Oxidation of Plant Allelochemicals by Phytophagous Sucking Insects

A THESIS

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ADDENDUM

Introduction - Choice of model enzyme system:

The model enzyme system used in this work (horseradish peroxidase and mushroom tyrosinase) was chosen on the basis that aphid saliva has both peroxidative and catechol oxidase activities. Catechol oxidases (including mushroom tyrosinase) are a type of diphenolase; i.e. those that are able to oxidise *o* -diphenols specifically.

DIMBOA Decomposition in aqueous solutions: (refer to pp. 23, 24, & 135)

DIMBOA has been shown in the literature (Pérez & Niemeyer, 1985; Bravo & Niemeyer, 1986) to decompose in aqueous solutions to a number of products. These include MBOA (6-methoxy-benzoxazolin-2-one), an isomer of DIMBOA (3,4-dihydroxy-7-methoxy-1,4-benz-oxazin-2-one), and the corresponding lactam of DIMBOA. It is possible that one or more of these compounds was formed during the aqueous oxidations carried out in the present work (both model enzyme and aphid salivary reactions); even more so, during the 24 hour choice test autoxidation of DIMBOA (p 135). It should be noted, however, that the decomposition reactions discussed in the literature were observed over much longer periods than the simple 2 hour reaction-time used here. Comparison of the chromatographic properties of these decomposition compounds with those observed from the oxidation of DIMBOA (section 1.2.4) would have proved interesting. Unfortunately samples of these compounds were not available, and the synthesis of them extends beyond the scope of the current work.

Minor Corrections

Page

32 Lines 10-17: The "rules of thumb" postulated here are based upon the comparison of UV-vis spectra, the sensitivity of which is limited. For this reason these suggestions should be treated as such - mere suggested reaction outcomes that require further investigation for individual compounds. Comparison of enzymic oxidation products by other analytical methods (such as TLC and HPLC) are not on their own conclusive, unless more than one analytical technique is used, or these analyses are performed using two or more different conditions (such as different solvent gradients).

35-36 Sections I, II and III: Reaction pH during the aphid salivary and model system oxidations was found to be 6.8. (The dilute reaction solutions used in these experiments were not buffered; pH remained steady throughout the 2 h reaction period).

68 Line 10: replace (Seigle-Murandi, *et al.*,) 1984 with 1981.

91 End of paragraph one, insert: "A negative result for the selective detection of hordenine oxidation products by TLC does not completely exclude the possibility of these (carboxyl) functional groups being present. (No indication of the sensitivity of the method used was obtained at the time of analysis)."

131 Legend to Table 4.1: The concentration of compounds used for these choice experiments in some cases was greater than that found in plants. In order to directly compare relative palatability of the allelochemicals tested in these simple choice tests similar concentrations of compound were used in each case.

References

The following references were incorrectly listed, the information outlined below replaces previous entries, and includes two new entries (see DIMBOA decomposition, above):

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Summary

Phytophagous sucking insects, aphids in particular, are common pests of plants. In response to insect attack, plants have evolved a variety of defensive mechanisms, including the induction and/or accumulation of deterrent phytochemicals. For their part, these insects are known to secrete salivary enzymes into their food material, apparently containing peroxidases and catechol oxidases as regular constituents. Possible interactions of the insect's oxidases with plant allelochemicals has received little attention to date, although it has been postulated that the oxidative action of aphid saliva may serve to detoxify these allelochemicals. Nevertheless only one such example has so far been demonstrated: namely the influence of catechin on the interaction of *Macrosiphum rosae* and roses (Peng & Miles, 1988a & 1988b).

In vitro, however, aphid saliva has been shown to oxidise a number of substrates including diphenols and selected alkaloids, although the very dilute nature of collected aphid salivary preparations has severely limited analysis of the products. In the present study, in order to obtain products in quantities sufficient for chemical analysis and bioassay of their effects on the insects, a model enzyme system was chosen to mimic the oxidative activity of the insect saliva (namely a mixture of horseradish peroxidase and mushroom tyrosinase). Products were analysed using UV spectroscopy and TLC. Many similarities between the two oxidising systems were observed. One significant difference found between aphid saliva and the model system was the surprising discovery that the aphid peroxidase was inhibited by copper chelators, indicating that it may be a hitherto undemonstrated copper-peroxidase (first suggested by Madhusudhan, 1994).

Close examination of allelochemical oxidations using the model system, in conjunction with various concentrations of plant reductants (glutathione and ascorbic acid), indicated that reductant levels in the plant play an important rôle in the oxidation-reduction interaction between plants and phytophagous insects. Increased levels of reductant(s) helped to maintain plant allelochemicals in the "unoxidised" (and presumably deterrent) state.

Isolation of the product(s) of plant allelochemicals after oxidation by the model system, followed by their structural identification by NMR and mass spectroscopy, was achieved for several substrates. As a result of detailed investigations, the reaction mechanisms of the oxidation processes involved have been proposed, and the likely oxidation products of other allelochemicals predicted.

Exposure of the insects to individual plant chemicals in feeding "choice" tests was performed. Results observed for the monophenol hordenine indicated the dimeric oxidation product to be less deterrent than the unoxidised substrate. Examination of some more complex compounds (e.g. diphenolics, indole alkaloids) appeared to give contrary findings, although in such instances there also appeared to be an inverse relation between the concentration and deterrency of the original substrate, suggesting that simple choice tests for the effects of allelochemicals on aphids may not always be valid.

In preliminary examinations, plants containing increased levels of reductants (i.e., genetically engineered potato plants, with elevated levels of glutathione reductase), and with high levels of phenolic allelochemicals (observed by HPLC analysis), tended to show correlated increases in resistance to attack by aphids.

Declaration

The work presented in this thesis is my own unless otherwise acknowledged, and has not previously been submitted to any University for the award of any degree or diploma. This thesis may be available for loan or photocopying provided that an acknowledgement is made in the instance of any reference to this work.

Debrah F. Lorraine.

May, 1995.

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I wish to sincerely thank Dr. Peter Miles (Dept. of Crop Protection) for his continual help, guidance and encouragement during the supervision of this work; particularly for his kind donation of time since his retirement in December, 1993. Special thanks, also, to Dr. David Ward (second supervisor; Dept. of Organic Chemistry) for helpful discussions and assistance with the chemical aspects of this project. To Dr. Andy Austin, who has officiated as Supervisor since Peter's retirement, your assistance has been greatly appreciated.

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Finally, (last, but by no means, least) to Phil, Mum, Dad, Andrea & Felicity, my sincere thanks and gratitude for your continual love and support throughout the period of this work, without which much of this would not have been possible.

LIST OF ABBREVIATIONS

Common abbreviations used throughout the text:

TLC	-	Thin Layer Chromatography
HPLC	-	High Performance Liquid Chromatography
^{1}H-NMR	-	Proton (^{1}H) Nuclear Magnetic Resonance spectroscopy
HRP	-	Horseradish peroxidase (enzyme from plant)
Tyrosinase	-	Mushroom tyrosinase (enzyme from fungi)
H_2O_2	-	Hydrogen peroxide
PTU	-	Phenyl thiourea
SBS	-	Sodium benzene sulphinate
PAC	-	Partially oxidised catechin
dd water	-	Double distilled water
ppt	-	Precipitate
DIMBOA	-	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one
2,4-DNPH	-	2,4-Dinitrophenylhydrazine

INTRODUCTION

Sucking Insects and Pests

Insects are known to be one of the most copious and successful of terrestrial animals, constituting approximately three-quarters of all defined animal species (Gillott, 1980). Phytophagous sucking insects (Homoptera and numerous Heteroptera) are considered by many to be the most economically important pests of the insect group. This is primarily due to their feeding habits, and is assisted in many cases by their extraordinarily high rates of reproduction (Dixon, 1987).

Aphids in particular are prominent pests of a vast number of agricultural plants. Approximately 4000 species of aphids are known at present, and all are phytophagous ("plant feeding"; Shaposhnikov, 1987). These insects (many of which are known as "greenfly" because of their colour) are quite small, generally 1-2 mm in length. Their food plants include ornamentals such as roses, as well as crops such as apple, potato, forest trees, and field crops such as lucerne, wheat, barley and maize (Davidson, 1923; Wensler, 1962; Todd, *et al.*, 1971; Gibson, 1974; Dreyer & Jones, 1981; Campbell & Binder, 1984; Leszczynski, *et al.*, 1989; Carver, 1991).

Aphids are an important pest of such plants due to their remarkable reproductive capacity, the drain they impose on plant resources, and their ability to transmit viral disease. As a result of infestation, crop yields may be severely decreased. In an effort to overcome aphid invasion insecticides are often applied, but this has resulted in the insects acquiring natural means of counteracting the chemicals used. Many attempts have been made to select aphid resistant crop

cultivars, but again aphids have developed biotypes able to overcome this resistance.

Plant Defences

The plants themselves are considered to have an abundance of natural means of defence against herbivores (including sucking insects). Such defences include plant surface texture and composition (e.g. hairiness in potato), the absence of nutrients required by the pest, the presence of hormone-like substances that affect insect development, unsuitable pH or osmotic pressure, or the accumulation of secondary products (antifeedants), (Levin, 1976; Paxton, 1991; Olaifa, *et al.*, 1991; Corcuera, 1993).

The proportion of total metabolic resources allocated by plants to defence is necessarily a compromise dictated by a number of selective pressures (e.g. severity of pest attack, availability of inorganic nutrients, loss of reproductive potential, etc.). With only finite sources of carbon and nitrogen, a trade-off or negative correlation results between allocation of resources to defence on the one hand, and to growth and reproduction on the other. The numerous strategies against insect herbivores adopted by plants are possibly an indication that no individual defence has proved inviolable.

The rôle of allelochemicals in plants, i.e., chemicals presumed to affect other species (whether plant competitors, animal herbivores, pollinators, or pathogens), remains controversial. For insects, the chemical composition of plants is generally assumed to play a major rôle in plant recognition (Rhoades, 1983; Schoonhoven & Derksen-Koppers, 1976) and, certainly with respect to aphids, in the choice of feeding site (Niemeyer, 1990). A major difficulty in assessing these effects arises, however, since the majority of secondary plant compounds are found in

parenchymal cells, often in cell vaccuoles, whereas most if not all aphids are believed to feed from phloem sieve tubes.

Strong evidence is nevertheless accumulating towards the involvement of plant allelochemicals in the defence system against even phloem-sucking insects, in that resistant cultivars are often found to possess higher concentrations than susceptible plants (Carrasco, *et al.*, 1978; Nicol, *et al.*, 1992) of compounds that can be shown to be deterrent *in vitro*. A variety of plant compounds have been tested on aphids (used in artificial diets to determine effects on insect feeding, growth and reproduction), these include flavonoids and phenolics, terpenoids, coumarins, and alkaloids* (Todd, *et al.*, 1971; Levin, 1976; Schoonhoven & Derksen-Koppers, 1976; Kubo & Klocke, 1983; Kubo, *et al.*, 1984; Dreyer, *et al.*, 1987; Niemeyer, 1990; Westcott, *et al.*, 1992). Notably, o-diphenolic groups have been well documented as showing feeding deterrency. Similarly gramine (Corcuera, 1984; Zuñíga & Corcuera, 1986; Lohar, 1989; Kawada & Lohar, 1989; Kanehisa, *et al.*, 1990; Rustamani, *et al.*, 1992) an indole alkaloid found in cereals, and DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) (Niemeyer, 1988; Niemeyer, *et al.*, 1989; Thackray, *et al.*, 1990; Barria, *et al.*, 1992; Givovich, *et al.*, 1992), a hydroxamic acid of wheat and maize, show potent anti-feeding activity. On the other hand, some species-specific plant compounds may be used by insects as non-nutritional markers (token substances) for recognition of food sources (Fraenkel, 1959). Moreover, secondary plant compounds may also be sequestered by the insects for use as pheromone precursors, as a basis for their own chemical defences against predators, or even as nutrients in their own right (Rothschild, 1972; Rothschild 1973; Duffey, 1980; Bernays & Woodhead, 1984; Blum, *et al.*, 1990); as well as acting as feeding phagostimulants or deterrents (Dreyer & Jones, 1981; Jones & Klocke, 1987).

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* The term alkaloid refers to nitrogen-containing organic bases, found in plants; phenol is a compound which has a hydroxyl group directly bonded to a carbon of the aromatic ring.

Phytoalexins ("mobilizable allelochemicals"), are antimicrobial substances produced by plants in response to various exogenous stimuli (including infection or stress), and are important in the natural defence of plants against disease. Recently, it has been suggested that phytoalexins may be involved in plant defence against insects (Sutherland, *et al.*, 1980; Smith, 1982; Paxton, 1991). These compounds are extremely diverse chemically (e.g. phenolics, isoflavonoids, sesquiterpenes, etc.), and can be found in many plant species (Sharma & Salunkhe, 1991). The defensive advantage of such compounds is that they are inducible, and hence divert plant metabolic resources to their production only when an insect or pathogen attack occurs. In contrast preformed defences, although having the advantage of being in place prior to pest attack, represent a significant investment of energy and metabolites devoted to a defence that may never be necessary. The plant thus diverts resources that might otherwise increase its ability to reproduce and exploit its ecological niche.

Aphid Feeding and Salivary Enzymes

Phytophagous sucking insects are known to feed on the phloem of their host plants (Gillott, 1980), and to secrete saliva into their food source (Miles, 1965; Lamb, *et al.*, 1967; McLean & Kirrel, 1968). Investigation of the content of this secretion has revealed the presence of salivary enzymes (Miles, 1965, 1968, 1969, & 1990; Miles & Peng, 1989; Miles & Harrewijn, 1991; Peng & Miles, 1988a), prominent among which are oxidases. Yet the function of these enzymes can be regarded as problematical. For many years one generally accepted concept of plant defence reported in the literature involved the conversion of diphenolic compounds to quinones by plant oxidases (Miles, 1968). Such products have long been considered as deterrent and/or toxic to sucking insects. That the insects should inject their own salivary oxidases into this system during feeding would seem to contradict their counter-defensive rôle. Work conducted in this laboratory,

however, has supported the conclusion that the insects' enzymes serve to further oxidise monomeric phenolics/quinones to non-toxic condensation products; e.g. catechin by the rose aphid (Peng & Miles, 1988a & 1988b; Peng's PhD Thesis, 1991) This complements work described by Georghiou (1972), where detoxification of alkaloids, primarily involving oxidations, reductions, hydrolyses, and conjugations, by sucking insects was reported.

Interaction of Insects and Plants

Resistance to insect attack in many plants appears to be based upon some chemically deterrent or toxic substance, rendering the plant unsuitable for insect infestation. Development of crop cultivars with additional resistance to insect pests may therefore be based on such a deterrent system, whether adventitiously (e.g. resulting from traditional methods of plant breeding) or deliberately (e.g. as a result of genetic engineering). The foreseeable problem with such strategies is the possibility that the insects will develop counter-resistance, in much the same way as many insects (including several species of aphids) have managed to overcome externally applied pesticides/insecticides.

A possible alternative to this *modus operandi* would be to alter the fine balance existing between natural plant defence chemicals and the insects' own biochemical counter-defences in such a way that the insects' counter-defenses become energetically uneconomic. As indicated above, aphids are able to utilise their own salivary oxidizing enzymes to effect the oxidation of plant defensive substances to non-toxic end-products. Thus the rose aphid, *Macrosiphum rosae*, has been found to oxidise the predominant rose phenol, catechin, into non-deterrent or even phagostimulant condensation products; it can do so, however, only up to limiting concentrations (Peng & Miles, 1988a).

The Salivary Oxidases of Aphids

The salivary oxidases of aphids have been characterised as both catechol oxidases (EC. 1.10.3.1) and peroxidases (EC. 1.11.1.7). The oxidation of catechin mentioned above could be carried out by catechol-oxidative functions alone, but the purely peroxidative function (as indicated by activity *in vitro* on aniline, for example; Miles & Peng, 1989), presumably also allows the aphids to attack certain classes of plant allelochemicals in addition to phenolics, e.g. terpenoids, aromatic amino compounds and alkaloids. So far the biological implications of these oxidations have been inferred, but left essentially untested. If it can be shown that such oxidations serve to make allelochemicals less toxic to insects, antioxidants rather than toxins *per se* may have an important potential in the development of crop cultivars resistant to aphids (Miles & Oertli, 1993). A novel feature of this approach is that it would seek, not to increase the concentration of inherently toxic components in the plant, but to increase their effectiveness as insect deterrents by augmentation of intrinsically nontoxic cofactors.

Aims of this Study

Hence, the objectives of this study were to:-

(1) Determine the function of oxidative enzymes found in aphid saliva, in relation to their possible involvement in overcoming plant allelochemical defences.

(2) Establish the substrate specificities and mode of action of these salivary oxidases, with a view to predicting their action on other, as yet untested phytochemicals.

(3) Determine the chemical nature of the oxidation products involved, and the effects of these products on the feeding behaviour of the insects.

Scope of the Work Undertaken

These objectives depended on the collection and isolation of aphid salivary enzymes. This presented considerable technical difficulties, however, and one of the major initial aims of the project was therefore to devise a model system whereby appropriate oxidation products could be prepared abiotically in amounts sufficient for analysis and for evaluation of their biological significance.

It may be appropriate to point out, in this context, the difficulties that had to be overcome even in testing the applicability of a candidate model system. Because of the very dilute nature of the preparations of aphid saliva that had to be used, the reactions that they mediated were never observed to go to completion *in vitro*. This meant that the products of such reactions were usually heavily overlayed by unreacted substrate, making definitive identifications of products technically very difficult. Moreover, the possibility arose that spectrophotometric differences between the product mixtures resulting from salivary activity and model enzyme systems were not necessarily due to qualitative differences in their

modes of action, but to the persistence of intermediates in the incomplete reactions in those mediated by the preparations of saliva. These complications had to be considered during interpretation of the data obtained.

Outline of Thesis Layout

As the work described in this thesis is a marriage between biochemical and chemical disciplines, presentation of the information within each chapter has also been a compromise in styles. The flow of each individual chapter is as follows:

Introduction - a brief explanation of the experiments undertaken, and the reasons behind such investigations.

Results & Discussion - observations and information obtained from the tests conducted.

Conclusion - summation of the results obtained, and the relevance of these to aphid-plant interactions as a whole.

Experimental - procedures used and recorded technical data, for all work undertaken.

GENERAL EXPERIMENTAL

The aphids used for all experiments were *Macrosiphum euphorbiae* (Thomas) cultured on young nasturtium (*Tropoleum majus*), unless otherwise stated. Collection of aphid salivary preparation was achieved essentially by the procedure illustrated by Miles & Harrewijn (1991). Aphids are normally restless and easily dislodged from plants in the early to mid-afternoon (P.Miles & J.Kitt, pers. comm.; personal observation), hence during the early afternoon aphids were brushed from their food plant, deprived of a food source for 1 h, and weighed out into feeding chambers (0.10-0.12 g per chamber) made from converted 35 mm film canisters (25 mm x 25 mm; see Miles & Harrewijn, 1991 for detailed diagram). Water (generally nanopure, 150 µl per chamber) was dispensed onto a Parafilm™ membrane covering the top of the chamber, which was enclosed above water in a black plastic container, and maintained at 25°C overnight. "Unfed controls" were aliquots of water dispensed and treated in the same way, but without aphids in the chambers. The following morning, samples of "fed" and "unfed" water were collected and utilised in the experiments.

Protein estimations performed on some samples of salivary preparations were carried out using Bovine Serum Albumin (BSA; 400 µl) as the protein standard. Samples to be tested, either protein standards or saliva preparations (320 µl), were mixed with sodium hydroxide (10 M; 2 µl), and BIO-RAD* (80 µl). The absorption of this mixture at 595 nm (with water "blank" as the internal reference) was then recorded. Salivary preparations were compared with the protein standard curve (Figure I), and active saliva was generally found to contain approximately 5-10 µg/ml of protein.

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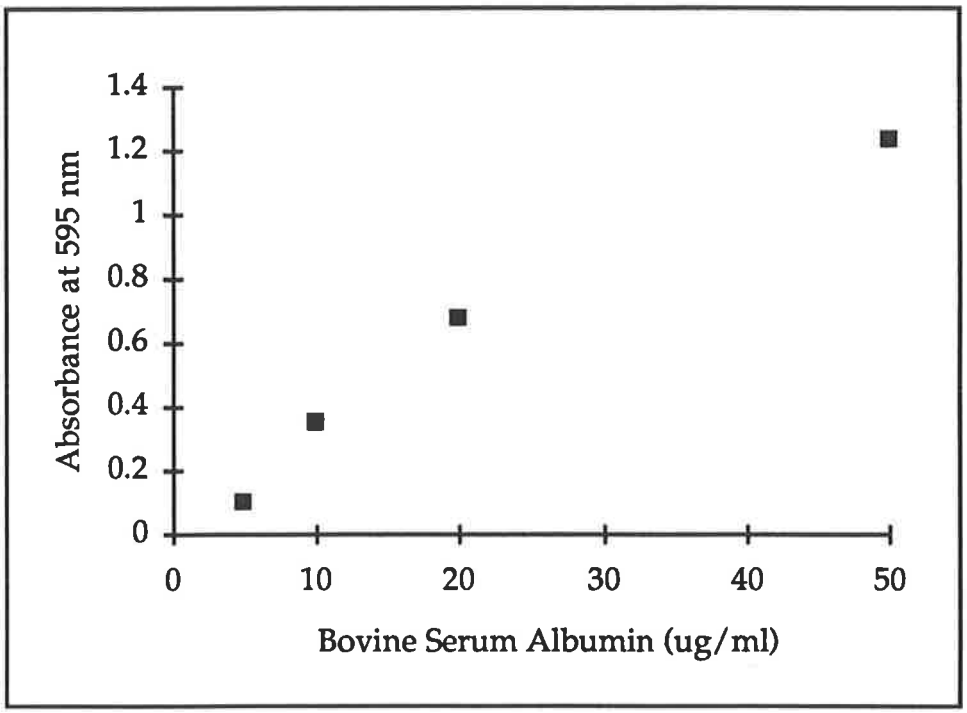
* BIO-RAD protein assay dye reagent concentrate, BIO-RAD laboratories.

Figure I: Protein standard curve (determined using bovine serum albumin solution).



Samples of neat saliva are directly obtainable from the larger sucking insects, and in this work the crusader bug, *Mictis profana F*, a large Heteropteran (*Coreidae*), was used as a suitable source. Individuals were collected during the morning, and placed inverted on foam boards (held securely by pins). Application of pilocarpine solution to the upturned insects' abdomen induced salivation (Miles & Slowiak, 1976; Madhusudhan, *et al.*, 1994). Yields of 10-20 µl/insect of pure saliva were obtainable, normally within 2-4 h. These preparations were used immediately in oxidation reactions.

UV Spectra were determined in quartz cuvettes over the range of 200-700 nm, unless otherwise stated. They were recorded on a Varian DMS 100 spectrophotometer, using a multiscan program and DS-15 Data station. Absorption maxima (λ_{max}) were recorded in nm. For all plant chemical analyses, reaction (compound + enzyme) and control (no enzyme) mixtures were incubated simultaneously. Where appropriate, H_2O_2 was included in both reaction and control solutions. The UV spectrum for the reaction was corrected for additives

(by subtracting both the control & enzyme spectra) to yield a "subtraction" spectrum, indicative of the reaction products formed.

High performance liquid chromatography (HPLC) was performed on an ICI instrument, (DP800 data station, 2x LC 1100 pumps). Separations were carried out on a Brownlee RP-18 column (spheri 5). UV Detection was achieved using an ICI LC 1200 UV detector, set to the appropriate wavelength (as stated in the text). Solvent systems used were as described for individual compounds (see Experimental).

Thin layer chromatography (TLC) was performed on silica gel 60 plates (with F_{254} indicator), unless otherwise stated. The solvent systems and visualisation techniques used are as indicated in the text; i.e., TLC (solvents; visualisation). Ammonium molybdate dip refers to a solution of ammonium molybdate (10 g) in sulphuric acid (1 M, 100 ml).

Gel electrophoresis was performed on Native PAGE gels (gradient 8-25), using a Pharmacia LKB™ Phastsystem separation unit as described by Madhusudhan, *et al.* (1994).

Melting points (m.p.) were determined using a Reichert hotstage microscope, and are uncorrected. ^{1}H NMR spectra were determined in deuterochloroform solutions (unless stated otherwise), and recorded at 300 MHz with a Bruker ACP 300 instrument.

Solvents used were generally analytical grade (HPLC grade used for HPLC analyses) and were freshly distilled; ether refers to diethyl ether. Nanopure water (18 MΩ) was obtained from a Millipore Q purification system.

CHAPTER 1

ENZYME SPECIFICITIES - APHID SALIVARY ENZYMES VS MODEL SYSTEMS

1.1 INTRODUCTION

The major aim of the work described in this chapter was to establish *in vitro* methods for the identification of the reaction products of aphid saliva on a number of representative phytochemicals. As indicated below, this study faced two major difficulties: lack of previous studies on the oxidation products of many of these compounds and the very dilute nature of the preparations containing aphid saliva.

Reactions Involving Plant Chemicals

Although the enzymic reactions of many plant chemicals, phenolics and alkaloids have been mentioned in the literature, very little (or no) product identification has been carried out. A typical example can be found for the plant alkaloid hordenine. The oxidation of hordenine by horseradish peroxidase (HRP) was previously investigated by Meyer & Barz (1978), who described the products simply as "insoluble polymeric material".

A number of other compounds have been treated similarly. Oxidation of tyramine, tyrosine and certain tyrosine derivatives (Gross & Sizer, 1959; Bayse, *et al.*, 1972) resulted in the formation of fluorescent products, the majority of which have, at best, been tentatively characterised by their UV spectral data. Many have been simply reported as "brown amorphous pigment" or "polymerised derivatives". Investigation into oxidative browning of grape musts has gone so far as to determine that grape phenolics are oxidised (by the polyphenoloxidases

present or via coupled oxidation) to o-quinones (Cheynier, *et al.*, 1989). These intermediates are then reported to react with hydroquinone to yield "condensation products".

The effects of aphid salivary enzymes on plant allelochemicals has, until recently, been only inferred; certainly no chemical identification of oxidation products has so far been made. Because of a lack of knowledge of how aphid salivary enzymes work, it has been possible only to speculate on the interactions that take place in the immediate vicinity of those regions in plant tissue that aphids penetrate with their mouth parts and from which they feed. Yet it is usually assumed that such interactions are the basis of plant reactions to aphid feeding.

Over the last decade, the work of Miles and associates (Miles, 1985, 1987, 1990; Peng & Miles, 1988a, 1988b, 1991; Miles & Peng, 1989; Miles & Harrewijn, 1991; Jiang & Miles 1993b; Miles & Oertli, 1993) has indicated that oxidation reactions caused in plants by the feeding of aphids could have importance in deciding whether the plant tissues concerned were susceptible or resistant to aphid attack. It was postulated that oxidases in the saliva and gut of aphids detoxified plant allelochemicals, although the products were not identified. Once again, however, oxidative polymerisation was assumed, although not necessarily to insoluble products (Peng & Miles 1988a).

Although the saliva of aphids can be obtained, presumably uncontaminated, in drops of water on which aphids have fed (Miles & Peng, 1989), these solutions are exceedingly dilute, and the saliva itself is of complex composition, at least with respect to proteins and amino acids (Madhusudhan, Ph D Thesis 1994; see also General Experimental of the present work). The collected saliva can be used to cause oxidation reactions, but the products tend to be mixtures, the individual components of which are either in very small quantities and/or almost impossible to separate in analysable amounts.

As foreshadowed in the Introduction, an attempt was therefore made to find a model system, using commercially prepared oxidising enzymes, that would produce analysable quantities of reaction products that had chromatographic mobilities identical with those produced by aphid saliva. The focus of this chapter is the comparison of the oxidations of several plant chemicals and phenolics with model enzyme systems such as HRP and mushroom tyrosinase and with aphid salivary preparations.

From the very large range of phytochemicals thought to play an allelochemical rôle, it was possible to deal with only a few. Their selection was to some extent dependent on previous work (hordenine), on alleged involvement in specific insect/plant interactions (DIMBOA, gramine, catechin) or their universal occurrence as "secondary compounds" in plants (the phenolic acids). Some specific plant toxins happened to be available (berberine, boldine). Betaine is accumulated as a nitrogenous storage compound in some plants stressed by drought. Ascorbate was included because of its postulated rôle in sparing oxidisable plant allelochemicals (Miles & Oertli, 1993). Other compounds were investigated as chemical models to establish general reaction principles (acetaminophen, aniline). The structural formulae of these compounds are illustrated in Figure 1.3.

1.2 RESULTS AND DISCUSSION

SECTION A - REACTION ANALYSES

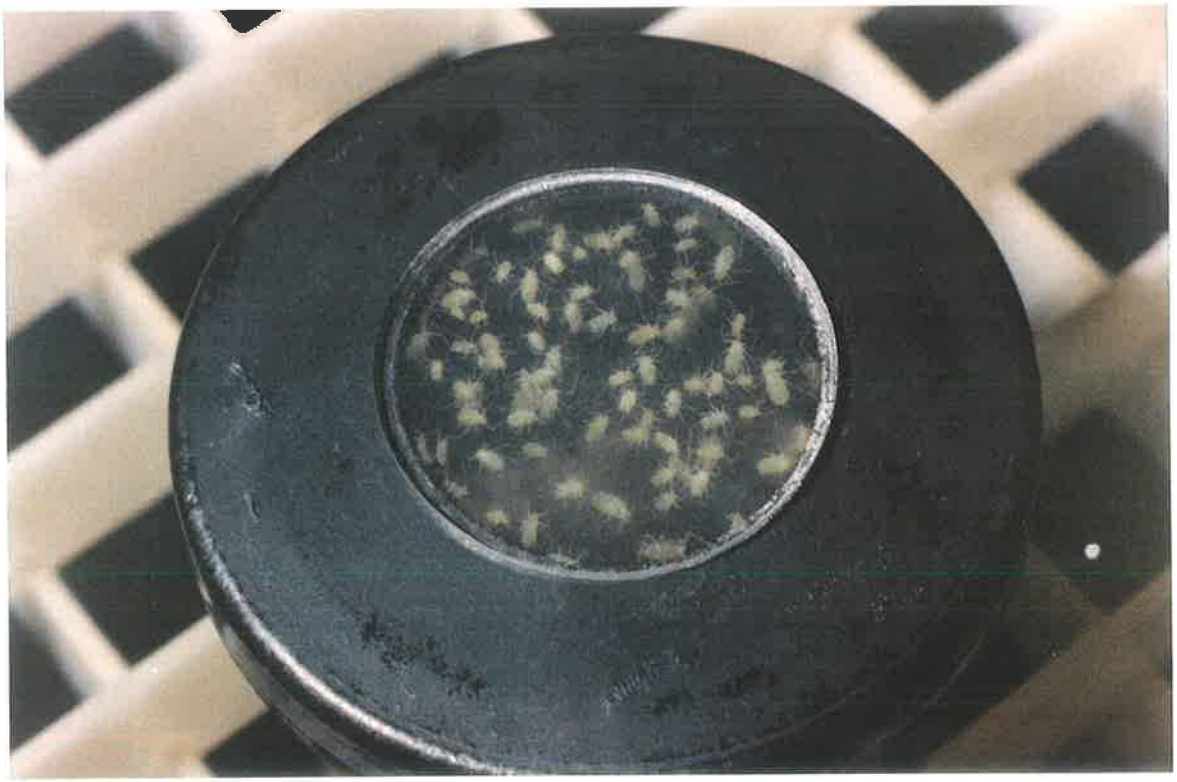
1.2.1 Collection of Aphid Salivary Preparations

It is possible to collect salivary secretions directly from the stylets of some of the larger phytophagous sucking insects, such as the Crusader Bug (see General Experimental of this thesis). This method is impossible with aphids, however, because of their much smaller size. For this reason Miles and associates developed a feeding technique that enables the collection of small quantities of dilute salivary preparations (Miles & Harrewijn, 1991).

Aphids (*ca*. 100-200 individuals) were weighed (*ca*. 0.10-0.12 mg) into feeding chambers, composed of a black film canister, modified as described in the General Experimental. Water (nanopure) was dispensed onto the parafilm membrane and the drop covered with a glass cover slip. The entire chamber was enclosed within a black plastic container (with a yellow lid), and left at a constant temperature of 25°C, illuminated with yellow light, for about 14-16 h.

Aphids are either negatively geotactic and/or attracted to yellow light and will climb up inside the feeding chamber and feed inverted upon the drop of liquid (see Figure 1.1). Aphids salivate while feeding, and some of their saliva accumulates in the solution. After an overnight feeding period, the drop of water becomes a dilute preparation of salivary enzymes. Previous work (Miles and co-workers) has shown that this solution contains enzyme activity (e.g. catecholase & peroxidase activity), although at a very low level. Attempts to concentrate these preparations have had only limited success, also the enzymic activity of the solutions tends to be lost after more than overnight storage.

Figure 1.1: Aphids (*M. euphorbiae*) inside a feeding chamber, ingesting from a droplet of water.



In this work the potato aphid, *M. euphorbiae*, was used as the source of salivary preparations, due to it's relatively large size, ease of handling, and the enzyme activity of drops on which it has fed. To maximise the activity in the collected solutions, each chamber of aphids was supplied with only 150 µl of water. Generally, about 100-125 µl of this could be recovered after overnight feeding (some loss occurs due to ingestion by the insects and/or evaporation*). These preparations were then used in enzymic reactions with phytochemicals.

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* Water can actually diffuse through stretched parafilm and evaporate.

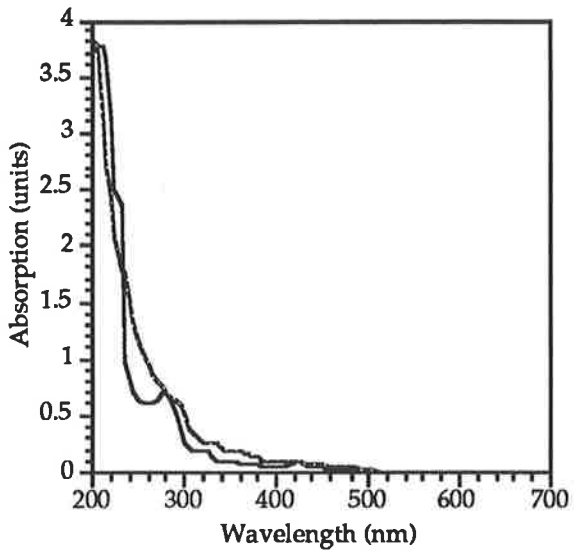
Random protein estimations (see General Experimental) performed on some enzymic preparations revealed them to contain between 5 & 10 μg/ml of protein. Hereafter when the reactions of aphid "saliva" are described or discussed, it is to such preparations that reference is made.

1.2.2 UV Spectral Analysis

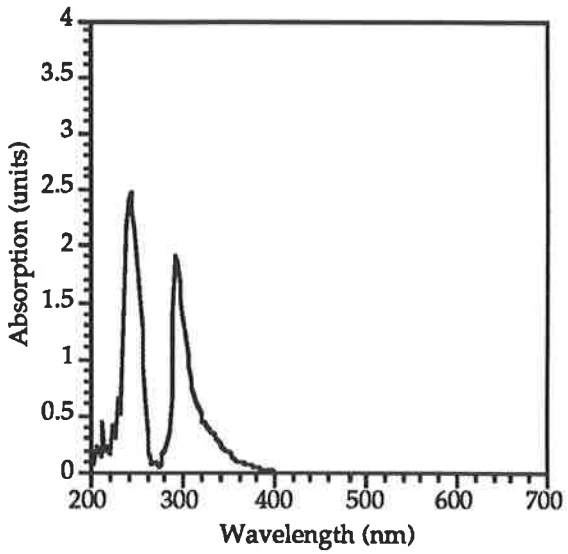
Each of the chemicals examined was treated with enzyme (either HRP and/or tyrosinase, or aphid saliva), generally in the presence of hydrogen peroxide (H_2O_2) as the electron acceptor for peroxidase activity. In each experiment, a control solution, also containing H_2O_2 but with no enzyme added, was subjected to the same conditions. The UV-visible spectra (200-700 nm) of these solutions were recorded, and the change in absorption of the substance ascertained by correcting the reaction spectrum for both the control solution and the enzyme system measured separately, both also incubated for the same length of time. The resulting corrected spectrum is here-in termed the "UV subtraction" spectrum. An indication of any oxidation product(s) formed was thereby obtained. The nature of the results so obtained is illustrated by a typical set of such spectra, shown below (Figure 1.2).

Figure 1.2: Set of UV reaction, control & subtraction spectra for the oxidation of hordenine by HRP/H_2O_2.

(a) Reaction & control (R = –; C = —)



(b) Subtraction spectrum



Initial experiments were performed with relatively concentrated solutions of both substrate and enzyme(s), in the hope of maximising product formation and simplifying peak determination. This proved to be an unfortunate misconception - at such higher concentrations, some absorption peaks were above the machine's upper absorbance limit, so that subtraction spectra became misleading. The experiments were later repeated using lower concentrations, to identify the true product peaks and compare the effects of machine limitations. The results using the higher concentrations are nevertheless given as Appendix 1, since some of the visible colours and precipitates produced provide additional evidence concerning reaction products.

Table 1.1: Typical UV subtraction spectra of dilute solutions of compounds following reaction with a low enzyme solution. All absorption peaks (nm) are based on recordings within the sensitivity limits of the spectrophotometer and can be considered a true indication of the formation of oxidation products. Data for autoxidations are after subtraction of spectra of freshly made solutions.

br = broad, sl = slight;

(-) = no reaction products observed;

negative numbers indicate negative peaks.

COMPOUND	HRP	TYROSINASE	APHID SAL.	AUTOXID.
Acetaminophen	268, 300, 350 to 420br	268, 300, 350 to 420br	243, -300	
Ascorbic Acid	(-)	-259, -388	-242, 280	
Berberine	(-)	(-)	(-)	
Betaine	(-)	(-)	(-)	
Boldine	246, 289, 326	(-)	(-)	
Caffeic acid	264, -291/-316, 410 yellow*	As for HRP yellow*	238, 269, -335	250, -277, -337, 370 to 500
Catechin	247, 293, 300 to 420br yellow-orange*	244, 378 orange-red*	246, 290sl, 434br, 483br	
Chlorogenic acid	261, -318, 403 yellow soln	260, -318, 403 yellow soln	260, -308, -336, 385sl	
DIMBOA	240, -275, 336, 420 yellow soln	(-)	-257, 304, 347 (with H_2O_2) 283, 298 (without H_2O_2) slight yellow	
Ferulic acid	253, -310, 367, 418 pink*	(-)	269, -342	230, -265
Gramine	300, 420	(-)	260, 303	257, -290, 306
Hordenine	234, 290, 333sl, 422sl	237, 289, 309	287	

* coloured products (precipitate) formed.

The observation that betaine did not react with HRP was not surprising as betaine is a quaternary amino compound, and as such no peroxidative reaction sites are present. Similarly, berberine, a complex, pentacyclic aromatic molecule, was unreactive towards the enzymes. It, too, has no free aromatic hydroxyl groups or other readily reactive sites.

Boldine provided an interesting result. This tetracyclic compound is acted upon by HRP, but shows no reaction with either mushroom tyrosinase or aphid salivary enzymes. Inactivity towards catecholoxidase is not unexpected in relation to boldine's chemical structure. The lack of reaction with aphid saliva, however, is intriguing. With virtually all other compounds investigated in this work, salivary enzymes were able to react with some functionality present on the molecule, at least in the presence of hydrogen peroxide.

Although the products of autoxidation of these compounds show similar absorption spectra to those produced with enzymes, little change to substrates took place under test conditions within 24 h in the absence of enzymes, and hence autoxidation did not interfere with the spectral analyses (taken after 2 h).

As can be seen from the absorption peaks observed, it seems likely that HRP and/or tyrosinase* and the aphid salivary enzymes may yield the same oxidation product(s) with some compounds. Similarly, several structurally related compounds appear to follow analogous reaction pathways. For instance, the UV data obtained implies that chlorogenic acid and caffeic acid are oxidised to the same compound(s). Similarly, some of the peaks observed in the ferulic acid subtraction spectrum appeared to correspond with the oxidation products of chlorogenic and caffeic acids. These speculations required further verification, and

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* This refers to the use of HRP + H_2O_2, HRP + H_2O_2 + tyrosinase, and tyrosinase alone.

for this purpose thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) techniques were employed.

The reaction of ascorbic acid with aphid saliva was also of interest because of the possibility that ascorbate could have a function in moderating the response of plants to aphid feeding (Miles & Oertli, 1993). Ascorbic acid was acted upon by aphid salivary enzymes to yield a UV subtraction spectrum with an absorption at 280 nm and a negative peak at 242 nm. Chemical oxidation of ascorbate (using iodine) resulted in the formation of dehydroascorbate, indicated by a loss of absorption in the UV-region of the spectrum. Ascorbate itself absorbs at 260 nm, hence the aphid saliva product(s) is neither unreacted ascorbate nor the dehydro-derivative. Work performed by Madhusudhan (PhD Thesis, 1994) has shown that the salivary enzymes from two other species of aphids, the spotted alfalfa aphid (*Therioaphis trifolii*) and the pea aphid (*Acyrthosiphon pisum*), are also able to react with ascorbic acid. These insects do not seem to secrete a specific ascorbate oxidase in their saliva, but their saliva showed non-specific oxidase dehydrogenase activity, with the ability *inter alia* to oxidise ascorbate.

1.2.3 Thin Layer Chromatography

To gain further knowledge of the number of oxidation products involved and their possible identity, and to enable comparisons of the different enzyme systems, many of the reactions were analysed by TLC. In general, analysis of reactions involving HRP and/or tyrosinase was readily achievable (see Table 1.2). Oxidations involving aphid salivary preparations, however, proved far more difficult to interpret. The enzymic solutions in these instances were so dilute that very little product formation occurred, and the major component detected was always the unreacted starting material.

Table 1.2: TLC analysis of reaction mixtures resulting from the action of HRP/Tyrosinase on some plant allelochemicals and acetaminophen. The solvent systems and visualisation techniques used are as described in the Experimental section.

COMPOUND	No. OF PRODUCTS	Rf VALUES
Acetaminophen	2	0.10, 0.38
Boldine	3	0.16, 0.34, 0.56
Caffeic Acid	2	0.06 & baseline
Chlorogenic Acid	2+	0.19, baseline to 0.10 (smear)
DIMBOA	2	0.13, 0.40
Ferulic Acid	2	0.33, 0.46
Gramine	2	0.25, 0.80
Hordenine	2	0.13 & base line

Attempts were made to concentrate aphid salivary reactions by freeze drying (oil pump vacuum), however autoxidation of the control solutions readily occurred under these conditions. As successful TLC analysis of the aphid enzymic oxidations was not possible, HPLC was chosen as an alternative analytical method for reaction comparisons.

1.2.4 High Performance Liquid Chromatography

HPLC investigations of the aphid salivary reactions were performed as indicated in the General Experimental. Reaction mixture components (oxidation products and starting materials) were visualised by UV detection, and this method proved very successful for the dilute enzymic reactions.

Hordenine reaction solutions clearly showed unreacted starting material, as well as two smaller product peaks. The chromatograms obtained, using a water-acetonitrile solvent system, were identical for HRP/tyrosinase* and aphid saliva reactions. Analysis of a 1:1 mixture of the two reaction types revealed a single peak for each product, thus giving strong evidence for the formation of common products by both enzyme systems.

Gramine, an indole alkaloid, similarly produced identical reaction products with either the model enzyme system or aphid saliva. Using the same solvent system as described for hordenine (above) the reaction of gramine with HRP/tyrosinase gave two major products, at 9.70 & 12.75 mins. Oxidation by aphid saliva also produced two compounds, with retention times identical to the model system products. This result was obtained using two different ion-pair reagents and gradients, eliminating the possibility of co-eluting compounds.

DIMBOA, when oxidised by the model enzyme system, was converted almost quantitatively to four products. The chromatogram obtained for aphid salivary reaction presumably also contained these compounds (retention times comparable), although a large amount of unreacted DIMBOA was still present. The dilute nature of the salivary preparations would account for a smaller overall yield of oxidation products. Analysis of the spectrophotometric data did not

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* The model enzyme system, i.e., HRP + H_2O_2 + mushroom tyrosinase.

confirm that all the absorption peaks produced by the saliva were the same as those produced by the model system. It is difficult to interpret these differences in view of the incompleteness of the observed reaction with saliva.

The diphenolic acids, and their derivatives, (caffeic, chlorogenic and ferulic acids) provided more interesting results. The oxidation of caffeic acid by HRP/tyrosinase yielded several products, whilst the reaction with aphid saliva gave only two. Both of these were observed in the model enzyme reaction, but only as minor oxidation products. Similarly, reaction of chlorogenic acid with HRP/tyrosinase gave a number of products, of which only the minor products were observed after the salivary oxidation. As with DIMBOA, it is possible to interpret these results as indicating a less complete stage of oxidation by the salivary preparation compared with the model enzyme system.

Ferulic acid gave considerable difficulty when oxidation by HRP and saliva was compared using both HPLC and UV spectra. Analysis of the HRP oxidation by HPLC showed no evidence of starting material, indicating that ferulic acid had been completely converted to other substances. Using UV detection at 270 nm, no product was found with absorption at this wavelength, although 271 nm was the peak of the main oxidation product with aphid saliva (UV spectroscopy).

Several attempts were made to analyse aphid saliva oxidations of ferulic acid by HPLC, but none were successful. The UV spectral data implied that the products from this reaction should be similar to those with caffeic acid, hence identical conditions had been applied. On all occasions, however, starting material was the only substance detected by HPLC. UV spectral analysis of these same salivary oxidation reactions confirmed product formation had indeed occurred, indicated by an absorption peak at 271 nm. Yet, HPLC analysis, with UV detection at 265-275 nm, revealed no compound(s) other than ferulic acid itself. Collection of the total solvent output from the column (5 minute fractions, and as one fraction), and subsequent UV analysis after concentration, also revealed no traces of

product(s). These observations can be explained if the ferulic acid/saliva oxidation products are retained on the guard column/column itself, and hence are not observed in fractions retrieved; or that the reaction product has the same retention time as ferulic acid, and hence starting material and product appeared as a single peak. Time constraints prevented further experimentation in this area. The use of a different solvent system or perhaps an alternative type of HPLC column may enable identification of all components of these reaction mixtures.

1.2.5 The Crusader Bug and it's Salivary Enzymes

The aphid saliva preparations used for the majority of this work are exceedingly low in enzyme concentration. In contrast, amounts of pure saliva can be collected directly from the mouthparts of larger sucking insects (see General Experimental). Quantities of saliva from Crusader Bug individuals were collected, and used in phytochemical oxidations, to provide a comparison between the different Hemipteran species.

Aniline was reacted with the pooled saliva from three fourth instar Crusader bugs. TLC analysis of the reaction mixture revealed quantities of unreacted aniline, and a non-polar product (Rf=0.87). This compound migrated to the same position as the product observed for oxidation of aniline by both the model enzyme system and aphid salivary preparations (concentrated reaction mixtures).

Oxidation of hordenine by Crusader bug saliva resulted in a UV subtraction spectrum with peaks at 262 & 312 nm. Again, these results are comparable with those found for aphid saliva and model enzymes. Thus it would seem that the saliva secreted by the (relatively much larger) Crusader bug has enzymic properties similar to that from the Aphididae. This observation could prove invaluable to future investigations of the direct interactions between insect feeding and plant response. Before such work could be instigated, however, a preliminary

investigation would be required to establish as far as possible that the salivary content of the two species would be comparable.

SECTION B - ENZYME INHIBITION STUDIES

Determination of the oxidation products formed by reaction of aphid saliva with plant allelochemicals was only one aspect of this investigation. Of equal importance is the need to establish the possible mechanisms involved in these reactions so that reasonable predictions can be made of the likely reactions that occur between aphid saliva and phytochemicals generally. The chemical composition of crop plants can be influenced, whether by traditional methods of plant breeding by selection, or by genetic engineering. Hence, a fore-knowledge of how changes in the chemical content of plant tissues might influence their interactions with sucking insects could provide an invaluable guide in efforts to produce crop plants that are either tolerant or resistant to aphid attack. One objective of the present work therefore was to establish as definitively as possible the nature of the salivary enzymes. With this purpose in mind a number of enzymic oxidation reactions were performed using specific enzyme inhibitors, in an attempt to characterise the salivary enzymes.

Catalase was chosen as a suitable inhibitor of peroxidative reactions. Even in the absence of added hydrogen peroxide, some peroxide may be present adventitiously or as a by-product of non-peroxidative oxidation of the substrate. Catalase is known to scavenge hydrogen peroxide from solutions very rapidly (Mason, 1957; Saunders, 1964), however, breaking it down to water and molecular oxygen.

Phenyl thiourea (PTU) is a convenient inhibitor of catechol oxidase (diphenolase) activity (Dubios & Erway, 1946). The PTU acts as a chelator of copper ions which act as the prosthetic group of most phenolases.

Reactions using the model enzyme system, as well as those using aphid salivary preparations, were also subjected to selective inhibition by catalase (10^5 units/ml; in the absence of added hydrogen peroxide) or PTU (3.3 mM and 10 mM). Control solutions, enzyme precluded, were subjected to the same conditions. Evidence for the enzyme mechanism involved was determined by analysis of the UV subtraction spectrum. Results are indicated in the following table (Table 1.3):

Table 1.3: Enzyme inhibition reactions performed on acetaminophen and a selection of plant chemicals:
x = No reaction occurred (i.e., complete inhibition).
√ = Reaction occurred (i.e., no or limited inhibition); products as for Section A unless specified otherwise.
- = Reaction not attempted.

COMPOUND	MODEL SYSTEM		APHID SALIVA		
	Catalase	PTU	Catalase	PTU (3.3 mM)	PTU (10 mM)
Acetaminophen	√	√	x	√	x
Ascorbic Acid	√	x	x	-	-
Caffeic Acid	√	√	√	√	x
Catechin	√ (246,333, 393,434,479)	√ (246,294, 338)	-	-	-
Chlorogenic Acid	√	√	√	√	x
DIMBOA	x	√	√ (283,298)	√	x
Ferulic Acid	x	√	x	√	x
Gramine	x	√	x	-	-
Hordenine	√	√	x	√	x

Examination of the results indicated in Table 1.3 shows that in the majority of cases the reactions proceed mainly via a peroxidative mechanism, although, in the absence of hydrogen peroxide for peroxidase activation, catechol oxidase activity enables the reaction to proceed. It should be noted here that, in the absence of exogenous H_2O_2 and catalase, phenolic substrates can be acted upon by a phenolase, possibly generating H_2O_2 (Jiang & Miles, 1993a) and thereby creating the conditions for a synergistic reaction between phenolase and peroxidase if the latter is also present. The obvious exceptions to these generalisations are gramine and DIMBOA. These two compounds are not acted upon by diphenolase, which complies with their chemical structure (no diphenolic sites); i.e., the nature of their oxidation is purely peroxidative.

When aphid saliva was used as the oxidising enzyme, the presence of 10 mM PTU (used to inactivate copper enzymes) prevented reaction occurring for all of the compounds tested. This result is comparable with similar experiments conducted using the spotted-alfalfa and pea aphids; the saliva from these insects was reported to contain a copper peroxidase (Madhusudhan, PhD Thesis, 1994); i.e., one that is completely inhibited by PTU.

PTU at a lower concentration (3.3 mM), however, did not yield total inhibition of the oxidation reactions, although a reduction in the expected peaks was observed in the UV subtraction spectra. This observation could be consistent with the presence of two enzymes or of an enzyme with more than one active site - one for diphenolase and one for peroxidase - both activities dependent on copper but with differing sensitivities to copper-chelating inhibitors.

SECTION C - GEL ELECTROPHORETIC STUDIES

Rigorous confirmation of copper-peroxidase activity in saliva from the potato aphid was obtained by electrophoretic analysis. Collected salivary

preparation was concentrated under vacuum (Speedy Vac™), and the resultant solution analysed by gel electrophoresis (see General Experimental). The total salivary protein profile, and identification of the bands responsible for catecholoxidase and peroxidase activity, were determined simultaneously, using the techniques developed by Madhusudhan (PhD Thesis, 1994); see also Madhusudhan, *et al.*, (1994).

At least seven protein bands were detected in concentrated saliva by silver staining of the gel (Rf's 0.65, 0.52, 0.46, 0.41, 0.26, 0.16 & 0.08), although only five of these were evident in the collected preparations prior to concentration. Incubation of the gel in a solution of L-DOPA (0.01 M) revealed only one band with diphenolase activity (Rf 0.46). Identification of a separate peroxidase protein (Rf 0.52) was established by incubation of the separated proteins in a 1:1 mixture of Western blotting reagents RPN 2109, this activity was inhibited by the presence of 0.01 M PTU.

These observations were consistent with aphid saliva containing two copper enzymes; i.e., with peroxidase and catecholoxidase activity respectively. At first glance these results appear inconsistent with the findings of Madhusudhan (PhD Thesis, 1994) which suggested the identity of peroxidase and catechol oxidase activity with the same proteins. It must be remembered, however, that investigations by Madhusudhan involved the use of saliva from the spotted alfalfa and pea aphids; species different from those used in the work described here. Differences between the oxidative enzymes of aphid species is an interesting concept, which clearly invites further investigation. Moreover, the confirmation of the existence of a copper-dependent peroxidase is itself noteworthy as being with little or no precedent before Madhusudhan's study. Nevertheless, further pursuit of these subjects was considered beyond the scope of the present study.

1.3 CONCLUSION

The reactions catalysed by aphid salivary enzymes is a topic that has received very little attention, until the present work. As indicated in the Results & Discussion of this chapter, aphid saliva is able to oxidise a considerable range of plant compounds. These include mono-phenolics (e.g. hordenine, acetaminophen and ferulic acid), di-phenolics (e.g. caffeic & chlorogenic acids), and other compounds capable of more complex chemical reactions such as indoles (gramine) and hydroxamic acids (DIMBOA), all of which have been postulated to be involved with plant defence against herbivores in some manner.

Reaction of aphid saliva with ascorbic acid could also be of fundamental significance. Ascorbate is a known plant reductant (or anti-oxidant), and has been suggested to inhibit the insects' ability to counteract plant defences (Miles & Oertli, 1993). Ascorbate, in plants, may help to maintain the redox balance between phenolic compounds and their oxidised counterparts (generally via quinones). By oxidising ascorbic acid the insects could therefore be affecting the sensitive redox balance of the plant, and may in consequence increase the ability of these insects to feed on a host (Jiang & Miles, 1993b).

The combination of horseradish peroxidase/hydrogen peroxide and mushroom tyrosinase as an enzyme system suitable for mimicking the combined peroxidase and catechol oxidase (diphenolase) activity of aphid saliva, shows promise. Nearly all the reactions catalysed by the model system gave oxidation products with UV spectral and chromatographic properties identical to those found following salivary oxidations. Although the model system cannot be assumed to conform precisely to aphid salivary reactivities, the application of this system would give a useful indication of the reactions possible, and a starting point for determining the nature of likely oxidation products.

Formation of oxidation products due to autoxidation (particularly when hydrogen peroxide may be present) occurs to a small extent with all the aphid salivary reactions outlined here. Although use of high concentrations of substrate (Appendix 1) gave reactions which may have indicated that autoxidation products were identical to those formed in the presence of aphid saliva or horseradish peroxidase, the results so obtained were not fully analysable because of the limitations of the spectrophotometric method employed. Analysis of the autoxidation of several compounds at lower concentration (Table 1.1), arguably more relevant to *in vivo* situations, indicated that only in some instances were autoxidation products similar to those produced in enzymic reactions (e.g. 306 nm peak for gramine). Autoxidation was only apparent in solutions after 24 h, whereas reaction analyses were performed after 2 h. In the experiments reported above, therefore, it may be assumed that autoxidation did not significantly affect results obtained with the enzyme systems involved.

Because of the many technical difficulties encountered in the use of preparations of aphid saliva, the results obtained offer at most an introductory glimpse into what may well prove to be a complex and vital biochemical link in the interaction between sucking insects and their food plants. It was therefore of particular interest to note similarities between oxidation reactions involving saliva from aphids and that of larger sucking insects (e.g. Crusader bug). If saliva from the larger sucking insects can be shown to react consistently in the same manner as that from aphididae, then it would be possible to use these insects as a much better, i.e., much more natural, model for the salivary activity of aphids than enzymes such as HRP and mushroom tyrosinase: the characteristics of the salivary enzymes of sucking insects are more likely to correspond with each other than with those derived from plants or fungi. Also, the usefulness of neat saliva of the large bugs compared with a model enzyme system may have other advantages. Salivary secretions have components other than oxidising enzymes, e.g. amino acids (Miles, 1972), which may affect the insect-plant interaction. A model enzyme system is

deficient in this way - it will never be able to simulate completely the complex reactions occurring within a plant. Investigations into the larger insects would therefore be most interesting and worthwhile, but would go well beyond the scope of the present project.

Biologically significant conclusions to be derived from the current research are the generalisations that can be made regarding the selectivity of aphid salivary enzymes. Analysis of the results obtained, and comparison with the structural features of the compounds involved (see Figure 1.3) mean that several "rules of thumb" can be postulated:

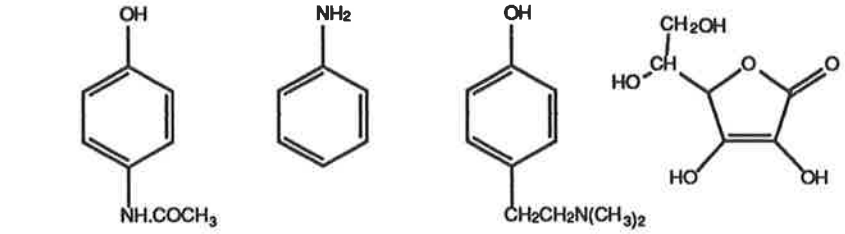
(i) Mono-phenols are oxidised by both HRP, mushroom tyrosinase and aphid saliva to give the same products, except where another functionality (i.e., amine) is present.

(ii) Non-phenolic or mono-phenolic amino/nitro-compounds are oxidised by both HRP and aphid saliva to give the same products (these compounds are not acted upon by catechol oxidase due to absence of an o-diphenol function).

(iii) Diphenols are oxidised by HRP, tyrosinase and aphid saliva to yield the same products.

From the information outlined above, and from the enzyme inhibition studies performed, it would seem that the aphid saliva examined here behaves predominantly as a peroxidase, with catechol oxidase (o-diphenolase) activity, and is similar in reactivity to the model enzyme system. The salivary reactions are inhibited by copper chelators (PTU), however, indicating the presence of a copper-based peroxidase, as well as a diphenolase. As indicated in the Results and Discussion, Section C, the existence of a copper-dependent peroxidase is of fundamental biochemical interest. Further investigation of this aspect of the results was considered beyond the scope of the present study.

Figure 1.3: Chemical structures of the compounds investigated.

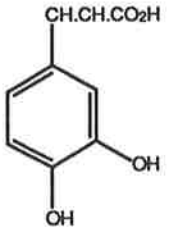


Acetaminophen

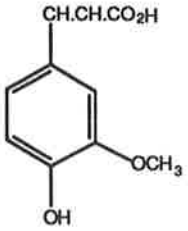
Aniline

Hordenine

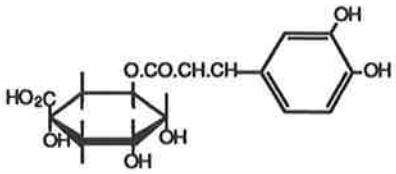
Ascorbic Acid



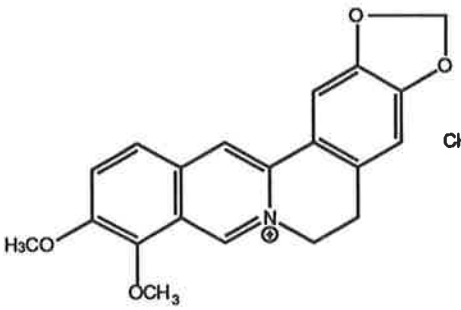
Caffeic Acid



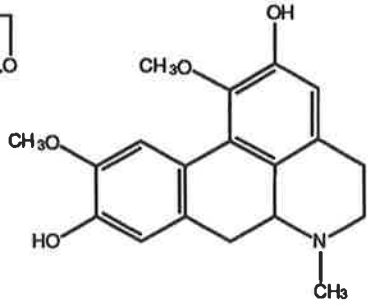
Ferulic Acid



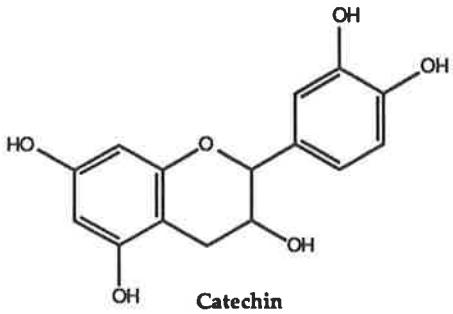
Chlorogenic Acid



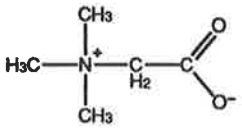
Berberine



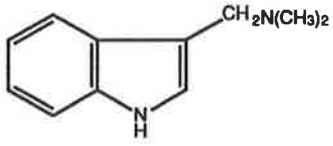
Boldine



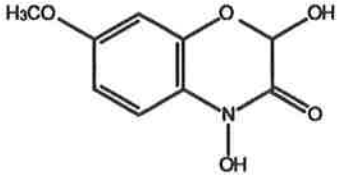
Catechin



Betaine



Gramine



DIMBOA

1.4 EXPERIMENTAL

Collection of aphid salivary enzymes, UV spectroscopy, gel electrophoresis, TLC & HPLC techniques are all described in the General Experimental section (see earlier). Each plant chemical was subjected to the same common reaction sequence, (described below), for both concentrated and more dilute oxidations. Information specific to the individual compounds is indicated separately. Any deviation from the general procedure is described in detail for the compound involved.

SECTION A - REACTION ANALYSES

In comparing the results given in Table 1.1 (page 19) and the more detailed accounts in the following Experimental section, it should be noted that for some compounds the UV spectral peaks for the different reaction mixtures were sufficiently similar as to be consistent with the production of identical products following oxidation by aphid saliva, by model enzymes and/or by autoxidation (e.g. for catechin and possibly gramine); whereas for other compounds significant differences in these respective reaction mixtures were apparently indicated. A number of points can be made in relation to these results.

Firstly, the UV data quoted in Table 1.1 were repeatable. The only qualification that needs to be made is that preparations obtained from different batches of aphids not surprisingly showed differences in activities and those with the greatest activities gave the highest and most easily measurable peaks. Secondly, because of the subtraction method used, the peaks quoted indicated at the very least genuine changes from the original starting materials. Thirdly, because all the oxidation reactions indicated can be progressive, differences in the spectra of reaction mixtures originally containing the same substrate could be due

to differing concentrations at analysis of intermediates in the same oxidative sequence leading to final (e.g. precipitated) oxidation products. It is noteworthy that even in autoxidation reactions, different peaks were recorded in concentrated and dilute solutions (e.g. of gramine). Fourthly, with respect to the saliva preparations, because of the very dilute nature of the participating enzymes, any indications of change in the reaction mixtures may be considered to indicate significant biochemical reactivity of the original secretion.

General Phytochemical Oxidations

I. HRP/H_2O_2 System.

(i) Concentrated solutions:

Plant chemical (1.0 mg/ml; 10% EtOH), H_2O_2 (0.03% in solution) and HRP (0.5 mg/ml) were reacted in a total volume of 1 ml. A control mixture (no enzyme added) was run simultaneously. After 2 h at room temperature the UV subtraction spectrum was calculated, and the mixtures analysed by TLC.

(ii) Dilute solutions:

Plant chemical (0.05 mg/ml, 10% EtOH) was reacted with HRP (0.10 mg/ml) and hydrogen peroxide (0.003%), in a total volume of 1 ml. A control solution, without enzyme, was treated similarly. After 2 h at room temperature the UV spectra of the solutions were recorded and the data analysed. The results were compared with the concentrated reaction outlined in (i) above.

II. HRP-Tyrosinase Model System.

Plant chemical (0.05 mg/ml; 10% EtOH) and H_2O_2 (0.003%) were reacted in the presence of HRP, tyrosinase (0.05 mg/ml each) or a 1:1 mixture of both, in a total volume of 1 ml. Control solutions (without enzyme) were treated similarly.

After 2 h at room temperature the UV subtraction spectra for each reaction was calculated.

III. Aphid Salivary Enzyme Oxidations.

(i) Concentrated solutions:

Plant chemical (1 mg/ml; 10% EtOH) and H_2O_2 (0.03% in solution) were reacted with aphid salivary preparation (usually 250-300 µl), in a total volume of 400 µl. Unfed control solutions were reacted similarly. After 2 h at room temperature the UV subtraction spectrum was obtained. TLC analysis was not generally performed.

(ii) Dilute solutions:

As for III (i) outlined above, but using only 0.05 mg/ml of plant chemical, and 0.003% hydrogen peroxide.

1.4a.1.1 Acetaminophen with Horseradish Peroxidase

1. With H_2O_2.

(i) Concentrated solutions:

In procedure I (i), immediately following enzyme addition a colour change (colourless to pale yellow) was observed. The control mixture (no enzyme added) remained colourless. The UV subtraction spectrum contained a product absorption at 306 nm and a lesser, broad absorption at 406 nm. The mixtures were analysed by TLC (ethyl acetate:acetic acid, 85:15; UV/iodine), revealing starting material (Rf 0.64) as well as traces of at least two products (Rfs 0.38 & 0.10).

(i) Dilute solutions:

In procedure I (ii), immediately upon enzyme addition the reaction solution underwent a colour change, colourless to yellow. A creamy-yellow suspension formed with time. The control solution remained colourless for the reaction period. The UV subtraction spectrum from the precipitated product* revealed sharp increases at 268 & 300 nm, as well as a broad peak over 350-420 nm.

2. Without H_2O_2.

The procedure outlined in I (i) was repeated, with the omission of hydrogen peroxide. The reaction and control solutions remained colourless for the reaction period, suggesting that reaction had not occurred. Analysis of the UV spectra obtained confirmed products to be absent.

1.4a.1.2 Acetaminophen with Aphid Salivary Enzymes

(i) Concentrated solutions:

In procedure III (i), the UV subtraction spectrum obtained showed an absorption peak at 309 nm, similar to that observed for the HRP reaction. TLC analysis (ethyl acetate:acetic acid 85:15; UV/iodine) of the concentrated solutions revealed starting material (Rf 0.62), but product components were not observed (concentration too low). The spectrophotometric results were identical with those found for the HRP reactions described in 1 and 2 above.

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* Reaction mixtures were centrifuged; the resultant precipitated pellet was removed, dissolved in EtOH (1 ml), and examined by UV spectroscopy.

(ii) Dilute solutions:

In procedure III (ii), the UV subtraction spectrum showed a peak at 243 nm, and a negative absorption about 300 nm, different to those observed for the more concentrated reaction.

1.4a.1.3 Reaction of Acetaminophen With HRP+Tyrosinase

Acetaminophen (0.05 mg/ml; 10% EtOH) and H_2O_2 (0.003%) were reacted in the presence of HRP + tyrosinase (1:1; 0.05 mg/ml of each), in a total volume of 1 ml. A control solution (without enzyme) was treated similarly. After 2 h at room temperature the UV subtraction spectrum contained peaks at 268 & 300 nm, with a slight broad absorption over 350-420 nm.

1.4a.2.1 Aniline With Horseradish Peroxidase

Procedure I (i) was performed using aniline. Immediately after enzyme addition a colour change was observed (colourless to red-brown). Control solutions (no enzyme added) did not undergo this colour change. The UV subtraction spectrum contained a large product absorption at 328 nm, shoulder at 408 nm. TLC analysis of the solutions ($CHCl_3$:MeOH, 95:5; UV/iodine) revealed aniline (Rf=0.57), as well as two products (Rfs=0.86 & 0.40).

1.4a.2.2 Aniline With Aphid Salivary Preparations

Procedure II (i) was performed using aniline. The UV subtraction spectrum revealed a product absorption at 305-313 nm. A slight absorption over the 400-500 nm range was present, but this was negligible in size. After concentration (freeze drying), the red-coloured reaction and control mixtures were analysed by TLC ($CHCl_3$:MeOH, 95:5; UV/iodine). This revealed the presence of starting material (aniline, Rf=0.57), and two products (Rfs=0.86 & 0.40) after reaction with

saliva. A control sample of aniline (1 mg/ml) subjected to the same concentrating conditions was also found to have only the Rf=0.40 product when analysed by TLC.

1.4a.2.3 Aniline with Crusader Bug Saliva

Three crusader bug nymphs were subjected to pilocarpine treatment (see General Experimental), and a total of 10 µl of watery saliva was collected. This was reacted with aniline (1 µl), and hydrogen peroxide (0.03% in solution), in a total of 50 µl. After 1.5 h at room temperature the reaction and control (double distilled water in place of saliva) solutions were analysed by TLC ($CHCl_3$-MeOH, 95:5; iodine/ammonium molybdate dip). The chromatography revealed unreacted aniline (Rf=0.58), as well as a slight trace of product in the reaction mixture at Rf=0.87.

1.4a.3.1 Ascorbate and Aphid Salivary Enzymes

(i) Concentrated solutions:

In procedure III (i), the UV subtraction spectrum revealed a product peak at 289 nm, suggesting that aphid saliva may be able to oxidise ascorbate.

(ii) Dilute solutions:

The procedure of III (ii) was repeated, except that the reaction was performed both with and without the addition of hydrogen peroxide. The UV subtraction spectrum was the same in either case - an absorption peak was observed at 280 nm, with a negative peak about 242 nm (possibly due to the loss of H_2O_2, see Madhusudhan's PhD Thesis, 1994).

1.4a.3.2 Ascorbic Acid With HRP &/or Tyrosinase

In procedure II, comparison of the UV spectra for the reaction and control solutions indicated a loss of absorption at 259 & 388 nm when tyrosinase was included in the mixture, presumably due to reaction of ascorbate to dehydroascorbate. Peroxidase alone did not alter the ascorbate spectrum in any way.

1.4a.3.3 Ascorbic Acid - Chemical Oxidation

Ascorbic acid (0.4 mg/ml, 1 ml) was used to prepare a control solution; consisting of ascorbate solution (50 µl) in double distilled water* (2 ml). The UV spectrum of this solution was recorded (200-900 nm), revealing a large peak at 260 nm. Iodine solution (12.7 mg/ml EtOH; 10 µl) was then added, and the cuvette shaken for 10 s. After this time sodium thiosulfate solution (124.0 mg/ml; 5 µl) was added, the yellow iodine colour quenched, and the UV spectrum again recorded, showing virtually no absorption.

1.4a.4.1 Berberine with Horseradish Peroxidase

(i) Concentrated solutions:

In procedure I (i), the UV subtraction spectrum obtained suggested that no reaction product(s) had formed, confirmed by TLC ($CHCl_3$:MeOH 1:4; iodine vapour).

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* Hereafter referred to as "dd water".

(ii) Dilute solutions:

In procedure I (ii), the UV subtraction spectrum obtained indicated that reaction had not occurred.

1.4a.4.2 Reaction of Berberine with Tyrosinase and/or HRP

In procedure II, the UV spectra for the reaction and control solutions revealed no change due to the presence of either enzyme had occurred, hence, berberine is not oxidised by these enzymes.

1.4a.4.3 Berberine with Aphid Saliva

(i) Concentrated solutions:

In procedure I (i), a peak at 468 nm was observed in the UV subtraction spectrum, suggesting product formation, although this product could have been spurious since the machine was recording near its upper absorbance limit* for both reaction and control solutions. TLC analysis ($CHCl_3$:MeOH 2:8; iodine vapour) was unable to show any evidence of product(s).

(ii) Dilute solutions:

In procedure I (ii), comparison of the reaction and control UV spectra indicated reaction product had not formed. This result is different from that observed in (i) above, but is more likely to be accurate considering the machine limits.

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* During the course of this experimental work it was discovered that the upper abosrbance limit of the UV spectrophotometer used was too low to record some peaks at lower wavelengths. Hence, the earlier concentrated experiments had to also be examined at lower concentrations, to determine if any mis-interpretation of spectra had occurred.

1.4a.5.1 Betaine with Horseradish Peroxidase

(i) Concentrated solutions:

In procedure I (i), betaine (1.0 mg/ml) and horseradish peroxidase (0.5 mg/ml) were reacted in the presence of H_2O_2 (0.03% in solution) at 42°C for 2h. After this time the UV subtraction spectrum was calculated. This showed no evidence of any reaction products. TLC analysis (butanol:EtOH:Ac.acid:H_2O 8:2:1:3; silver nitrate*) confirmed this result.

(ii) Dilute solutions:

In procedure I (ii) comparison of the observed reaction and control UV spectra indicated reaction product(s) had not formed.

1.4a.5.2 Reaction of Betaine with Tyrosinase and/or HRP

In procedure II, the UV spectra for the reaction and control solutions revealed no change due to the presence of either enzyme had occurred. Betaine is thus not oxidised by these enzymes.

1.4a.5.3 Betaine with Aphid Salivary Preparation

Procedure III (ii) was employed upon betaine. Comparison of the observed reaction and control UV spectra showed no evidence of reaction product.

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* Stahl, (1965).

1.4a.6.1 Boldine with Horseradish Peroxidase

(i) Concentrated solutions:

In procedure I (i), immediately upon enzyme addition a cream precipitate formed. The reaction and control mixtures were centrifuged, and the pellets and supernatants separately analysed by UV spectroscopy. The UV subtraction spectrum, for the combined pellet and supernatant data, yielded peaks at 381 and 453 nm. TLC analysis ($CHCl_3$:MeOH 3:2; iodine vapour) of the pellet revealed 3 components (Rfs = 0.56, 0.34 & 0.16), whilst boldine was retained in the aqueous supernatant (Rf = 0.40). In an alternative solvent system ($CHCl_3$:MeOH 5:4) boldine was seen at Rf = 0.59, product components at Rfs = 0.79-baseline (streaked).

(ii) Dilute solutions:

In procedure I (ii), the UV subtraction observed showed absorptions at 246, 289, & 326 nm.

1.4a.6.2 Boldine with Mushroom Tyrosinase

Boldine (0.05 mg/ml; 10% EtOH) and mushroom tyrosinase (0.10 mg/ml) were reacted in a total volume of 1 ml for 2 h at room temperature. After this time the UV spectrum of each solution was recorded. Comparison of the control and reaction spectra indicated that product formation had not occurred in this case.

1.4a.6.3 Reaction of Boldine with Tyrosinase & HRP

Procedure II was repeated, with slight modification, only the mixture of HRP and tyrosinase (1:1; 0.05 mg/ml each) was used in the reaction. Formation of reaction product(s) was indicated, with absorptions at 246, 289, & 326 nm.

1.4a.6.4 Boldine With Aphid Saliva

(i) Concentrated solutions:

In procedure III (i), the calculated UV subtraction spectrum was found to contain a peak at 345 nm, quite different from that observed for boldine and horseradish peroxidase. However, TLC analysis ($CHCl_3$:MeOH 5:4; iodine vapour/UV) revealed boldine (Rf = 0.52) and other components (Rf = 0.70 & 0.34), similar to those observed for HRP (i). These are presumably due to autoxidation of boldine during the concentration procedure (occurred to boldine in control solution subjected to the same concentrating conditions).

(ii) Dilute solutions:

In procedure III (ii), comparison of the UV spectra obtained showed no difference between the reaction and control solutions, suggesting oxidation had not occurred.

1.4a.7.1 Caffeic Acid and Horseradish Peroxidase

1. With Hydrogen Peroxide.

(i) Concentrated solutions:

In procedure I (i), immediately upon enzyme addition a colour change was observed, (colourless to yellow/orange). After 2 h the UV subtraction spectrum was calculated, revealing absorptions at 383 & 408 nm. TLC analysis (ethyl acetate:isopropanol:water, 65:25:10; UV/iodine) revealed unreacted caffeic acid (Rf=0.75), and a possible product component with Rf=0.82.

(ii) Dilute solutions:

Caffeic acid (0.5 mg/ml; 20% EtOH) and HRP (0.025 mg/ml) were reacted in the presence of H_2O_2 (0.03%), in a total volume of 400 µl. Immediately upon enzyme addition a colour change was observed, colourless to yellow-orange. After 2 h at room temperature the UV spectra of the diluted solutions (50 µl with 450 µl water) were recorded and the data analysed. The UV subtraction spectrum showed a substantial loss in absorption about 316 nm , with an increase at 264 nm. The solutions/ppt were subjected TLC analysis (silica; ethyl acetate:isopropanol:H_2O, 13:5:2; UV/iodine). This revealed the reaction mixture to contain unreacted caffeic acid, as well as two other components (base line and Rf = 0.06) which were not observed in the control solution. The precipitate from the reaction did not move from the base line, an observation compatible with the presence of polyphenolic compound(s).

2. Without Hydrogen Peroxide.

The reaction described in 1 (ii) above was repeated, without the addition of H_2O_2. The solutions remained colourless for the reaction period, compatible with oxidation not occurring. After 2 h at room temperature the UV spectra of the diluted solutions (as above) were recorded. These confirmed that no reaction had occurred.

1.4a.7.2 Reaction of Caffeic Acid with Tyrosinase and/or HRP

In procedure II, immediately after enzyme addition all of the reaction solutions changed from colourless to yellow. The reactions containing peroxidase were quicker in this colour change than when tyrosinase was the only enzyme present. After 2 h at room temperature the UV spectra for the reaction and control solutions were recorded. The UV subtraction spectra revealed peaks at

260 & 410 nm, with negative absorptions at 291/330 nm, for all three enzyme systems.

1.4a.7.3 Caffeic Acid with Aphid Salivary Enzymes

(i) Concentrated solutions:

Caffeic acid (1 mg/ml; 20% EtOH), without hydrogen peroxide, was reacted with aphid salivary preparation (usually 300 µl), in a total volume of 400 µl. Unfed control solutions were reacted similarly. After 2 h at room temperature the UV subtraction spectrum was obtained. The product peak observed, at 376 nm, was similar to that found for the HRP oxidations of ferulic acid, but slightly different from that of HRP/caffeic acid.

(ii) Dilute solutions:

Caffeic acid (0.5 mg/ml; 10% EtOH), with hydrogen peroxide (0.003%), was reacted with aphid saliva (300 µl), in a total volume of 400 µl. After 2 h at room temperature the UV spectra of the diluted solutions (50 µl added to 450 µl of dd water) were recorded. The UV subtraction spectrum showed slight positive peaks at about 238 & 269, with negative peak at -335 nm. These results are different from those obtained for caffeic acid and horseradish peroxidase.

1.4a.7.4 Caffeic Acid Autoxidation

(a) A solution of caffeic acid (1 mg/ml, 10% EtOH), with 0.03% H_2O_2, was left at room temperature for 24 h. The UV spectrum of the mixture was recorded periodically, at 0, 2, 4, 7 & 24 h intervals. Comparison of the spectra revealed the formation of an absorption peak at about 388 nm. The formation of this peak was very slow, only obvious after 24 h, and hence should not interfere with reaction analyses after 2 h enzyme-mediated oxidation.

(b) Caffeic acid (0.05 mg/ml) with hydrogen peroxide (0.03%) in dd water (1 ml, 10% EtOH) was left to autoxidise overnight. The UV spectrum was recorded at specific time intervals, after 0, 2, 4, 8 & 24 h. Comparison of the UV spectra obtained showed the T=0, T=2 and T=4 h spectra to again be virtually identical. No change in caffeic acid was observed until after 8 h. Comparison of the T=0 and T=8 h spectra reveal some oxidation of caffeic acid had occurred. The UV subtraction spectrum (T=8 - T=0) revealed small negative absorptions at 277 & 337 nm, with positive peak at 250 nm, and a slight absorption from 370-500 nm.

1.4a.7.5 HPLC Analysis of the Caffeic Acid/HRP Reaction

HPLC analysis of the caffeic acid/HRP reaction mixture was performed using a water-acetonitrile solvent system, (solvent A - 0.1% formic acid in nanopure water; solvent B - 0.1% formic acid in acetonitrile), with UV detection at 270 nm. Good traces were obtained for both caffeic acid and the HRP reaction mixture, with one major product being observed (6.5 min). Traces of starting material, and other products, were also seen. Gradient used: 5% B for 5 min; 5-40% B over 20 min; 40-100% B over 5 min; maintained at 100% B for 5 min.

1.4a.7.6 HPLC Analysis of the Caffeic Acid/Aphid Saliva Oxidation

Caffeic acid (0.05 mg/ml; 10% EtOH) and H_2O_2 (0.003% in solution) were reacted with aphid salivary preparation (300 µl), in a total volume of 400 µl. After 2 h at room temperature the UV subtraction spectrum was obtained, confirming reaction had occurred. This solution was analysed by HPLC, following the gradient and solvent system outlined above. The caffeic acid oxidation product peaks were observed at 15.2, 16.9, 17.5 & 18.7 min. These were identical with those of some of the minor products from the HRP oxidation (above).

1.4a.8.1 Oxidation of Catechin with Horseradish Peroxidase

Procedure I (i) was used to oxidise catechin. Immediately upon addition of enzyme to the reaction mixture, a colour change was observed (colourless to yellow-orange). Although an immediate colour reaction was not noted in the control solution (no enzyme added), some yellow colouring was present after 2 h at room temperature. The reaction solution was centrifuged, and the UV spectrum of each solution (reaction supernatant and precipitated pellet; control mixture) was then recorded. The supernatant and pellet spectra were combined, and the UV subtraction spectrum (control reference) was calculated. This revealed several product absorptions: sharp peaks at 251 and 294 nm, as well as broad absorptions at 392 and 434 nm.

1.4a.8.2 Catechin with Aphid Salivary Enzymes

(i) Concentrated solutions:

In procedure III (i), the UV subtraction spectrum contained absorptions at 251 and 292 nm, as observed for the HRP oxidation of catechin, suggesting similar products are formed.

(ii) Dilute solutions:

In procedure III (ii), the UV subtraction spectrum showed peaks at 246 & 290 nm, with smaller broad absorptions at 434 &483 nm. These results are identical with those found for the HRP/H_2O_2 oxidation of catechin, but different from the tyrosinase reaction.

1.4a.8.3 Oxidation of Catechin by Tyrosinase

Catechin (1.0 mg/ml; 10% EtOH) and H_2O_2 (0.03%) were reacted in the presence of mushroom tyrosinase (0.5 mg/ml). Immediately upon enzyme addition to the reaction mixture, a colour change was observed (colourless to

orange-red). An immediate colour change was not observed in the control solution (no enzyme added), although some colouring was present after 2 h at room temperature. The reaction solution was centrifuged, but no precipitate was seen, indicating that the colour was not caused by product in suspension. The UV spectrum of each solution (reaction and control) was then recorded, and the UV subtraction spectrum calculated. This revealed several product absorptions, at 320 and 400-500 nm. TLC analysis was attempted, but the components were found to streak across the plate. Best results were obtained with silica plates ($CHCl_3$-MeOH, 1:1 + trace NH_3).

1.4a.8.4 Reaction of Catechin With HRP and/or Tyrosinase

In procedure II, immediately after enzyme addition colour changes were observed, the reaction containing HRP developed a pale yellow colour, whilst the reaction with tyrosinase only produced a much brighter yellow. The control solutions remained colourless throughout. After 2 h at room temperature the UV spectra for the reaction and control solutions were recorded. The oxidation of catechin by HRP/H_2O_2 (either with or without tyrosinase) resulted in a subtraction spectrum with peaks at 247, 293 and 300-420br nm. When tyrosinase was used as the sole oxidising enzyme, the subtraction spectrum showed similar absorption peaks at 244 & 378 nm, but the 293 nm absorption was not present.

1.4a.8.5 Catechin Autoxidation

A solution of catechin (1 mg/ml) in dd water (2 ml) was left to react at room temperature for several days. The UV spectrum of the solution was recorded at specific time intervals: 0, 1, 2, 4, 6, & 24 h. Analysis of the spectra obtained showed that little change took place during the first 6 h. After 24 h, however, comparison of the T=24 h spectrum with that for T=0 h revealed the presence of peaks at 251 and 292 nm that were also observed after enzymic oxidation (concentrated solutions).

1.4a.9.1 Chlorogenic Acid and Horseradish Peroxidase

1. With Hydrogen Peroxide.

Chlorogenic acid (0.5 mg/ml; 20% EtOH) and HRP (0.025 mg/ml) were reacted in the presence of H_2O_2 (0.03%), in a total volume of 400 µl. Immediately upon enzyme addition a colour change was observed, colourless to bright yellow. After 2 h at room temperature the UV spectra of the diluted solutions (50 µl with 450 µl water) were recorded and the data analysed. The UV subtraction spectrum showed a substantial loss in absorption about 318 nm, with increases at 261 & 403 nm.

2. Without Hydrogen Peroxide.

The procedure outlined for (1) above was repeated with the omission of the H_2O_2. Comparison of the UV spectra obtained showed no difference between the reaction and control solutions, indicating that oxidation did not occur.

1.4a.9.2 Reaction of Chlorogenic Acid with Tyrosinase and/or HRP

In procedure II, immediately after enzyme addition all of the reaction mixtures changed from colourless to yellow. This occurred much faster for reactions containing peroxidase than when tyrosinase alone was the enzyme present. After 2 h at room temperature the UV spectra for the reaction and control solutions were recorded. The UV subtraction spectra contained peaks at 260 & 403 nm, with a negative absorption at 318 nm, for all three systems. TLC analysis (ethyl acetate:isopropanol:water, 55:25:20; UV/iodine) revealed chlorogenic acid (Rf=0.26) as the only component of the control solution. The reaction mixture, however, revealed a product component at Rf=0.19, with further product(s) in a smear from the baseline to Rf=0.10. Baseline component was observed as a brown precipitate in the reaction solution.

1.4a.9.3 Chlorogenic Acid and Aphid Salivary Preparation

Procedure III (ii) was used for the salivary oxidation of chlorogenic acid. The resultant UV subtraction spectrum showed absorptions at 260 & 385 nm, with negative peaks at 308 & 336 nm.

1.4a.9.4 HPLC Analysis of Chlorogenic Acid/HRP Reaction

Analysis of the chlorogenic acid/HRP reaction mixture was performed as for the caffeic acid reactions (1.4a.7.5). Excellent traces for the separation of chlorogenic acid and oxidation products were obtained, with one major product and at least five minor products observed.

1.4a.9.5 HPLC Analysis of the Chlorogenic Acid/Aphid Saliva Oxidation

Chlorogenic acid (0.05 mg/ml; 10% EtOH) and H_2O_2 (0.003% in solution) were reacted with aphid salivary preparation (300 µl), in a total volume of 400 µl. After 2 h at room temperature the UV subtraction spectrum was obtained, confirming reaction had occurred. This was then used in HPLC examinations, following the gradient and solvent system outlined for caffeic acid. The chlorogenic acid major oxidation product was observed at a retention time of 12.5 min in the reaction chromatogram, with two minor products at 7.2 & 13.9 min, that also were not observable in control solution chromatograms. The retention times of the 7.2 & 12.5 min peaks were the same as for products of HRP oxidation (above).

1.4a.10.1 DIMBOA and Horseradish Peroxidase

In procedure I (i), immediately upon enzyme addition the reaction mixture underwent a colour change (colourless to yellow), which was not observed for the control solution (no enzyme added). The UV subtraction spectrum revealed a

large peak at 337 nm. TLC analysis ($CHCl_3$-ethyl acetate 1:1 + trace NH_3; UV/iodine) showed DIMBOA (base line), and at least two other components with Rfs=0.13 & 0.40.

1.4a.10.2 DIMBOA with Aphid Salivary Enzymes

1. With Hydrogen Peroxide.

(i) Concentrated solutions:

In procedure III (i), the reaction solution appeared slightly yellow with time, and the UV subtraction spectrum obtained revealed product peaks at 314 & 337 nm. These were similar to those observed for DIMBOA and HRP (above). TLC analysis of the concentrated solutions detected only starting material, however, indicating that any product(s) formed were not in high enough concentration for detection.

(ii) Dilute solutions:

In procedure III (ii), the UV subtraction spectrum contained peaks at 304 & 347 nm, with a negative absorption at 257 nm.

2. Without Hydrogen Peroxide.

(i) Concentrated solutions:

Procedure III (i) was repeated, with the omission of hydrogen peroxide. After a 2 h reaction period the UV subtraction spectrum was calculated. In the absence of H_2O_2 an absorption at 312 nm was observed, whereas in the presence of H_2O_2 (see above) two peaks are seen (314 & 337 nm).

(ii) Dilute solutions:

In procedure III (ii), when hydrogen peroxide was not included in the mixture, DIMBOA oxidation showed peaks in the UV subtraction spectrum at

283 & 298 nm, different from those observed above (1.ii. - hydrogen peroxide present).

1.4a.10.3 Reaction of DIMBOA with Tyrosinase and/or HRP

In procedure II, immediately after enzyme addition the peroxidase-containing solutions underwent a gradual colour change, from colourless to yellow. The tyrosinase reaction and control solutions did not alter. After 2 h at room temperature the UV spectra for the reaction and control solutions were recorded. The UV subtraction spectra revealed that peroxidase was capable of reacting with DIMBOA, whilst tyrosinase was not. The HRP/H_2O_2 reaction gave product peaks in the subtraction spectra at 240, 336 & 420 nm, with negative absorptions at 275 nm. The reaction containing tyrosinase alone showed no difference between reaction and control spectra.

1.4a.10.4 HPLC Analysis of DIMBOA/HRP Reaction

The oxidation of DIMBOA (0.1 mg/ml) by HRP (trace)/H_2O_2 (0.003%) was analysed by HPLC (see General Experimental), using a modification of the method outlined by Niemeyer, *et al.*, (1989). Solvent A: 0.05% H_3PO_4 in water:methanol (95:5); Solvent B: methanol; UV detection was performed at 305 nm. Good separation of DIMBOA and oxidation products was obtained, with retention times as follows: starting material (8.0 min), products (5.67, 6.13, 10.03 & 10.36 min). Gradient used: 25% B for 5 min; 25-45% B over 9 min; 45-100% over 5 min; return to 25% over 8 min.

1.4a.10.5 HPLC Analysis of the Aphid Salivary Oxidation of DIMBOA

DIMBOA (0.1 mg/ml; 10% EtOH) and H_2O_2 (0.003% in solution) were reacted with aphid salivary preparation (235 µl), in a total volume of 400 µl. After 2 h at room temperature the UV subtraction spectrum was obtained, confirming reaction had occurred. This was then used in HPLC examinations, following the

gradient and solvent system outlined above for the HRP oxidation. DIMBOA and oxidation products were observed at retention times identical to those following model enzyme oxidation. Only unreacted starting material (8.0 min) was observed in the control solution.

1.4a.11.1 Ferulic Acid and Horseradish Peroxidase

(i) Concentrated solutions:

Ferulic acid (1.0 mg/ml; 20% EtOH) and HRP (0.5 mg/ml) were reacted in the presence of H_2O_2 (0.03% in solution) at room temperature. Immediately upon enzyme addition a colour change was observed, (colourless to magenta/pink). After 2 h the UV subtraction spectrum was calculated, revealing absorptions at 379 & 408 nm. TLC analysis (ethyl acetate:isopropanol:water, 65:25:10; UV/iodine) showed unreacted ferulic acid (Rf=0.75), and at least two other components with Rfs=0.33 & 0.46.

(ii) Dilute solutions:

Procedure I (ii) was repeated with one slight change. The mixtures were made up both with and without hydrogen peroxide. Immediately upon enzyme addition the reaction containing hydrogen peroxide underwent a colour change (to pink), the other solutions remained colourless. Analysis of the UV spectra obtained showed that in the absence of H_2O_2 reaction did not occur, whilst when it was included a subtraction spectrum with peaks at 254 & 368 nm, with negative absorption over 260-350 nm, was observed.

1.4a.11.2 Ferulic Acid with Aphid Salivary Enzymes

(i) Concentrated solutions:

Ferulic acid (1 mg/ml; 20% EtOH) and H_2O_2 (0.03%) were reacted with aphid salivary preparation (usually 300 µl), in a total volume of 400 µl. Unfed control solutions were reacted similarly. After 2 h at room temperature the UV subtraction spectrum was obtained. The product peak observed, at 371 nm, was identical to that found for the HRP oxidation of ferulic acid.

(ii) Dilute solutions:

In procedure III (ii), the UV spectra obtained gave a subtraction spectrum with a peak at 269 nm and negative absorption at 342 nm.

1.4a.11.3 Reaction of Ferulic Acid with Tyrosinase and/or HRP

In procedure II, immediately after enzyme addition the reaction solutions containing peroxidase changed from colourless to pale pink. The reaction containing tyrosinase alone and the control solutions did not appear to change colour. After 2 h at room temperature the UV spectra for the solutions were recorded. When HRP/H_2O_2 were present in the reaction mixture the UV subtraction spectrum contained peaks at 253, 367 & 418 nm, with a negative absorption at 310 nm. The reaction containing tyrosinase alone showed no differences between the reaction and control spectrum.

1.4a.11.4 Ferulic Acid Autoxidation

(a) A solution of ferulic acid (1 mg/ml, 10% EtOH) was left at room temperature overnight. The UV spectrum of the solution was recorded at specific time intervals, (0, 2, 4, 8, & 24 h). Comparison of the spectra revealed the formation of a peak at 376 nm, similar to that seen for enzymic oxidation, although only after 24 h.

(b) The method outlined in (a) above was repeated using ferulic acid (0.05 mg/ml) and H_2O_2 (0.003%). Comparison of the UV spectra obtained showed little change within the first 8 h. The 24 h spectrum indicated a loss of absorption at 265 nm, with small increase at 230 nm.

1.4a.11.5 HPLC Analysis of the Ferulic Acid/HRP Reaction

HPLC analysis of the ferulic acid/HRP reaction mixture was performed as for caffeic acid reactions (1.4a.7.5). Good traces for the identification of ferulic acid were obtained. Analysis of reaction mixtures indicated all starting material to be utilised, however, peaks for reaction product(s) were not detected at 270 nm (UV detection).

1.4a.11.6 HPLC Analysis of the Ferulic Acid/Aphid Saliva Oxidation

Ferulic acid (0.05 mg/ml; 10% EtOH) and H_2O_2 (0.003% in solution) were reacted with aphid salivary preparation (250 µl), in a total volume of 400 µl. After 2 h at room temperature the UV subtraction spectrum was obtained, confirming reaction had occurred. This solution was then used in HPLC examinations, following the gradient and solvent system outlined for caffeic acid. The oxidation product(s) from this reaction were not detected after repeated attempts. Fractions of the column eluant were collected (5 min fractions, and a second attempt as one complete fraction), evaporated to dryness, and analysed by UV spectroscopy. No evidence of oxidation product(s) was observed.

1.4a.12.1 Gramine and Horseradish Peroxidase

Gramine was treated as for the reaction of ferulic acid and HRP (i). The UV subtraction spectrum obtained showed a peak at 306 nm and a smaller absorption at 425 nm. TLC analysis (MeOH + trace NH_3; UV/iodine) revealed

unreacted gramine (Rf=0.15), and at least two other components with Rfs=0.25 & 0.80.

1.4a.12.2 Gramine with Aphid Salivary Enzymes

(i) Concentrated solutions:

Gramine was treated as for the reaction of ferulic acid and aphid salivary preparation outlined previously. The observed UV subtraction spectrum showed a product peak at 301 nm, identical to that found for gramine & HRP. TLC analysis (MeOH + trace NH_3; UV/iodine) revealed two components: unreacted gramine (Rf=0.10) and a product (Rf=0.21). These results are very similar to those found for the gramine/HRP reaction described above.

(ii) Dilute solutions:

The procedure outlined for ferulic acid and aphid saliva ((ii), above) was repeated using gramine (0.05 mg/ml). In the absence of H_2O_2 reaction did not occur, but when H_2O_2 was included in the mixture the UV subtraction spectrum showed peaks at 260 & 303 nm.

1.4a.12.3 Reaction of Gramine with Tyrosinase and/or HRP

In procedure II, when HRP/H_2O_2 were present in the reaction mixture the UV subtraction spectrum contained increases in absorption at 300 & 420 nm. When tyrosinase was used as the sole oxidising enzyme no differences between the reaction and control spectra were observed.

1.4a.12.4 Autoxidation of Gramine

(a) A solution of gramine (1 mg) in dd water-EtOH (3:2, 1 ml) was left at room temperature and exposed to the atmosphere for 1 day. During this time the UV spectrum of the solution was recorded immediately and after 2, 4, 8, & 24 h. The spectral data were collated and compared. A small absorption at 302 nm was

observed after 2 h, which decreased with time, broadening over the 325-450 nm range. TLC analysis (MeOH + trace NH_3; UV/iodine) after 24 h revealed unreacted gramine, with no evidence of reaction product(s).

(b) The experiment outlined in (a) above was repeated, the only exception being the addition of H_2O_2 (0.03%) to the solution. Analysis of the time course UV spectra revealed a product peak at 307 nm. This absorption increased with time, as well as broadening slightly. The spectrum appears to be identical with the reaction product observed for enzymic oxidations of gramine.

(c) Gramine (0.05 mg/ml; 10% EtOH) and H_2O_2 (0.003%) were left at room temperature overnight. The UV spectrum of this solution was recorded at specific time intervals, 0, 2, 4, 8 & 24 h. Comparison of these spectra showed that very little change took place during the initial 8 h. After 24 h, however, a loss of absorption at 290 nm, with increases at 257 & 306 nm, was apparent.

1.4a.12.5 HPLC Analysis of Gramine/HRP Reaction

1. Pic B7 Ion-pair Reagent.

HPLC analysis of the gramine/HRP reaction was performed using a water-acetonitrile solvent system, (A) Water:MeOH:Pic B7* (90:10:2.5), (B) Acetonitrile:MeOH:Pic B7 (90:10:2.5), with UV detection at 300 nm. Gramine was observed as a broad peak (12.5-14.5 min) in the reaction chromatogram, with oxidation products at 9.0, 9.4 & 10.2 min. Gradient used: 45% B for 5 min; 45-70% B over 15 min; 70% B for 4 min; return to 45% B.

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* Pic B7 is a Millipore-Waters commercial ion-pairing reagent, consisting of 1-heptane sulfonic acid as a calcium salt.

2. Heptafluorobutyric acid (HFBA).

An alternative solvent system was used, (A) H_2O:MeOH 9:1 + 0.05% HFBA, (B) AcCN:MeOH 9:1 + 0.05% HFBA. Better peak separations were achieved, with gramine (16.0 m) and two major products (9.70 & 12.75 min) observed as sharp peaks. Gradient used: 5% B for 5 min; 5-60% B over 26 min; 60-100% B over 4 min; remain at 100% B for 2 min; return to 5% B in 8 min.

1.4a.12.6 HPLC Analysis of the Gramine/Aphid Saliva Oxidation

1. Pic B7 Ion-pair Reagent.

Gramine (0.5 mg/ml; 10% EtOH) and H_2O_2 (0.00015% in solution) were reacted with aphid salivary preparation (500 µl), in a total volume of 1 ml. After 2 h at room temperature the UV subtraction spectrum was obtained, confirming reaction had occurred. This was then used in HPLC examinations, following the gradient and solvent system outlined in (1) above. The peaks observed for gramine and oxidation products were identical with those seen for the HRP oxidation (see (2)).

2 Heptafluorobutyric acid (HFBA).

The solvent system outlined in (2) above was used. Gramine (15.8 min) and two products (9.50 & 12.5 min) were identified, corresponding to those found for gramine/HRP in this solvent system.

1.4a.13.1 Hordenine with Horseradish Peroxidase

(i) Concentrated solutions:

(a) In procedure I (i), the UV subtraction spectrum revealed a major peak at 300 nm and a smaller absorption at 245 nm. TLC analysis (MeOH + trace NH_3;

UV/iodine) showed three components: hordenine (Rf=0.31), and two products (Rf=0.13 & base line).

(b) Procedure I (i) was repeated, with the omission of hydrogen peroxide. In the absence of H_2O_2 no product peak was observed in the subtraction spectrum. Similarly, TLC analysis (MeOH + trace NH_3; UV/iodine) confirmed reaction did not occur.

1.4a.13.2 Hordenine with Aphid Salivary Enzymes

(i) Concentrated solutions:

(a) In procedure III (i), the UV subtraction spectrum contained a product peak identical to that found for hordenine & HRP at 300 nm. TLC analysis (MeOH + trace NH_3; UV/iodine) was unsuccessful in determining reaction products (only unreacted starting material was observed) presumably due to the small quantity of product produced.

(b) Procedure III (i) above was repeated, with the omission of hydrogen peroxide. The UV subtraction spectrum did not contain any product absorption, nor was the hordenine/HRP reaction product detectable by TLC (MeOH + trace NH_3; UV/iodine). The lack of perceptible reaction in the absence of H_2O_2 was similar that found for mixtures of HRP and hordenine.

(ii) Dilute solutions:

Procedure III (ii) was used to oxidise hordenine freebase (0.05 mg/ml). The UV subtraction spectrum showed a peak at 287 nm.

1.4a.13.3 Hordenine with Aphid Saliva from Potato Cultured Aphids

Saliva was collected from potato aphids (*M. euphorbiae*), cultured on potato plants, and was reacted with hordenine freebase (1 mg/ml) and hydrogen peroxide (0.03% in solution) in a 2 h oxidation. After this time the resultant

UV subtraction spectrum revealed peaks at 245 & 289 nm, slightly shifted from those observed for "nasturtium cultured" aphids, but with very similar appearance.

1.4a.13.4 Oxidation of Hordenine by Tyrosinase

Hordenine hemisulphate (1.0 mg/ml; 10% EtOH) and mushroom tyrosinase (0.5 mg/ml) were reacted in the presence of H_2O_2 (0.03% in solution), in a total volume of 1 ml, for 2 h at room temperature. After this time the UV subtraction spectrum was calculated, revealing a peak at about 300 nm and a smaller absorption at 243 nm. TLC analysis (MeOH + trace NH_3; UV/iodine) revealed three components: hordenine (Rf=0.25), and two products (Rf=0.12 & base line). These results are consistent with those observed for the horseradish peroxidase oxidation of hordenine.

1.4a.13.5 Reaction of Hordenine with HRP/Tyrosinase

(i) Concentrated solutions:

Hordenine hemisulphate (1.0 mg/ml; 10% EtOH), H_2O_2 (0.03% in solution) were reacted with mushroom tyrosinase (0.5 mg/ml) and HRP (0.5 mg/ml), in a total volume of 1 ml, for 2 h at room temperature. After this time the UV subtraction spectrum was calculated, revealing a peak at about 325 nm. TLC analysis (MeOH + trace NH_3; UV/iodine) revealed three components: hordenine (Rf=0.32), and two products (Rf=0.19 & base line). These results are consistent with those observed for both individual HRP and tyrosinase oxidations of hordenine (in the presence of H_2O_2).

(ii) Dilute solutions:

In procedure II, the UV subtraction spectrum showed peaks at 234, 290, 333(small) & 422(small) nm.

1.4a.13.6 Hordenine Autoxidation

A solution of hordenine freebase (1 mg) in dd water-EtOH (9:1, 1 ml) was left at room temperature and exposed to the atmosphere for 1 day. During this time the UV spectrum of the solution was recorded at the following intervals: 0, 1, 2, 4, 7 & 24 h. The spectral data were collated and compared, showing a gradual increase in the width and height of the product peak at 307 nm. TLC analysis (MeOH + trace NH_3; UV/iodine) revealed three components: hordenine (Rf = 0.29) and two products (Rfs = 0.11 & base line). These appear to be the same as those observed for the enzymic oxidations of hordenine, although not formed in such large quantities.

1.4a.13.7 Hordenine Reaction with Crusader Bug Saliva

Salivary secretion collected directly from *Mictus profana* (2 µl) was reacted with hordenine (1 mg/ml; 10% EtOH) and H_2O_2 (0.03% in solution), for 2 h at room temperature. A reaction control (dd water) was run simultaneously. After this time the UV subtraction spectrum was obtained. A product peak (312 nm) was observed, similar to that found previously for hordenine oxidation by either HRP or aphid salivary preparations.

1.4a.13.8 HPLC Analysis of Hordenine/HRP Reaction

1. Methanol-water.

HPLC analyses of the hordenine/HRP reaction mixture were performed on an RP-C18 column, UV detection at 300 nm. Initial solvent system used was (A) Water:MeOH:Pic B7 (90:10:2.5), (B) MeOH:Pic B7 (100:2.5). Excellent separation of hordenine was observed (elution time 22 min), however, the reaction product(s) were eluted during the column wash (37 min). Gradient used: 5% B for 5 min; 5-40% B over 30 min; 40-100% B over 5 min; return to 5% B.

2 Acetonitrile-water.

An alternative system was then used, (A) Water:MeOH:Pic B7 (90:10:2.5), (B) Acetonitrile:MeOH:Pic B7 (90:10:2.5). Better results were obtained, and both hordenine and its reaction products were well separated (5 min), with gradient as follows: 45% B for 5 min; 45-95% B over 40 min; 95-100% B over 2 min; return to 45% B. The chromatogram indicated hordenine (5 min), major product (10 min), minor product (12 min).

1.4a.13.9 Analysis of the Hordenine/Aphid Saliva Oxidation

Hordenine freebase (1 mg/ml; 10% EtOH) and H_2O_2 (0.03% in solution) were reacted with aphid salivary preparation (300 µl), in a total volume of 400 µl. After 2 h at room temperature the UV subtraction spectrum was obtained, confirming reaction had occurred. This was then used in HPLC examinations, following the gradient and solvent system outlined in (1) above. The hordenine and oxidation product peaks observed were identical with those seen for the HRP oxidation (see (2)).

SECTION B - ENZYME INHIBITION STUDIES

General Inhibition Methods

I. Peroxidase Inhibition.

(i) Model enzyme reaction:

Plant chemical (0.05 mg/ml; 10% EtOH) and HRP/tyrosinase (0.05 mg/ml of each) were reacted in the presence of catalase (10^3 units/ml), without H_2O_2, in a total volume of 1 ml. After 2 h at room temperature the UV subtraction spectrum was calculated.

(ii) Aphid saliva:

As for (i) above, using aphid salivary preparation (250-300 µl) in a total volume of 400 µl, in place of the model enzymes.

II. Tyrosinase Inhibition.

(i) Model enzyme reaction:

Plant chemical (0.05 mg/ml; 10% EtOH), H_2O_2 (0.003% in solution), and HRP/tyrosinase (0.05 mg/ml of each) were reacted in the presence of phenylthiourea (3.3 mM; 0.05 mg/ml), in a total volume of 1 ml. A control solution (without enzyme) was treated similarly. After 2 h at room temperature the UV subtraction spectrum was calculated.

(ii) Aphid saliva:

As for (i) above, using aphid salivary preparation (250-300 µl) in a total volume of 400 µl, in place of the model enzymes. This procedure was also repeated using 10 mM PTU.

The UV subtraction spectrum for each compound was as indicated in Table 1.3 (page 27). Colour changes, observed upon addition of model enzymes to the reaction mixtures, are indicated in Table 1.4 below. The control solution remained colourless for all. Aphid salivary oxidations were not observed to develop colouration, this is likely due to the dilute nature of any reaction products formed.

Table 1.4: Colour changes observed during model enzyme inhibition studies: 0.05 mg/ml HRP and tyrosinase with 0.003% H_2O_2 ± catalase (10^3 U/ml) or PTU (3.3 mM).

COMPOUND	NO INHIBITOR	CATALASE	PTU
Acetaminophen	yellow	peach	yellow
Ascorbic Acid	-	-	pale peach
Caffeic Acid	yellow	yellow	yellow
Catechin	yellow	orange	yellow-brown
Chlorogenic Acid	yellow	orange	yellow
DIMBOA	yellow	orange	yellow
Ferulic Acid	pink	-	pink
Gramine	-	-	-
Hordenine	-	-	-

The different colours observed for the reactions in the presence of inhibitor are presumably a result of the specific enzyme activity remaining in operation; i.e., in the absence of H_2O_2 and presence of catalase, only the catecholoxidase (o-diphenolase) acts upon the compound.

SECTION C - GEL ELECTROPHORETIC STUDIES

Aphid saliva was collected as described in the General Experimental, with one minor modification. Each chamber of insects was provided with only 100 µl of nanopure water. The pooled salivary preparation (six chambers, *ca*. 500 µl) was concentrated in a Savant Speed Vac™ evaporative concentrator to *ca*. 20 µl.

Aliquots of 5 µl each of concentrated salivary preparation and high molecular weight markers* were applied to a pair of wells in a 6/4 Phastgel applicator. Another 3 pairs of wells were similarly applied to a second applicator. These were each loaded on a non-denaturing Phastgel and subjected to electrophoresis simultaneously (see General Experimental; Madhusudhan, *et al*., 1994). One gel was placed in a Pharmacia LKB™ Phastsystem development unit for the detection of proteins using the Pharmacia silver staining method. The second gel was cut into three equal sections so that each contained one pair of gel lanes (one lane each of salivary extract and MW standard proteins). One gel section was incubated in 0.01 M L-DOPA in Tris-HCl buffer (0.1 M; pH 7.4) at 4°C overnight (band development was observed during the first 2 h at room temperature). With the two remaining sections, one of the gels was immersed for 3 mins in 0.01M PTU, placed in a 1:1 mixture of Western blotting reagents RPN 2109 (contain luminol and H_2O_2) in which 0.01 M PTU was dissolved, while the other gel was simultaneously immersed for 3 mins in water only then transferred to the Western blotting reagents for 3 mins (both in the absence of PTU). Both gels were drained, wrapped in Glad Wrap™ and exposed to X-ray film for 4 h in the dark. The film was developed using an AGFA Curix 60™ machine.

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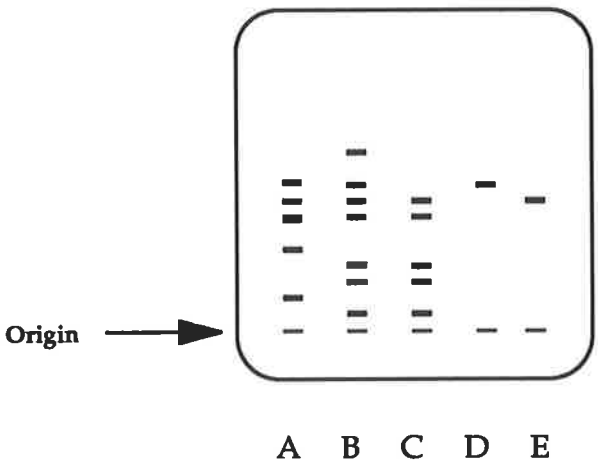
* Pharmacia high molecular weight markers (1 vial dissolved in 1 ml water, then 100 µl taken and made up to 300 µl).

Up to seven bands were detected by silver staining of the total proteins (Rfs 0.65, 0.52, 0.46, 0.41, 0.26, 0.16, 0.08), of which the 0.65 and 0.52 bands were not detected in dilute saliva (see Figure 1.4, below). When stained with the chemiluminescence reagents for peroxidase activity only one band was observed (Rf 0.52, corresponding to a molecular weight of *ca*. 675,000). This activity was abolished in the presence of PTU, indicative of a copper-peroxidase. Rf values for the High Molecular Weight standards used were as follows: 0.50 - thyroglobulin (669,000), 0.43 - ferritin (440,000), 0.39/0.38 - catalase (232,000), 0.27 - lactatedehydrogenase (140,000), 0.11 - albumin (67,000). Incubation with L-DOPA yielded a reaction at the Rf 0.46 locus (corresponding to a molecular weight of *ca*. 550,000) in the aphid salivary concentrate, presumably due to catechol oxidase activity.

Figure 1.4: Enzyme activity bands observed during gel electrophoresis of aphid salivary preparation; A = molecular weight markers, B = aphid saliva, C = concentrated aphid saliva, D = peroxidase activity (chemiluminescence), E = catechol oxidase activity (DOPA).



CHAPTER 2

REACTION KINETICS

2.1 INTRODUCTION

The identification of classes of compounds that are capable of reacting with aphid salivary enzymes is only one part of this work. Further information that was considered of major importance are the mechanism(s) involved in these reactions, and the identities of the oxidation products formed. The UV spectroscopic analyses discussed in Chapter 1 outline the oxidation reactions that can occur with aphid saliva and model enzymes with respect to the possible products, but do not give any information regarding the kinetics or mechanisms involved.

Examination of the literature reveals that although the oxidation and/or degradation of plant chemicals by fungi has been investigated (Steiman, *et al.*, 1982; Seigle-Murandi, *et al.*, 1984; Steiman & Seigle-Murandi, 1984; Rahouti, *et al.*, 1989; Gams, *et al.*, 1990), very little work has been undertaken on the reaction processes occurring during plant-insect oxidations. Hordenine, a deceptively simple phenylethylamine alkaloid, was chosen as a starting compound for these studies. This choice was based on previously demonstrated reaction with aphid saliva (Miles & Peng, 1989), because samples of hordenine freebase were immediately available, because of its apparent structural simplicity and because it combined both mono-phenolic and alkaloid properties. As it turned out, the choice was probably unfortunate. From the outset, separation of its reaction products met with seemingly inexplicable difficulty, yet as more and more time was invested in their investigation, it became more and more imperative to bring it to some kind of resolution.

It should be noted that the aspect of enzyme kinetics relevant to this Chapter was the influence on rate and magnitude of product formation, determined from the appearance and height of spectral peaks, of relative concentrations of substrate, enzyme and cofactors (H_2O_2; reductants). Calculation of rate or affinity constants as such will not be considered.

2.2 RESULTS & DISCUSSION

2.2.1 Hordenine Reactions

As indicated in the previous chapter, UV spectral analysis of the reaction of hordenine with both model enzymes and with aphid salivary preparations appear to yield the same end-product(s). This was confirmed by HPLC comparisons of the two enzyme systems. In order to obtain suitable quantities of these products for structural identification it was first necessary to maximise the reaction performance.

Horseradish peroxidase was chosen as the most effective enzyme for oxidation, as the aphid salivary preparations were very dilute, and consequently caused proportionately low product formation. Initial comparisons of the hordenine/HRP reaction with various enzyme concentrations indicated that only small quantities of HRP were necessary for good product yields. This result prompted a study of oxidation with time (at approximately 5-10 min intervals). An intriguing aspect of the reaction was that the majority of hordenine oxidation was observed in an initial, rapid reaction (0-1 min). After this time oxidation of the remaining substrate was comparatively slow and the reaction never approached completion. Thus although good yields could be obtained in absolute terms, the product was always mixed with large quantities of unchanged hordenine, and separation of the two proved difficult (see Chapter 3 of the present work).

Dependence on the presence of hydrogen peroxide (H_2O_2) was initially considered the probable cause of the rapid decline in rate of oxidation. Further investigation, however, showed that addition of H_2O_2 during the course of the reaction did not maintain rapid oxidation, nor yield significantly increased product formation with time.

An alternative explanation considered was enzyme inhibition or degradation during the reaction (Ator & Ortiz De Montellano, 1989; Kahn, 1989). Once again, however, addition of more HRP and H_2O_2 during the course of the oxidation was not observed to alter the rate or outcome significantly. Even the addition of mushroom tyrosinase was not able to 'push' the reaction to completion.

It has been noted that the addition of phenolics to reaction mixtures may enhance oxidation of certain substrates in some instances (Danner, *et al.*, 1973). With this in mind, a final attempt to elucidate the cause of reaction deceleration was made. Additional small amounts of hordenine were added to the oxidation mixture. Observation over time did not, however, indicate any increase in the reaction rate.

Inhibition of the enzyme(s) by one of the oxidation products formed during the reaction, i.e., feedback inhibition, was the next possibility explored. Formation of an ammonium compound during the reaction could inhibit the enzymes, gradually limiting the number of active enzyme sites present. The reaction pH during hordenine oxidation was monitored, as changes would be indicative of formation of such an ammonium compound, thus accounting for the slowing in oxidation speed. Observation of reaction pH both before, during and after 30 min of oxidation nevertheless showed no change during the reaction (pH 6-7). Hence, it seems that the sudden drop in reaction rate is not due to enzyme poisoning by ammonium reaction intermediates and/or products.

2.2.2 Hordenine Equilibrium Investigations

Due to the so far inexplicable deceleration of the hordenine-HRP oxidation reaction the concept of an oxidation equilibrium was considered. Although reversible, oxidation equilibria are generally considered to proceed almost totally in one direction, and the reverse reaction is often disregarded. It is possible nevertheless that the oxidised products of the substrates may have themselves

been sufficiently reducing to stop the forward reaction. If this were the case, then the addition of more enzyme would have no effect on the reaction equilibrium. On the other hand, whilst addition of more substrate would not alter the ratio of reduced/oxidised substrate, it would affect the overall quantity of product formed.

To determine if such an equilibrium system were in operation in the hordenine reaction, the UV-spectra of solutions of unoxidised and HRP-oxidised substrate were compared. The peak maxima for the unoxidised and oxidised solutions were recorded, and the changes in the reaction spectrum followed until a (more or less) steady state was reached (approximately 35 min). At this stage a further portion of substrate was added to the reaction mixture, and the absorbance at λ_{max} (oxidised) noted. The ratio of absorbances at the wavelengths of the unoxidised and partly-oxidised peaks were calculated for the initial oxidation reaction, and compared with that observed after addition of extra hordenine to the mixtures (see 2.4.3).

This experiment was repeated a number of times, and all attempts yielded similar results. It was found that the ratio of oxidised to unoxidised peak heights decreased after further substrate additions, implying that subsequent oxidation of hordenine does not take place. These findings are consistent with a regular oxidation 'equilibrium', i.e., one favouring product formation with minimal amounts of the reverse reduction occurring. Unfortunately, however, this did not help to explain the persistent problem of a reaction deceleration that prevented yields of analysable amounts of products free from excessive quantities of unreacted substrate.

All attempts to overcome oxidation deceleration described above, using additions of H_2O_2, H_2O_2 + HRP, tyrosinase or further additions of substrate, were thus unsuccessful. The concept of enzyme inhibition, perhaps by an oxidation product or intermediate appeared unlikely, as no obvious enzyme inhibitors (e.g. HCN, ammonium compounds) from this reaction could be envisaged or

detected. Work conducted simultaneously in our laboratory by Dr Jiang suggested that the relatively high concentration of substrate used in these experiments (hordenine = 1 mg/ml) may be the cause of the observed slow oxidation, as he noted that product formation did approach completion at very dilute concentrations of substrate. Partial inhibition of the enzyme by loosely-held oxidation product hindering the entrance of new substrate molecules to the active site, was a possible cause; i.e., after the initial oxidation, the enzyme active sites would become "blocked". This would occur faster in concentrated solutions where the interaction of enzyme molecules with substrate would be more rapid, whereas there would be only a small amount of substrate per enzyme active site in dilute solutions. Unfortunately, as product isolation was the overall aim

in these experiments, it was not practical to use the dilute hordenine concentrations necessary for continual enzymic oxidation. Hence, a low product yield in relation to unchanged hordenine had somehow to be accommodated.

2.2.3 More Detailed Analysis of Hordenine Reaction Mechanism

Investigations into the changes in the redox balance of lucerne tissue under aphid attack, by Jiang & Miles (1993b), gave surprising results. They observed that extracts of infested lucerne leaves showed immediate oxidation of PAC solutions* (by observation of the UV absorbance at 438 nm) independent of the quantity of extract added. When healthy leaf extracts were used, however, the amount of oxidation was approximately inversely proportional to the amount of extract added. From this it was hypothesised that while substrate-limited oxidation may

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* PAC - Partially Autoxidised Catechin; 10 mM catechin in phosphate buffer that has been autoxidised for several days. Can be used as a highly sensitive redox indicator by observing the oxidation or reduction of this compound at 438 nm.

occur in all plant leaves, it is limited in healthy leaves by the quantities of reductant(s) that they are able to maintain.

As Jiang's work was the first known suggestion of such an occurrence *in vivo*, it was decided in the present study to determine if similar results could be obtained with the hordenine/model enzyme system. As both HRP and mushroom tyrosinase had been shown to oxidise hordenine to the same product (Chapter 1), tyrosinase was chosen as a suitable enzyme for further investigation, thereby eliminating the need to include hydrogen peroxide in the reaction mixtures. A control solution, without enzyme added, was used as a standard for spectrophotometric measurements. Observation of the hordenine/tyrosinase oxidation was performed at 285 nm. Immediately upon enzyme addition to the reaction mixture a rapid increase at this wavelength was observed. The oxidation quickly slowed down, with no change in absorption after approximately 20 minutes. With further enzyme addition (addition of water to the control solution) faster oxidation was again stimulated, but for a very small period. These results (Appendix 2a) are similar to those observed for the HRP oxidations mentioned for hordenine above.

When reductants (e.g. glutathione or ascorbic acid) were included in the reaction mixtures, a constant oxidation rate was observed for an extended period of time. Once all of the reductant had been used (oxidised), however, the reaction quickly came to a stand-still. Also, at times (during the early stages of the reactions, when the quantity of reductants were high) the amount of hordenine oxidation occurring was less than the amount of product being reduced (due to the oxidised reductants), hence the concentration of reductant is an important factor.

Thus, for this system, it was shown that reductants were able to prolong the period of hordenine oxidation. This longer oxidation time relied upon the reduction of small quantities of oxidised hordenine by the anti-oxidant present (either glutathione or ascorbate). In effect this slowed down the hordenine

oxidation reaction, resulting in a slower, but more constant oxidation rate. Once all the reductant present had itself been oxidised, however, the remaining hordenine was only partially converted, since the reaction soon ceased. When the concentration of reductant was greater than that of substrate, of course, the rate of substrate reduction became greater than the 'forward' substrate oxidation reaction. Hence, a suitable balance of substrate:reductant concentrations is required for optimum oxidation to proceed.

2.2.4 "PAC" Oxidation in a Model System

Hordenine is found in the rootlets of barley (Liu & Lovett, 1993), and thus is unlikely to be exposed to aphid attack. It seemed suitable, therefore, to perform these experiments with a compound accessible to the insects. PAC (as used in Jiang & Miles, 1993b) was an obvious choice since catechin is a phenolic present in many plant tissues and it's redox reactions are readily detectable. It was reacted with mushroom tyrosinase under conditions similar to those described above for hordenine.

Observation of the reaction of PAC with tyrosinase at 438 nm showed rapid oxidation (increase in absorption at this wavelength). Inclusion of reductant (glutathione or ascorbate) in the mixtures limited this reaction somewhat, until the reductant had been completely oxidised (Appendix 2b).

All of these observations are compatible with Jiang's findings, and can be applied to the interaction of plants and insects (Miles & Oertli, 1993). Aphids feeding on plants of low reductive capabilities should readily be able to oxidise putatively defensive chemicals present (by reaction with salivary enzymes), whereas plants that contain more reductants should be less affected by injection of these enzymes, in that they would have some capacity to reverse oxidation of allelochemicals. Thus, the plant would prolong the action of oxidisable defensive compounds, and should therefore be less susceptable to insect attack. This redox

system would be substantially affected by factors that would alter the relative amounts of reductants and oxidisable allelochemicals in the plant and activity of the insects' salivary oxidases, as mentioned in Chapter 1 (1.3).

2.3 CONCLUSION

In order to obtain suitable quantities of product(s) for structural identification, the yields from aphid salivary oxidations of plant defence chemicals needed to be optimised. This could not be achieved without a thorough understanding of the reaction mechanisms. Hordenine was chosen as a starting point for detailed examination, due to the ease of handling, availability, and water-solubility of this compound. As aphid salivary preparations were so dilute, model enzymes were used to enable greater product formation.

When the HRP oxidation of hordenine was closely observed at substrate concentrations thought to be suitable for recovery of analysable quantities of product, it was noted that oxidation occurred only within the first few minutes of reaction. After this time, subsequent hordenine oxidation was minimal, and the reaction did not go to completion. A variety of factors that may have affected the oxidation were considered, including dependence upon H_2O_2, enzyme degradation, and inhibition by a reaction intermediate or product. Attempts were made to interpret and overcome these problems and to prolong rapid oxidation, namely by: addition of H_2O_2, HRP/H_2O_2 and tyrosinase, further substrate additions, and observation of the reaction pH (to detect formation of inhibitory compounds); none proved successful. Investigation of the equilibrium balance between hordenine oxidation and the reverse reduction was also performed. Comparison of the absorbance ratios of unoxidised and partly-oxidised hordenine solutions after initial oxidation, and again after further enzyme additions and reaction, showed nevertheless that the 'forward reaction', or formation of oxidation product, rather than the reverse reduction is the predominant reaction occurring between hordenine and the model enzymes.

No reasonable explanation for this reaction deceleration was apparent until experiments conducted in this laboratory by Dr. Jiang revealed that very low

concentrations of substrate gave more quantitative product formation. "Blocking" of the enzyme active site, by loosely-held product molecules, was thought to be the cause of this. As product isolation was the main aim of the investigation, however, high substrate concentration and slow oxidation had to be accommodated if good overall product yields were to be obtained.

Confirmation *in vitro* of Jiang's novel hypothesis "that a substrate limited oxidation occurs in all plant leaves, but that this is increased in infested leaves; whilst healthy leaves contain significantly more reductant(s)" has been achieved. Analysis of the mushroom tyrosinase oxidations of hordenine and PAC (partially autoxidised catechin) revealed that the presence of reductant(s) can prolong substrate oxidation. Both compounds were quickly oxidised by the enzyme; the oxidation reached a steady-state but could be re-started by addition of further enzyme. Nevertheless, the period of rapid oxidation until steady-state was re-attained shortened with successive additions of enzyme.

An interpretation of these findings can be presented as follows. Inclusion of reductant in the mixture of hordenine or catechin greatly affects the redox balance. While suitable quantities of reductant are present, oxidation of substrate proceeds with small amounts of product being reduced back to starting material. Once all of the reductant has itself been oxidised, uncontrolled substrate oxidation begins but quickly ceases. Too great a concentration of reductant results in the back reaction (substrate reduction) taking prominence, and net substrate oxidation is therefore slow. The relative reductant and substrate concentrations are therefore of critical importance in the net oxidation rate of the latter.

These observations are consistent with the general hypothesis - that the injection of aphid salivary enzymes into their hosts during feeding results in the oxidation of defensive chemicals to products of less insect deterrence/toxicity. Also that the reductants present in plants serve to counter-balance these oxidation reactions. Since the presence of reductants serves, nevertheless, to prolong

oxidation, but may increase the resistance of plants to aphids (Miles & Oertli, 1993; Jiang & Miles, 1993b) it must be assumed that it is the intermediates that exist during oxidation rather than the oxidation products themselves that are significant in the insect-plant interaction. Thus plants that contain greater reductive capacity should be less affected by the insects, as they are able to prolong the action of their defensive chemicals.

Thus the importance of the relative activities of the oxidising enzymes in aphid saliva and the reducing systems in the tissues immediately affected by aphid feeding (see Chapter 1; 1.3) cannot be under-stated, as this overall redox system would seem to be a major factor in determining the degree of susceptability, tolerance and/or resistance of plants to attack by these insects.

2.4 EXPERIMENTAL

2.4.1 Hordenine Reactions

1. Various Enzyme Concentrations.

Hordenine hemisulphate (1.0 mg/ml; 10% EtOH) and H_2O_2 (0.03% in solution) were reacted with HRP (0.1, 0.5, & 1.0 mg/ml), in a total volume of 1 ml. The three reactions (and associated control mixtures) were treated as per hordenine/HRP reactions (2 h at room temperature). The UV subtraction spectra obtained all contained the 300 nm peak. The peak width varied somewhat with enzyme concentration - the more dilute enzyme reaction yielding the broader peak, suggesting greater relative product formation.

2. HRP Reaction Over Time.

(i) Hordenine hemisulphate (1.0 mg/ml; 10% EtOH), H_2O_2 (0.03% in solution), and HRP (0.5 mg/ml) were reacted at room temperature for 2 h. The UV spectra of the reaction & control solutions were recorded at a series of time intervals (0, 15, 30, 45, 60, 90, & 120 min). Analysis of the resultant UV subtraction spectra (correcting for hordenine without enzyme) revealed the 300 nm product absorption. The shape of this peak was not found to alter significantly after the first 15 min.

(ii) The above procedure was repeated, using a more dilute enzyme concentration, HRP (0.005 mg/ml). Formation of the product peak (300 nm) was found to increase slowly with time (both in peak height & width), after a rapid initial oxidation.

(iii) Using the procedure outline in (i), the oxidation spectra were recorded at specific time intervals over a 2 day period (0, 2, 4.5, 24, & 47 h). Maximum oxidation of hordenine was obtained within the first minute (T=0 h spectrum).

Subsequent increases in width/height of the subtraction peak were relatively small.

3. Supplementation of Hydrogen Peroxide Over Time.

Hordenine hemisulphate (1.0 mg/ml; 10% EtOH) was reacted with HRP (0.1 mg/ml) in the presence of H_2O_2 (0.03%), in a total volume of 4 ml. A control solution (no enzyme added) was used as the internal reference, and the UV subtraction spectrum for these solutions was recorded at 15 min intervals. Additions of further portions of H_2O_2 (30% solution; 5 µl) were made periodically, but were not found to influence the peak height (305 nm) significantly.

4. Supplementation of HRP Over Time.

(i) The procedure outlined in (3) above was repeated, with one modification. Portions of HRP (0.1 mg/ml, 5 µl) were added periodically, as well as the H_2O_2 solution. These additions during the course of the reaction did not appear to increase product formation to any extent, as judged by the peak height/width at 305 nm.

(ii) The method of (i) was used, with UV subtraction spectra recorded every 7 min. The results observed were identical.

(iii) Dilute enzyme solution (HRP = 1×10^{-3} mg/ml) was also used in the previous method (ii). As before, the product peak was not found to increase significantly after the initial rapid oxidation of hordenine.

5. Addition of HRP & Tyrosinase.

A mixture of HRP and tyrosinase (0.8 mg/ml of each) was used as the oxidising enzyme solution in the procedure outlined in 4(ii). Results identical to those found for the HRP addition experiments were obtained; i.e., after the initial rapid oxidation of hordenine, further fast reaction could not be induced.

6. Supplementation of Substrate (Hordenine) Over Time.

Hordenine freebase (1.0 mg/ml; 10% EtOH) was reacted with HRP (0.1 mg/ml) in the presence of H_2O_2 (0.03%), in a total volume of 4 ml. A control solution (no enzyme added) was used as the internal reference, and the UV subtraction spectrum for these solutions was recorded at 5 min intervals. Additions of further portions of hordenine (1 mg/ml; 10 µl) were made periodically, but were not found to influence peak height (305 nm) significantly.

7. Tyrosinase Reaction Over Time.

Hordenine freebase (1.0 mg/ml, 10% EtOH), H_2O_2 (0.03% in solution), and tyrosinase (0.5 mg/ml), in a total volume of 4 ml, were reacted at room temperature. The UV subtraction spectrum (corrected for control - no enzyme present) was recorded every 7 min. Additions of fresh enzyme solution (4 µl), followed by further amounts of H_2O_2 (3% solution; 4 µl) were made periodically. The change in peak height/width at 295 nm was observed. This peak was not found to increase rapidly after the initial oxidation of starting material, as was found in the HRP reaction.

2.4.2 pH Observations

Hordenine freebase (1 mg/ml; 10% EtOH) was reacted with a trace of HRP in the presence of H_2O_2 (0.03% in solution), in a total volume of 400 µl. The pH of the reaction and control solutions was recorded prior to and immediately after enzyme addition, and also after 30 min at room temperature. No change in solution pH was observed: It remained between pH 6-7 throughout. After the 30 min reaction period, the UV subtraction spectrum for the two solutions was calculated, revealing product absorption at 305 nm. The mixtures were then acidified (0.1 M HCl; 100 µl), further enzyme added (10 µl), and again left to react for 30 min. After this time the UV subtraction spectrum had not altered, indicating that acidification does not recommence rapid hordenine oxidation.

2.4.3 Hordenine Equilibrium Investigations

1. Hordenine/HRP Oxidation.

Hordenine hemisulphate (2 mg dissolved in 400 µl dd water, 10% EtOH) and H_2O_2 (0.03% in solution) were made up to 3.6 ml with dd water. The UV spectrum of this solution was recorded, and the absorbance/wavelength of the hordenine peak noted. To this solution was added HRP (2 mg in 400 µl dd water), and a control solution (no enzyme added) was used as the internal reference. The UV subtraction spectrum was recorded, product peak noted, and the development of the peak height in the product spectrum recorded every 5 min. Once a steady-state had been reached (approx. 30 min) another portion of hordenine (400 µl) was added (to both reaction and control mixtures), and the UV spectrum again followed. Comparison of the peak heights for the starting material and product peaks before and during oxidation, and then again after a second addition of hordenine suggested that a normal reaction equilibrium situation was in place, i.e., oxidation equilibrium favours product formation. The comparisons were performed and presented using the following equations:

$$R_1 = A_{O1} / A_{R1} \qquad R_2 = A_{O2} / A_{R2}$$

Where: R_1 and R_2 are the ratios of absorbance at the wavelengths of unoxidised and part-oxidised peaks.

R_1 = initial oxidation ratio, R_2 = further oxidised ratio.

A_{Ox} = oxidised peak absorbance (after enzyme addition).

A_{Rx} = unoxidised peak absorbance (prior to enzyme addition).

2. Various Enzyme Concentrations.

The procedure outlined in 1 (above) was repeated, using four different concentrations of HRP: 0.025, 0.050, 0.100, 0.200 mg/ml in the total volume (4 ml). Comparison of peak heights revealed similar results to the previous calculations (see 1 above). A regular oxidation equilibrium seems to be involved in the hordenine-HRP reaction, favouring product formation, i.e., R1> R2.

2.4.4 Detailed Analysis of Hordenine Reaction Mechanism

1. Tyrosinase - Various Enzyme Concentrations.

Hordenine hemisulphate (0.5 mg/ml) and H_2O_2 (0.03%) were made up in a total volume of 3.92 ml. Tyrosinase (10 mg/ml; 40 µl) was added at time T=0 min, the solutions mixed, and the UV spectrum recorded (control, i.e., no enzyme added, was used as the internal reference solution). The λ_{max} was determined from the T=0 spectrum, and the absorbance at this wavelength (290 nm) was recorded every 5 min. After 20 min no further increase was observed, hence a further portion of enzyme (40 µl) was added. Observations were continued every 5 min until the experiment was completed (35 min).

2. Various Concentrations of Tyrosinase with Reductant Present.

Hordenine (0.5 mg/ml) was reacted with tyrosinase (either 0.01, 0.05 or 0.10 mg/ml) in the presence of either no reductants, or a variety of concentrations of ascorbate (asc) or glutathione (glu) (0.05, 0.10 or 0.20 mg/ml), in a total volume of 4 ml.

The different reactions (no reductant; glu 0.1; glu 0.2; asc 0.05; asc 0.1; asc 0.2) were made up individually in 4 ml quartz cuvettes. Prior to enzyme addition the cuvettes were inserted into the spectrophotometer (control solution as the internal reference) and the machine was zeroed (285 nm). After 20 min the

absorbance at 290 nm was recorded. This procedure was repeated for each of the six reactions, and performed for all three tyrosinase concentrations. The absorbance data obtained was then analysed (Appendix 2a).

2.4.5 PAC Oxidations

PAC (partially autoxidised catechin; 10 mM) was produced as described by Jiang & Miles (1993b), i.e., catechin (116 mg) was stirred in phosphate buffer (50 mM, pH 7, 40 ml) at room temperature for 3 days. After this time, it was diluted with a further portion of buffer (40 ml), and this solution used in subsequent experiments.

PAC (5 mM) was reacted with tyrosinase (either 0.001, 0.01, 0.05 or 0.10 mg/ml) in the presence of either no reductant or a variety of concentrations of the two reductants, but in the absence of hydrogen peroxide. The different reactions (no reductant; glu 0.1; glu 0.2; asc 0.05; asc 0.1; asc 0.2) were made up individually in 4 ml quartz cuvettes. Prior to enzyme addition the cuvettes were placed in the spectrophotometer (control as internal reference solution) and the machine was zeroed (438 nm). After 20 min the absorbance at 438 nm was recorded. This procedure was repeated for each of the six reactions, and performed for all four tyrosinase concentrations. The absorbance data obtained was then analysed (Appendix 2b).

CHAPTER 3

PRODUCT IDENTIFICATION AND ISOLATION

3.1 INTRODUCTION

Isolation and/or identification of the products formed from the oxidation of plant allelochemicals was a major aim of this project. As discussed in Chapter 1 little examination of the products of peroxidase and catechol oxidase reactions involved in the immediate interaction of plants with insects and pathogens has been performed previously, and work with aphid salivary oxidations seems confined to this laboratory.

Due to the small quantities of product that result from the enzymic oxidation of phytochemicals (as a consequence of enzyme inhibition problems and the dilute nature of the obtained salivary preparations, outlined in Chapter 2), a variety of techniques was employed in attempts to identify possible functional groups or structural features of the products.

It has long been believed that the oxidation of most plant and natural phenolics proceeds via o-quinone intermediates. These quinones are thought to react either with another molecule of hydroquinone (or that two semiquinone radicals react) to form polymerised phenolic product(s). Insect cuticle, condensed tannins, oxidative browning and deterioration of food stuffs (including wine, fruit, etc.) all occur via such mechanisms (Manthey, *et al.*, 1988; Cheynier, *et al.*, 1988 & 1989; Richard, *et al.*, 1991). The involvement of o-quinones as intermediates in these reactions has been established by trapping experiments. The reagent commonly used is sodium benzene sulfinate (Piretti, *et al.*, 1977; Davies & Pierpoint, 1975; Sato, *et al.*, 1993), which can quickly combine with intermediate quinones to yield the corresponding sulphones, thus preventing further oxidation.

To obtain definitive structures for the oxidation product(s) it was first necessary to isolate enough material for detailed identification and analysis, e.g. NMR & mass spectroscopic studies. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) are tools commonly employed for this purpose (Halpaap, 1969; Krstulovic´ & Brown, 1982; Goodenough, *et al.*, 1983; Heftmann, 1983; Potter, *et al.*, 1985), and were used in the current investigations.

3.2 RESULTS & DISCUSSION

SECTION A

3.2a.1 Quinone Identification

Trapping experiments were conducted to determine if quinones were produced (perhaps as intermediates) during the oxidation of the phytochemicals studied. For this purpose reactions were performed on the plant chemicals indicated below (Table 3.1) using sodium benzene sulfinate (see 3.1).

Table 3.1: Observation of quinone-type intermediates during oxidation.

COMPOUND	MODEL ENZYME SYSTEM	APHID SALIVA
Caffeic Acid	Some quinone formation	Some quinone formation
Chlorogenic Acid	Some quinone formation	Quinones form
Ferulic Acid	Quinones form	Some quinone formation
Hordenine	None	None

As can be seen from Table 3.1, diphenolic compounds are generally able to pass through o-quinone intermediates during oxidation. Analysis of the UV subtraction spectra for these reactions showed little or no evidence of product formation in the presence of benzene sulfinate. Derivatives of diphenols, such as ferulic acid, must presumably first undergo hydrolysis (of the ester moiety in this instance, giving caffeic acid), to the corresponding diphenol which can then be oxidised to an o-quinone.

Hordenine, a mono-phenol, did not seem to follow such a pathway. If quinones were produced during the HRP oxidation of hordenine, then they should quickly react with the benzene sulfinate salt present, preventing further oxidation to reaction products (as for the diphenolic compounds). Attempts were made using 1.8, 3.5 and 10 equivalents of sodium benzene sulfinate (SBS), but the UV subtraction spectra for these reactions all contained a product peak at 318 nm, similar to that produced in the absence of the quinone-trapping agent. The presence of this product in all these reactions was also confirmed by TLC.

Similarly, the reaction between hordenine and aphid salivary enzymes (using 1.05 equivalents of SBS) yielded reaction product in the UV subtraction spectrum, again confirmed by TLC analysis. Thus, it would seem that o-diphenolic (quinone) intermediates are not formed during the oxidation of hordenine (and possibly other monophenols) by either HRP or aphid salivary enzymes.

SECTION B - HORDENINE OXIDATION

3.2b.1 Extraction of Hordenine Oxidation Product(s)

The ease of handling hordenine (good solubility, availability and relatively low mammalian toxicity) made it an apparently suitable choice for initial isolation experiments to determine appropriate general procedures.

To identify the type of product(s) involved in the hordenine oxidation (i.e., acidic, neutral or basic), preferential extractions of hordenine-HRP reaction mixtures were performed. The aqueous and organic phases were analysed by TLC after each extraction to determine their composition.

Chloroform and ether extractions were carried out on the neutral reaction mixture. Chloroform was very successful, ether to a lesser extent, in removing the product(s) and some unreacted hordenine from the aqueous phase. After

acidification of the aqueous solution (pH 1), ether and chloroform extractions did not yield any product(s) or hordenine. Similarly, the extractions performed on the basicified reaction mixture (pH 14) were unsuccessful. It was determined that the oxidation product(s) formed are soluble in organic solvents at or near a neutral reaction pH, and that chloroform is the most suitable solvent for extracting the components from hordenine-HRP reaction mixtures.

3.2b.2 Thin Layer Chromatographic (TLC) Analysis of Hordenine Reactions

A variety of techniques and chromatographic media were utilised in attempts to find the most suitable system for analysis of oxidation reactions of hordenine. As hordenine is an aromatic compound both UV visualisation and exposure to iodine vapour were suitable methods of detection. It was also possible to detect hordenine using silver nitrate staining (Stahl, 1965), although this technique was more complex to perform. In general, UV visualisation and exposure to iodine were the preferred methods used.

A variety of solvent systems were investigated on both silica gel F_{254} and kieselguhr F_{254} plates. Alternative systems, such as the use of cellulose plates and paper chromatography were also tested. The best results, however, were obtained on silica gel, using methanol (with traces of ammonia) as the development solvent.

In the majority of the early experiments, hordenine hemisulphate (i.e., $[C_{10}H_{15}NO]_2H_2SO_4.2H_2O$) was used as the substrate. Once TLC analyses began, however, it was found that the purchased hemisulphate was contaminated by the freebase of hordenine. This resulted in two component spots for the starting material, unnecessarily complicating the reaction chromatograms. Hence, the freebase was produced (in excellent yield) from hordenine hemisulphate, via reaction with aqueous ammonia. This was then used in subsequent reactions.

3.2b.3 Detection of Functional Groups

Attempts were made to determine if the hordenine oxidation products contained a carbonyl group (aldehyde or ketone, perhaps formed by oxidation of the hydroxyl) or a peroxide moiety. The detection techniques used were those of 2,4-dinitrophenylhydrazine (2,4-DNPH) and potassium iodide-starch (Stahl, 1965). Neither method revealed a product "spot" on silica chromatograms, suggesting that the hordenine oxidation products do not contain either a carbonyl or peroxide group.

3.2b.4 Preparative TLC

As good separation of hordenine and its oxidation products were obtained on silica (see reaction analyses in Chapter 1), attempts were made to isolate the major product for structural identification. Experiments thus far had shown that the enzymic oxidation of hordenine in concentrations sufficient for analysis could not be induced to go to completion (Chapter 2), hence it was necessary to combine and extract several small reactions rather than one large scale reaction.

The individual mixtures (10 x 1 ml) were combined and extracted*, and the sample was then purified by preparative TLC (silica). The separated components could finally be extracted from the silica ($CHCl_3$-MeOH and MeOH only), and structural analyses conducted. In the two attempts using this method, however, only unreacted hordenine was successfully recovered. The product component appeared to be bound to the silica plate; thus an alternative method was sought.

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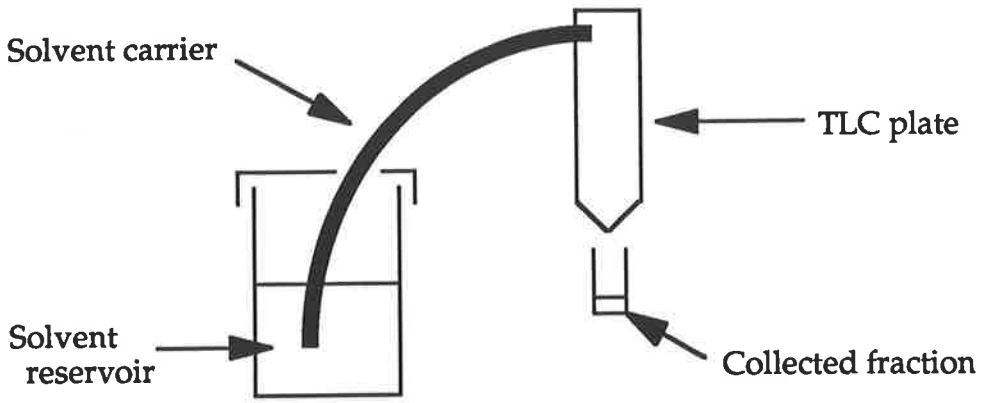
* Initially using $CHCl_3$-MeOH, later CH_2Cl_2-MeOH was found to yield better results.

A sample of the hordenine and oxidation products extracted from an HRP oxidation was applied to several different types of TLC plates (silica, kieselguhr and alumina), and extractions of the separated components attempted. Of the three types of TLC media used kieselguhr was found to be unsuitable (impractical solvent system and difficulties in visualisation of components), whereas good separations were obtained with the other two. Extraction from silica plates with methanol recovered hordenine with only traces of product. Alumina plates gave excellent separations of hordenine and products, however, and were used for subsequent preparative TLC attempts.

In the hope of removing the separated components more quantitatively from the alumina plate, hot solvent extractions were performed, but they gave little improvement on cold extraction yields of product, judged by TLC and UV spectroscopic analysis. Likewise, continual extraction with boiling solvent (for 30 min) did not extract all compounds from the alumina. Similar experiments conducted on silica plates were also unsuccessful, hence other preparative separation methods were investigated.

As the oxidation products could not be successfully extracted from the TLC media used, the concept of descending chromatography was considered. This involved running a peaked alumina TLC plate in the reverse direction (see Figure 3.1) and collecting fractions as they were eluted.

Figure 3.1: Peaked TLC plate used for descending chromatography.

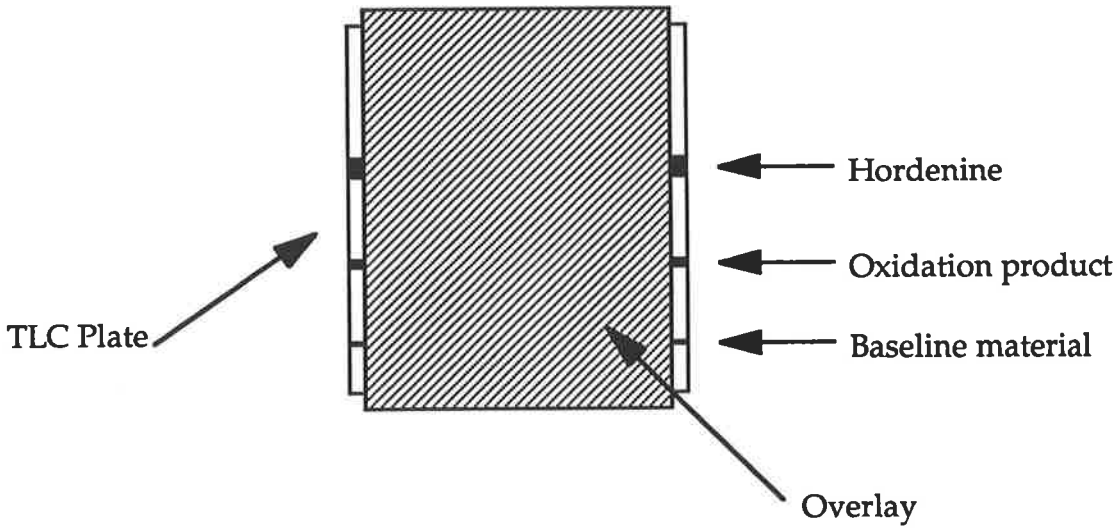


After 1.5 h of development only unreacted hordenine had been obtained, and the desired product was not in a position on plates for elution. With the excessive run time involved, and the difficulties experienced in maintaining the correct solvent gradient (the carrier paper was exposed to the atmosphere), this technique was considered to be impractical.

The apparent binding of the hordenine oxidation products to the alumina (and silica) TLC plates was puzzling. One possible cause that seemed worth consideration was the exposure of the separated components to light, and in particular to UV light (during visualisation of the separated components), resulting in some interaction between the chromatographic media and the reaction products.

In order to investigate this possibility, a hordenine-HRP mixture (10 x 1 ml portions) was reacted in the dark for 2 h, and all subsequent procedures (including extraction and preparative TLC) were carried out in the absence of light. Once developed, the preparative plate was covered with aluminium foil, with only test strips at the two side edges of the plate exposed. These were examined under UV light, and the positions of the ends of the reactive bands marked (see Figure 3.2). From this, the position of the complete band was judged, and the extraction of the compound performed.

Figure 3.2: Selective UV detection of separated TLC bands.



The results of TLC when light was excluded showed a significant improvement on previous attempts under normal laboratory illumination. UV analysis of the separated components revealed recovered hordenine to be the higher Rf compound (as predicted by TLC), and the oxidation product as the other component (peak at 319 nm). These compounds were found to decompose with overnight storage at -4°C (nitrogen atmosphere), however, hence all subsequent separations and analyses were performed on freshly isolated substances.

Small quantities of material were obtained using the method outlined immediately above, and these were subjected to both NMR and mass spectral analysis. The analytical data obtained for the major component confirmed it to be recovered hordenine, whilst the mass spectrum observed from the product indicated a "2M-2" dimer of hordenine. The ^{1}H-NMR spectrum of this component was contaminated by traces of starting material (hordenine), however, and hence definitive confirmation of the proposed structure was not possible. Such a result is consistent with several reported attempts to isolate the products of peroxidase oxidation of phenols (Markwalder & Neukom, 1976; Potter, *et al.*, 1985).

3.2b.5 Column Chromatographic Investigations

Separation of hordenine and associated HRP oxidation products by flash chromatography (Still, *et al.*, 1978) was attempted. A flash silica column, with methanol as the running solvent, was successful in obtaining purified hordenine from the reaction mixtures, but, as in silica TLC, oxidation products could not be eluted. Again it appears possible that binding of the products to the column was occurring. Alternative packing material would therefore be needed.

LH 20 (reverse phase) sephadex was used in both small, medium and large column lengths (1 cm x 6, 12, or 30 cm, respectively). Purification of hordenine-HRP oxidations by the smaller columns resulted in some separation of the components, but most of the fractions obtained were of mixed content. The 30 cm column yielded greater separation of the starting hordenine and oxidation products, but still the product fractions collected were of a mixed nature. As sufficient quantities of pure components (for product identification techniques) were not isolated, this method was abandoned.

3.2b.6 Preparative High Performance Liquid Chromatography (HPLC)

Good separations of starting hordenine and oxidation product were obtained during analytical reverse phase HPLC, hence a preparative run was attempted. As with the preparative TLC, several small hordenine-HRP reaction mixtures were combined, then separated by HPLC. The solvent system used (water-acetonitrile) contained an ion-pairing reagent (Pic B7), and after the preparative samples were concentrated this solid remained. Attempts to selectively extract or wash the reagent from the samples were unsuccessful. Mass spectral analyses of the contaminated samples were dominated by pairing reagent fragmentations, preventing identification of either hordenine or product information. For this reason alternative HPLC solvent systems and pairing reagents were investigated.

Trifluoroacetic acid (TFA) is a common additive used in chromatographic separations (Olieman & Voskamp, 1984). HPLC analyses using water-methanol (A; 90:10) and acetonitrile-methanol (B; 90:10), with 1% TFA included as the ion-pair reagent, were performed. Although separation of the hordenine and major oxidation product peaks were obtainable by this method, the relatively close proximity of the two peaks made the use of TFA unsuitable for preparative scale work.

A similar, more volatile compound, heptafluorobutyric acid (HFBA), has also been shown to have good ion-pair properties (Olieman & Voskamp, 1984). This was included in the HPLC solvent system at a concentration of 0.05%. Excellent separations of hordenine and oxidation product were obtained using this system, hence it was used for subsequent preparative scale investigations.

3.2b.7 Hordenine dimerisation

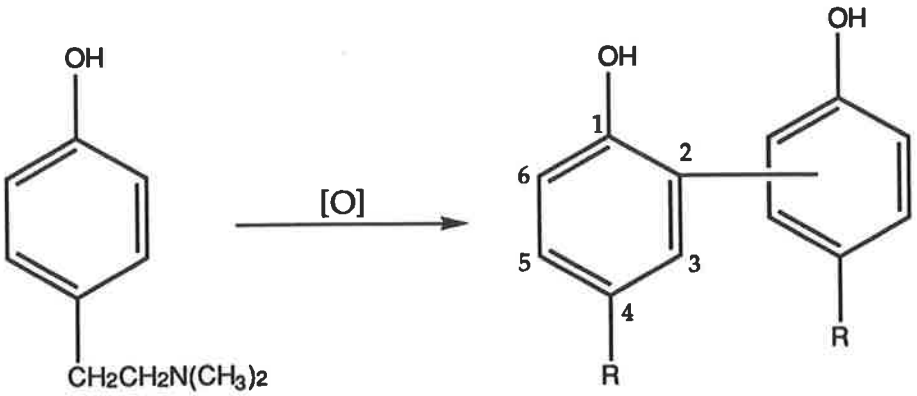
The dimerisation of phenols via radical reactions has been reported in the literature; the method of Bacon and Munro (1960) was essentially followed, and used to produce the dimer of hordenine. After work-up, the crude reaction mixture was found (by TLC) to contain unreacted hordenine, as well as some of the desired product. Analysis of this crude mixture by RP-HPLC confirmed that the product obtained was identical to that from the enzymic oxidations of hordenine. Attempts of this reaction on a preparative scale were unsuccessful.

Small-scale reaction of hordenine with ferric chloride (Bowden & Reece, 1950a & 1950b; Bhattacharjee & Mahanti, 1985) was also shown by TLC and HPLC to produce the desired phenolic dimer. Large-scale production, however, proved unsuitable for isolating the dimerised product. The large quantities of unreacted starting hordenine and other reaction products present in these mixtures made purification difficult. Increasing reaction time did not improve the yield of hordenine dimer, and only served to increase the number of overall products

formed (e.g. trimers, polymers, etc). For this reason hordenine was oxidised by a dilute HRP solution (0.2 mg/ml), in an attempt to maximise oxidation and limit the amount of remaining starting material.

Overnight oxidation in this system at last resulted in almost quantitative reaction (as estimated by TLC analysis), and the crude reaction products (approximately 20 mg in total) were purified by semi-preparative HPLC over several days. From this the major oxidation product was obtained in sufficient yield to undergo proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) & mass spectral analyses. The structural features of this compound were determined from NMR spectra, obtained in two different solvents (deuterated water and deutero-pyridine) as good resolution of all peaks was not obtained in either one. The aromatic region (D_2O spectrum) consisted of signals at δ 7.30 (1H,m), 7.16 (1H,m), and 6.82 (1H,d,7Hz), attributed to H3,H5 and H6 respectively (based upon the NMR of hordenine; Pouchert, 1993). The aliphatic portion of the compound (D_5-pyridine spectrum) showed two multiplets, δ 2.82 & 2.56 (2H), assigned to the methylene hydrogens, and a singlet at δ 2.21 (6H) for the amino-methyls. All of this information, and the observed mass spectrum molecular ion (M^+ 328), was consistent with the proposed dimeric (2"M" - 2) structure.

i.e.:



Hordenine

Hordenine "Dimer" (2M-2)

where R = $CH_2CH_2N(CH_3)_2$

SECTION C - OTHER ALKALOIDS

3.2c.1 Acetaminophen - Preparative TLC & HPLC Analysis

The reaction of 4'-hydroxyacetanilide (acetaminophen) with HRP has been well documented (Fischer & Mason, 1984; Potter, *et al*, 1985; Mc Cormick & Shihabi, 1990). The products obtained from such reactions are condensation products, primarily dimers of acetaminophen (joined at the ortho-hydroxy position). The structural similarity of acetaminophen and hordenine, and the difficulties experienced in isolating hordenine oxidation products, lead to the investigation of the acetaminophen-HRP reaction described by Potter, *et al.*, (1985). The oxidation products were easily detected on silica TLC plates using either chloroform-methanol or ethyl acetate-acetic acid mixtures (three products observed), and the UV-subtraction spectrum obtained for this reaction revealed sharp peaks at 268 & 300 nm.

Acetaminophen and associated oxidation products were extracted from aqueous reaction mixtures, and separated by preparative TLC. From the silica

plates were recovered unreacted acetaminophen and at least four product components. The UV spectra of these components all contained a peak at about 255 nm; component 1 (highest Rf; acetaminophen) also showed a broad peak at 280 nm, whilst the other components showed absorption at 290-300 nm.

The HPLC traces obtained from analysis of the reaction mixture and individual components were very similar to those reported in the literature (Potter, *et al.*, 1985). Acetaminophen was the largest peak observed, whilst two products (the acetaminophen 'o,o'- & 'o,N'- dimers, based upon UV & HPLC data; Potter, *et al*, 1985) were quite prominent. Hence it would seem that the preparative TLC isolation methods used here are suitable for obtaining phenolic oxidation products, without altering their composition. Quantitative extraction yields, however, have not yet been obtained.

3.2c.2 Gramine Oxidations - Extraction and Preparative TLC

Chloroform extraction of gramine-HRP oxidation mixtures proved very successful in recovering quantities of the starting material and oxidation products. After concentration, these samples were subjected to preparative TLC on silica plates (methanol + trace NH_3), and five components were obtained. Component 4 was shown to be unchanged gramine (by TLC and mass spectral analysis). The major products, components 2 & 5, were designated as products A & B respectively (identifiable by their TLC and UV spectroscopic data).

3.2c.3 Column Chromatographic Isolation of Gramine-HRP Oxidation Products

Sephadex chromatography (LH 20) of the HRP oxidation of gramine was successful in removing unreacted starting material from the reaction products. Separation of the individual products, unfortunately, was not achieved, as mixed fractions were obtained. The combined product fractions were subjected to semi-preparative TLC and five compounds were recovered, but none was obtained in sufficient quantity for structural identification. As this process gave inadequate

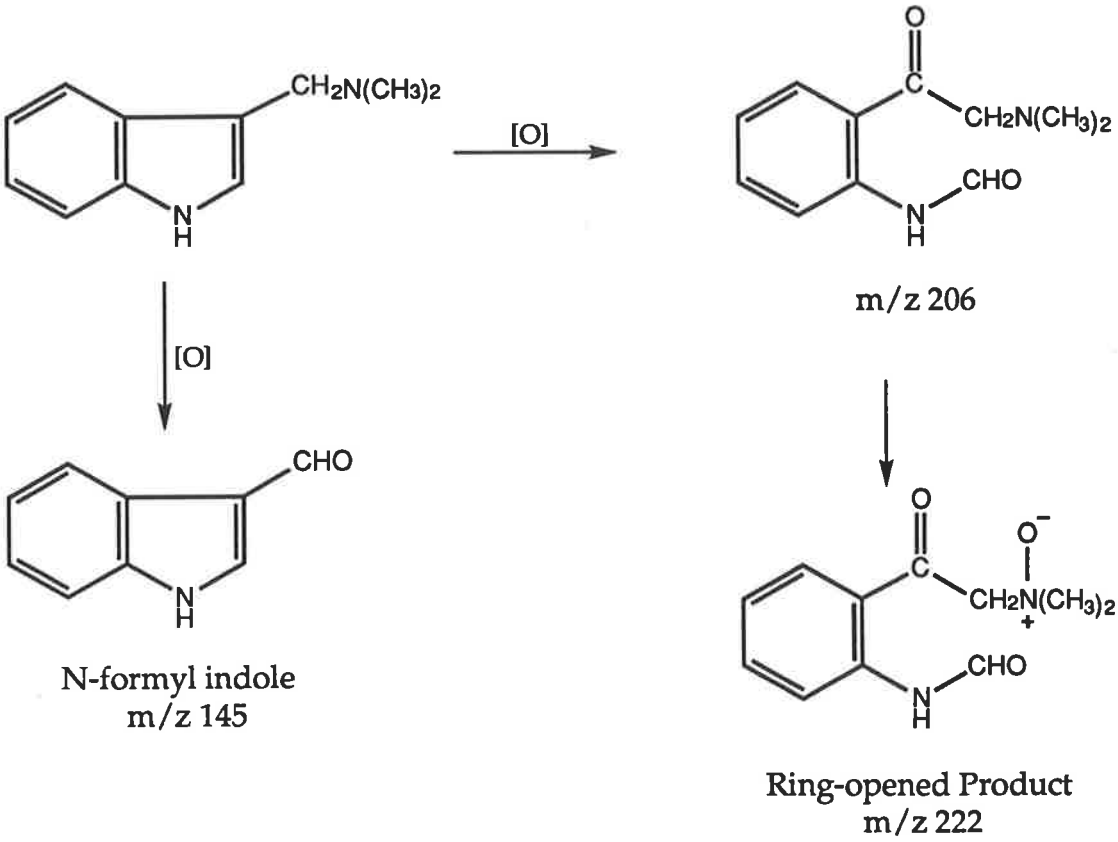
yields of product, preparative HPLC was chosen as a more suitable means of purification.

3.2c.4 Preparative HPLC of Gramine Oxidations

An HRP-enzymic oxidation of gramine was performed, and the resultant crude product material (containing some unreacted gramine) was purified by semi-preparative HPLC (see 3.4c.2.4). The major reaction products (retention times 9.0 & 11.0 min) were collected, and analysed for content by TLC. This revealed component 2 to be of mixed content, whilst component 1 was obtained in sufficient yield for structural analysis. The ^{1}H-NMR data obtained suggested that even component 1 contained two compounds, as the signals observed were consistent with both formyl-indole and an N-oxide ring opened compound, as determined by comparison with similar compounds in the literature. Signals observed at δ 12.20 (1H,s) and 9.85 (1H,s) can be attributed to the aldehyde and amine protons, respectively, of 3-formyl indole (Pouchert, 1993). The integration obtained for aromatic signals at δ 8.57, 7.49 and 7.13 suggested these signals were due to more than one compound, and irradiation at 7.35 ppm agreed with this. Signals at δ 8.57 (1H,d,8Hz,ArH), 8.08 (1H,s,NHC<u>H</u>), 7.49 (1H,t,8Hz,ArH), and 7.13 (2H,m,ArH) were also attributed to 3-formyl indole; whilst δ 9.00 (1H,s,NH), 8.57 (1H,d,8Hz,ArH), 8.44 (1H,s,CHO), 8.08 (1H,d,8Hz,ArH), 7.49 (1H,t,8Hz,ArH) and 7.13 (1H,m,ArH) were attributed to the ring-opened compound (*cf.*, gramine; Pouchert, 1993). Aliphatic signals at 2.27 (3H,s,CH_3) and 2.69 (3H,s,CH_3), were compatible with those observed for the amino-side chain of gramine; whilst the δ 4.55 (2H,s) was consistent with the C<u>H</u>$_2$N of an N-oxide (determined by comparison of the shifting affects of bromoacetophenone *cf.* acetophenone; Pouchert, 1993). Similar products have been reported from transition metal oxygenation of gramine and other 3-substituted indoles (Dufour, *et al.*, 1980).

Formation of these molecules can be envisaged as occurring via oxidative attack at the amino-sidechain and either the 2- or 3-aromatic carbons of gramine.

i.e.:



The mass spectral data observed for component 1 corresponds to the m/z 206 intermediate, and the corresponding N-oxide (m/z 222; 223 - FAB conditions). Thus, although definitive confirmation of the postulated structures has not been obtained, strong evidence suggests that such reaction pathways are in operation here.

3.3 CONCLUSION

Identification of the products formed from aphid salivary oxidation of plant allelochemicals is an involved and difficult task. The dilute nature of the salivary enzyme preparations obtained from the insects limits the total amount of product formed from such reactions, hence the use of model systems capable of producing the same compounds but in greater yields is necessary. Even so, the nature of the enzyme-substrate interaction was found to be one that precluded obtaining some products in yields both adequate for analysis and free from gross contamination with unreacted substrate.

The quinone-intermediate trapping studies conducted here gave some evidence of similar reactivities of aphid salivary enzymes and commercially available horseradish peroxidase. Oxidation of diphenolic acids (and their derivatives) by HRP was found to proceed predominantly via o-quinone intermediates (indicated by the trapping of such compounds with sodium benzene sulfinate). The reaction of aphid salivary preparations with these compounds was shown to pass similarly through such intermediates, but not exclusively, since other oxidation product(s) still form in the presence of sodium benzene sulfinate.

Analysis of the mono-phenol, hordenine, oxidised by either HRP or aphid saliva, showed sodium benzene sulfinate (at either 1.8, 3.5 or 10 molar equivalents) did not affect the outcome of the reaction. A hordenine oxidation product was always observed at about 300 nm. This result is not surprising, as mono-phenols require oxidation to di-phenols before quinone-type reactions can take place. If formation of some alternative oxidation product is a faster reaction, then it is unlikely that oxidation to the diphenol could occur to a significant extent.

Thus, it can be expected that, in general, oxidation of diphenolic compounds would proceed via o-quinone intermediates (at least in part), whilst mono-phenolic

compounds may or may not undergo such reactions, depending upon the ease of formation of other oxidation products relative to hydroxylation to the diphenol.

Observation of the hordenine oxidation enabled formulation of a general procedure for analysis of other allelochemicals, although detailed examination of this reaction proved more difficult than first anticipated.

Extractions of aqueous hordenine-HRP reaction mixtures revealed the oxidation products to be essentially neutral (not extractable under acidic or basic conditions). Selective TLC detection tests performed on these reactions to determine if either carboxyl or peroxide moieties were present proved negative. Optimisation of TLC media and solvent systems showed either silica or alumina to be the most suitable chromatographic material, using methanol or methanol-chloroform mixtures for development, hence these were used for the subsequent analysis of other plant chemicals.

Of the various preparative TLC techniques investigated for hordenine, including both silica and alumina plates, hot solvents, continual extraction, and descending chromatography, none was particularly successful in isolating purified oxidation products. The apparent binding of the hordenine products to the chromatographic media was thought to be caused or enhanced by exposure of the developed chromatograms to UV light. By performing the TLC procedures in the absence of light, and using selective UV detection of the chromatograms (see Figure 3.2), quantities of hordenine and oxidation products were obtained. These could not be stored for any length of time, however, (they began to decompose), hence all analyses were performed on freshly isolated material. ^{1}H-NMR spectroscopy of the major oxidation product isolated by this method revealed it to be contaminated by traces of hordenine. The mass spectral analysis of this compound nevertheless gave fragmentations corresponding to a hordenine dimer, which were not found for samples of unreacted hordenine subjected to the same mass spectral conditions.

NMR spectroscopic identification was necessary for definitive confirmation of the proposed dimeric structure, thus other methods of purification were investigated. Column chromatography (using either flash silica or reverse-phase sephadex) of hordenine-HRP oxidation mixtures was not successful. Semi-preparative HPLC, however, proved a suitable alternative.

Initial attempts using the Pic B7 solvent system were unable to yield purified product, as the ion-pair reagent could not subsequently be separated. Chemical synthesis of the hordenine dimer, using either ferric chloride or radical dimerisation, was able to give the desired compound, but never totally free of unreacted starting material or other products (hordenine polymers). Similarly, purification of these reaction mixtures proved difficult, and enzymic oxidation was reconsidered.

The use of long reaction times, and time-consuming preparative HPLC separations (see Experimental section), resulted in quantities of oxidation product suitable for spectral analysis. The ^{1}H-NMR spectrum observed corresponded with a hordenine (2M-2) dimer, as did the mass spectral data (FAB; glycerol matrix).

The difficulties encountered during examination of the hordenine oxidation reaction were a useful guide to the analysis of other compounds. Oxidation of acetaminophen, a mono-phenol structurally similar to hordenine, is well documented in the literature (Potter, *et al.*, 1985). Application to the acetaminophen-HRP oxidation of the preparative TLC and analytical HPLC techniques perfected for the hordenine reaction, gave results identical with those of Potter and co-workers. This indicated that these techniques are probably appropriate for the analysis of allelochemical oxidations generally.

When gramine, an indole alkaloid, was subjected to similar examination, two major and at least two minor products were identified by analytical TLC of the HRP-oxidation mixtures. Although column purification of these solutions

removed unreacted gramine, the many products formed were not, however, separable by this method.

Attempts at preparative TLC of the gramine-HRP oxidation resulted in the isolation of five product components, but unfortunately not in high enough yield for structural identification. Preparative HPLC of these mixtures yielded a major product fraction, that when analysed by ^{1}H-NMR and mass spectroscopic techniques, appeared to be in fact two compounds, an N-oxide ring opened gramine derivative and 3-formyl indole. These compounds have been found previously from the reaction of transition metals with 3-substituted indoles (Dufour, *et al.*, 1980).

From the results presented here some general guidelines for oxidation of aromatic compounds by the enzymes present in aphid saliva can be postulated.

(i) The oxidation of di-phenolic and related compounds occurs via o-quinone intermediates (by hydrolysis to the diphenol compound if necessary, e.g. in the case of ferulic acid).

(ii) Compounds with mono-phenolic sites are likely to form ring adducts such as dimers, trimers, etc., by direct conjugation of the phenol radical with another molecule of starting material. Oxidation of mono-phenols via o-quinone intermediates would occur only when formation of the necessary diphenol is more favoured than the alternative oxidation pathway (e.g. hordenine forms dimers, rather than diphenol).

(iii) Complex ring aromatics, such as gramine, that have no hydroxyl reactive sites present, may undergo ring opening reactions.

3.4 EXPERIMENTAL

SECTION A

Quinone Trapping - General Method

1. Plant Phytochemical with HRP.

Plant compound (0.50 mg/ml, 10% EtOH), hydrogen peroxide (0.03%) and HRP (0.25 mg/ml) were reacted together in a total volume of 1.0 ml. A similar reaction was carried out in the presence of sodium benzene sulfinate (5 molar equivalents), and all solutions were left at room temperature for 2 h. For most compounds, colour changes were observed immediately after enzyme addition. In the presence of sodium benzene sulfinate, however, the colour change was generally less, yielding either a paler or colourless solution. After this time the UV spectra of the diluted solutions (50 µl diluted with 450 µl water) were obtained (after removal of any ppt.), and the subtraction spectra calculated. TLC analysis was performed.

2. Plant Phytochemical Chemical with Aphid Salivary Preparations.

Plant compound (0.05 mg/ml) was reacted with H_2O_2 (0.003%) and aphid saliva in the presence and absence of sodium benzene sulfinate (5 equiv.), in a total volume of 0.5 ml. Control mixtures (using unfed water in place of saliva) were reacted simultaneously. After 2 h at room temperature the UV subtraction spectra were calculated.

3.4a.1 Caffeic Acid with Sodium Benzene Sulfinate

1. Immediately upon enzyme addition to the reaction solutions colour changes were evident. The caffeic acid-HRP solution changed from colourless to yellow/orange, becoming pink (with a precipitate forming) over time, whereas in

the presence of SBS the mixture became only pale yellow. The control solutions without HRP remained colourless throughout. The UV subtraction spectrum for the reaction showed a broad absorption over 260-350 nm, with negative absorption at 360-500 nm. When SBS was included in the mixtures these absorptions were not as prominent.

TLC analysis (ethyl acetate:isopropanol:water, 13:5:2; UV/iodine) of the solutions showed the caffeic acid oxidation to contain a baseline product (precipitate - possibly polyphenolic in nature), with little evidence of starting material. The SBS reaction mixture contained unreacted caffeic acid and SBS, and two components not evident in the corresponding control solution that were presumably SBS-caffeic acid conjugates.

2. The UV subtraction spectra contained peaks at 224 & 266 nm, with negative absorption at 337 nm, indicative of caffeic acid oxidation, both in the presence and absence of SBS. When SBS was present, however, the magnitude of the peaks observed was smaller, suggesting that a portion of the oxidation reaction proceeds via quinone intermediates.

3.4a.2 Chlorogenic Acid with Sodium Benzene Sulfinate

1. Addition of enzyme to the reaction solutions containing chlorogenic acid resulted in a colour change, colourless to bright yellow, with a brown precipitate developing over time. In the presence of sodium benzene sulfinate the addition of enzyme yielded only a pale yellow solution; the control mixtures without enzyme remained colourless. The UV subtraction spectrum contained a broad decrease over 265-370 nm and an increase at 255 nm indicative of chlorogenic acid oxidation. When SBS was included, the spectrum contained a smaller absorption from 270-350 nm, but increases at 257 & 376 nm presumably indicative of conjugates with SBS were observed.

TLC analysis (as for caffeic acid) of the separated solutions (reaction supernatant; reaction precipitate dissolved in EtOH) showed the precipitate to be a baseline product, whilst the SBS reaction contained unreacted chlorogenic acid and SBS, and a component which was not observed in the control solution (presumably the SBS-diphenol conjugate). These results are very similar to the caffeic acid oxidation observations outlined above.

2. The UV subtraction spectrum for oxidation of chlorogenic acid and aphid saliva showed peaks at 287 & 339 nm, indicative of oxidation products. When SBS was included in the mixtures, however, no difference between the reaction or control spectrum was observed. Thus the oxidation of chlorogenic acid by aphid saliva would seem to proceed totally through quinone intermediates.

3.4a.3 Ferulic Acid and Sodium Benzene Sulfinate

1. Immediately after enzyme addition, both of the reaction mixtures underwent a colour change, colourless to pink without SBS; colourless to yellow in the presence of SBS. Both paled in colour with time, whilst the control solutions remained colourless throughout. The UV subtraction spectra obtained for the reaction showed a broad decrease over 300-340 nm, with an increase over 350-600 nm, indicative of ferulic acid oxidation. When SBS was included in the mixture, some absorption over 300-350 nm was apparent, with only a slight increase at 377 nm.

TLC analysis (as for chlorogenic acid; 3.4a.2.1) revealed the pink precipitate and reaction solution to contain a base-line product (possibly polyphenolic), with little evidence of unreacted ferulic acid. In the presence of SBS, the mixtures contained unreacted ferulic acid and SBS, as well as slight evidence of a higher Rf product (0.52) not observed in the corresponding control (presumably the SBS-ferulic acid conjugate).

2. The subtraction spectrum for the reaction with aphid saliva contained a peak at 268 nm, with a negative absorption at 341 nm, indicative of ferulic acid oxidation. When SBS was included in the mixtures these peaks were observed, but were smaller. Thus ferulic acid oxidation in this case proceeds at least partially via quinone-type intermediates, presumably after hydrolysis of the methoxy side chain.

3.4a.4 Hordenine with Sodium Benzene Sulfinate

1. Hordenine with HRP.

(i) Hordenine (1.0 mg/ml; 10% EtOH), H_2O_2 (0.03% in solution), and HRP (0.5 mg/ml) were reacted in the presence of sodium benzene sulfinate (1.4 mg, 1.8 molar equivalents). After 2 h at room temperature the UV subtraction spectrum was calculated, revealing a peak at 300 nm. TLC analysis (MeOH + trace NH_3; UV/iodine) revealed three components: hordenine (Rf=0.30), and two products (Rf=0.09 & base line), indicative of hordenine oxidation.

(ii) The procedure outlined in (i) was repeated, with the one modification - 3.5 equivalents of SBS were used. UV spectral and TLC analyses produced results essentially identical with the previous attempt (i).

(iii) The procedure outlined in (i) was followed, using 10 equivalents of SBS. As before, the benzene sulphinate was not found to affect the hordenine oxidation significantly, confirmed by both UV spectroscopic and TLC analyses.

2. Hordenine with Aphid Salivary Preparations.

Hordenine hemisulphate (1.0 mg/ml; 10% EtOH), H_2O_2 (0.03% in solution), and aphid saliva (400 µl), in a total volume of 500 µl, were reacted in the presence of SBS (1.4 mg, 1.5 molar equivalents). After 2 h at room temperature the UV subtraction spectrum was calculated, revealing two product peaks, one at 242 nm, and a second at 295 nm. TLC analysis (MeOH + trace NH_3; UV/iodine)

revealed three components: hordenine, and two products, consistent with those previously observed for the hordenine-HRP and hordenine-saliva reactions.

SECTION B - REACTION PRODUCTS OF HORDENINE

3.4b.1 Extraction of Product(s)

1. Chloroform Extractions.

Hordenine free base (1.0 mg/ml; 10% EtOH), with H_2O_2 (0.03% in solution), was reacted with HRP (0.5 mg/ml), in a total volume of 1 ml. After 2 h at room temperature the reaction and control mixtures were diluted with dd water to 2.5 ml, and extracted with chloroform (2.5 ml). TLC analysis of the organic and aqueous phases was performed (MeOH + trace NH3; UV/iodine). The aqueous phase was then acidified (pH 1, 0.1 M HCl), re-extracted with chloroform (2.5 ml), and the two phases analysed by TLC (as before). Lastly, the aqueous phase was treated with base (pH 14, 1.0 M NaOH), extracted, and examined by TLC. The majority of the unreacted starting material & products were removed from the aqueous phase under neutral conditions. Acidic and basic extractions of the aqueous reaction mixture were unsuccessful.

2. Ether Extractions.

The procedure outlined in (1) above was repeated, using ether as the extracting organic solvent. Hordenine and some products were extracted, however, the majority of the oxidation products remained in the aqueous phase, regardless of the solution pH.

3.4b.2 Thin Layer Chromatographic (TLC) Analysis

1. Visualisation.

Several visualisation techniques (see below) were tested on the TLC analysis (1-butanol:EtOH:Acetic acid:H_2O 8:2:1:3) of hordenine hemisulphate solutions (0.5, 1.0 & 2.0 mg/ml).

	Technique	Observ^n of Cmpd
1	UV light exposure	+
2	Silver nitrate reagent*	+
3	Ammonium molybdate/sulphuric acid*	+
4	Phosphomolybdic acid solution*	-
5	Iodine vapour (exposure)	+

Only technique 4 was not successful in detecting hordenine.

2. Choice of Solvent System/TLC Media.

A typical enzymic oxidation of hordenine hemisulphate (1.0 mg/ml) and HRP (0.5 mg/ml) in the presence of 0.03% hydrogen peroxide, was analysed using several types of chromatographic media and a variety of solvent systems, as indicated below. All visualisation of starting material & product(s) was performed by exposure of the plates to iodine vapour. The separation values (Rf's) obtained are as indicated.

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* Stahl, (1965).

MEDIUM	SOLVENT SYSTEM	Rf	
		HORDENINE	PRODUCTS
Silica (F_{254})	As for 3.4b.2.1 (BEAW)	0.45	0.08, baseline
	Acetone:MeOH (2:1)	0.11	baseline
	1-Propanol:EtOH (1:10)	0.09	baseline
	H_2O:acetone (1:2)	0.09	base line
	H_2O:EtOH (2:5)	0.60	0.13, baseline
	MeOH + trace NH_3	0.31	0.13, baseline
Kieselguhr (F_{254})	H_2O:EtOH (50:1)	0.16	base line
	H_2O:EtOH:CH_3CO_2H (4:1:tr)	0.60	0.19, baseline
Cellulose	Ethyl acetate:MeOH (1:4)	0.30	streaked
Paper (#1)	MeOH (100%)	No movement	No movement

3. Freebase vs Hemisulphate TLC Analysis:

TLC analysis (kieselguhr F_{254}; MeOH + trace NH_3; UV/iodine) of hordenine hemi sulphate solutions showed two component spots for hordenine. Similar analysis of the freebase in solution was consistent with only one component.

3.4b.3 Selective Detection of Functional Groups

A hordenine-HRP reaction (standard conditions) was used for TLC examinations. Analyses were performed on silica plates (MeOH + trace NH_3),

visualisation was achieved by UV irradiation, then selective detection tests were carried out (as indicated below).

TEST	FUNCTION GROUP DETECTED	RESULT
2,4-DNPH*	Free aldehydes/ketones	-
PI/starch*	Peroxides	-

3.4b.4 Preparative TLC

1. Silica F_{254} Plates.

Hordenine (1.0 mg/ml) - HRP (0.5 mg/ml) reaction mixtures (10 x 1 ml) were made up, and reacted at room temperature for 2 h. After this time the individual reaction mixtures were combined, and extracted with $CHCl_3$-MeOH (4:1, 4 x 5 ml). The combined organic extracts were concentrated (N_2 gas) and subjected to preparative TLC (silica; MeOH + trace NH_3). After development the plate was air-dried and visualised under UV light. The crudely separated products were scraped from the plate, and attempts made to remove them from the silica using $CHCl_3$-MeOH (4:1). TLC analysis of the isolated components revealed the presence of small amounts of hordenine in the fraction from the appropriate position on the plate. The two reaction products were not observed, however, suggesting their affinity for silica.

2. Alumina Plates:

Experiments similar to that outlined in (1) above were performed using alumina TLC plates.

(i) Initial attempts to remove the separated components from the TLC plates, using CH_2Cl_2-MeOH (4:1), CH_2Cl_2 (100%), and MeOH-H_2O (1:1), isolated

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* Stahl, (1965).

only the starting material. Reaction products were still unobtainable from the alumina surface.

(ii) The extractions were then performed using hot solvents; CH_2Cl_2 (100%), CH_2Cl_2-MeOH (3:1), and MeOH (100%), 500 µl portions of each. Similarly, only hordenine was obtained from the alumina.

(iii) Extractions were carried out by refluxing in CH_2Cl_2-MeOH (3:1; 4 ml), i.e., continual extraction, for a period of 30 min. This method yielded no improvement on the previous attempts (i & ii).

3. Continual Extraction of Silica Plates:

The continual extraction outlined in 2(iii) above was performed on silica plate separations of the hordenine-HRP oxidation. Again, adequate quantities of the desired compounds were not obtained.

4. Peaked TLC Plate Extractions:

An alumina TLC plate, with "peaked" end shape (see Figure 3.1, page 93) was run in a descending manner. This involved loading extracted hordenine and reaction products from an HRP reaction mixture to the top of the plate, and allowing the solvent system ($CHCl_3$-MeOH 3:1, gravity fed) to develop downwards. Fractions (*ca.* 100 µl) were collected from the tip of the plate, and these were subsequently analysed for content by ascending TLC.

After 1.5 h of elution and collection, the analysed fractions were found to contain large quantities of hordenine. Development (UV/iodine vapour) of the peaked TLC plate revealed the base line peak unremoved from the starting point. The major product component had shifted down the plate, and seemed to be positioned to elute eventually. This method was not favoured, however, due to the extremely long run time involved.

5. Darkroom Separations:

Hordenine (1.0 mg/ml) - HRP (0.5 mg/ml) reaction mixtures (10 x 1 ml) were made up, and reacted at room temperature for 2 h, in total darkness. After this time the individual reaction mixtures were combined, and extracted with $CHCl_3$-MeOH (4:1, 4 x 5 ml). All subsequent work was performed under a nitrogen atmosphere, in complete darkness (whenever possible). The combined organic extracts were concentrated (N_2 gas), then loaded onto a semi-preparative alumina plate and developed ($CHCl_3$-MeOH 3:1, trace NH_3).

After drying (N_2 gas) the positions of the separated components were determined using selective UV exposure (see Figure 3.2, page 94). The three bands observed were excised from the plate, and the compounds extracted with CH_2Cl_2-MeOH (1:3, 1 ml for each), still in the absence of white light (it was necessary to use photographic yellow light for some procedures). The UV spectrum of each organic extract was obtained, confirming component 1 to be hordenine. Component 2 was the major product, with UV absorption at 318 nm, whilst component 3 (base line product) was a minor peak at 277 nm. These results were consistent with the TLC analysis of the components (silica; MeOH + trace NH_3). The compounds were stored under nitrogen at -4° overnight, but UV and TLC analysis the following morning revealed all to be further oxidised/decomposing.

This method was repeated (as outlined above), and the samples obtained were analysed by ^{1}H-NMR and mass spectral techniques. All attempts were unsuccessful in isolating the major product completely free of hordenine, however, and the contamination of peaks in the ^{1}H-NMR spectrum prevented definitive structure determination.

Examination of the components by mass spectroscopy proved more rewarding.

Component 1: m/z 165 (M^+), 121, 107, 91, 77, 58 (literature hordenine values, Meyer & Barz, 1978).

Component 2: m/z 328, 270, 152, 105, 58 (dimeric 2"M^+"-2 structure).

3.4b.5 Column Chromatography

1. Flash Silica:

A flash silica column (Still, *et al.*, 1978) was attempted. The solvent used (MeOH + 0.5% NH_3) was successful in eluting hordenine freebase from the column. When the crude products from enzymic oxidations were run, however, they could not be eluted.

2. Sephadex Chromatography:

(i) 6 cm Column.

A small reverse phase sephadex column (LH 20; 6 cm) was set up, and a hordenine-HRP oxidation mixture was passed through (MeOH + 1% acetic acid). Fractions (20 x 1 ml) were collected, and examined for content by TLC (MeOH + trace NH_3; UV). It was found that all the hordenine and products had eluted in the initial column volume (3 ml), collected prior to the 1 ml fractions.

(ii) 12 cm Column.

A 12 cm LH 20 column, similar to (i) above, was attempted. Fractionation of the crude reaction mixture revealed some separation of hordenine and products, but mixed fractions were still obtained.

(iii) 30 cm Column.

A final attempt with LH 20, using a 30 cm column, was made. Hordenine hemisulphate (24 mg), HRP (8 mg) and hydrogen peroxide (3% solution; 25 µl) were reacted in a total volume of 2.5 ml, at room temperature overnight. The reaction was then extracted with chloroform (3 x 1 ml), and the combined organic extracts concentrated and checked by TLC (MeOH + trace NH_3; iodine/UV). The remaining solids were dissolved in the running solvent (MeOH + 1% acetic acid; 2 ml), and this sample was used on the column.

An LH 20 column (15 g LH 20 swollen in MeOH (75 ml) at room temperature overnight) of 30 cm length (burette) was used. The sample (see above) was loaded onto the column, followed by a 1 ml wash. Two initial fractions (zero fractions: 0=0-15 min, 0'=15-20 min) were collected, then an automatic fraction collector (equipped with test-tubes containing 1.5 ml eppendorfs) was used. Fractions were collected (50 s intervals; 50 fractions), and these were then analysed for content by TLC. This revealed that some separation of hordenine and the oxidation products was obtained, although the majority of fractions were of mixed content. The quantities of pure sample resulting from this experiment were not sufficient to enable product identification.

3.4b.6 High Performance Liquid Chromatography

1. Pic B7 Ion Pairing System:

(i) Hordenine (1.0 mg/ml) - HRP (0.5 mg/ml) reaction mixtures (5 x 1 ml) were made up, and reacted at room temperature for 2 h. After this time, the individual reactions were combined and extracted with $CHCl_3$-MeOH (4:1, 4 x 2.5 ml). The organic extracts were concentrated (N_2 gas), and dissolved in MeOH (5 ml). This mixture was then run on the HPLC column in small aliquots (1 ml), and fractions collected (approx. 500 µl). The solvent was evaporated under vacuum

(Speedy Vac), and like fractions (labelled at the time of collection) were combined using dichloromethane. The solvent was removed (N_2 gas), and a white precipitate remained (presumably Pic B7). The samples were subjected to mass spectral analysis, but only fragmentations involving the Pic B7 ion-pairing reagent were distinguishable.

(ii) The reaction method of (i) immediately above was repeated, and the resultant crude reaction purified by preparative HPLC. After collection of the product fractions they were re-submitted to preparative HPLC, without Pic B7 ion-pair reagent in the solvent system. The peaks obtained from these runs were of poor shape, and difficult to collect quantitatively, thus the amount of resultant pure samples obtained was not sufficient for structural identification.

(iii) The method of (i) was followed precisely. The HPLC product fractions obtained were passed through an alumina squat column (1 x 1 x 2 cm), and washed with a series of solvents (1 ml each of $CHCl_3$-MeOH 4:1; MeOH; MeOH-H_2O; H_2O). Pic B7 was not removed from the desired products by this method.

(iv) The method of (i) was repeated, the semi-purified product fractions were dissolved in $CHCl_3$-MeOH (4:1, 1 ml) and washed with sodium bicarbonate solution (10%, 2 x 0.4 ml). This was not successful in removing the acidic Pic B7 pair-reagent from the fractions.

(v) The method of (i) was repeated, and the resultant crude product mixture was loaded onto a silica squat column (1 x 1 x 2 cm; $CHCl_3$-MeOH 1:1). The column was developed with a series of solvents (2 ml each of $CHCl_3$-MeOH 1:1; $CHCl_3$-MeOH 1:4; MeOH-NH_3 50:1; Methanol-H_2O 1:1). All fractions obtained were contaminated with differing amounts of Pic B7, and hence structural determination was not possible.

2. Alternative HPLC Systems:

(i) Trifluoroacetic acid.

Samples of crude hordenine-HRP reactions were examined by HPLC, using water-methanol (A; 90:10), and acetonitrile-methanol (B; 90:10), with 1% trifluoroacetic acid as the ion-pair reagent. Good separations of the hordenine and reaction products were obtained.

(ii) Heptafluorobutyric acid.

Heptafluorobutyric acid (HFBA; 0.05%) was used in place of TFA in the solvent system outlined in (i) above. Excellent separations of hordenine and the oxidation products were obtained.

3.4b.7 Hordenine Dimerisation

1. Chemical Dimerisation:

(i) Solutions of hordenine freebase (0.025 M, 10 ml), sodium persulphate (0.050 M, 5 ml), and silver nitrate (0.005 M, 5 ml) were equilibrated to 40°C. The silver nitrate was then combined with the sodium persulphate solution, and the mixture stirred for 5 min at 40°C. Hordenine solution was added to the stirring reagent, and the reaction maintained at 40°C overnight. A colour change (colourless to yellow-brown) was observed after approximately 20 min and persisted throughout the reaction thereafter.

The aqueous reaction mixture was slowly cooled to room temperature, adjusted to pH 14 (1.0 M NaOH) and the precipitated silver removed. The filtrate was neutralised (pH 7, 1 M HCl) and extracted with $CHCl_3$ (10 x 10 ml). The combined organic extracts were concentrated (rotary evaporator) and analysed by HPLC (see 2, below).

(ii) With the previous attempt ((i) above) some of the reaction product was retained in the aqueous phase after chloroform extraction. Hence the method was repeated. After removal of the silver precipitate the aqueous reaction mixture was portioned (58 x 1 ml aliquots), and all evaporated to dryness under vacuum (Speedy Vac). The samples were combined ($CHCl_3$-MeOH 7:3), and solvent removed (N_2 gas). The crude reaction products were then ready for separation, (see section 3, below).

(iii) Ferric chloride was also used to produce the hordenine dimer. Hordenine freebase (25 mg), in EtOH (100%, 1 ml), and ferric chloride hexahydrate (39 mg) were stirred together in dd water (14 ml) at 40-45°C for 17 h. After this time the aqueous reaction was cooled to room temperature and extracted with chloroform (5 x 5 ml). The combined organic extracts were evaporated to dryness (rotary evaporator) and the crude product analysed by TLC (MeOH + trace NH_3; UV/iodine). This revealed quantities of unreacted hordenine (Rf=0.28), the suspected dimer (Rf=0.06), as well as base line products.

2. HPLC Analysis:

Reaction samples (from 1(ii) and (iii), above) were run on a RP-C18 column, gradient and solvent system identical to that used for analysis of hordenine enzymic oxidations. Comparison of the chemical reaction with HRP-oxidised hordenine revealed the products to be identical. Strong evidence was obtained by the analysis of a 50:50 mixture of chemical and enzymic oxidations, revealing a single peak.

When the compounds were stored in the freezer overnight (or for longer periods), HPLC analysis revealed a change in composition. It is thus not possible to retain these compounds for any length of time.

3. Preparative Isolation of Hordenine Dimer:

The crude products were produced and obtained as described in 1(ii), above. Several methods of product purification were then attempted.

(i) Preparative TLC was performed on silica plates (MeOH + trace NH_3). The separated components were extracted from the plate ($CHCl_3$-MeOH 1:1), and analysed by UV spectroscopy. This revealed recovered hordenine (major component), as well as small quantities of the desired product (UV 317 nm). This method proved very inefficient, however, and alternative separation methods were investigated.

(ii) Preparative HPLC - Trifluoroacetic acid solvent system.

Attempts made using trifluoroacetic acid as the ion-pairing reagent were successful in obtaining small quantities of isolated oxidation product. Contamination of the product with hordenine was observed, however, as a result of the high concentrations used (poor peak separation).

(iii) Preparative HPLC - Heptafluorobutyric acid solvent system.

Crude products from both 1 (ii) and 1 (iii), above, were produced and subjected to HPLC analysis using heptafluorobutyric acid as the ion-pair reagent. Solvent A: Water-MeOH 9:1; solvent B: Acetonitrile-MeOH 9:1; both containing 0.1% heptafluorobutyric acid. Good separations of hordenine and product peaks were observed, and this solvent system was used for semi-preparative HPLC, using the following gradient: 40% B for 5 min; 40-75% B over 8 min; 75-80% B over 8 min; 80-100% B over 2 min; return to 40% B; flow rate 0.8 ml/min. Fractions obtained proved to be in low concentration, hence structural analyses were not performed.

3.4b.8 Oxidation Product Isolation and Identification

Hordenine freebase (20 mg) was reacted with HRP (5 mg) and H_2O_2 (30% solution; 20 µl), in a total volume of 20 ml. The reaction was stirred at room temperature overnight (23 h). After this time the reaction was checked by TLC (silica; MeOH + trace NH_3), the solution frozen (-4°C; 3 h), and evaporated to dryness (freeze drier/oil pump; overnight). The sample was then defrosted & extracted with MeOH (2 x 5 ml), the combined organic extracts were centrifuged, and the supernatant evaporated to dryness (N_2 gas).

HPLC separation of the crude material was performed. The sample was separated into two (approximately 10 mg halves), each portion diluted with water/acetonitrile (1:1; 1 mg/ml concentration), and run over two consecutive days. The solvent system and gradient used was as described above for 3(iii), injection volume was 1 ml.

Collected 'like' fractions (test-tubes) were combined, and evaporated to dryness (Speedy Vac, oil pump). TLC analysis revealed that fractions 1 & 2 were the hordenine oxidation products (HPLC peaks 8.0 & 9.5 min respectively). The fractions were stored at -4°C (N_2 atmosphere) during overnight periods. Fraction 1 (presumably dimer) showed contamination by component 2, and was resubmitted to preparative HPLC on day 3. This purified sample, and component 2, were subjected to NMR & mass spectral analyses.

NMR analysis of component 2 revealed not enough sample was present for structural identification. As this was the minor oxidation product, further attempts concentrated on the determination of the structure of component 1. High field ^{1}H-NMR was performed in several solvent systems, in order to obtain the best results (MeOD, $CDCl_3$, D_2O, and D_5-Pyridine). The different structural features of this compound were identified using a combination of spectra: aromatics from the D_2O spectrum, and aliphatics from the D_5-pyridine spectrum; ^{1}H-NMR

δ 7.30 (1H,m,H3), 7.16 (1H,m,H5), 6.82 (1H,d,7Hz,H6), 2.82 (2H,m,CH_2), 2.56 (2H,m,CH_2), 2.21 (6H,s,$N(CH_3)_2$). Mass spectra analysis of the sample (FAB; glycerol matrix) revealed ion 329 (M+H), consistent with any "dimer" of hordenine.

SECTION C - OTHER ALKALOIDS

3.4c.1.1 Acetaminophen With HRP - TLC Analyses

1. Chloroform - Methanol:

An acetaminophen (1.0 mg/ml) - HRP (0.5 mg/ml) reaction mixture was analysed by TLC ($CHCl_3$:MeOH, 9:1 + 2% acetic acid; UV/iodine vapour). This revealed acetaminophen (Rf=0.25), as well as three reaction products, Rfs=0.14, 0.07, & 0.02.

2. Ethyl acetate - Acetic acid:

An acetaminophen (1.0 mg/ml) - HRP (0.5 mg/ml) reaction mixture was subjected to TLC on silica plates (ethyl acetate-acetic acid; 85:15; UV/iodine vapour). Acetaminophen (Rf=0.57) and the three reaction products (Rfs=0.38, 0.23, & 0.10) were detected, slightly better separated than for (1) above.

3.4c.1.2 Acetaminophen - Preparative TLC Analysis

Acetaminophen (1.0 mg/ml) - HRP (0.5 mg/ml) reaction mixtures (10 x 1 ml) were made up, and reacted at room temperature for 2 h. After this time the individual reaction mixtures were combined, and extracted with ethyl acetate (4 x 5 ml). The organic extracts were concentrated (N_2 gas) and subjected to preparative TLC (ethyl acetate-acetic acid, 85:15; UV). The separated compounds (six components) were scraped from the plate, and removed from the silica using ethyl acetate extraction. TLC analysis of the isolated components revealed them to

be unreacted acetaminophen (fraction 1), three major products (fractions 2,3, & 5), and two minor products (fractions 4 & 6). These were stored under nitrogen at -4°C overnight, and appeared unchanged the following day (TLC analysis). UV spectral analysis of each component revealed the following:

Fraction	UV absorption	Possible structure*
1	280 nm (broad)	Acetaminophen
2	257, 288 nm	Dimer
3	254, 300 nm	Dimer
4	NOT ENOUGH SAMPLE RECOVERED	
5	256, 291 nm	
6	256, 289 nm	

3.4c.1.3 Acetaminophen - HPLC Analysis

1. Analytical Analysis of the Acetaminophen-HRP Oxidation:

Acetaminophen (0.4 mg/ml) was reacted with HRP (0.2 mg/ml) in the presence of H_2O_2 (0.03%), at room temperature for 1 h. This sample was then used in HPLC analyses; solvent A: water-MeOH-acetic acid (88:10:0.2), solvent B: MeOH (100%), with UV detection performed at 254 nm. Excellent separation of all components was obtained, gradient as follows: 10% B for 5 min; 10-70% B over 42 min; 70-100% B over 3 min; return to 10% B. The results observed confirm those found for TLC analysis, quantities of acetaminophen were recovered together with several major components, and two minor components.

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* Structures postulated from the UV data observed, & from the products identified by Potter & coworkers (1985).

2. Analysis of Separated Reaction Components:

The procedure used for the preparative TLC separation of acetaminophen and its HRP oxidation products, outlined previously (see Preparative TLC), was repeated. The separated components were then individually analysed by HPLC to confirm their structural identities (notation as for Potter, *et al.*, 1985).

TLC Fraction	Possible Identity (HPLC Component)
1	Acetaminophen
2	B (major product)
3	A (major product)
5	traces of E & F
6	NOT ENOUGH SAMPLE

Mass spectral analysis of component 1 confirmed it to be acetaminophen, m/z 151 (M^+), 109, 80, 71, 42.

3.4c.2.1 Gramine With HRP - Extraction of the Reaction Mixture

Gramine-HRP reaction mixtures were made up (see 1.4a.12.1), and left for 2 h at room temperature. After this time extractions of the aqueous phase were performed using chloroform (4 x 250 µl). The combined organic extracts were concentrated (N_2 gas), and examined for content by TLC, confirming the presence of gramine and at least two products.

3.4c.2.2 Gramine Oxidation - Preparative TLC

Gramine (1.0 mg/ml) - HRP (0.5 mg/ml) reaction mixtures (5 x 1 ml) were made up, and reacted at room temperature for 2 h. After this time the individual reaction mixtures were combined, and extracted with $CHCl_3$ (5 x 2 ml). The organic extracts were concentrated (N_2 gas) and subjected to preparative TLC (MeOH + trace NH_3). After development, the plate was air-dried and visualised under UV light. The crudely separated products were scraped from the plate, and

extracted from the silica using MeOH (1 ml). TLC and UV spectral analysis of the isolated components revealed the presence of gramine in the expected fraction (#4), whilst the other components contained reaction products.

i.e. Component	λ_{max} (nm)	Possible content
1	200-300 br	Negligible (Rf=0.80)
2	238; 291	Product A (Rf=0.76)
3	242; 294	"
4	200-300 br (lge)	Gramine (Rf=0.08)
5	277	Product B (Rf=0.02)

Component 4 (gramine) was isolated in high enough yield for mass spectral identification, (m/z: 174 (M^+), 130, 103, 77, 44), confirming it to be recovered starting material. TLC analysis of these components after storage at -4°C for 48 h revealed some decomposition, hence reaction mixtures/products cannot be stored for any length of time.

3.4c.2.3 Gramine and Horseradish Peroxidase - Isolation of Oxidation Products

Gramine (22.5 mg) was dissolved in EtOH (100%; 4 ml), and the solution made up to 40 ml with nanopure water. To this was added HRP (8 mg) and H_2O_2 (0.003%), and the reaction was stirred at room temperature. After 24 h the reaction mixture was frozen, then evaporated to dryness (freeze drier; O/N). The resultant crude product was extracted with MeOH (2 x 2 ml), centrifuged, and the supernatant evaporated to dryness. This material was examined by TLC (MeOH + trace NH_3), revealing unreacted gramine and products.

The sample was dissolved in MeOH (2 ml) and subjected to column chromatography (LH20 column; 20 cm in length; MeOH + 1% acetic acid). The

sample was loaded, and a zero fraction (10 ml) collected. A fraction collector (10 s per fraction) was used to collect the remaining fractions (1-69), and a further 10 ml of solvent was passed through the column. TLC analysis of the fractions revealed gramine to elute quickly (fractions 4-42), with the major products located in fractions 49-70. Like fractions were combined, the solvent removed (N_2), and these were stored at -4°C awaiting further analysis.

The product fraction was dissolved in MeOH (200 µl) and subjected to semi-preparative TLC (silica; $CHCl_3$-MeOH, 3:1 + trace NH_3). From this five compounds were extracted (MeOH, 1 ml), with Rf values: 1=0.95, 2=0.84, 3=0.69, 4=0.58, 5=0.24. None of these was obtained in sufficient quantity for structural identification.

3.4c.2.4 Preparative HPLC of Gramine Oxidations

The gramine oxidation method outlined for product isolation (above) was repeated. After overnight reaction and removal of the water, the resultant crude reaction products (and unreacted gramine) were dissolved in MeOH:H_2O (1:9) and separated by semi-preparative HPLC (sample volume = 100 µl; column, solvents, gradient and UV detection as for analytical examination, see 1.4a.12.5.2).

The two major product peaks (9 & 11 min) were collected, and the solvent removed under vacuum (Speedy Vac). TLC analysis revealed product peak 2 to be a mixture, hence this was not analysed further. Product peak 1 was subjected to ^{1}H-NMR and mass spectroscopic analyses, indicating the presence of two likely components. Total data observed:^{1}H-NMR (MeOD) δ 12.20 (1H,s), 9.85 (1H,s), 9.00 (1H,s), 8.57 (2H,d,8Hz), 8.44 (1H,s), 8.08 (2H,d,8Hz), 7.49 (2H,t,8Hz), 7.13 (3H,m),4.55 (2H,s), 2.69 (3H,s), 2.27 (3H,s); m/z (glycerol matrix) 223 (base peak), 207, 131, 115, 75, 61, 57, 45, 31. This data is consistent with both indole-3-carboxaldehyde and a ring-opened gramine N-oxide derivative (see structure postulations, 3.2c.4).

CHAPTER 4

BIOLOGICAL SIGNIFICANCE OF OXIDATION OF PHYTOCHEMICALS BY APHID SALIVA

4.1 INTRODUCTION

As discussed in the introductory chapter of this work, plant allelochemicals including alkaloids, phenolics and related compounds, have been thought to play a major rôle in the defence against insect attack, particularly sucking insects. A large range of plant compounds has been tested on aphids to determine their effect upon insect feeding (Todd, *et al.*, 1971; Schoonhoven & Derksen-Koppers, 1976; Jones & Klocke, 1987; Niemeyer, *et al.*, 1989; Kawada & Lohar, 1989; Givovich, *et al.*, 1992; Westcott, *et al.*, 1992). Some reports have suggested that deterrency by such compounds is a result of their high degree of unsaturation, with detoxification reactions involving hydroxylations (Haug & Hoffman, 1986). Yet other evidence points to different degrees of deterrency/acceptability in successive stages of oxidation (Peng & Miles, 1988a).

Catechin was shown by Peng & Miles (1988a) to be deterrent to the feeding of the rose aphid, *M. rosae*. After exposing insects to various diets, oxidised polymers of catechin were observed in the test solutions. Further, when offered a choice between a diet of sucrose alone and one containing autoxidised catechin, the insects showed preference for the autoxidised phenol. These results led to the suggestion that the first formed polymeric products were less deterrent, while the larger polymers were either non toxic or, if soluble or bound to solids, were no longer ingested by the insect.

The phenolic content of a plant is known to have an effect upon the type and number of insects that can infest it, and at what growth stage such infestations can

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The phenolic content of a plant is known to have an effect upon the type and number of insects that can infest it, and at what growth stage such infestations can

occur. Many examples of insect-deterrent and/or toxic compounds have been reported (above), and the phenolic profiles of a number of plant species have been determined (Oleszek, *et al.*, 1990; Pérez-Ilzarbe, *et al.*, 1991; P.W.Miles, pers. comm.). Some workers have also suggested (Miles & Oertli, 1993; Jiang & Miles, 1993b) that the availability of reductants in plants may play a critical rôle in the defence of plants against insect attackers (also see Chapter 2).

Miles & Oertli (1993) postulated that plants with greater amounts of reductants may be better able to combat the oxidative attack of sucking insects. Miles (pers. comm.) has suggested that the incorporation of additional genes coding for enhanced reductive systems into a plant's genome might therefore lead to cultivars with greater resistance or tolerance to aphid infestation. The difficult process of inserting genes for increased synthesis of reductant enzymes into potato and lucerne genomes is currently being undertaken by Miles & co-workers (pers. comm.). It will be some years, however, before comprehensive testing of such transformed plants against insect attack is possible.

In Section A of this chapter, a number of plant allelochemicals, with various structural features, were investigated for their possible feeding deterrency. In some cases both unoxidised and oxidised compounds were studied. Section B describes investigations into the phenolic content of plants (with specific reference to potato plants), and the direct effects caused by reaction of these phenolics with oxidative enzymes. A number of potato lines were also examined to determine what effect (if any) transgenic inclusion of elevated levels of an enzyme involved in the recycling of reductants had on their phenolic profiles.

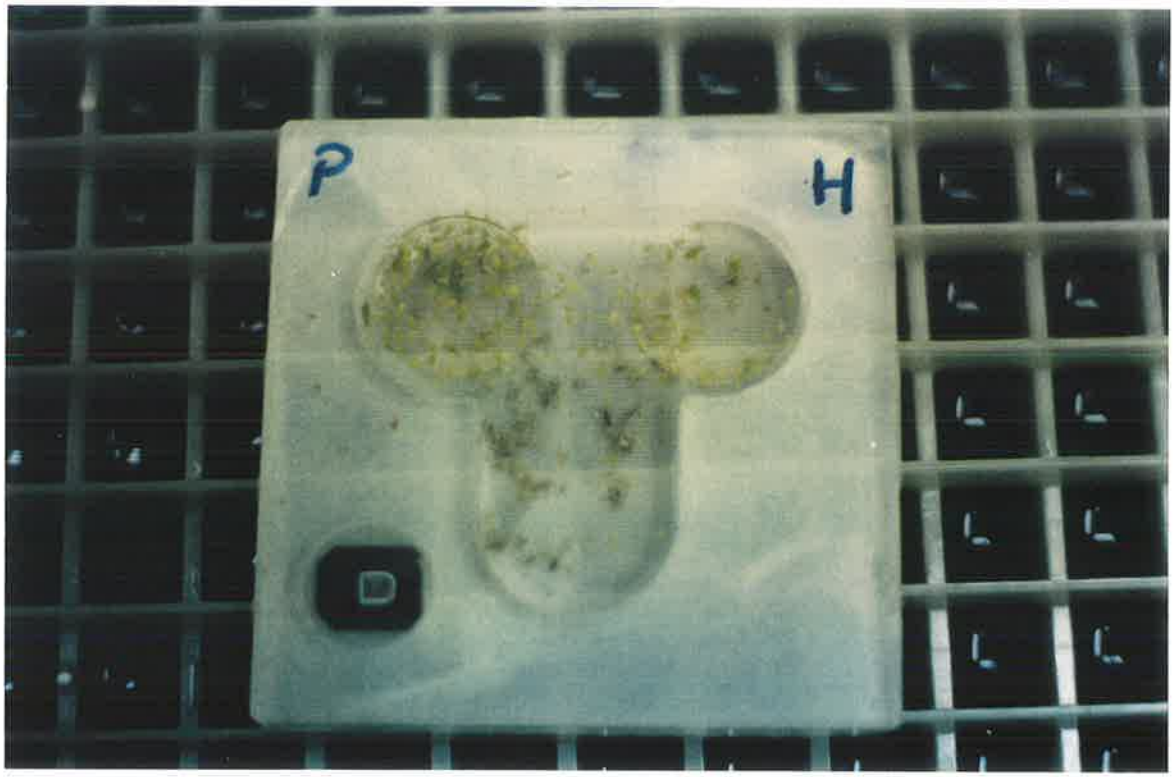
4.2 RESULTS AND DISCUSSION

SECTION A

4.2a.1 Plant Chemicals - Deterrency to Aphid Feeding

A selection of the compounds investigated in the present work were tested for their affects on the feeding of aphids on artificial diet. In general, the compound was included in a sucrose diet, at a variety of concentrations, and offered to insects enclosed in a T-chamber (Figure 4.1). Sucrose solution without the plant chemical (control solution) was used as the alternative "choice" diet, and the number of aphids feeding on the two diets was recorded at specific time intervals. During periods of low potato aphid availability, experiments were conducted using the rose aphid, found in abundance on roses at the Waite Campus (The University of Adelaide). When adequate numbers of the potato aphid had accumulated these tests were repeated to check whether both species behave in a similar way.

Figure 4.1: Choice test T-chamber containing aphids. Aphids are dispensed into the base of the chamber, diets positioned at the top left and right-hand sides.



All compounds tested showed some deterrent effect upon the aphids, in that preference was shown for feeding on sucrose control solutions rather than diet containing the plant chemical (see Table 4.1). Hordenine, unlike caffeic acid, gramine and DIMBOA, showed increased deterrency with increased concentration.

Table 4.1: Apparent deterrency of various amounts of plant chemical (number of aphids feeding on test diet as a % of total feeding insects in the control & test diets). All data shown corresponds to 8 h observation time (see data in Appendix 3); overall differences significant to the 2% level (χ^2 heterogeneity; n=1). Concentrations chosen based upon earlier investigations by Peng & Miles (1988a).
NB: (-) = not tested.

COMPOUND	1 mg/ml	2 mg/ml	5 mg/ml	10 mg/ml
Caffeic Acid	27%	27%	38%	(-)
DIMBOA	35%	37%	32%	(-)
Gramine	26%	40%	34%	(-)
Hordenine	26%	21%	16%	14%

The identification of phytochemicals capable of acting as feeding deterrents to aphids is of continuing interest to crop protectionists and many examples of such chemicals have been reported in the literature (Leszczynski, *et al.*, 1985; Corcuera, 1984; Zuñíga, *et al.*, 1985, 1988; Zuñíga & Corcuera, 1986; Niemeyer, *et al.*, 1986; Niemeyer, 1988; Lohar, 1989; Niemeyer, *et al.*, 1989; Kanehisa, *et al.*, 1990; Thackray, *et al.*, 1990; Barria, *et al.*, 1992). A major goal of the current work was to

determine if the oxidation products of such compounds have a lesser effect upon the aphids' feeding.

4.2a.2 Hordenine as a Feeding Deterrent

Hordenine was again investigated in some detail, as the products of aphid salivary oxidation of this compound have already been identified (see 3.2b.7), and can easily be produced by enzymic reaction (HRP/H_2O_2). In choice tests with hordenine, observed deterrency increased with increasing concentration and these results therefore appeared subject to straight forward interpretation. Further tests were then conducted using hordenine and reductants in various combinations. If reductants enhance the deterrency of oxidisable compounds to aphids the inclusion of reductant in diets presented to aphids should maintain the chemical under investigation in an unoxidised state, i.e., theoretically - deterrent to sucking insects.

Glutathione and ascorbate are known plant reductants, and both were shown to be non-deterrent to aphids in choice experiments at concentrations of 2 mg/ml (see Table 4.2). When either of these reductants was incorporated with hordenine (5 mg/ml) in solution, and the mixture was tested against hordenine alone in an aphid choice experiment, no difference between the two test diets could be found. Nevertheless, examination of the diets by UV spectroscopy at the conclusion of the experiment showed hordenine was oxidised by the feeding aphids, even in the presence of reductant. Thus, glutathione and ascorbate at the concentrations tested were unable to prevent the oxidation of hordenine solutions under the test conditions, and this experiment did not provide direct confirmation of an interaction of oxidising and reducing systems as determining factors in allelochemical deterrency to aphids.

Table 4.2: Choice experiments conducted with *M. rosae* and *M. euphorbiae*, using sucrose control solution, and either glutathione or ascorbic acid at various concentrations.
ND = not deterrent to the aphids,
D = deterrent to the aphids,
A = somewhat attractive or palatable to aphids.

APHIDS	GLUTATHIONE			ASCORBIC ACID		
	2 mg/ml	5 mg/ml	10 mg/ml	2 mg/ml	5 mg/ml	10 mg/ml
Rose	ND	A	ND	ND	ND	D
Potato	ND	A	ND	ND	D	D

Previous work (3.2b.7) has shown that the hordenine oxidation products could be produced in almost quantitative yield from hordenine by reaction with horseradish peroxidase. Quantities of these products were obtained and tested in a choice experiment against sucrose control, and also tested directly against hordenine itself. When offered sucrose as an alternative to the oxidation products (5 mg/ml) the insects showed no significant preference for either solution. The oxidation products were also not as deterrent to insect feeding as unoxidised hordenine, and direct comparison of the products with unreacted starting material showed the insects greatly favoured the product diet (70% of aphids feeding on this diet, $P<0.001$, see Appendix 3). The UV spectral analyses of the fed test-solutions revealed that at both concentrations, the hordenine exposed to the aphids had been oxidised somewhat, compared with unfed controls, indicating that they were indeed feeding on and causing the oxidation of the solutions. These observations are similar to those found by Peng & Miles (1988a) for catechin and the feeding of rose aphids, and appear to add support to the notion that there are degrees of deterrency in plant defences.

4.2a.3 The Investigation of Other Plant Chemicals

The investigation of the feeding deterrency of gramine, caffeic acid and DIMBOA, however, did not prove to be straightforward. These compounds are of low solubility in water but can be dissolved or dispersed in an aqueous diet by inclusion of ethanol. It follows that all diets presented in a choice experiment must then include the same quantities of ethanol, including the sucrose control diet.

Choice experiments conducted for gramine and caffeic acid indicated the compounds were deterrent to the aphids at concentrations of 1, 2, & 5 mg/ml (Table 4.1), but with the lower concentrations generally having the greater deterrency. Higher concentrations that seem to be less deterrent suggests that (i) the oxidation products more readily formed at low concentration may be more deterrent, (ii) ingestion at higher concentrations immobilises the insects, or (iii) ingestion only occurs at low concentration and makes them more restless. Any one of these possibilities renders the test result inconclusive and uninterpretable, and casts severe doubt as to the validity of choice tests in general.

With catechin (Peng & Miles, 1988a) very low concentrations were found to be phagostimulant, and as concentration increased so did the deterrence towards insects until a limiting value was reached. Similarly, in this investigation hordenine was observed to become more deterrent with increased concentration. This suggests that the relationship between concentration and deterrency may possibly indicate the validity of a particular choice test. The observed inconsistencies with other compounds (as discussed above), however, suggests all results obtained using simple choice tests must be interpreted cautiously.

The high concentration of ethanol needed for solubility of gramine and caffeic acid (20% ethanol compared with 5% for the hordenine experiments) was also a point of concern, and the effects of this upon the insects was therefore addressed. An experiment was conducted to compare the feeding of insects

offered a choice between sucrose solutions containing either 10% or 20% ethanol. To confirm that the two diets (i.e., left & right-hand sides of the choice chamber; see Figure 4.1) had equal probability of being chosen by the insects, chambers offering no choice but only 10% ethanol or 20% ethanol as the two diets were observed. The insects showed no preference for the left- or right-hand side of the T chamber (52:48 left *vs* right; $P>0.75$), indicating that palatability of the diet was the deciding factor involved. When offered a direct choice between sucrose solutions containing either 10% or 20% ethanol, however, the insects had an apparent preference for the 20% ethanol solution.

Whether the apparent preference for the higher alcohol content was due to the solution being more palatable to the aphids, or that after the insects ingest the higher alcohol solutions they become incapacitated and unable to move from it, was not determined. These experiments did, however, demonstrate that 20% ethanol in test solutions may well mask some of the effects of plant phenolics in feeding choice tests. The same concentration of ethanol was also included in the sucrose control solution, with the intention that any preferential effect of the alcohol should be counter-balanced; nevertheless the possibility cannot be excluded that a combination of alcohol and toxin could result in a synergistic reduction of the insects' movement and a false "preference" for the toxin.

This was confirmed by comparison of the feeding effects of DIMBOA, using both 10% and 20% ethanol in sucrose. In these two experiments DIMBOA 1 mg/ml was apparently more deterrent to the insects than the 2 (or 5) mg/ml solution. The DIMBOA diets became coloured during the 24 h experiment period, whether exposed to feeding aphids or simply as control solutions. Thus, autoxidation of DIMBOA occurred during this time in both. Comparison of these solutions by UV spectroscopy showed the control solutions had even greater absorptions than the aphid-exposed solutions at corresponding wavelengths,

casting doubt on the extent to which the insects had attempted to feed on the DIMBOA solutions, irrespective on any apparent preferences displayed.

Aged solutions (DIMBOA autoxidised at room temperature overnight), were therefore offered in choice tests, with freshly prepared material offered as the control solution. Initially the unoxidised DIMBOA appeared to be more palatable to the insects (68% of aphids feeding on freshly prepared DIMBOA; $P<0.01$). Once autoxidation of both the fresh and aged DIMBOA solutions had proceeded further (i.e., after 24 h on the diets), however, the proportion of aphids feeding upon the older solution had increased (i.e., there was no longer a significant difference between the acceptability of the two diets). While the results of these experiments are clearly open to various interpretations, there is at least a possibility that as oxidation progressed the later oxidation products began to precipitate out of the solutions, making diets more tolerable.

The results of preference tests for DIMBOA, and also caffeic acid (which similarly appeared less palatable to the aphids after 24 h of exposure to the diet), irrespective of the interpretation difficulties involved, would seem to negate the "degrees of deterrency" hypothesis of Miles and Oertli (1993). What must be remembered here, is that the simple, if not problematical, choice tests employed in the current study are a gross oversimplification of the complex interactions occurring *in vivo* between aphids and plant tissues. One of the results of oxidation of phenolics in the presence of proteins, for instance, is the formation of relatively insoluble protein-phenol complexes (Haslam, 1988). Thus, in the section that follows, experiments are reported in which the immediate oxidation products of reactive phytochemicals, such as caffeic acid, were shown to complex readily with other components of naturally occurring biochemical systems. Such interactions lead to conjugates or copolymers that precipitate out of solution and thereby rapidly reduce chemical deterrency (to sucking insects) of the systems overall.

SECTION B

Oxidation of Phenolic Allelochemicals *in vivo* During Aphid-Plant Interactions

The work reported in this section is of a highly preliminary nature, and depends in part on materials and incomplete results contributed by other workers. It has been included only because of its potential significance (in the production of transgenic plants with resistance to aphids) and because it illustrates one of the directions in which work of the kind attempted in the present investigation could be utilised and developed in future.

4.2b.1 Extraction of Phenolic Compounds from Plants

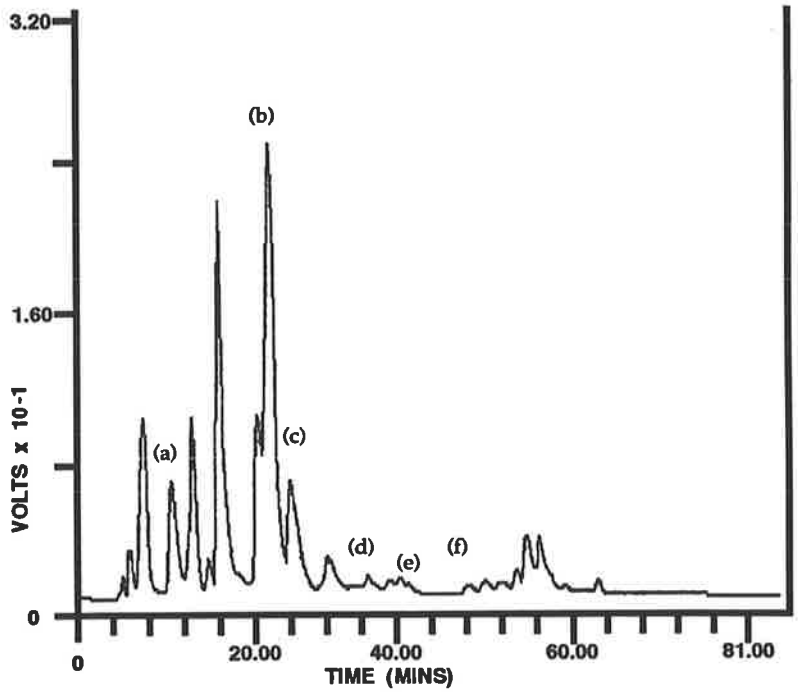
The two extraction solvents used in initial experiments, hot 80% methanol and 100% methanol, were successful in extracting phenols from dried potato leaves. Quantities of plant pigment (chlorophyll) were also removed, as indicated by the green colour of the extracts. Hot 100% methanol was chosen as the most suitable extracting solvent, as this eliminated subsequent extraction of an aqueous phase, thus simplifying the method. The optimum fresh weight of plant material required for drying was found to be approximately 5 g. Samples prepared in this way gave excellent phenolic profiles when examined by HPLC, showing many constituent compounds (e.g. see Figure 4.2).

Of the different types of potato leaves tested in these experiments (obtained from various regions of the plant, at different growth stages, see 4.4b.3), the first fully expanded and lower adjacent leaves were found to give the best results. This relatively new growth also supported aphid populations in the field. All leaves tested gave very similar phenolic profiles (types of compounds present), although the relative amounts of each compound (compared with the internal standard) varied somewhat. The youngest plant growth was found to have high levels of all compounds, making observation and identification of the phenolic profile easier

(Figure 4.2). These leaves were used for subsequent investigations, and were also the basis of the freeze-dried transformed material obtained from Dr. P. Miles.

Figure 4.2: HPLC trace of phenols extracted from potato plant, newest growth; (a) - catechol (internal std), (b) - caffeic acid, (c) - esculin, (d) - ferulic acid, (e) - p-coumaric acid, (f) - chlorogenic acid.

(i)



4.2b.2 Reaction of Plant Extracts with Oxidising Enzymes

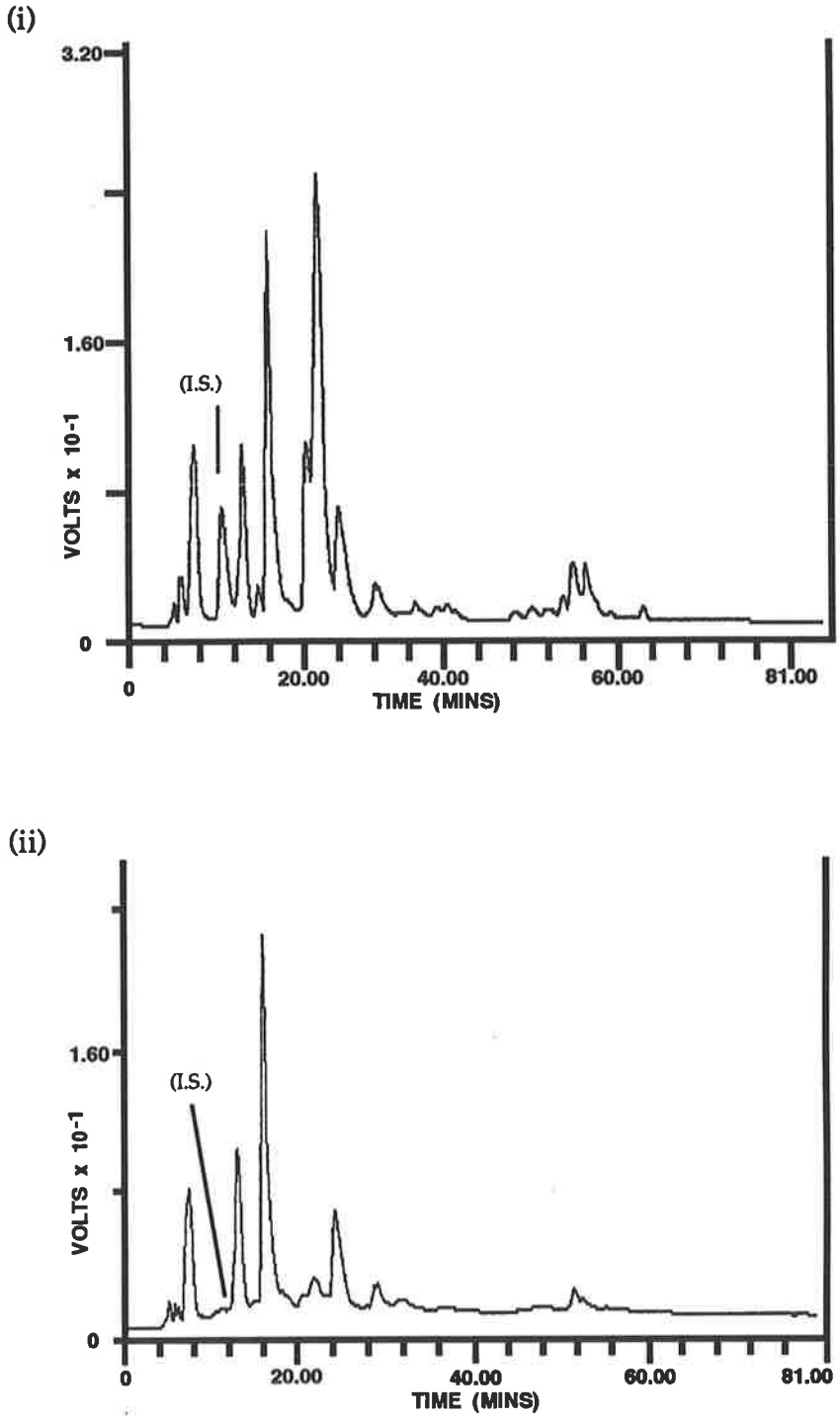
Investigations into the effect of aphid salivary enzymes on plant phenolics is a major component of the current work. The effect that these enzymes may have on the phenolic content of their host plant is not known. Earlier experiments conducted using individual plant allelochemicals and aphid saliva (Chapter 1) were isolated systems, i.e., involving a single plant compound and the enzymes in a one-pot reaction. The effects of combinations of known chemicals on such a reaction were not investigated since the almost endless possibilities for fully

defined, *in vivo* combinations would have gone far beyond the objectives of this study and the resources available to it. Nevertheless, use was made here of a readily obtainable natural combination from a relevant living source, namely the potato plant. In particular, it was hoped that such an attempt might throw some light on the problems that emerged from the aphid choice tests on artificial diets.

When aphids feed on a plant they do not generally encounter any one compound alone, but rather face a multitude of compounds, some of which may together constitute the plant's allelochemical defence. It is likely that the oxidation reactions occurring in the presence of such a variety of compounds would involve integrated reactions, i.e., the oxidation of one compound may lead to the subsequent oxidation of another, or conjugation of the oxidation product with another compound. For this reason the effect of oxidising enzymes on the total phenolic extracts of a plant was of substantial interest.

Extracts of new potato growth as defined above were prepared and used in reactions with both aphid salivary enzymes and the model enzyme system. Addition of model enzymes (HRP and mushroom tyrosinase) to the plant solution greatly altered the observed HPLC trace (Figure 4.3). All phenolic peaks found for the extract were substantially reduced, and some were no longer present. In particular, the peaks of caffeic and chlorogenic acids, esculin and p-coumaric acid had decreased significantly. These observations imply oxidation of the compounds, possibly to more conjugated (higher wavelength UV absorption) products, and would explain the appearance of precipitates that were observed as the reaction progressed.

Figure 4.3: HPLC trace of extract (i), and extract + HRP/tyrosinase (ii); (I.S.) - internal standard (catechol).



When aphid saliva was used as the oxidising reagent changes in the phenolic profile of the extract were again seen, but none so dramatic as those found

for the more concentrated model enzymes. Several peaks were diminished in size, and these were not affected by reaction of the extract with a control solution consisting of 'unfed' water (see General Experimental) used in place of the aphid saliva preparation. Hence, the changes that were observed did indeed appear to be due to the action of saliva on the extracted phenolics.

From the results obtained, it seems that the extraction and HPLC analysis of potato phenolics performed here is a suitable method for determining plant phenolic profiles. Moreover, it allowed determination of the effects of oxidising reagents (particularly aphid saliva) on a system approximating more closely the complex *in vivo* environment than any *in vitro* reaction with individual substrates.

4.2b.3 Phenolic Content of Transformed Potato Lines

Miles and co-workers (pers. comm.) have recently been able to introduce a bacterial gene for glutathione reductase into tissue cultures of an aphid-susceptible potato, *c.v.* Coliban. The significance of this work lies in the involvement of the enzyme in the regeneration cycle of the reductive system of plants, which can be exhausted in compatible (susceptible) reactions of plants with aphids (Jiang & Miles, 1993b). Although experiments with transformed lines of potatoes have only just begun, and the plants were as yet few in number and still very young at the time of the present study, Miles kindly made small samples available for phenol analysis. Preliminary data on their glutathione reductase content and an initial indication of the susceptibility to aphid infestation (*Myzus persicae*) of the plants from which they were obtained was also given by Miles & co-workers.

Analysis of the samples, both control (untransformed original clone) and lines transformed with the bacterial genes for cytoplasmic glutathione reductase, revealed similar HPLC phenolic profiles for all of the lines tested. The total and relative amounts of phenolics present nevertheless varied from plant to plant. Only limited amounts of transformed lines were available, sufficient for single

determinations of their phenolic profiles, but replicate analyses of the control material were sufficiently similar to give confidence to the differences noted in the transformed material. Relatively high concentrations of phenols were observed in lines 1, 2, 5, 8, and the control. Conversely, the other lines were found to have either quite low (line 6) or mid-range phenol concentrations (lines 3, 4 & 7). Thus, it would seem that incorporation of reductant genes into the potato genome has in some instances altered the plant's phenolic profile.

Investigations by Miles and co-workers (pers. comm.) have revealed 10-15% increases of glutathione reductase activity in all of the transformed lines (when compared with those of the control). Preliminary testing of the resistance of the plants to aphid infestation has also shown increased resistance significant at the 5% level (n=3) in several of the lines (specifically 8, 4, 1, & 7), whilst the remaining lines behave similarly to the control (i.e., 2, 6, 3, & 5). The single phenolic profile obtained in the present study correlates reasonably well with the resistance data, in that lines containing both increased levels of reductant and high concentrations of phenolics appear better able to withstand insect attack (e.g. 1 & 8, 4 & 7). Conversely, a plant with elevated reducing capacity, but a low phenolic content (line 6), showed high susceptibility when infested with aphids. It must be stressed that the preliminary data quoted here are far from definitive, due to the very small amounts of material available and the non-replicated results for phenol and glutathione reductase content. They require confirmation and rigorous testing, but are nevertheless reported here with the permission of Miles (pers. comm.) because of their far reaching implications.

4.3 CONCLUSION

Many plant allelochemicals have been reported in the literature as being deterrent, or indeed toxic, to feeding insects. All of the compounds investigated here using choice tests (hordenine, gramine, caffeic acid, & DIMBOA), showed evidence of deterrency at concentrations of 1-5 mg/ml. Closer examination of these simple tests indicated that apparent feeding deterrencies cannot always be relied upon, as many factors are involved in the "decision" of an insect to move from and/or feed on a diet.

Results obtained for the monophenol hordenine showed an expected increase in feeding deterrency with increased concentration (up to a limiting level). Further investigations apparently revealed the oxidation product (from the reaction of aphid saliva upon hordenine) to be more palatable to the insects than the starting compound. Subsequent experiments with the other compounds, however, did not follow such straightforward paths.

Results with hordenine were obtained by direct comparison of solutions of hordenine and oxidation products, offered as choice diets to caged insects. Surprisingly, however, the inclusion of known plant reductants (e.g. glutathione and ascorbic acid) in hordenine solutions did not increase their deterrency. Under the conditions of the test, these reductants were not able to maintain unoxidised hordenine, i.e., both hordenine plus reductant and hordenine alone underwent oxidation at comparable rates. The insects correspondingly showed no preference for either solution.

From the investigations carried out here it seems that direct comparison of the unoxidised plant chemical and its preformed oxidation product(s) is the most suitable method for determining the relative deterrency of the two. This result is significant whether applied to simple choice tests or perhaps to more reliable

testing methods (such as the use of radioactive diets and detection of ingested radioactivity). The incorporation of reductants (for maintaining unoxidised starting material) in one of the choice diets would give useful results only if the reductant used does indeed prevent oxidation or influence the oxidation pathway under the test conditions.

The results observed for the hordenine investigations were nevertheless consistent with the idea of decreasing degrees of deterrence in successive stages of oxidation of defensive allelochemicals. What must be stressed here is that the investigations into other compounds (such as caffeic acid, gramine and DIMBOA) have shown inadequacies in the simple choice test analyses. A degree of uncertainty in the interpretation of these results was noted when it was found that low concentrations (1 mg/ml) of these phytochemicals were more deterrent than higher concentrations. This was presumably due to behavioural effects of ingestion of compound by the aphids, which were likely to occur more readily at low concentrations. A further complicating factor was the need for inclusion of ethanol as a solvent in the choice solutions. The possibility therefore existed that ethanol might induce ingestion from toxic solutions causing immobilisation on, indicating an apparent but spurious "preference" for, the more toxic of the diets offered.

It must be remembered too, that the tests performed here involved offering aphids a single phytochemical in a simple sucrose diet. The situation *in vivo* is far more complex, and many other components (e.g. other phytochemicals, proteins, amino acids, membranes, etc.) are present which may well interact with the aphid salivary oxidation products from these phytochemicals. Investigations performed into the types and quantities of phenolics found in potato plants, determined by HPLC analysis, and the effects caused by the inclusion of reductant genes into the potato genome on the phenolic profile, give an insight into the complexity of these interactions.

Turning to methods for extraction of naturally occurring phytochemical mixtures in growing plants, hot methanol (100%) proved a suitable solvent for extracting the phenolics from freeze-dried potato material. This material could then be simply prepared and used in HPLC analyses to determine phenolic content, and the procedure is applicable to many different types of plant species.

Within plant choice of material is important in such studies. Larger leaves from the newest growth proved an excellent source of phenols for HPLC examination. The phenolic profile obtained from such material was typical for most regions of the plant, but had the advantage in potato, at least, of providing higher phenol concentrations for easier examination.

Reaction of plant extract with oxidising enzymes had a profound effect upon the peaks observed in the HPLC trace. Use of "model enzymes", such as HRP and tyrosinase, gave dramatic reductions in the height of the HPLC peaks, with many of the phenols disappearing completely. Aphid saliva had a less noticeable affect upon the plant extracts, although changes in peak heights and ratios were observed. Analysis of such interactions, where many plant phytochemicals are present in one mixture, gives a situation more closely resembling that found in the intact plant. Although a study of oxidation by aphid saliva of individual allelochemicals *in vitro* is a necessary preliminary to determine the reaction products that might be formed *in vivo*, the actual reactions that occur in living plant tissue could well be significantly different.

Transgenic potato plants with increased reductive capacity (glutathione reductase) were analysed for phenolic content by the same procedure. Preliminary investigations indicated that the transformed lines contained similar phenolic compounds, but in different ratios. Lines with high phenolic concentrations, and increased levels of reductant demonstrated elevated resistance to aphids in preliminary experiments (Miles & co-workers). In contrast, plants with higher

reductant levels, but of low phenol concentration, were still severely affected by aphid infestation.

These observations, although of a preliminary nature and not yet definitively tested, are of great importance. Should such trends prove consistent, then the application of this information to plant breeding would allow for a relatively easy selection of transgenic production of cultivars with increased resistance to aphids, and possibly other sucking insects.

4.4 EXPERIMENTAL

SECTION A

4.4a.1 Hordenine Deterrency

1. Hordenine Fed to Rose Aphid:

M. rosae were collected from the Waite Campus (The University of Adelaide) rose garden during the afternoon, and deprived of a food source overnight, prior to use in choice experiments.

Sucrose (15%) in phosphate buffer (50 mM, pH 7), with EtOH (5%) was used as the standard diet solution, and from this a hordenine stock solution (10 mg/ml, 2 ml; adjusted to pH 7.0 (1.0 M HCl)) was produced. The remaining sucrose solution was then corrected for Cl^- ion additions with KCl (1.0 M). Hordenine dilutions (1, 2, & 5 mg/ml) were made up using the sucrose solution, and these were tested in subsequent choice tests.

The collected aphids were portioned into choice test T-chambers (30-50 aphids each)* , and the chambers were covered with parafilm. The hordenine solutions (1, 2 , 5 or 10 mg/ml; 200 µl) were dispensed onto the parafilm, along with a sucrose control for each chamber. The position of the sucrose and hordenine drop was alternated on each chamber, and four replicates of each hordenine *vs* sucrose concentration were performed.

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* Aphids are attracted to light and also to heat, so care was taken during dispensing to ensure conditions did not favour any one of the test diet positions.

The choice chambers were placed inside black plastic containers, over water and covered with yellow lids. They were left at 25°C under a yellow lamp, and the number of aphids feeding inverted on each drop of liquid was counted after 2, 4, 8, & 24 h*.

2. Hordenine Fed to Potato Aphid:

M. euphorbiae were collected from glasshouse-grown potatoes, and treated as for the rose aphid in (1) above. The choice test outlined was repeated with one modification, only hordenine solutions of 2, 5, and 10 mg/ml were tested, due to space and resource limitations. Results similar to those for the rose aphid were observed.

4.4a.2 Glutathione Deterrency

1. Glutathione Fed to Rose Aphids:

The method used for hordenine (4.4a.1.1) above, was repeated, using reduced glutathione stock solution (10 mg/ml, 2 ml) in place of hordenine.

2. Glutathione Fed to Potato Aphids:

The method outlined in (1) above was repeated, using the potato aphid, *M. euphorbiae*.

4.4a.3 Ascorbic Acid Deterrency

1. Ascorbic Acid Fed to Rose Aphids:

The method outlined for hordenine (4.4a.1.1) above was repeated, using ascorbic acid in place of hordenine.

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* Raw data for all choice experiments is collated in Appendix 3.

2. Ascorbic Acid Fed to Potato Aphids:

The method of (1) above was followed precisely, using potato aphids.

4.4a.4 Hordenine in Combination With Reductants

1. Hordenine with Reduced Glutathione:

Hordenine (5 mg/ml) and hordenine/glutathione solutions (5 and 2 mg/ml respectively) in 15% sucrose/phosphate buffer, were offered to rose aphids in a choice test.

Hordenine and mixed hordenine/glutathione solutions were adjusted to pH 6.8 (1.0 M HCl, 1.0 M KOH). Aphids were dispensed into T-chambers (30-50 individuals), and each chamber was supplied with a sucrose control (200 µl), and a test solution (either H or H/G; 200 µl). The chambers were then set up in black containers with yellow lids under lights, as for the individual deterrency tests described previously.

Four replicates of the controls *vs* H and *vs* H/G were performed. The number of feeding aphids was counted after 2, 4, 8, and 24 h. After completion of the observations the fed solutions were collected, and the UV spectrum of the diluted solutions (1/10 dilution) recorded.

2. Hordenine in Combination With Ascorbic Acid:

The experiment outlined in (1) above for hordenine/glutathione in combination was repeated, using ascorbate (2 mg/ml) in place of reduced glutathione, and potato aphids in place of the rose aphids.

4.4a.5 Hordenine Compared With Its Oxidation Product

M. euphorbiae were collected from the glasshouse during the afternoon, and deprived of a food source overnight, prior to use in the choice experiment.

Oxidation products were produced by reaction of hordenine freebase (21 mg) with HRP (5 mg) in the presence of H_2O_2 (0.03%), in total volume of 20 ml (dd water:EtOH 19:1). The reaction was stirred at room temperature overnight, frozen in liquid N_2 and evaporated to dryness (freeze drier/oil pump; 24 h). The resultant powder was extracted with MeOH (2 x 5 ml) and the combined organic extracts dried (N_2 gas). The remaining brown solid was examined by TLC (MeOH + trace NH_3), revealing quantities of oxidation products and small traces of hordenine. This crude product material was stored overnight (-20°C), and used in a choice test on the following day.

Hordenine freebase (5 mg) was dissolved in EtOH (100 µl), and made up to 2 ml with sucrose solution (15% in 50 mM phosphate buffer; pH 7). Oxidation product (5 mg) was similarly dissolved in EtOH (100 µl), and made up to 2 ml with sucrose solution (15% in 50m M phosphate buffer; pH 7). These two solutions were then adjusted to pH 7.0 (1.0 M HCl, 1.0 M KOH). For use as a choice control, an identical sucrose solution was prepared (15% in 50 mM buffer, pH 7; containing 5% EtOH).

Aphids were dispensed into T-chambers (30-50 individuals in each), and the chambers were then covered with parafilm. The hordenine (H), oxidation product (O), and sucrose (S) solutions were dispensed onto the parafilm. In test A, each chamber was supplied with a sucrose control (200 µl), and the test solution (either H or O; 200 µl). For test B, the aphids were offered either hordenine or oxidation product (H or O; 200 µl each) but no sucrose solution. The choice chambers were placed inside black plastic containers, covered with yellow lids, and kept at 25°C under a yellow lamp. For determination of the effects of aphids on solutions dispensed to them in choice tests, some test solutions were dispensed to choice chambers containing no aphids, and maintained under the same test conditions as the concurrent choice test.

Four replicates of S *vs* H, S *vs* O, and H *vs* O were performed. The number of aphids feeding inverted on each drop of liquid was counted after 2, 4, 8, and 24 h. After 24 h, the fed solutions were collected, and the UV spectra of the diluted solutions (1/10 dilution) were recorded.

4.4a.6 Gramine and its Deterrency to Feeding Insects

Sucrose (15%) in phosphate buffer (50 mM, pH 7), with EtOH (20%) was used as the standard diet solution, and from this gramine stock solution (5 mg/ml, 2 ml; adjusted to pH 7.0 (1.0 M HCl)) was produced. The remaining sucrose solution was then corrected for Cl^- ion additions with KCl (1.0 M). Gramine dilutions (1 & 2 mg/ml) were made up using the same standard sucrose solution, and these were tested in subsequent choice experiments, as for hordenine (1).

4.4a.7 Caffeic Acid and Insect Feeding

The method outlined for gramine (4.4a.6) was repeated, using caffeic acid (5 mg/ml stock solution) in place of gramine.

4.4a.8 The Effects of Ethanol Concentration

Sucrose solutions (10% & 20% EtOH, 5 ml each) were made up in pH 7.0 phosphate buffer. Aphids were portioned into choice test T-chambers (30-50 aphids each), and these were covered with parafilm. Sucrose solution (10% or 20%; 200 µl) was dispensed onto the parafilm, the position of the different drops was alternated for each chamber. Four replicates of each sucrose test were performed, ie: 10% *vs* 10%, 20% *vs* 20%, and 10% *vs* 20%, as for the previous experiments.

4.4a.9 DIMBOA Deterrency

(i) The method outlined for gramine previously (4.4a.6) was repeated, using DIMBOA (5 mg/ml stock solution) in place of gramine. All solutions became coloured with time.

(ii) The method of (i) above was repeated, with slight modification. Only 10% EtOH in sucrose was used for the experiment, and 1 & 2 mg/ml DIMBOA were the only concentrations investigated. The colour of the DIMBOA diets was observed to darken (pink-brown colour) with time, whether exposed to aphids or not, as for (i) above.

(iii) Solutions of DIMBOA (2 mg/ml in 10% EtOH sucrose solution) were aged for 24 h (room temperature overnight), and then tested against freshly prepared DIMBOA in an aphid choice test. Concentrations of 1 & 2 mg/ml were given to aphids in T-chambers as for previous choice experiments. Four replicates were set up; D1 *vs* D1', D2 *vs* D2' (Dx = freshly prepared, Dx' = aged solution; x = x mg/ml DIMBOA). The number of aphids feeding on each drop of diet was observed after 2, 4, 8 & 24 h.

SECTION B

4.4b.1 Extraction of Plant Phenols

(i) Leaves (1.1 g fresh weight) were collected from potato plants (*c.v.* Coliban), and dried (freeze drier, oil pump) overnight. The samples were crushed slightly, and extracted with hot 80% MeOH (65°C). The green extracts were centrifuged, the supernatant collected, and the methanol removed (evaporated, N_2 gas). The remaining aqueous solution was extracted with chloroform (2 x 2 ml), the combined organic extracts were evaporated to dryness, and the resultant solid taken up in MeOH (2 ml). This was centrifuged and the supernatant evaporated to an approximate volume of 0.5 ml. This extract was examined by reverse phase HPLC (as described by Pérez-Ilzarbe, *et al.*, 1991), UV detection at 280 nm, catechol (10 mM) as the internal standard.

(ii) Fresh leaf material (1.48 g) was treated as for (i) above, with slight modification. Extraction of the plant phenolics was achieved using 100% methanol (2 x 10 ml), and the combined extracts filtered. The green methanolic solution was evaporated to dryness (N_2), the residue taken up in MeOH (2 ml), centrifuged and the supernatant evaporated. The resultant solid was dissolved in MeOH:H_2O (1:1; 0.6 ml), sonicated & centrifuged, and kept under a N_2 atmosphere. The supernatant was analysed by HPLC as outlined in (i) above.

(iii) Leaf material (approximately 5 g) was treated as for (ii) above. The dried material was extracted with 100% methanol (2 x 20 ml), and the extracts filtered. The resulting green methanolic solution was evaporated to dryness (rotary evaporator), the residue taken up in MeOH (5 ml), centrifuged and the supernatant evaporated. The resultant solid was dissolved in MeOH:H_2O (1:1; 2.5 ml), sonicated & centrifuged, then maintained under a N_2 atmosphere. The supernatant was analysed by HPLC as outlined in (i).

4.4b.2 Phenol Standards

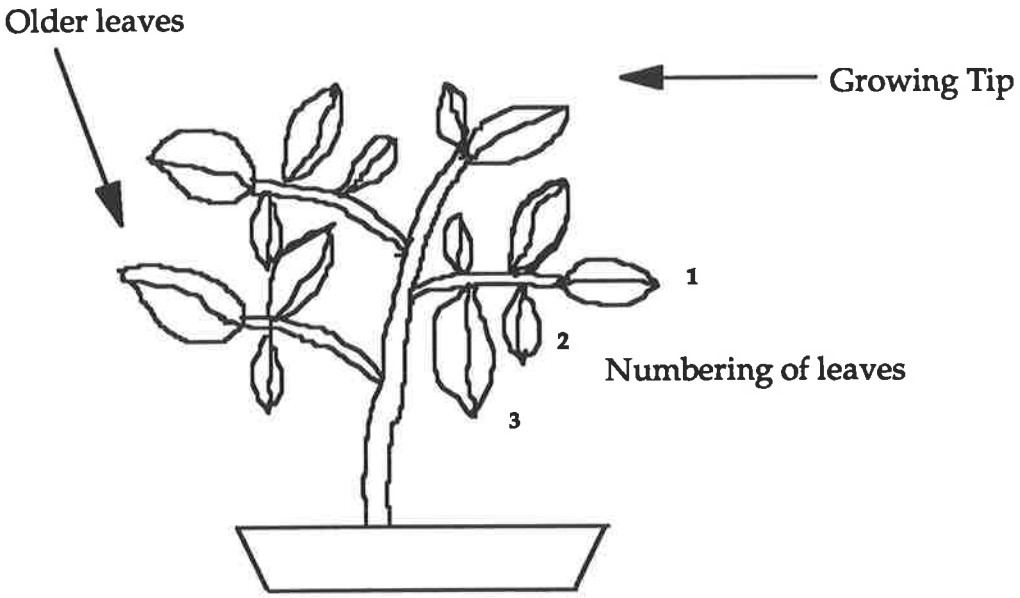
Phenolic standards (0.5 mg/ml; H_2O:MeOH, 9:1) were analysed by HPLC, using the conditions and method outlined for the potato extracts (4.4b.1.i) above. The observed peaks and retention times are as follows:

COMPOUND	APPROXIMATE ELUTION TIME (MIN)
Catechin	17.72
Caffeic Acid	20.88
Esculin	22.71
Epicatechin	30.37
Ferulic Acid	32.91
p Coumaric Acid	36.28
Chlorogenic Acid	43.54
t-Cinnamic Acid	61.01

4.4b.3 Choice of Plant Leaf Type - Phenolic Content

Potato leaves from different regions of the same plant were collected (see table and figure following), each type of sample was dried and extracted as for (4.4b.1.iii).

Figure 4.4: Potato plant, with numbered leaves.



SAMPLE	LEAF TYPE	FRESH WEIGHT (g)
A	Young - stage 2/3	4.47
B	Old - stage 1	5.16
C	New growth - stage 1/2	5.00

The extracts were examined for phenolic content by HPLC as for (4.4b.1.i) previously. Good separation of most peaks was observed, and a large number of compounds was detected. Caffeic acid (22 min), ferulic acid (33 min), and chlorogenic acid (44 min) were observed in all samples tested. All of the phenolic profiles for the different leaf types were similar, however newest plant growth (large leaves, 1&2) showed the greatest quantity of phenolics relative to the internal standard.

4.4b.4 Reaction of Potato Extracts With Oxidising Enzymes

1. Reaction with Model Enzymes:

Extracts of new potato growth (leaves 1 & 2) were prepared as described previously (4.4b.1.iii). Extract (110 µl) and H_2O_2 (0.003%) were reacted with HRP and mushroom tyrosinase (0.05 mg/ml of each) for 2 h at room temperature, in a total volume of 500 µl. The mixture was analysed by HPLC (conditions as described for potato extracts), and catechol standard (10 mM; 10 µl) was added to the mixture immediately prior to injection. Plant extract reaction mixtures, after being left overnight at room temperature, were dark brown in colour and contained a precipitate.

2. Reaction with Aphid Salivary Preparation:

Extract (110 µl) and H_2O_2 (0.003%) were reacted with aphid salivary preparation (250 µl). A control reaction was performed simultaneously, using unfed water in place of aphid saliva. After 2 h at room temperature the reaction mixtures were analysed by HPLC, and catechol standard (10 mM; 10 µl) was added immediately prior to injection.

4.4b.5 Phenolic Extracts of Transformed Potato Lines

Freeze dried potato leaves* (*ca.* 150 mg of dried material; see table, below) were extracted as for the method outlined previously (4.4b.1.iii). The resultant phenolic solid was taken up in MeOH:H_2O (1:1, 0.5 ml) and used in HPLC analyses. Catechol standard (10 mM; 10 µl) was added to the 0.5 ml sample just prior to injection.

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* Obtained from Miles & co-workers.

SAMPLE	DRIED WEIGHT (mg)	COLOUR CHANGE
Control	200	
1	150	m
2	100	i
3	150	s
4	100	s
5	150	s
6	150	i
7	150	i
8	150	i

After several days at room temperature colour changes and precipitate formation were noted. These could be described as:

m	-	minor	(yellow-brown liquid)
i	-	intermediate	(coloured solution, light brown ppt.)
s	-	substantial	(dark brown/blk ppt., brown liquid).

GENERAL DISCUSSION

When originally commenced it was hoped that this study would enable a better understanding of the function and specificities of the oxidative enzymes found in aphid saliva. The elucidation of the chemical nature of the oxidation reactions occurring between aphid saliva and plant allelochemicals, and to determine the effects (if any) of resultant oxidation products upon this interaction, was of primary interest. As with most research projects, some of these questions were answered, whilst other intriguing possibilities emerged. In a sense, a major result of this investigation is to pose new questions and set some of the constraints within which they need to be answered.

Enzymes in Aphid Saliva

The saliva of aphids contains enzymes with oxidative capabilities. The range of compounds affected by such enzymes was found here to be considerable. Not only did oxidation reactions occur with both mono- and di-phenols, but also with functionally more complex compounds such as indoles and hydroxamic acids. From the oxidation reactions observed in this study, it would seem that aphid saliva behaves primarily as a peroxidase, with additional catechol oxidase activity; closely approximated by a model enzyme system that combined horseradish peroxidase/hydrogen peroxide with mushroom tyrosinase. Of biochemical and physiological importance was the confirmation of dependence upon copper as a chelating group for this peroxidase activity, as suggested by Madhusudhan (1994). Such findings are of fundamental interest since similar observations have been reported for only two other aphid species, both by Madhusudhan (1994).

Saliva + Plant Allelochemical - Oxidation Products

The oxidation of di-phenols and related compounds, by aphid salivary enzymes, was observed in this study to proceed predominantly via o-quinone intermediates (by hydrolysis of related compounds to the diphenol if necessary). Compounds with mono-phenolic sites, however, appear more likely to form conjugate compounds such as "2M-2" dimers, "3M-3" trimers, etc., by direct conjugation of the phenol radical with another molecule of substrate; unless formation of the necessary diphenol is more favoured than the alternative oxidation pathway. Complex ring aromatics that do not have hydroxyl reactive sites present, on the other hand, may undergo ring opening reactions.

Feeding Deterrency of Allelochemical Oxidation Products

All of the plant allelochemicals tested were deterrent to the feeding of aphids, albeit using simple, and in further analysis somewhat inappropriate, choice test methods applied here. Preliminary investigations using the monophenol hordenine showed classical increases in deterrency with increased compound concentration by this method. Nevertheless, investigations of other compounds cast severe doubt upon the validity of simple choice tests based on movement between diets, since such tests would fail if toxic diets caused immobility of the insects. More definitive testing of the acceptability of diets requires a better criterion, perhaps use of radioactive-labelled constituents within the test diets, and comparison of ingested activity by the feeding insects.

What was apparent from the present study, was the palatability or tolerance of the hordenine oxidation product over the unreacted starting compound. This gives worth to the notion of degrees of deterrency within a series of oxidation products of defensive phytochemicals.

Plant Defences - Redox Reactions

In vitro confirmation of Jiang's novel hypothesis "that a substrate limited oxidation occurs in all plant leaves, but that this is increased in infested leaves; whilst healthy leaves contain significantly more reductant(s)" was achieved. Observation of the mushroom tyrosinase oxidations of both hordenine and PAC (partially autoxidised catechin) has shown that the inclusion of reductant(s) can prolong (i.e., delay) substrate oxidation. These findings are consistent with the general hypothesis - that the injection of aphid salivary enzymes into their host plants during feeding results in the oxidation of defensive chemicals to products of less insect deterrence/toxicity, and that the reductants present within plants serve to counteract such oxidative reactions. Thus, plants containing greater reductive capacity should be less affected (in theory) by the feeding of insects, as such plants should be better able to maintain the effectiveness of their (oxidisable) defensive chemicals.

The Complex Situation *in vivo*

Testing such simple relations in the *in vivo* environment, which is complicated by the many other factors and constituents present, became of paramount importance. Suitable methods for the analysis of phenolic content of potato leaf extracts were developed and optimised. Reaction of plant extracts with both aphid salivary preparations and the model enzyme system showed obvious differences in the phenolic HPLC traces when compared with no reaction controls. These more complex interactions, with many plant allelochemicals present within one mixture, gives a situation more closely approximating the nature of the intact plant.

The application of these methods to the analysis of transgenic plants containing elevated reductant capacity showed promise in the preliminary investigations undertaken here. Those plants containing an additional glutathione

reductase gene (for maintaining active glutathione - a known plant reductant), and also containing high levels of phenolics, showed greater resistance to aphid infestation than those containing only one or the other "defence system". If increasing a plant's reductive capacity, whilst maintaining a suitable level of phenolics, consistently yields plants that are better equipped to combat aphid infestation, then plant breeders may have a new approach to producing resistant plant cultivars. One that is based not on introduction of more toxic components into plants, but on non-toxic reductants that would have a deleterious affect only on insects that rely on oxidative detoxification of potential antifeedants in their food sources.

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APPENDIX 1

UV Spectral Data - High Substrate and Enzyme Concentrations

Compound	HRP	Tyrosinase	Aphid Sal.	Autoxid.
Acetaminophen	306, 406br		309	
	pale yellow soln.			
Aniline	328, 408 red-brown*	305 to 313, 400 to 500sl		
Ascorbic Acid		289		
Berberine	(-)		468	
Betaine	(-)		(-)	
Boldine	381, 453 cream*		345	
Caffeic acid	383, 408		376	388
	yellow-orange*			
Catechin	251, 294, 392br, 434br	320, 400 to 500	251, 292	251, 292
	yellow-orange*	orange-red*		
DIMBOA	337		(314, 337) or (312)	
	yellow soln		slight yellow	
Ferulic acid	379, 408		371	376
	pink*			
Gramine	306, 425sl		301	307
Hordenine	245sl, 300	243sl, 300	300	305

* Formation of coloured product or precipitate.

N.B. DIMBOA + Saliva - two sets of information are provided, one for with and one for without added hydrogen peroxide.

APPENDIX 2

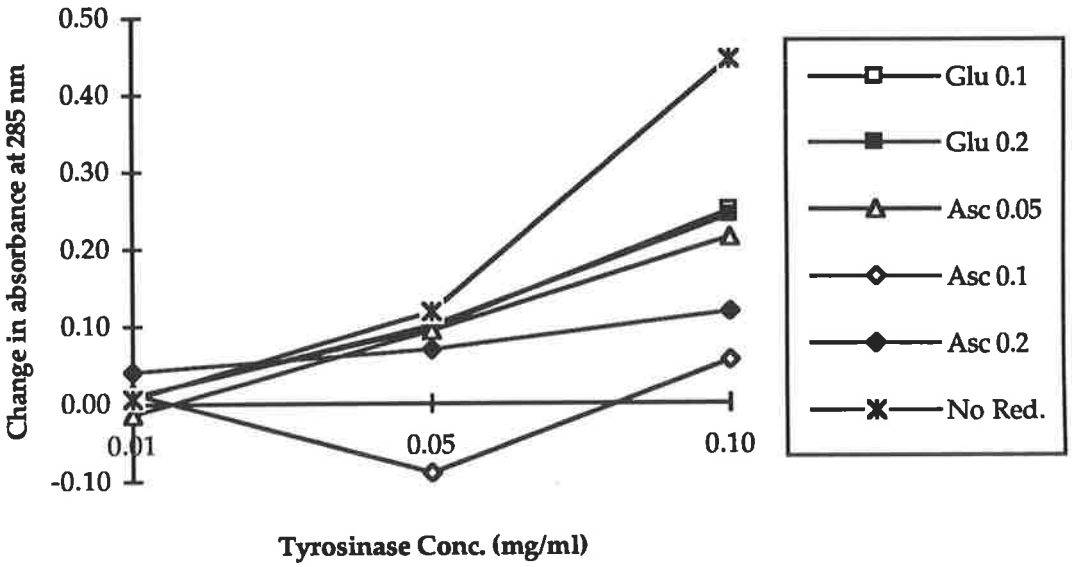
Appendix 2a

Graphical data obtained from time course oxidations of hordenine and mushroom tyrosinase, in the presence and absence of various amounts of reductants.

N.B.: Glut = glutathione

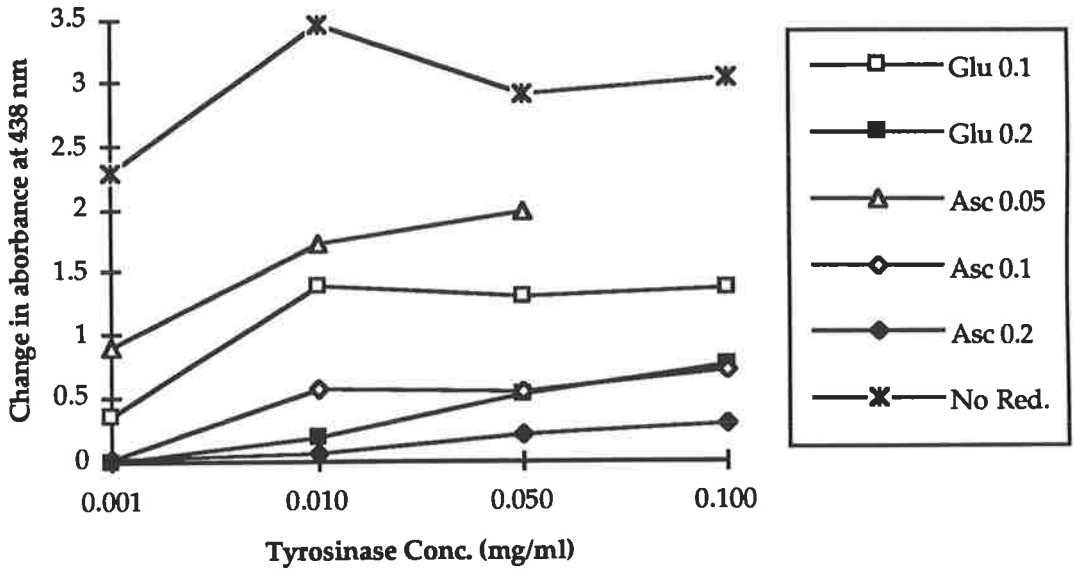
Asc = ascorbate/ascorbic acid

Red. = reductant.



Appendix 2B

Graphical data obtained from time course oxidations of PAC (partially oxidised catechin) and mushroom tyrosinase, in the presence and absence of various amounts of reductants.



APPENDIX 3

Aphid Feeding Choice Tests

The following tables contain the raw data observed for the feeding of aphids offered two solutions in a simple choice test (control and test diet). All control *vs* test solutions were tested using χ^2 methods, and χ^2 heterogeneity methods, to determine significance of differences and the applicability of combining replicates from different days (x, x', x" = experimental times for different days).

Time (hrs)	Sucrose	CA=1mg/ml	Av. sucrose	Av. CA	1 mg/ml	Pooled 1 mg/ml	Sucrose	CA=2mg/ml	Av. sucrose	Av. CA	2 mg/ml	Pooled 2 mg/ml	Sucrose	CA=5mg/ml	Av. sucrose	Av. CA	5 mg/ml	Pooled 5 mg/ml
2	18	18	25.5	12.25	32.45	30.12	18	18	25.5	12.25	32.45	30.12	21	10	22.5	16.75	42.68	43.15
	37	15					37	15					26	20				
	20	12					20	12					28	6				
	27	4					27	4					15	31				
2'	13	13	33	12.25	27.07		13	13	33	12.25	27.07		32	10	29.75	16.5	35.68	
	59	11					59	11					26	16				
	30	15					30	15					20	3				
	30	10					30	10					41	37				
2*	21	12	30.25	13.75	31.25		21	12	30.25	13.75	31.25		17	21	18.25	20.25	52.60	
	40	15					40	15					21	8				
	16	5					16	5					16	7				
	44	23					44	23					19	45				
4	22	23	26	13.25	33.76	28.48	22	23	26	13.25	33.76	28.48	27	7	23.75	11.25	32.14	39.24
	41	18					41	18					30	13				
	10	6					10	6					16	3				
	31	6					31	6					22	22				
4'	15	1	34.25	6.25	15.43		15	1	34.25	6.25	15.43		30	8	32.75	17	34.17	
	61	6					61	6					36	19				
	19	14					19	14					20	5				
	42	4					42	4					45	36				
4*	22	12	25.75	14.75	36.42		22	12	25.75	14.75	36.42		21	17	19.75	21	51.53	
	33	18					33	18					16	14				
	14	11					14	11					26	11				
	34	18					34	18					16	42				
8	18	20	22.25	10.25	31.54	26.92	18	20	22.25	10.25	31.54	26.92	27	10	24.5	13.5	35.53	36.95
	33	14					33	14					18	20				
	11	6					11	6					26	5				
	27	1					27	1					27	19				
8'	20	6	32.75	8.25	20.12		20	6	32.75	8.25	20.12		23	5	29.25	12.25	29.52	
	55	5					55	5					34	9				
	20	16					20	18					19	6				
	36	6					36	6					41	29				
8*	18	14	25.75	11.25	30.41		18	14	25.75	11.25	30.41		18	14	17.5	16	47.76	
	27	14					27	14					22	7				
	14	6					14	6					13	12				
	44	11					44	11					17	31				
24	15	10	24.5	7.25	22.83	24.45	15	10	24.5	7.25	22.83	24.45	27	9	23.75	14	37.09	36.34
	41	8					41	8					30	23				
	15	4					15	4					14	8				
	27	7					27	7					24	16				
24'	14	4	16.75	7.25	30.21		14	4	16.75	7.25	30.21		15	8	19.5	11.5	37.10	
	23	7					23	7					18	12				
	13	14					13	14					15	4				
	17	4					17	4					32	22				
24*	27	8	27.5	7.75	21.99		27	8	27.5	7.75	21.99		19	5	18.5	9.75	34.51	
	31	6					31	6					15	14				
	17	3					17	3					15	10				
	35	14					35	14					25	10				

* The sucrose and test solution averages corresponding to all 1, 2 & 5 mg/ml tests are significantly different ($P<0.01$), as determined by Chi-squared and Chi-squared heterogeneity tests.

A3.1 - Caffeic Acid Data

Time (hrs)	Sucrose	D=1mg/ml	Av. sucrose	Av. DIM	1 mg/ml	Pooled 1 mg/ml	Sucrose	D=2mg/ml	Av. sucrose	Av. DIM	2 mg/ml	Pooled 2 mg/ml	Sucrose	D=5mg/ml	Av. sucrose	Av. DIM	5 mg/ml	Pooled 5 mg/ml
2	21	4	28	13	31.71	35.38	21	5	22.25	15.5	41.06	41.10	35	8	26.5	16.75	38.73	37.95
	29	13					35	27					36	31				
	19	16					18	10					22	7				
	43	19					15	20					13	21				
2'	21	25	22.75	18	44.17		25	15	23	19.75	46.20		19	8	20.25	10.5	34.15	
	29	15					31	12					24	10				
	11	9					19	6					17	8				
	30	23					17	46					21	16				
2"	13	10	25.5	10.75	29.66		18	11	24.25	13.25	35.33		16	7	26	17.25	39.88	
	36	6					30	18					33	19				
	13	15					18	11					25	15				
	40	12					31	13					30	28				
4	22	13	29.25	13.5	31.58	31.81	12	4	22	11.25	33.83	37.42	26	13	24.5	13.25	35.10	37.05
	22	16					33	16					32	27				
	25	14					21	8					22	6				
	48	11					22	17					18	7				
4'	20	18	24.75	14.25	36.54		26	12	26.5	19	41.76		16	4	21	11.75	35.88	
	26	8					38	19					27	14				
	14	10					21	6					22	10				
	39	21					21	39					19	19				
4"	14	7	24.25	8.75	26.52		19	11	24.25	13.25	35.33		18	3	20.75	14	40.29	
	35	5					37	18					22	24				
	15	11					20	11					21	10				
	33	12					21	13					22	19				
8	20	12	26	13.75	34.59	35.36	16	4	22	12.5	36.23	37.47	26	9	25.75	15.5	37.58	32.14
	19	22					33	17					43	28				
	20	10					13	10					19	5				
	45	11					26	19					15	20				
8'	19	20	24.25	15	38.22		21	17	26.5	17.75	40.11		17	5	24	7.5	23.81	
	21	17					36	16					33	10				
	14	7					33	8					24	4				
	43	16					16	30					22	11				
8"	10	9	18.75	9	32.43		7	2	13.25	6.75	33.75		13	3	16.75	8.5	33.66	
	29	10					22	10					18	16				
	11	7					11	6					20	5				
	25	10					13	9					16	10				
24	18	13	26.25	11.75	30.92	25.06	11	5	28	8.75	23.81	24.94	31	10	35.5	11.25	24.06	26.84
	25	11					43	15					47	18				
	26	14					27	6					40	3				
	36	9					31	9					24	14				
24'	20	11	27.5	8.5	23.61		23	11	29	11.75	28.83		12	6	16.25	6.75	29.35	
	28	7					35	12					20	7				
	18	3					29	11					16	4				
	44	13					29	13					17	10				
24"	19	3	28.5	7.25	20.28		17	10	25.75	7	21.37		22	9	20.5	8.5	29.31	
	44	3					37	6					19	7				
	18	11					23	4					26	7				
	33	12					26	8					15	11				

* The sucrose and test solution averages corresponding to all 1, 2 & 5 mg/ml tests are significantly different ($P<0.02$), as determined by Chi-squared and Chi-squared heterogeneity tests.

A3.2 - DIMBOA Data

Time (hrs)	Sucrose	Gr=1mg/ml	Av. sucrose	Av. Gr	1 mg/ml	Pooled 1 mg/ml	Sucrose	Gr=2mg/ml	Av. sucrose	Av. Gr	2 mg/ml	Pooled 2 mg/ml	Sucrose	Gr=5mg/ml	Av. sucrose	Av. Gr	5 mg/ml	Pooled 5 mg/ml
2	7	14	26.25	12.25	31.82	33.26	7	9	16.25	11.5	41.44	41.00	19	10	22	11.5	34.33	35.63
	40	14					29	10					31	12				
	13	10					9	4					23	7				
	45	11					20	23					15	17				
2'	16	8	22.75	12	34.53		8	7	17.5	10.5	37.50		20	10	24	13.75	36.42	
	37	8					22	7					29	2				
	14	5					23	10					22	15				
	24	27					17	18					25	28				
4	15	19	26.75	13.5	33.54		8	13	16.25	12.75	43.97		15	11	24	13.5	36.00	
	35	15					18	12					33	17				
	17	9					15	6					25	1				
	40	11					24	20					23	25				
4'	16	9	25.25	12.75	33.55	28.51	19	8	20.5	10.75	34.40	33.98	13	6	23	11.75	33.81	34.00
	41	11					37	13					37	4				
	17	13					16	5					17	12				
	27	18					10	17					25	25				
7	13	4	26.5	9.75	26.90		5	14	15.25	11	41.90		12	6	20.5	11.25	35.43	
	35	18					24	12					25	16				
	20	11					9	3					17	6				
	38	6					23	15					28	17				
8'	10	3	27.25	9	24.83		19	8	23.5	8.75	27.13		22	5	22.5	11	32.84	
	41	14					28	8					26	7				
	20	3					25	7					14	10				
	38	16					22	12					28	22				
24	21	14	25	7	21.88	26.10	0	2	17.5	6	25.53	25.46	11	4	19.75	8.5	30.09	32.23
	32	5					19	8					18	7				
	20	5					26	3					25	8				
	27	4					25	11					25	15				
24'	12	16	25.25	10.75	29.86		18	8	22.75	7.75	25.41		15	10	21.25	11	34.11	
	36	9					28	7					25	8				
	13	7					23	6					20	3				
	40	11					22	10					25	23				
	* The sucrose and test solution averages corresponding to all 1, 2 & 5 mg/ml tests are significantly different (P<0.02), as determined by Chi-squared and Chi-squared heterogeneity tests.																	

Time (hrs)	Sucrose	Hor=1mg/ml	Av. sucrose	Av. Hord	1 mg/ml	Pooled 1 mg/ml	Sucrose	H=2mg/ml	Av. sucrose	Av. Hord	2 mg/ml	Pooled 2 mg/ml	Sucrose	H=5mg/ml	Av. sucrose	Av. Hord	5 mg/ml	Pooled 5 mg/ml	Sucrose	H=10mg/ml	Av. sucrose	Av. Hord	10 mg/ml	Pooled 10 mg/ml
2	4	13	15	5.25	25.93	32.19	10	3	12.5	9	41.86	27.37	23	3	30.75	3.5	10.22	12.92	13	6	23.5	6	20.34	17.05
	11	3					14	12					26	3					10	1				
	9	5					12	3					28	2					26	4				
	36	0					14	18					46	6					45	13				
2*	25	10	39.25	20.5	34.31		6	4	17	6	26.09		21	2	18.5	3.5	15.91		17	17	24.5	7.75	24.03	
	32	6					30	4					3	5					22	3				
	41	40					27	9					24	1					32	4				
	59	26					5	7					26	6					27	7				
2*							40	3	36.75	21.25	36.64		29	3	38.5	7.25			26	3	41	9	18.00	
							25	36					50	3					55	10				
							52	22					43	17					40	2				
							30	24					32	8					43	21				
2 (spud)							48	9	45	5.6667	11.18		60	8	62.25	8	11.39		48	5	46	5	9.80	
							37	4					58	5					43	4				
							52	4					54	10					47	6				
													77	11										
4	8	17	22.75	8.25	26.61	27.37	12	8	18.75	6.75	26.47	24.86	26	3	36	4.75	11.66	14.06	17	3	29.5	6	16.90	15.24
	15	4					18	8					30	7					17	1				
	29	10					21	1					46	5					38	7				
	39	2					26	10					42	4					46	13				
4*	31	5	44.25	17	27.78		24	4	28.75	7.5	20.69		24	4	28.75	7.5	20.69		42	19	31.75	10	23.95	
	30	4					29	7					29	7					20	8				
	52	28					45	10					45	10					28	6				
	64	31					17	9					17	9					37	9				
4*							37	4	39	24.25	38.34		38	5	44	8			30	1	48	7.75	13.90	
							27	33					56	7					62	11				
							59	24					47	14					53	5				
							33	36					35	6					47	14				
4 (spud)							44	5	48	5.3333	10.39		56	4	54.75	8.5	10.61		49	4	47	4.3333	8.44	
							38	9					48	5					36	6				
							56	2					45	5					56	3				
													70	12										
8	18	9	26.5	10	27.40	26.18	18	4	24.5	6	19.67	21.40	39	2	40.75	5.25	11.41	15.24	21	5	30.5	7.5	19.74	13.91
	14	9					30	2					29	8					19	3				
	34	7					26	7					50	8					35	7				
	40	15					24	11					45	3					47	15				
8*	30	4	44	15	25.42		29	11	28	10.25	26.80		28	11	22.5	10.25	31.30		45	14	36.75	8.5	18.78	
	28	8					24	11					15	11					27	6				
	55	35					39	4					16	4					42	4				
	63	15					20	15					31	15					33	10				
8*							42	2	46.5	18.5	28.46		42	1	46.25	7.25	13.55		32	5	46.5	5.75	11.00	
							35	33					58	12					50	8				
							68	16					51	11					52	2				
							41	23					34	5					52	10				
8 (spud)							41	8	43.33333	4	8.45		47	8	58.75	7.5			45	4	45.66667	4	8.05	
							38	4					60	6					34	3				
							51	2					59	4					58	5				
													69	12										
24	25	7	30.75	7.75	20.13	23.11	28	5	20.5	4.25	17.17	21.16	33	3	39	4.25	9.83	15.91	20	4	22.25	3	11.88	12.36
	24	5					13	3					27	3					12	2				
	32	3					21	5					53	3					22	2				
	42	16					20	4					43	8					35	4				
24*	39	14	53.25	17.5	24.73		33	8	33.5	6.25	15.72		48	8	28.75	6.25	17.86		48	10	39	4	9.30	
	40	8					33	6					18	6					36	2				
	71	30					43	8					33	8					39	1				
	63	20					25	3					16	3					33	3				
24*							53	13	55	18.75	25.42		44	7	52.5	12.25	18.92		42	7	52.25	9	14.69	
							61	24					69	18					64	12				
							78	19					64	15					48	4				
							28	19					33	9					55	13				

* The sucrose and test solution averages corresponding to all 1, 2, 5 & 10 mg/ml tests are significantly different ($P<0.01$), as determined by Chi-squared and Chi-squared heterogeneity tests.

A3.4 - Hordenine Data

Time (hrs)	Hordenine	Product	Av. HORD	Av. OX PROD	5 mg/ml	Pooled 1 mg/ml				
2	3	2	8.25	12	59.26	72.81				
	15	22								
	5	13								
	10	11								
2'	3	13	7.25	29.5	80.27					
	3	53								
	14	22								
	9	30								
4	7	5	5.75	12.25	68.06	77.31				
	8	20								
	6	9								
	2	15								
4'	4	15	6.5	29.5	81.94					
	5	41								
	13	25								
	4	37								
8	4	4	7.75	10.75	58.11	71.98				
	7	16								
	12	9								
	8	14								
8'	6	26	6.75	26.5	79.70					
	9	38								
	11	19								
	1	23								
24	11	13	7.5	14.5	65.91	71.08				
	6	17								
	6	18								
	7	10								
24'	7	18	7.25	21.75	75.00					
	7	28								
	11	23								
	4	18								
	* Test results significant for all observation times (P<0.01), as determined by Chi-squared and Chi-squared heterogeneity tests.									

A3. 5 - Hordenine vs Oxidation Product Data