a strong generator of superoxide radicals. As well, both the CAT inhibitor 3AT and the SOD inhibitor DEDC, agents which would be expected to increase the level of oxidative stress in tissues, have been shown in houseflies to increase GSH while having little effect on GSSG levels (Allen *et al.*, 1983; Sohal *et al.*, 1984). Both the α-T-induced and paraquat-induced increases in GSH were not accompanied by corresponding increases in GR, ruling out an induction of GR activity as the reason for the increased GSH. The increased GSH could also be the result of increased GST activity and this area should be investigated further. The relative insensitivity of GR to photo-oxidation (Figure 7) could mean that any photo-oxidation of GSH to GSSG by α-T is quickly compensated for by high GR activity. The lack of a photo-oxidative effect on GSH levels might also be related in part to the localization of the very lipophilic α-T molecule in membrane compartments where GSH levels would be expected to be lower than in the cytosol. Increased GSH is however, clearly in response to the allelochemical alone since the photosensitizing conditions (α-T +UV) were not significantly different from non-photosensitizing (α-T -UV) conditions.

In summary, we have demonstrated that the phototoxin α-T causes a strong

increase in LPO in midgut tissue of *M. sexta* and this is likely an important factor in its toxicity. It may also explain in part the previous observation of damaged midgut epithelial cells. The relatively low levels of GPOX, and the failure of GPOX, GR, and PER to adequately respond (i.e. with no observed induction and only declines in activity under photosensitizing conditions), would contribute to increased LPO and oxidation of other important biomolecules. These results are consistent with the sensitivity of *M. sexta* to phototoxins. This work also demonstrates the ability of photo-oxidants to destroy important enzymatic targets. α-T clearly perturbs cellular thiol status although the mechanism by which this occurs is not fully understood.

Chapter 5: Effect of Antioxidant Enzyme Inhibitors and Glutathione Depletion on the Toxicity of α-Terthienyl.

5.1 Introduction

Insect herbivores must overcome a diverse array of defensive phytochemicals in their diets. The ability of insects to deal with these often toxic compounds has been accomplished primarily through the evolution of highly developed detoxification enzyme systems (e.g. PSMOs, GST) which metabolically degrade foreign substances to more hydrophilic and hence more readily excretable compounds (Brattsten, 1979). Some of these phytochemicals are, however, also phototoxic, that is their toxicity is greatly enhanced in the presence of light (usually in the near UV range). These phototoxins produce highly toxic, activated species of oxygen such as singlet oxygen and free radicals such as superoxide and hydroxy anions which are capable of serious cellular and genetic damage. Thus many insect herbivores must face the double challenge of dealing with these allelochemicals as potentially lethal poisons as well as the possibility that they may also create severe oxidative stress.

All aerobic organisms have evolved both enzymatic and non-enzymatic defense mechanisms to remove activated oxygen species and free radicals which arise through normal respiratory and metabolic processes or in response to a number of environmental agents (Fridovich, 1977; Larson and Berenbaum, 1988). In mammals, major antioxidative defense enzymes include SOD, CAT, GR, and GPOX which act to remove potentially harmful oxygen species and /or peroxides (Halliwell and Gutteridge, 1985). In insects, a

number of these antioxidant enzymes have recently been examined in terms of their ability to provide protection to insect herbivores feeding on phototoxin-containing plants. Insect herbivores specializing on phototoxin-containing plants appear to have higher constitutive levels of antioxidant enzymes (Ahmad *et al.*, 1988a; Aucoin *et al.*, 1991a). Phototoxin ingestion may also induce higher activities of some of these enzymes. For example, xanthotoxin induces higher levels of SOD, CAT, and GPOX activity when fed to the cabbage looper *T. ni.* (Lee and Berenbaum, 1989). Larvae of the phototoxin specialist *A. plagiata* have been observed to have higher levels of CAT and GR activity when fed the hypericin-containing *H. perforatum* but not when fed the hypericin-free *H. calycinum* (Aucoin *et al.*, 1991a). It appears that some antioxidant enzymes may be inducible defenses against phototoxins in insects although it remains to be shown that they are in fact necessary defenses.

Endogenous antioxidants are another important line of defense insect herbivores have against phototoxins (Larson, 1986; Aucoin *et al.*, 1990). Beta-carotene and vitamin E, for example are excellent scavengers of activated oxygen species (Foote, 1986) and have been shown to completely protect against the phototoxicity of the thiophene α-T to *M. sexta* (Aucoin *et al.*, 1990). In the case of vitamin E this may be due to its ability to strongly protect against lipid peroxidation (Aucoin *et al.*, 1991b). Many other antioxidants in insects may also be important defenses against the ingestion of phototoxins including such compounds as uric acid and reduced glutathione (Larson, 1986; Aucoin *et al.*, 1990) however the role of these antioxidants has not been explored.

In this report we investigate the role of two antioxidant enzymes, SOD and CAT,

and whether they offer some degree of protection to lepidopterous larvae challenged with the phototoxin α-T. As well, we examine the role of reduced glutathione (GSH). It may be a particularly important molecule because of its antioxidant function as well as its role in the GSH-dependent antioxidant enzymes GR and GPOX and the detoxification enzyme GST. With the use of relatively specific inhibitors, we examine whether SOD, CAT, and GSH are important defenses against exposure to α-T.

5.2 Methods and Materials

5.2.1 Chemicals

All chemicals, reagents, and co-factors were obtained from Sigma Chemical Co., St.Louis, Miss.

5.2.2 The Effectiveness of Antioxidant and Antioxidant Enzyme Inhibitors

To assess the effectiveness of antioxidant and antioxidant enzyme inhibitors, third instar larvae of *M. sexta* were raised for up to 72 h on diets containing either the SOD inhibitor diethyldithiocarbamate (DEDC)(0, 2, 20 ug/g), the CAT inhibitor 3-amino-triazole (3AT)(0, 20, 200 ug/g), or the GSH synthesis inhibitor buthionine sulfoximine (BSO)(500 ug/g). Larvae were then dissected and midgut tissue examined for SOD and CAT activity as previously described (Aucoin *et al.*, 1991a; section 2.2). Separate samples

were used to examine the effectiveness of BSO in depleting GSH levels in midgut tissue. GSH levels were assayed according to the method of Griffith (1980). In separate, 72 h experiments, larvae were raised on similar diets and the growth and cumulative mortality examined to ensure the compounds were not overly toxic.

5.2.3 Phototoxicity of α-T

In order to examine the toxicity of α-T to larvae with impaired antioxidant defenses, groups of ten, third instar larvae (mean weight 60-80 mg) were raised on diets containing the inhibitors for up tp 72 h and then sprayed with α-T using a Potters tower (see Aucoin *et al.*, 1990; section 3.2.2). In the case of DEDC and 3AT, the level of compound used was that which caused approximately a 50% reduction in enzyme activity within 48 h. The level of α-T used was an application rate of 1.31 $\pm$ 0.37 ug/cm^2, a level which results in approximately 50% mortality in treated groups. After treatment, larvae were placed under a bank of near-UV lamps (300-400nm) for 8 h and then returned to the incubator. Mortality assessments were then made at 24 and 48 h after spraying.

5.2.4 Long-term Toxicity of α-T to Larvae with Depleted GSH

To assess the role of GSH in preventing the longer-term toxicity of α-T to *M. sexta*, larvae were raised for 72 h on diets containing α-T in the presence or absence of BSO (500 ug/g)(control $\pm$ UV, BSO $\pm$ UV, α-T $\pm$ UV, and BSO + α-T $\pm$ UV).

Growth and development of larvae on these diets was observed as was cumulative mortality. The effect of GSH depletion on LPO was also examined using the method of Dillard and Tappel (1984) as previously described (section 4.2.2).

5.3 Results

5.3.1 Effectiveness of the Inhibitors DEDC, 3AT, and BSO.

Both DEDC and 3AT were found to be excellent inhibitors of SOD and CAT activity respectively (figures 14 and 15). Within 48 h, 20 ug/g DEDC reduced the level of SOD activity in midgut tissue by approximately 50% (from 9.6 $\pm$ 1.6 to 5.2 $\pm$ 1.7 units)(figure 14). 3AT caused a 50% decline in CAT activity after 24 h using 200 ug/g of the compound in the diet although there was some recovery of activity at 48 h.

BSO caused a strong reduction in GSH levels in midgut tissues (table 11) with the treated groups (72 h) showing 98% lower GSH levels than controls. GSSG levels were also reduced by 84%.

Inhibitor treatments with DEDC (20 ug/g), 3AT (200 ug/g), and BSO (500 ug/g) had only slight effects on the growth and development of larvae (table 12). Weight gain was inhibited by a maximum of only 20% in treated groups, possibly due to antifeedant effects. As well, cumulative mortality in the treated groups was never more than 5%, a level of mortality commonly seen in controls.

Figure 14: Effect of the inhibitor diethyldithiocarbamate (DEDC) on superoxide dismutase (SOD) activity in third instar larvae of *M. sexta*.

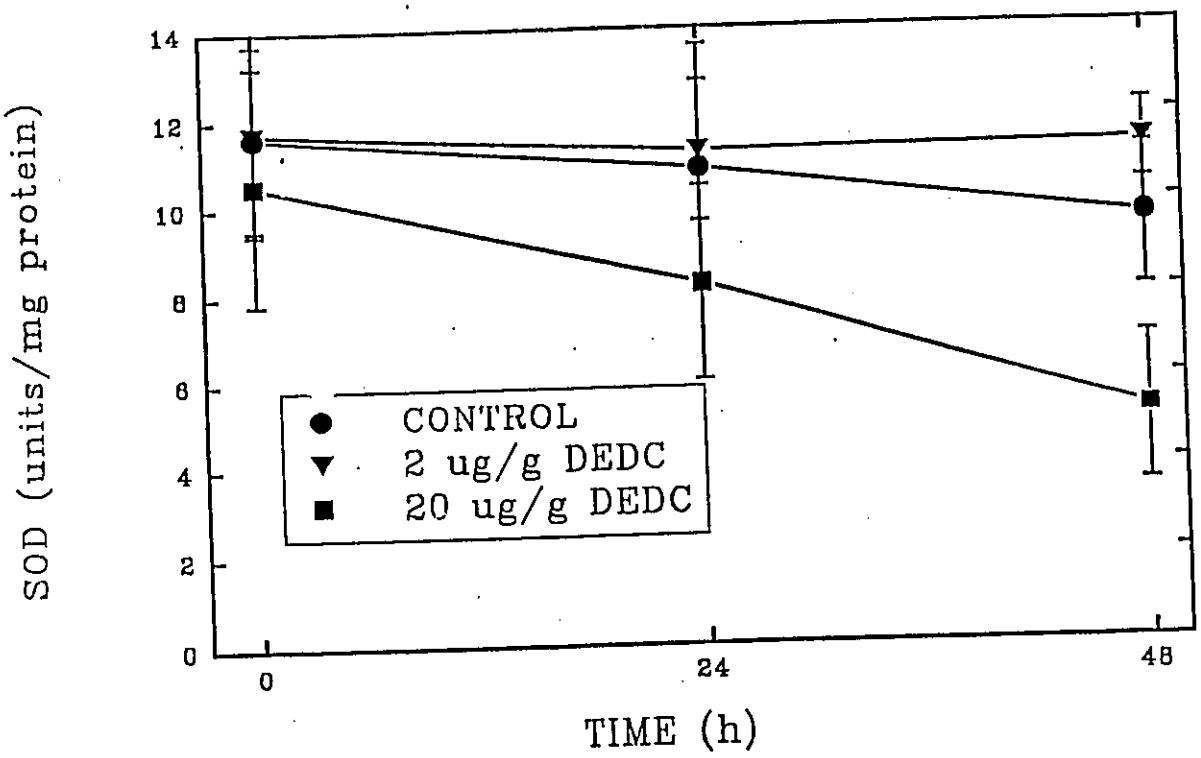

SOD (units/mg protein)
14
12
10
8
6
4
2
0
0
24
48
TIME (h)
CONTROL
2 ug/g DEDC
20 ug/g DEDC

Figure 15: Effect of the inhibitor 3-amino-triazole (3AT) on catalase (CAT) activity in third instar larvae of *M. sexta*.

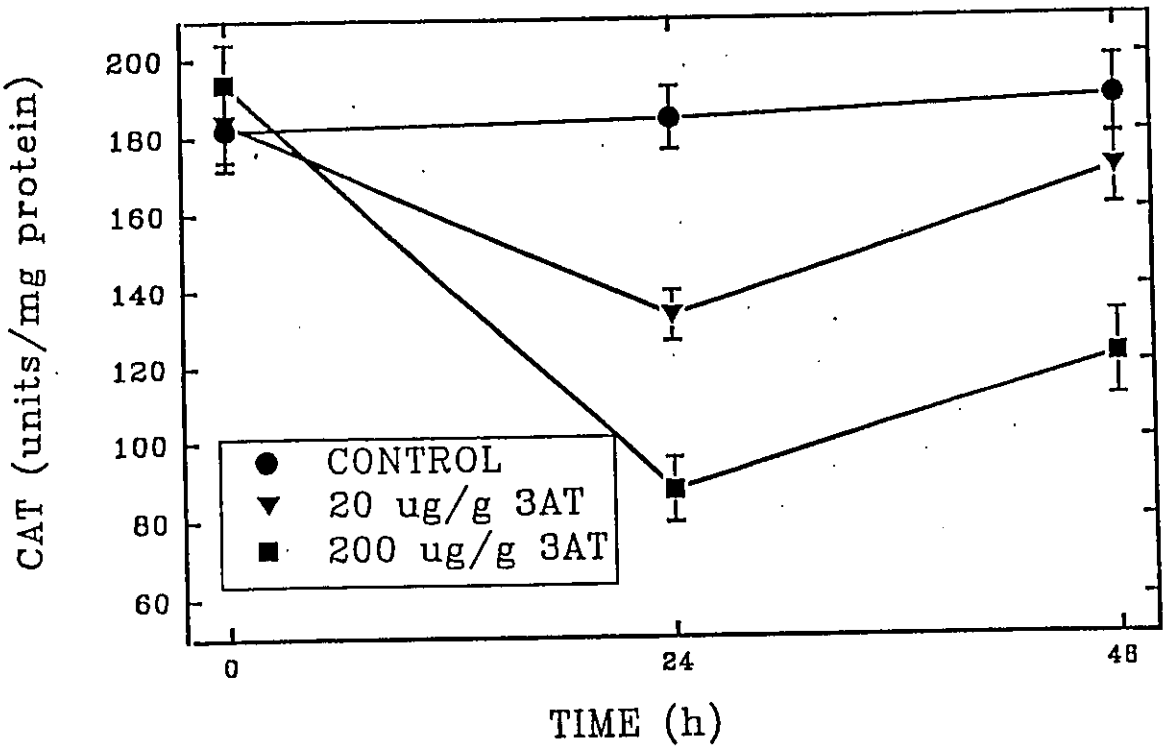

CAT (units/mg protein)
200
180
160
140
120
100
80
60
0
24
48
TIME (h)
CONTROL
20 ug/g 3AT
200 ug/g 3AT

Table 11: Effect of buthionine sulfoximine (BSO) on glutathione levels in midgut tissue of third instar *Manduca sexta*.*

Treatment	Total	GSH	GSSG	GSSG as % Total	GSH:GSSG
Controls	49.6 ± 1.6	48.8 ± 1.9	0.86 ± 0.38	1.7	58:1
BSO	3.4 ± 0.8	3.3 ± 0.8	0.14 ± 0.07	4.1	24:1
% inhibition	93	98	84	-	-

* values represent means ± S.D. of 4 samples and are ug equivalents GSH per 100 mg tissue.

Table 12: Effect of antioxidant and antioxidant enzyme inhibitors on the growth of third instar *Manduca sexta* larvae.

Treatment	72 h weight gain (mg)*	as % of controls	cumulative mortality (%)
Control	85.9 ± 6.2	100	0
DEDC	68.2 ± 5.8	79	5
3AT	71.3 ± 3.6	83	5
BSO	69.9 ± 5.2	81	5

*weight gain values represent means ± S.D. of 37 to 45 larvae per treatment.

5.3.2 Effect of Antioxidant/Antioxidant Enzyme Inhibitors on the Acute Toxicity of α-T.

In order to assess the effects of impaired antioxidant defenses on the short-term toxicity of α-T, treated larvae were sprayed with an LD_{50} level of the phototoxin. Table 13 shows the results of dietary inhibitors on the 24 and 48 h mortalities of larvae treated in the spray tower. In all cases the groups receiving the inhibitors did not show significantly higher levels of mortality when compared to controls.

5.3.3 Effect of GSH Depletion on the Toxicity of Ingested α-T and on Lipid Peroxidation in Midgut Tissues

The presence of dietary inhibitors did not affect the acute toxicity of topically applied α-T, however it is possible that antioxidant defenses may be more important under long-term, chronic oxidative stress. Therefore we decided to investigate the longer term effects of one inhibitor, BSO, on the chronic toxicity of ingested α-T. In order to ensure BSO was itself not overly toxic, it was first necessary to examine the effect of BSO on growth and cumulative mortality. Table 14 indicates that BSO causes approximately a 25% reduction in weight gain however it does not increase mortality. α-T itself causes a substantial decline in weight gain over the 72 h but only in the presence of UV was significant mortality (25%) observed. The combination of BSO and α-T (+UV) had the most dramatic effect on weight gain (a reduction to only 5% of controls) and cumulative

Table 13: Effect of antioxidant and antioxidant enzyme inhibitors on the acute (24 h) toxicity of topically applied α-T to *Manduca sexta*.

	% Mortality			
Treatment	24 h	(n)*	48 h	(n)
Control	62.2	18	65.5	8
DEDC	59.0	10	64.0	10
3AT	61.0	8	62.0	8
BSO	52.0	10	70.0	10

* (n) = number of replicate groups of ten larvae treated with α-T in the Potters spray tower.

Table 14: Effect of buthionine sulfoximine on the chronic (72 h) toxicity of α-T fed third instar *Manduca sexta* larvae.

Treatment	72 h weight gain (mg)*	as % controls	cumulative mortality
-UV	79.2 ± 7.8	100	0
+UV	77.6 ± 8.6	98	0
BSO (-UV)	58.3 ± 7.3	74	0
BSO (+UV)	61.5 ± 6.9	78	5
α-T (-UV)	55.0 ± 5.8	69	0
α-T (+UV)	18.8 ± 5.9	24	25
BSO+α-T (-UV)	18.4 ± 5.1	23	0
BSO+α-T (+UV)	3.6 ± 3.0	5	45

* values represent the combined results of 2 experiments and are means ± S.D. of 21 to 40 larvae per treatment.

mortality (45%).

GSH depletion also strongly influenced αT-induced LPO in midgut tissues of *M. sexta*. Table 15 demonstrates that BSO alone does not cause increased formation of fluorescent products associated with LPO whereas BSO plus α-T causes a clearly significant, four-fold increase in fluorescence.

5.4 Discussion

Both the SOD inhibitor DEDC, and the CAT inhibitor 3AT were very effective at reducing the level of activity of these enzymes. DEDC, a copper chelator which has been shown to inhibit SOD activity *in vitro* and *in vivo* (Heikkila *et al.*, 1976), reduced SOD activity in whole body samples by approximately 50% in 48 h without any apparent toxicity to *M. sexta*. A similar reduction in CAT activity was obtained using 200 ug/g 3AT, an indirect and irreversible inactivator of this enzyme (Williams *et al.*,1985). However neither inhibitor significantly affected the level of mortality associated with topical treatment of larvae with α-T.

In *M. sexta*, we observed (results not shown) that long-term treatments with SOD and CAT inhibitors resulted in very high mortality making an evaluation of the long-term, protective effects of these antioxidant enzymes against phototoxin exposure very difficult. The inability of SOD and CAT activity reduction to increase the toxicity of α-T may be due to a number of factors. For example it is possible that the inhibitors were localized mainly in sites different from those occupied by α-T. Mortality due to topical α-T may

Table 15: Effect of glutathione depletion on α-T-induced lipid peroxidation in midgut tissues of *Manduca sexta*.*

Treatment	-UV	+UV
Controls	68.2 $\pm$ 5.7[a]	61.9 $\pm$ 7.1[a]
BSO	59.3 $\pm$ 4.1[a]	63.6 $\pm$ 7.0[a]
α-T	56.6 $\pm$ 5.0[a]	158.6 $\pm$ 21.8[b]
BSO+α-T	51.5 $\pm$ 6.0[a]	416.1 $\pm$ 38.7[c]

* values represent means $\pm$ S.D. of 4 samples and are relative fluorescence units per 100 mg tissue. A standard solution of 1 ug/ml quinine sulfate in 0.1 N H_2SO_4 gave a fluorescence intensity of 94.0. Values followed by the same letter are not significantly different.

be closely related to the striking black cuticular lesions caused by this phototoxin (Downum, 1984; Champagne *et al.*, 1986) while inhibitors may be acting primarily on midgut tissues. Perhaps more importantly, there may be a threshold level of SOD and CAT activity which is sufficient to overcome the added oxidative stress induced by α-T. In *D. melanogaster* for example, a number of catalase-deficient mutants have been isolated whose viability is not affected until nearly 98% of CAT activity is lost (MacKay and Bewley, 1989). Insect herbivores with high constitutive levels of antioxidant enzymes (e.g. many specialist feeders on phototoxin-containing plants) or those with inducible activities may be suitably protected against phototoxin ingestion. Another factor is the relative amounts (and sensitivities to) free radicals and/or singlet oxygen produced by the phototoxin. α-T is known to be a strong producer of singlet oxygen (Scaiano *et al.*,1989) however a significant amount of superoxide anions are also produced (Kagan *et al.*, 1989)

The relatively non-toxic GSH synthesis (glutamylcysteine synthetase) inhibitor BSO (Griffith and Miester, 1979) was very effective at depleting tissue GSH levels in *M. sexta* without demonstrating any adverse toxic effects. Dietary administration of BSO did not, however, affect the toxicity of α-T in the short term. In the longer term (72 h) tests however, BSO strongly affected both weight gain and mortality of larvae exposed to dietary α-T. The fact that GSH depletion in midgut tissues strongly increased mortality suggests that this is a major site of α-T-induced damage. These results demonstrate the importance of GSH in the toxicity of α-T, although we cannot determine whether GSH itself is acting as an antioxidant, or whether the increased toxicity is due to an impairment of GSH-dependent enzymatic defenses such as GPOX,GR and/or GST. GST may be an

important detoxification enzyme which could reduce the toxicity of ingested α-T through a GSH-conjugation mechanism. Both enzymes are also capable of removing a wide range of potentially harmful organic and lipid peroxides (Mannervik, 1985). One of the apparent manifestations of oxidative stress is increased LPO so any impairment of these defenses might lead to increased susceptibility to LPO and potentially lethal cellular damage. Our results in this study confirm our previous findings that α-T induces increased LPO in midgut tissues of *M. sexta* (Aucoin *et al.*,1991b) and demonstrate that GSH depletion strongly enhances this effect. Thiol status is known to be an important factor in the proper function and integrity of the cell (Halliwell and Gutteridge, 1985) and any perturbation of this status (such as might be caused by α-T) might be expected to affect the sensitivity of the cells to oxidative stress. α-T does affect cellular thiol status in *M. sexta* midgut tissue (Aucoin *et al.*, 1991b) however the mechanism by which this occurs is not known.

In summary, we have demonstrated that neither inhibition of the antioxidant enzymes SOD and CAT nor depletion of GSH affects the acute toxicity of topically applied α-T to *M. sexta*. In contrast, depletion of GSH levels strongly enhances both the extent of LPO in midgut tissues as well as the overall toxicity of α-T when larvae were fed the toxin over a 72 h period. Further research is necessary to determine the relative importance of GSH as an antioxidant and the GSH-dependent activities of GPOX and GST in protecting insect herbivores against oxidative stress.

CHAPTER 6: GENERAL DISCUSSION

6.0 Introduction

The rationale for this study was based on the knowledge that insect herbivores have evolved a wide variety of biochemical and physiological mechanisms (e.g. inducible PSMOs) which have enabled them to exploit plants containing potentially toxic chemicals. Phototoxins are a unique class of these phytochemicals and we felt it logical to hypothesize that insect herbivores feeding on plants containing these photo-oxidants (particularly specialist feeders) should have evolved specific defensive mechanisms. In the case of strong photo-oxidants such as α-T or hypericin, which produce highly toxic activated species of oxygen, specific defenses would likely include antioxidant enzymes (e.g. SOD, CAT, GR, GPOX, PER) as well as endogenous antioxidants (e.g. vitamin E and beta-carotene) which may or not be derived from the insect's diet. The mode of action of these photo-oxidants (i.e. oxidation of lipids, proteins and nucleic acids) led us to investigate whether these antioxidants and antioxidant enzymes might offer a significant level of protection against this damage at the biochemical level.

The results reported in the previous chapters support and provide strong evidence for our contention that insect herbivores make use of both antioxidants and antioxidant enzymes to protect against phototoxicity. In those insects adapted to

specialize on phototoxic plants, these defenses may be particularly important in reducing the potential toxicity of these compounds and may in some cases have accounted for their ability to develop successfully on these plants. In the following discussion, the significance and implications of key results will be addressed.

6.1 The Role of Antioxidant Enzymes

The antioxidant enzymes examined in this study were SOD, CAT, GPOX, GR, and PER. In the case of the GSH dependent GPOX, no activity was found using H_2O_2 as substrate (sect 2.4.1) whereas considerable activity was observed using cumOOH as substrate (Chapter 4) and this is consistent with the work of others (e.g. Pritsos *et al.*, 1988, Lee and Berenbaum, 1990, Simmons *et al.*, 1989). The GPOX activity appears to be closely associated with a non Se-dependent GST which accepts H_2O_2 and a wide variety of peroxides (Ahmad and Pardini, 1989, Simmons *et al.*, 1989). Because a major consequence of photo-oxidative damage is LPO, both GSH-dependent peroxidases (GPOX) and non GSH-dependent peroxidases (e.g. PER) may be important cellular defenses.

It has been suggested (Pritsos *et al.*, 1988, Pardini, 1989) that specialist feeders on phototoxic plants might have higher constitutive levels of antioxidant enzyme activity as compared to non-specialists that act as a defense against ingestion of photo-oxidants. Pardini *et al.* (1988) have, for example, shown that in the specialist feeder *P. polyxenes*, SOD activity is generally higher than in the generalists *T. ni* and *S. eridania*. Our results (Chapter 2) support this hypothesis as the hypericin specialist

A. plagiata was found to have significantly higher levels of SOD and CAT activity when compared to the two generalists *O. nubilalis* and *M. sexta*. Although these studies only represent a small number of species, they suggest that high constitutive levels of antioxidant enzyme activity may be a defense mechanism. Many plant compounds have been shown to induce enzymatic detoxification mechanisms (e.g. PSMOs, GST) in insect herbivores (see for example Yu, 1983) and so it is logical that antioxidant enzyme defenses might also be inducible.

Recently, a number of plant-derived photo-oxidants have been examined for their ability to induce antioxidant enzyme activity in lepidopterous larvae (see Table 16). In many cases it appears that the activity of a number of enzymes are increased upon ingestion of the compounds, particularly in the longer-term studies. Our results with hypericin (Chapter 2) indicate that this compound induces both CAT and GR activity in *A. plagiata* and this may be an adaptation which may has allowed this species to specialize on hypericin-containing plants. In many cases however, particularly in the short-term studies, it is apparent that many antioxidant enzymes are inhibited by these compounds. We have suggested (section 2.4.2) that this inhibition is caused by the generation of free radicals or singlet oxygen leading to photo-oxidation or perhaps direct inhibition. Our results with α-T (Chapter 4) have also demonstrated inhibition of the antioxidant enzymes PER, GPOX and GR; enzymes which may be particularly important defenses against $^1O_2$-induced LPO. α-T rapidly induces LPO in M. sexta midgut tissue (Chapter 4) while inhibiting antioxidant enzyme activity. The fact that phototoxins can strongly inhibit these

Table 16: Effect of Plant-Derived Pro-oxidants on Antioxidant Enzyme Activity in Lepidopterous Larvae.*

Species	Compound	Time Period and Effect on Enzymes (+, -, N/C)		References
		Short Term (1-12h)	Long Term (12-48h)	
Papilio polyxenes	Quercetin	SOD +		Pritsos *et al.*, 1988
		CAT -		
		GR -		
Trichoplusia ni	Xanthotoxin	SOD +	SOD +	Ahmad *et al.*, 1988b
		CAT -	CAT +	Lee and Berenbaum, 1989
		GR -	GPOX +	
Spodoptera eridania	Quercetin, Xanthotoxin	GPOX -		Ahmad *et al.*, 1989
Anaitis plagiata	Hypericin	SOD -	SOD N/C	Aucoin *et al.*, 1991a
		CAT -	CAT +	
		GR -	GR +	
Depressaria pastinacella	Xanthotoxin**		SOD +	Lee and Berenbaum, 1990
			CAT N/C	
			GR N/C	
	Bergapten, Imperatorin		ALL N/C	
Manduca sexta	α-T		CAT _	Aucoin *et al.*, (unpublished observations)
			GR -	Aucoin *et al*, 1991b
			GPOX -	
			PER -	

* all results represent the effect of compounds administered in the diet in the presence of UV.
** includes piperonyl butoxide

antioxidative defenses implies that many phytophagous insects, particularly those specialized on phototoxic plants, may need high constitutive or inducible levels of enzyme activity if they are to act as an effective defense. Insect herbivores which are very sensitive to phototoxins (e.g. *M. sexta*) may lack sufficient antioxidative defenses. In *M. sexta* at least, LPO appears to be an important manifestation of α-T toxicity and is associated with reduced antioxidant enzyme activity.

In a number of cases it has been shown that phototoxins, as with many other allelochemicals, are capable of inducing detoxification enzyme activity (see section 3.2.2.1) and it has been suggested (Lee and Berenbaum, 1989, Aucoin *et al.* 1991a) that the antioxidant enzyme system and the P450-based detoxification system work in concert to prevent oxidative damage. In the case of furanocoumarins, it appears that the activity of P450-based detoxification mechanisms are crucial in determining the sensitivity and tolerance of insect herbivores (Lee and Berenbaum, 1989). In *D. pastinacella*, xanthotoxin is rapidly metabolized by the P450 system (Nitao, 1989) and appears to have little effect on antioxidant enzymes (Lee and Berenbaum, 1990). However if xanthotoxin is administered together with the P450 inhibitor piperonyl butoxide, SOD activity is significantly increased. This suggests that unmetabolised xanthotoxin may be accumulating and generating activated oxygen species which in turn induce antioxidant enzyme activity. A similar close association between P450 activity and α-T toxicity is known to exist (section 3.2.2.1). In the case of *M. sexta*, relatively low elimination and metabolism rates (Iyengar et al., 1987; 1990) may account in part for its sensitivity to this phototoxin, as would its relatively low levels

of antioxidant enzyme activity (Aucoin *et al.*, 1990). Further evidence of a close cooperation between P450 and antioxidant enzymes is provided by the fact that GPOX in insects appears to be closely associated with GST (Simmons *et al.*, 1989). Thus activation of GST detoxification mechanisms by allelochemicals may also lead to increased protection against oxidative stress and LPO.

6.2 The Role of Antioxidants

We have pointed out that in insect herbivores (section 3.2.2.3; Table 1) there are many plant-derived or endogenous antioxidants which are excellent scavengers of the activated oxygen species generated by phototoxins. As well, we have shown that supplementing diets with antioxidants can dramatically alter the toxicity of α-T (Aucoin *et al.*, 1990). This implies that for insects feeding on phototoxin-containing plants, phototoxicity may depend in part on the antioxidative constituents of the plants themselves. There are however wide variations in both the plant content of antioxidants and the ability of insects to absorb antioxidants into various tissues (Aucoin *et al.*, 1990). The effectiveness of various antioxidants to act as a defense also depends on a number of other factors including lipophilicity and the nature of the oxygen species produced. Beta-carotene and vitamin E are both good scavengers of $^1O_2$, however vitamin E is also particularly effective at interrupting the free radical initiated chain reactions of LPO. The presence of reducing agents in insect diets may also affect the nature of the oxidative stress. Hartman *et al.* (1988) have, for example, shown that the presence of reducing agents (e.g. uric acid) decreases the

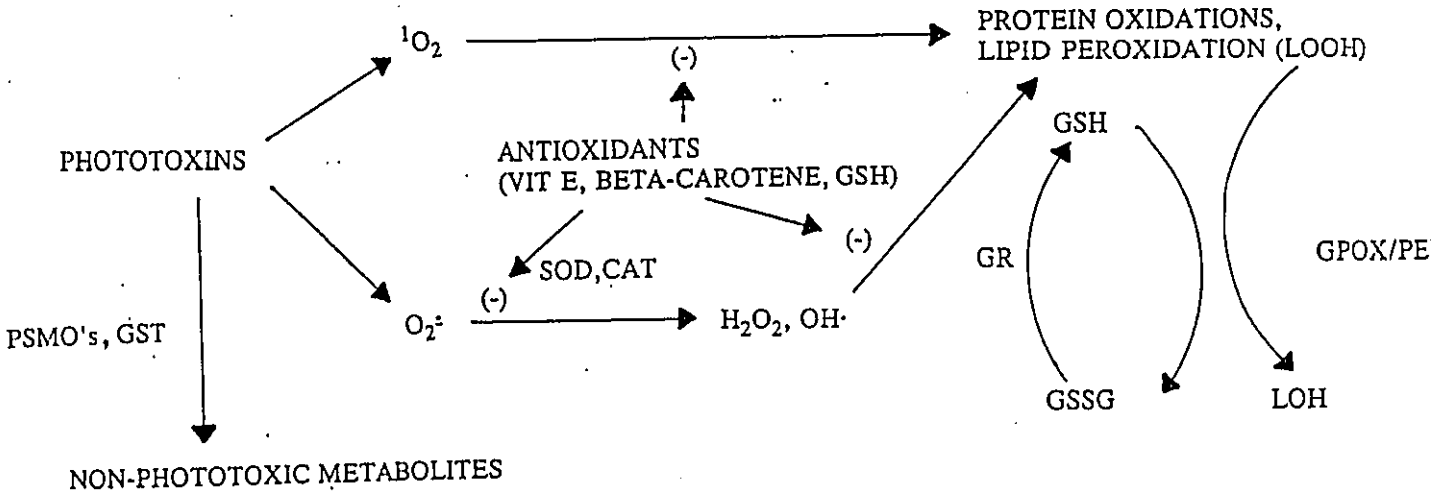
production of singlet oxygen by cercosporin (a fungal pigment closely related to hypericin) while eliciting superoxide production. Thus considerable mechanistic complexity is involved in the effectiveness of antioxidants in preventing or reducing the oxidative stress imposed by ingested phototoxins.

Our results with the GSH-depleting agent BSO (Chapter 5) also implicate GSH as a potentially important antioxidant. GSH has, however, multiple functions in the cell including its role in supplying reducing power for GPOX and GST activity and so its depletion from the cell could have indirect effects as well. The fact that GSH depletion is associated with increased LPO and increased mortality due to α-T treatment strongly suggests that GSH-dependent enzymatic defenses (GST and GPOX) have an important role in the detoxification of α-T. In the case of *M. sexta*, this is particularly likely given this species' inability to respond to α-T ingestion with increased PSMO activity. Our results also provide further evidence that the biochemical mode of action of α-T is due to LPO.

6.3 Sensitivity to Phototoxins

We have been investigating antioxidant and antioxidant enzyme defenses in phototoxin-tolerant and phototoxin-sensitive insect herbivores in an attempt to understand the role of these defenses in insect herbivory. A large number of factors, including enzymatic and non-enzymatic defenses, appear to influence phototoxicity (see figure 16). In the case of the phototoxin specialist *A. plagiata*, it is clear that this species has developed behavioural and biochemical adaptations to overcome the

Figure 16: Enzymatic and non-enzymatic defenses in insect herbivores against the oxidative stress caused by phototoxins.


PHOTOTOXINS
$^1O_2$
PROTEIN OXIDATIONS,
LIPID PEROXIDATION (LOOH)
(-)
ANTIOXIDANTS
(VIT E, BETA-CAROTENE, GSH)
GSH
(-)
SOD,CAT
GR
GPOX/PE
PSMO's, GST
$O_2^{\cdot}$
(-)
H_2O_2, OH·
GSSG
LOH
NON-PHOTOTOXIC METABOLITES

potentially lethal effects of hypericin ingestion. *A. plagiata* has high constitutive levels and inducible levels of antioxidant enzyme activity (Chapter 2) as well as high tissue levels of lipid soluble antioxidants (Chapter 3) which likely allow it to counteract the oxidative stress caused by hypericin. The ability of this species to metabolize and eliminate hypericin remains to be investigated.

In the case of the phototoxin-sensitive *M. sexta*, our results indicate that not only does this species have relatively low antioxidant enzyme activity, but it also obtains little antioxidant protection from its artificial diet. α-T is a strong generator of $^1O_2$ and, coupled with the inability of *M. sexta* to adequately detoxify this molecule, this would lead to development of LPO and tissue damage. As well, α-T treatment results in the inhibition of key defensive antioxidant enzymes which would likely exacerbate this damage.

6.4 Implications

Insect herbivores which have well adapted detoxification enzyme systems as well as antioxidant and antioxidant enzyme defenses are most likely to be capable of utilizing and/or developing successfully on phototoxin-containing plants. These defenses may have allowed some species (e.g. *A. plagiata*) to specialize on these plants and perhaps gain a selective advantage from reduced competition. Development of behavioural, physiological, and biochemical adaptive strategies are likely an attempt to circumvent the phototoxic defenses of plants and as such may be an important step in the coevolution of insects on plants.

6.5 Future Research

Suggested areas of future research are as follows:

1. To investigate fully the role of other antioxidants (e.g. uric acid) in providing protection against oxidative stress due to phototoxin ingestion.

2. To examine the relative contribution of P450 metabolism and antioxidative defenses, particularly in phototoxin-tolerant specialists such as *A. plagiata*. This would allow us to understand more fully the role of antioxidant enzymes.

3. Determine more precisely the role of GSH in preventing oxidative damage.

4. Examine the role of non GSH-dependent peroxidases and their ability to provide protection against LPO.

5. Examine more closely the light-dependency of induction of antioxidant enzyme activity to determine if they are responding to the phototoxin itself or to the activated oxygen species.

REFERENCES

Aebi H (1984). Catalase *in vitro*. Meth Enzymol 105: 121-124.

Ahmad S, Brattsten LB, Mullin CA, Yu SJ (1986). Enzymes involved in the metabolism of plant allelochemicals. In: Brattsten LB, Ahmad S (eds). Molecular aspects of insect-plant associations. Plenum Press, N.Y.pp 73-127.

Ahmad S, Pardini RS (1988). Evidence for the presence of glutathione peroxidase activity toward an organic hydroperoxide in larvae of the cabbage looper moth, *Trichoplusia ni*. Insect Biochem 18: 861-869.

Ahmad S, Pardini RS (1989). Corrigendum. Insect Biochem 19: 109.

Ahmad S, Pritsos CA, Bowen SM, Kirkland KE, Blomquist GJ, Pardini RS (1987). Activities of enzymes that detoxify superoxide anions and related oxyradicals in *Trichplusia ni*. Arch Insect Biochem Physiol 6: 85-92.

Ahmad S, Pritsos CA, Bowen SM, Hiesler CR, Blomquist GJ, Pardini RS (1988a). Subcellular distribution and activities of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase in the southern armyworm, *Spodoptera eridania*. Arch Insect Biochem Physiol 7: 173-183.

Ahmad S, Capps C, Pardini RS (1988b). Response of the antioxidant enzymes of the cabbage looper moth (*Trichoplusia ni*) larvae to the superoxide-generating plant pro-oxidants, quercetin and xanthotoxin. J Cell Biol 107:418a.

Ahmad S, Beilstien MA, Pardini RS (1989). Glutathione peroxidase activity in insects: a reassessment. Arch Insect Biochem Physiol 12: 31-37.

Allen RG, Farmer KJ, Sohal RS (1983). Effect of catalase inactivation on levels of inorganic peroxides, superoxide dismutase, oxygen consumption, and life span in adult houseflies (*Musca domestica*). Biochem J 216: 503-510.

Allen RG, Farmer KJ, Sohal RS (1984). Effect of diamide administration on longevity, oxygen consumption, superoxide dismutase, catalase, inorganic peroxides, and glutathione in the housefly, *Musca domestica*. Comp Biochem Physiol 78C: 31-38.

Ames BN (1981). Uric acid provides an antioxidant defense in humans against oxidant and radical caused aging and cancer: a hypothesis. Proc. Nat. Acad. Sci.U.S.A. 78:6858.

Armstrong D, Rinehart R, Dixon L, Reigh D.(1978). Changes of peroxidase with age in *Drosophila*. Age 1:8-12.

Arnason JT, Chan GFQ, Wat K, Downum K, and Towers GHN (1981a). Oxygen requirement for near-UV mediated cytotoxicity of alpha-terthienyl to *Escherchia coli* and *Sacharomyces servisiae*. Photochem. Photobiol. 33:821-824.

Arnason JT, Swain T, Wat CK, Graham EA, Partington S, Towers GHN, and Lam J (1981b). Mosquito larvicidal activity of polyacetylenes from species in the Asteraceae. Biochem. Sys. Ecol. 9:63-68.

Arnason JT, Philogène BJR, Morand P, Scaiano JC, Werstiuk N, Lam J (1987). Thiophenes and acetylenes: phototoxic agents to herbivorous and blood-feeding insects. In: Hietz JR, Downum KR (eds). Light-Activated Pesticides. ACS Symposium Series 339. American Chemical Society, Washington D.C.pp255-265.

Arnason JT, Philogène BJR, Towers GHN (1991) Phototoxins in plant-insect interactions. In:Rosenthal G, Berenbaum MR (eds). Herbivores (2nd ed.). Academic Press (in press).

Arnason JT, Philogène BJR, Morand P, Imrie K, Iyengar S, Duval F, Soucy-Breau C, Scaiano JC, Werstiuk NH, Hasspieler B, Downe AER (1989). Naturally occuring and synthetic thiophenes as photoactivated insecticides. In: Arnason JT, Philogène BJR, Morand P (eds). Insecticides of Plant Origin. American Chemical Society Symposium Series 387, ACS Washington, D.C. p.164-172.

Arnason JT, Guerin B, Kraml MM, Mehta B, Redmond RW, Scainano JC (1991). Phototoxic and photochemical properties of sanguinarine. Submitted - Photochem Photobiol.

Ashwood-Smith MJ, Ring RA, Liu M, Phillips S, Wilson M (1984). Furanocoumarin resistance in the larvae *Phytomyza spondylii* feeding on *Herculeum lanatum* is associated with the enzymatic breakdown of 8-methoxypsoralen. Can J Zool 62: 1971-1976.

Aucoin RR, Fields P, Lewis M, Philogène BJR, and Arnason JT (1990). The protective effect of antioxidants to a phototoxin-sensitive insect herbivore *Manduca sexta*. J

Chem Ecol 16:2913-2924.

Aucoin RR, Philogène BJR, and Arnason JT (1991a). Antioxidant enzymes as biochemical defenses against phototoxin-induced oxidative stress in three species of herbivorous lepidoptera. Arch Insect Biochem Physiol 16:139-152.

Aucoin RR, Philogène BJR, Arnason JT (1991b). Biochemical mode of action of the phototoxin α-terthienyl in *Manduca sexta*: Lipid peroxidation and the role of antioxidants and antioxidant enzymes. (in preparation).

Aucoin RR, Schnieder E, Arnason JT (1991c). Evaluating the phototoxicity and photogenotoxicity of plant secondary compounds. In: Linskens HF, Jackson JF (eds). Modern Methods of Plant Analysis. New Series Vol.13 Springer-Verlag, Berlin (in press).

Averbeck D (1989). Recent advances in psoralen phototoxicity mechanisms. Photochem Photobiol 50: 859-882.

Bakker JA, Gommers EJ, Niewenhuis I, and Wynberg H.(1979). Photoactivation of the nematicidal compound alpha-terthienyl from roots of marigolds (*Tagetes* species). J. Biol. Chem. 254:1841-1845.

Bell RA, Joachim FG (1976). Techniques for rearing laboratory populations of tobacco hornworms. Ann Ent Soc Am 19:365-367.

Berenbaum MR (1978) Toxicity of a furanocoumarin to armyworms: a case of biosynthetic escape from insect herbivores. Science 201: 532-534.

Berenbaum MR (1981). Patterns of distribution and insect herbivory in the Umbelliferae: Plant chemistry and community structure. Ecology 62: 1254-1266.

Berenbaum MR (1987) Charge of the Light Brigade: Phototoxicity as a Defense Against Insects. In: Light-Activated Pesticides. Hietz JR, Downum KR, eds. ACS Symposium Series No. 339. American Chemical Society, Washington, D.C., pp. 206-216.

Berenbaum MR, Feeny PP (1981). Toxicity of angular furanocoumarins to swallowtail butterflies: escalation in a coevolutionary arms race? Science 212: 927-929.

Berenbaum MR, Lee, K.W. (1991). Sensitivity of parsnip webworms to non-hostplant phototoxins. Photochem. Photobiol.(in press).

Boulter CJ, Chefurka W (1990). The effect of phosphine fumigation on superoxide dismutase, catalase, and peroxidase in the granary weevil, *Sitophilus granarius*. Pest Biochem Physiol 36:52-63.

Boveris A, Oshino N, Chance B (1972). The cellular production of hydrogen peroxide Biochem J 128:617-621.

Bramhall SN, Noack N, Wu M, Loenberg JR (1969). A simple colorimetric method for the determination of protein. Anal Biochem 31:146-153.

Brattsten LB (1979). Ecological significance of mixed function oxidases. Drug Metab. Rev. 10: 35-58.

Brattsten LB, Ahmad S (1986). Molecular aspects of insect-plant associations. Plenum Press, N.Y.

Bureau J, Bushway RJ (1986) HPLC determination of carotenoids in fruits and vegetables in the United States. J.Food Sci. 51(1):128-130.

Bull DL, Ivie GW, Beier RC, Pryor NW, Oertli EH (1984). Fate of photosensitizing furanocoumarins in tolerant and sensitive insects. J Chem Ecol 6: 893-911.

Camm EL, Towers GHN, Mitchell JC (1975) UV-mediated antibiotic activity of some compositae species. Phytochemistry 14: 2007-2011.

Carrol CR, Hoffman CA (1980). Chemical feeding deterrent mobilized in response to insect herbivory and counter adaptation by *Epilahcna tredecemnotata*. Science 209: 414-416.

Champagne DE, Arnason JT, Philogène BJR,Campbell G, McLaughlin (1984). Photosensitization and feeding deterrence of *Euxoa messoria* by alpha-terthienyl, a naturally occurring thiophene from the asteraceae. Experientia 40: 577-578.

Champagne DE, Arnason JT, Philogène BJR, Morand P, Lam J (1986). Light-mediated allelochemical effects of naturally occuring polyacetylenes and thiophenes from Asteraceae on herbivorous insects. J Chem Ecol 12: 835-845.

Cohen MB, Berenbaum MR, Schuler MA (1989). Induction of cytochrome P-450-mediated detoxification of xanthotoxin in the black swallowtail. J Chem Ecol 15: 2347-2355.

Conn EE (1979) Cyanide and cyanogenic glycosides. In:Herbivores: their interaction with secondary plant metabolites. Rosenthal GA, Janzen DH (eds). Academic Press, N.Y. pp 387-412.

Dadd RH (1985). Nutrition: organisms. In: Kerkut,G.A. and Gilbert, L.E. (eds). Comparative insect physiology, biochemistry, and pharmacology. Academic Press, Vol.4, pp313-380.

David WAL, Ellaby S (1975). The viability of eggs of the African armyworm *Spodoptera exempta* in laboratory cultures. Ent. Exp. App. 18:269-280.

Dethier VG (1970) Chemical interactions between plants and insects. In: Chemical Ecology. Sondhiemer E, Simeone JB (eds). Academic Press, N.Y.pp83-102.

Dillard CJ, Tappell A (1984). Flourescent damage products of lipid peroxidation. Methods Enzymol 105:337-341.

Downum KR (1986) Photoactivated biocides from higher plants. In: Natural Resistance of Plants To Pests: Roles of Allelochemicals. Green HB, Hedin PA, eds. ACS Symposium Series No.296. American Chemical Society, Washington, D.C., pp.197-205.

Downum KR, Rosenthal GA, Towers GHN (1986). Phototoxicity of the allelochemical alpha-terthienyl to larvae of *Manduca sexta*. Pest Biochem Physiol 22:104-109.

Duffey S (1980). Sequestration of plant natural products by insects. Ann Rev Entomol 25: 447-477.

Duran N, Song PS (1980). Hypericin and its photodynamic action. Photochem Photobiol. 43: 677-680.

Ehrlich PR, Raven PH (1964). Butterflies and plants: a study in coevolution. Evolution 18: 586-608.

Feltwell J, Rothschild M(1974) Carotenoids in thirty-eight species of lepidoptera. J. Zool. 174:441-465.

Feltwell J. (1978). The distribution of carotenoids in insects. In: Harbourne, J.E. (ed). Biochemical aspects of plant and animal coevolution. Academic Press, N.Y. pp 277-293.

Fields P, Arnason JT, Philogene BJR (1990). Behavioural and physical adaptations of three insects that feed on the phototoxic plant *Hypericum perforatum*. Can J Zool 68: 339-347.

Fields P, Arnason JT, Aucoin RR, Philogene BJR, Morand P (1991). Photo-activated insecticides. Mem Ent Soc Can (in press).

Flohe L (1982) Glutathione peroxidase brought into focus. In: Free Radicals in Biology, Vol.I. Pryor WA, ed. Academic Press, N.Y. pp. 223-254.

Flohe L, Otting F (1984). Superoxide dismutase assays. Meth Enzymol 105:93-104.

Foote CS (1987). Type I and type II mechanisms of photodynamic action. In: Hietz JR and Downum KR (eds). Light Activated Pesticides. ACS Symposium Series No. 339. American Chemical Society, Washington, D.C. pp 22-38.

Foote CS, Denny RW (1968).Chemistry of singlet oxygen.VII.Quenching by beta-carotene. J.Amer. Chem. Soc.90:6233-6235.

Foote CS, Clough RC, and Yee BG (1978). Photooxidation of tocopherols. In: deDuve, C. and Hayaishi, O. (eds). Tocopherol, oxygen, and biomembranes. Elsevier, Amsterdam.pp 182-193.

Fridovich I (1977). Oxygen is toxic. Bioscience 27:462-466.

Fridovich I (1983) Superoxide radical: an endogenous toxicant. Ann Rev Pharmacol Toxicol 23: 239-244.

Giese AC (1980) Hypericism. In: Photochemical and Photobiological Reviews, Vol. V. Smith KC, ed. Plenum Press, N.Y. pp. 229-255.

Gilbert LE, Raven PH (1975). Coevolution of animals and plants. University of Texas Press, Austin. 446p.

Giles A, Wamer A and Kornhauser A. (1984). *In vivo* protection by beta-carotene against phototoxicity. Photochem. Photobiol. 395:3(ABSTR).

Goodwin TW. (1980). The biochemistry of carotenoids. Vol.II. Chapman and Hall, London. pp 97-121.

Griffith OW. (1980). Determination of glutathione and glutathione disulphide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106:207-212.

Griffith OW, Miester A(1979). Glutathione: interorgan translocation, turnover, and metabolism. Proc Nat Acad Sci USA 76:5606-5610.

Guthrie WD (1971) Resistance of maize to second-brood European corn-borers. Rep 26th Ann Corn Sorghum Res Conf. American Seed Association Publication No.26, pp. 165-179.

Gutteridge JMC, Halliwell B (1990). The measurement and mechanism of lipid peroxidation in biological systems. Trends Biol Sci 15:129-135.

Habeeb AFSA. (1972). Reaction of protein sulfhydryl groups with Ellman's reagent. Methods Enzymol XXVB: 457-464.

Halliwell B, Gutteridge JMC (1985). Free Radicals in Biology and Medicine. Clarendon Press, Oxford, p.302.

HarborneJB (1978). Introduction to ecological biochemistry. Academic Press, N.Y. 243p.

HarborneJB (1989). Recent advancesin chemical ecology. Natural Products Reports 6(1): 85-109.

Hartman PE, Dixon WJ, Dahl TA, Daub ME (1988). Multiple modes of action by cercosporin. Photochem Photobiol 47: 699-703.

Hasspieler BM, Arnason JT, Downe AER (1988). Toxicity, localization, and elimination of the phototoxin alpha-terthienyl in mosquito larvae. J Mosq Control Assoc 4:479-484.

Hasspieler BM, Arnason JT, Downe AER (1990). Modes of action of the plant-derived phototoxin α-terthienyl in mosquito larvae. Pest Biochem Physiol 38: 41-47.

Heikklia RE, Cabbat FC, Cohen G (1970). In vivo inhibition of superoxide dismutase in mice by diethyldithiocarbamate. J Biol Chem 251:2182-2185.

Hodnick WF, Kung FS, Roettger WJ, Bohmont CW, Pardini RS (1986). Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. Biochem Pharmacol 35: 2345-2350.

Ivie GW, Bull DL, Pryor NW, Oertli EH (1983). Metabolic detoxification: mechanisms of insect resistance to plant psoralens. Science 221: 374-376.

Iyengar S, Arnason JT, Philogène BJR, Morand P, Werstiuk NH, Timmins G (1987). Toxicokinetics of the phototoxic allelochemical alpha-terthienyl in three herbivorous lepidoptera. Pest Biochem Physiol 29: 1-9.

Iyengar S, Arnason JT, Philogène BJR, Werstiuk NH, Morand P (1990). Comparative metabolism of the phototoxic allelochemical alpha-terthienyl in three species of lepidopteran larvae. Pest Biochem Physiol 37:154-164.

Janzen DH (1980). When is it coevolution? Evolution 34: 611-612.

Joshi PC, Pathak MA (1983). Production of singlet oxygen and superoxide radicals by psoralens and their biological significance. Biochem Biophys Res Commun 112: 638-646.

Kagan J, Bazin M, Santus R (1989). Photosensitization with alpha-terthienyl: The formation of superoxide ion in aqueous media. J. Photochem. Photobiol. B: Biology 3, 165-174.

Kapin MA, Ahmad S (1980). Esterases in larval tissues of the gypsy moth *Lymantria dispar*: optimum assay conditions, quantification, and characterization. Insect Biochem 10: 331-337.

Kayser H. (1985). Pigments. In: Kerkut, G.A. and Gilbert, L.E. (eds). Comparative insect physiology, biochemistry, and pharmacology. Vol.10. Academic Press. pp368-415.

Knox JD, Dodge AD (1985). Isolation and activity of the photodynamic pigment hypericin. Plant Cell Environ. 8: 19-25.

Knox JP, Samuels RJ, Dodge AD (1987). The photodynamic activity of hypericin. In: Hietz J, Downum KR (eds). Light-activated pesticides. ACS Symposium Series No.339., American Chemical Society, Washington, D.C.pp 265-270.

Kramer, K.J., Hendricks, L.H., Liang, Y.T., and Sieb, P.A. (1978). Effect of ascorbic acid and related compounds on the tobacco hornworm *Manduca sexta* Johannson(Lepidoptera:sphingidae).J. Agric. Food Chem. 26(4): 874-878.

Krinsky NI, Deneke SM (1982). Interaction of oxygen and oxy-radicals with carotenoids. J. Nat. Cancer Inst. 69:205-207.

Larson RA (1986). Insect defenses against phototoxic plant chemicals. J. Chem. Ecol. 12: 859-870.

Larson RA, Berenbaum M. (1988). Environmental phototoxicity. Env. Sci. Technol. 22(4): 354-360.

Larson RA, Marley KA, Tuveson RW, Berenbaum MR(1988). Beta-carboline alkaloids: Mechanisms of phototoxicity to bacteria and insects. Photochem Photobiol. 48: 665-674.

Lee K, Berenbaum MR (1989). Action of antioxidant enzymes and cytochrome P-450 mono-oxygenases in the cabbage looper in response to plant phototoxins. Arch Insect Biochem Physiol 10: 151-162.

Lee K, Berenbaum MR (1991). Defense of parsnip webworm against phototoxic furanocoumarins: Role of antioxidant enzymes. J Chem Ecol (in press).

Levine SA, Kidd PM (1986). Antioxidant adaptation: Its role in free radical pathology. Biocurrents Division, Allergy Research Group, San Leandro, California. p.367.

Lowry O, Rosebrough NJ, Farr AL, Randall RJ. (1951). Protein measurement using the folin phenol reagent. J. Biol Chem 193:265-272.

MacKay WJ, Bewley GC (1989). The genetics of *Drosophila melanogaster*: Isolation and characterization of acatalasemic mutants. Genetics 122:643-652.

MacRae WD, Irwin DAJ, Bisalputra T, Towers GHN (1980) Membrane lesions in human erythrocytes induced by the naturally occurring compounds alpha-terthienyl and phenylheptatryne. Photobiochem Photobiophys 1: 309-318.

Mannervik B (1985). Glutathione peroxidase. Meth Enzymol 113: 490-495.

Mathis C, Ourisson G (1963). Etude chimio-taxinomique du genre hypericum:I: Repartition de l'hypericine. Phytochem 2: 157-171.

McCord JM, Fridovich I (1969). Superoxide dismutase: an enzymic function for erythrocuprein. J Biol Chem 244: 6049-6057.

McFarlane JE (1972). Vitamin E, tocopherol quinone, and selenium in the diet of the house cricket *Ascheta domestica*. Israel J. Ent. 3:7-14.

McKey D (1979). The distribution of secondary compounds in plants. In: Rosenthal GA, Janzen DH (eds). Herbivores: their interactions with plant secondary metabolites. Academic Press, N.Y. pp 55-122.

McLachlin DG, Arnason JT, Philogène BJR, Champagne D (1982). Antifeedant activity of the polyacetylene phenylheptatryne (PHT) from the Asteracea to *Euxoa messoria* (Lepidoptera:noctuidae). Experientia 38: 1061-1062.

McRae DG, Yamamoto E, Towers GHN (1985). The mode of action of polyacetylene and thiophene photosensitizers on liposome permeability to glucose. Biochim. Biophys. Acta. 821:488-496.

Mead JF (1972). Free radical mechanisms of lipid damage and consequences for cellular membranes. In: Free Radicals in Biology, Vol. 1., Pryor WA, ed. Academic Press, N.Y. pp. 51-67.

Miller KW, Yang CS (1985). An isocratic HPLC method for the simultaneous determination of plasma retinol, alpha-tocopherol, and various carotenoids. Anal. Biochem. 145:21-26.

Mullin CA, Wilkinson CF (1980). Insect epoxide hydrolases: properties of a purified enzyme from the southern armyworm *Spodoptera eridania*. Pest. Biochem Physiol. 18: 101-106.

Nitao J (1989). Enzymatic adaptation in a specialist herbivore for feeding on furanocoumarin-containing plants. Ecology 70(3): 629-635.

Nitao J (1990). Metabolism and excretion of the furanocoumarin xanthotoxin by parsnip webworms. J Chem Ecol 16: 417-428.

Pace N, MacKinney G (1941). Hypericin, the photodynamic pigment from St. John's wort. J Am Chem Soc 63: 2570-2577.

Paglia DE, Valentine WN (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70: 158-167.

Pardini RS, Pritsos CA, Bowen SM, Ahmad S, Blomquist GJ (1989). Adaptations to plant pro-oxidants in a phytophagous insect model: Enzymic protection from oxidative stress. In: Oxygen Radicals in Biology and Medicine. Simic MG, Taylor KA, Ward JF, von sonntag C, eds. Plenum Publishing Corp., New York. pp. 725-728.

Philogène BJR, Arnason JT, Duval F (1984a) Effect of khellin and 8-methoxypsoralen on the development and survival of *Aedes atropalpus*. Can Ent 17: 1153-1157.

Philogène BJR, Arnason JT, Towers GHN, Abramowski Z, Campos F, Champagne D, McLaughlin D (1984b) Berberine: a naturally occurring phototoxic alkaloid. J Chem Ecol 10:115-123.

Philogène BJR, Arnason JT, Berg CW, Duval F, Champagne D, Taylor RG, Leitch LC, Morand P (1985) Synthesis and evaluation of the naturally occurring phototoxin alpha-terthienyl as a control agent for larvae *Aedes atropalpus, Aedes intrudens* (Diptera: Culicidae) and *Simulium verecundum* (Diptera: Simulidae). J Econ Ent 78: 121-126.

Poff J (1976). Carotenoids of some aposematic and cryptic *Leptinotarsa* (Coleoptera: chrysomelidae). Am. Zool. 16:271.

Pritsos CA, Ahmad S, Bowen SM, Elliot AJ, Blomquist GJ, Pardini RS (1988). Antioxidant enzymes of the black swallowtail butterfly, *Papilio polyxenes*, and their responses to the pro-oxidant allelochemical quercetin. Arch Insect Biochem Physiol 8: 101-113.

Racker E (1955). Glutathione reductase (liver and yeast). Meth Enzymol 2: 722-732.

Reese JC (1979). Interactions of allelochemicals with nutrients in herbivore food. In: Rosenthal GA, Janzen DH (eds). Herbivores:their interaction with plant secondary metabolites. Academic Press, N.Y. pp 387-412.

Rhoades DF (1979). Evolution of plant chemical defenses against herbivores. In: Rosenthal GA, Janzen DH (eds). Herbivores:their interaction with plant secondary metabolites. Academic Press, N.Y. pp3-54.

Robinson JR (1983). Photodynamic insecticides: a review of studies on photosensitizing dyes as insect control agents, their practical applications, hazards, and residues. Residue Reviews 88: 69-93.

Robinson JR, Beatson EP (1985). Enhancement of dye-sensitized phototoxicity to house fly larvae *in vivo* by dietary ascorbate, diazabicyclooctane, and other additives. Pest. Biochem. Physiol. 24:375-383.

Rosenthal GA (1977) The biological effects and mode of action of canavanine, a structural analogue of arginine. Q. Rev. Biol. 52: 155-178.

Rothschild M.(1974). Coevolution of plants and animals. In: Gilbert, L.E. and Raven, P.H. (eds).University of Texas Press. pp20-51.

Ryan CA (1979) Proteinase inhibitors. In: Herbivores: thier interaction with secondary plant metabolites. Rosenthal GA, Janzen DH (eds). Academic Press, N.Y. pp 599-618.

Samuels R, Knox P (1989). Insecticidal activity of hypericin towards *Manduca sexta* larvae. J Chem Ecol 15: 855-861.

Sandberg SL, Berenbaum MR (1989). Leaf-tying by tortricid larvae as an adaptation for feeding on phototoxic *Hypericum perforatum*. J Chem Ecol 15: 875-885.

Scaiano JC, Evans C, Arnason JT (1989). Characterization of the alpha-terthienyl radical cation: evidence against electron transfer to oxygen *in vitro*. J. Photochem. Photobiol. B:Biology Vol. 3: 411-418.

SchoonhovenLM (1968) Chemosensorybasis of host plant selection. Ann. Rev. Entomol. 13: 115-136.

Schoonhoven LM (1982) Biological aspects of antifeedants. Ent. Exp. Appl. 31: 57-69.

Sedlak J, Lindsay RH (1968). Estimation of total, protein-bound, and non protein-bound sulfhydryls in tissue with Ellman's reagent. Anal Biochem 25: 192-205.

Sen A, Iyengar S, Arnason JT, Craig DA, Philogène BJR, Morand P (1990). Cytotoxic effects of alpha-terthienyl on the midgut of the tobacco hornworm *Manduca sexta*. Can. J. Zool.68:2010-2019.

Simmons TW, Jamall IS, Lockshin RA (1989). Se-independent glutathione peroxidase associated with glutathione-S-transferase from the housefly *Musca domestica*. Comp Biochem Physiol 94:323-327.

Sohal RS, Farmer KJ, Allen RG, Ragland SS (1984). Effects of diethyldithiocarbamate on life span, metabolic rate, superoxide dismutase, catalase, inorganic peroxides, and glutathione in the male adult housefly, *Musca domestica*. Mech Age Dev 24:175-180.

Song PS, Tapley KJ (1979) Photochemistry and photobiology of psoralens. Photochem Photobiol 29: 1177-1197.

Spikes, J.D. (1977). Photosensitizers. In: Smith, K.C.(ed). The science of photobiology. Plenum Press. N.Y. pp87-112.

Strauss RS, Snyder EL, Wallace PD, Rosenberger TG (1980). Oxygen detoxifying enzymes in neutrophils of infants and their mothers. J Lab Clin Med 95: 897-906.

Swain T (1977) Secondary compounds as protective agents. Ann Rev Plant Physiol 284: 479-536.

Towers GHN (1979) Photosensitizers in plants and their photodynamic action (a review). Prog Phytochem 6: 183-202.

Towers GHN (1984). Interaction of light with phytochemicals in some natural and novel systems. Can. J. Bot. 62:2900-2911.

Turunen, S. (1976). Vitamin E effect of lipid synthesis and accumulation of linolenate in *Pierris brassicae*. Ann. Zool. Fenn. 13:148-152.

Tuveson RW, Berenbaum MR, Heininger EE (1986) Inactivation and mutagenesis by phototoxins using *Escherichia coli* strains differing in sensitivity to near- and far-ultraviolet light. J Chem Ecol 12: 933-947.

Tuveson RW, Larson RA, Marley KA, Wang G, Berenbaum MR (1989). Sanguinarine, a phototoxic H_2O_2-producing alkaloid. Photochem Photobiol. 50: 733-738.

Veerman A. (1980). Functional involvement of carotenoids in photoperiodic induction of diapause in the spider mite, *Tetranychus urticae*. Physiol. Ent. 5:297-300.

Waldbauer GP (1968) The consumption and utilization of food by insects. Adv Insect Physiol 5: 229-288.

Wat CK, Biswas RK, Graham EA, Bohm L, Towers GHN, Waygood ER (1979) Ultraviolet-mediated cytotoxic activity of phenylheptatrynefrom *Bidens alba*. J Nat Prod 42: 103-111.

Wat CK, MacRae D, Yamamoto E, Towers GHN, Lam J (1980) Phototoxic effects of naturally occurring polyacetylenes and alpha-terthienyl on human erythrocytes. Photochem Photobiol 32: 167-172.

Whittaker RH, Feny PP (1971). Allelochemicals: chemical interactions between species. Science 171: 757-770.

Williams RN, Delamere NA, Paterson CA (1985). Inactivation of catalse by 3-amino-triazole: an indirect irreversible mechanism. Biochem Pharmacol 34:3389-3391.

Witting LA (1976). Vitamin E and lipid antioxidants. In: Pryor, W. (ed). Free radicals in biology. Vol.IV. Academic Press. N.Y. pp 295-315.

World Health Organization (1963) Insecticide resistance and vector control. WHO 13th Rept. No.265.

Yamamoto E, Wat CK, MacRae WD, Towers GHN (1979). Photoinactivation of human erythrocyte enzymes by α-terthienyl and phenylheptatryne, naturally occuring compounds in the Asteraceae. FEBS Lett 107: 134-136.

Yu SJ (1982). Host plant induction of glutathione transferase in the fall armyworm. Pest. Biochem. Physiol. 18: 101-106.

Yu SJ (1983). Induction of detoxifying enzymes by allelochemicals and host plants in the fall armyworm. Pest. Biochem Physiol. 19: 330-336.

Zaspel BJ, Csallany AJ (1983). Determination of alpha-tocopherol in tissues and plasma by HPLC. Anal. Biochem. 130: 146-150.

APPENDIX I: Phototoxins from higher plants.

Biosynthetic Class	Precursor	Examples[1]	Occurrence (Families)	Principal Mode of Action[2]
furanocoumarins	shikimic acid	psoralen angelicin	Rutaceae Umbelliferae Leguminosae Moraceae Solanaceae Pittosporaceae Thymeleaceae Orchidaceae Asteraceae	DNA monoadducts and crosslinking
extended quinones	acetate	hypericin	Hypericaceae	singlet oxygen, free radicals
polyiines	oleic acid	PHT alpha-T	Asteraceae Apiaceae Campanulaceae Pittosporaceae Olacaceae Euphorbicaceae Valerianaceae Annonaceae Opiliceae Sapindaceae Araliaceae	singlet oxygen, free radicals
furanochromones	acetate	khellin visnagin	Umbelliferae	DNA monoadducts
furanoquinolines	tryptophan	dictamnine	Rutaceae	photoadducts
isoquinolines	tyrosine, phenylalanine	berberine sanguinarine	Annonaceae Papaveraceae Berberidaceae Juglandaceae Magnoliaceae Menispermaceae Ranununculaceae Rubiaciae Rutaceae	singlet oxygen, free radicals, photogenotoxicity
beta-carbolines	tryptophan	harman	Simaroubaceae Rutaceae Cyperaceae Fabaceae Polygonaceae Rubiaceae Sapindaceae Passifloraceae Zygophyllaceae Solanaceae	singlet oxygen, photoadducts
isoflavonones	shikimic acid	pisatin	Fabaceae	free radicals

Source: Aucoin RR, Schnieder E, Arnason JT (1991c). Evaluating the phototoxicity and photogenotoxicity of plant secondary compounds. In: Linskens HF, Jackson JF (eds). Modern Methods of Plant Analysis, New Series Vol 13. Springer-Verlag, Berlin (in press)

APPENDIX II: Single leaf of *Hypericum perforatum* illustrating the black, marginal glands containing the phototoxin hypericin

APPENDIX III: Comparative toxicity of alpha-terthienyl and 8-methoxypsoralen to *A. plagiata*, *O. nubilalis* and *M.sexta.*

Table A

24 h toxicity of topically applied α-T and 8-MOP

Species	α-T		8-MOP	
	LD_{50}(ug/g)	Fiducial limits	LD_{50}(ug/g)	Fiducial limits
A. plagiata	1045.0	775.9-1432.9	557.9	398.4-781.6
M. sexta	61.4	38.8-93.9	56.5	32.7-101.5
O. nubilalis	>2000	N.D.	>2000	N.D.

Table B

48 h toxicity of topically applied α-T and 8-MOP

Species	α-T		8-MOP	
	LD_{50}(ug/g)	Fiducial limits	LD_{50}(ug/g)	Fiducial limits
A. plagiata	806.8	31.2-9865.2	508.3	356.6-717.0
M. sexta	19.9	11.3-31.8	50.6	29.3-89.5
O. nubilalis	>2000	N.D.	>2000	N.D.

The 24 h and 48 h LD50s of α-T and 8-MOP were determined in order to establish whether there are differences in the sensitivities of these insects to different classes of phototoxins. Fifth instar larvae of *A. plagiata* and *O. nubilalis* and third instar larvae of *M. sexta* were topically treated with the compounds in acetone using either a Hamilton syringe alone or together with a microapplicator to deliver the required doses in a volume not exceeding 3 ul/larva. After treatment the larvae were placed under UV lights for 8 h and then mortality assessed after 24 and 48 h. LD50s and fiducial limits were determined using SAS probit analysis.