A COMPARISON BETWEEN THE SALIVARY PHYSIOLOGY OF THE CRUSADER BUG, *Mictis profana* Fabricius (COREIDAE) AND THE GREEN LUCERNE MIRID, *Creontiades dilutus* (Stål)

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SUMMARY

The saliva of phytophagous Heteroptera, secreted into plant substrates during feeding, is implicated in the etiology of the often characteristic lesions produced by these insects. Similarities in salivary components within Heteropteran families allow for comparisons and conclusions drawn on any one representative species to be extended to other species of the same family. In this context, the salivary physiology of *Mictis profana* Fabricius (Coreidae) and *Creontiades dilutus* (Stål) (Miridae), representatives of two significantly important families of the Heteroptera, is investigated.

Feeding by *M. profana* causes initial water soaked lesions, followed by a concurrent increase in water content at the feed site, and acropetal terminal wilting of small diameter shoots of the host plant. Cells in the vicinity of the vascular bundles are selectively targeted by branches of the stylet tracks. Successive feeding punctures, indicated externally by the presence of stylet sheath flanges, occur at intervals of about 0.3 mm invariably in a basipetal direction. It is postulated that this successive basipetal adjustment of feed sites is necessary for the insect to counter the advancing tip senescence.

The posterior lobe contributes sucrase (sucrose α-D-glucohydrolase, EC 3.2.1.48) as the only carbohydrase in the watery saliva. The salivary sucrase was found to have a pH optimum of 7.25, with molecular weight (MW) ca 66,000, and exhibited weak maltase activity. It appeared in the saliva in variable dilution (recorded activities were from 0.002 to 0.01 Units/µl), consistent with a moderately diluting function by the structurally undifferentiated accessory gland. The only other enzyme secreted in the watery saliva was a catechol oxidase derived from the accessory gland. Given that sugars are not a limiting dietary resource it is postulated that the salivary sucrase, localised within the food plant by stylet sheath, induces a strong osmotic gradient by the conversion of endogenous sucrose to glucose and fructose, thus creating a general flow of nutrients to the region from which the insect is imbibing. This is supported by a concurrent increase of detectable amino nitrogen at the feed site. In attempts to simulate this process in pressure bomb experiments, infiltration of glucose and fructose, stoichiometrically equivalent to 0.5 M sucrose *via* a cut end of a lucerne, *Medicago sativa* L. shoot caused an increase in both the quantity and the amino acid content of tissue sap over that extractable by 0.5 M sucrose alone.

The feed site of *M. profana* is clearly a sink site, where phloem unloading of solutes actively takes place (previous work on phloem transport, while still to some extent controversial, has indicated the importance of sugars and plant invertases as a

stimulus for phloem unloading). The insect destroys the acropetal meristematic sink (its competitor), possibly by the sheer volume of liquids removed and certainly by causing acropetal necrosis. This may well be promoted by the secretion of salivary oxidases which should be capable of inducing a general 'leakiness' to plant cell membranes. At the same time, the insect creates a local stimulus that elicits phloem unloading presumably by elevating monosaccharides by action of the salivary sucrase. This, effectively is an enzymic mimic of the plants own invertase. The term "osmotic pump feeding" is proposed for such a process.

Like *M. profana*, *C. dilutus* causes water soaked lesions around the feed site. It feeds preferentially on the flower racemes and seed heads, a food source apparently obligatory for juvenile survival. Abscission of the flower buds and seed pods occurs with remarkably few feeding punctures.

Both a pectinase (endopolygalacturonase, EC 3.2.1.15) and an amylase were detected in the salivary glands of *C. dilutus*. The MW of the pectinase band was estimated at 105,000. In the salivary glands its presence was restricted to the posterior lobe. As with *M. profana*, a catechol oxidase was secreted into artificial agar diets and was detected from the accessory gland. Fundamentally distinct from the coreid, however, is absence from the feeding punctures of *C. dilutus* of any recognisable stylet sheath. In addition, almost certainly relevant to its feeding strategy, is the presence of a vesicular reservoir on the accessory gland. The lack of a functional stylet sheath would undoubtedly assist in the ability of the salivary pectinase to infiltrate plant tissues, and with enzymic maceration of the intercellular pectic matrix, allow pockets of cells, beyond the reach of the stylets, to be made available to the feeding bug. This is consistent with the need for large volumes of watery saliva (readily available from the accessory gland reservoir) which could be secreted then reingested during feeding. Although there is no reason to believe that pectic substances subserve a specific nutritional requirement for the mirid, there was a demonstrable release of bound proteins, neutral polysaccharides and other plant chemicals when stem sections were incubated in pectinase.

DECLARATION

The work described in this thesis has not previously been submitted to any university for the award of any degree or diploma.

Some of the work has already been published in scientific journals (Appendices One to Three).

The paper by Madhusudhan *et al.* (Appendix One) is a compilation of methods developed cooperatively by the three authors following frequent discussion and comparison of our research results, and it is difficult in retrospect to disentangle how much was contributed by whom. My main contributions were the development of standard protocols for colorimetric analyses and to some extent development of methods for demonstrating enzymic activity in electrophoresis gels.

In the paper by Miles and Taylor (Appendix Two), the part describing the pressure bomb experiments was entirely my own contribution, since I both suggested and developed the method and I obtained all the results using it. Conversely, the part describing the infiltration of stem section using a water pump was entirely the contribution of Dr Miles and, in consequence, I have not included it in the body of this thesis.

In the paper by Taylor and Miles (Appendix Three), some of the theoretical concepts that it tests and develops had previously been put forward in publications by Dr Miles, but otherwise the paper describes my own unaided work.

Publications notwithstanding, I believe the work described in the body of this thesis accurately describes my personal contribution to all the topics addressed.

This thesis may be made available for loan or photocopying provided that an acknowledgment is made of any reference to the work it describes.

Gary S. Taylor

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Firstly, to my parents, Glen and Margaret Taylor, for actively encouraging an early interest in entomology and other things biological.

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PREFACE

In Chapter One, the literature that provides a general background for the present study is reviewed. The Chapters that follow describe observations and experiments undertaken by the author. Each is divided into a brief abstract of the chapter, followed by a specific introduction, sections detailing materials and methods, results and a discussion. Chapter Two deals with the relevant aspects of the biology of the insect species primarily investigated including details of feeding processes. Chapter Three is a comparative description of the salivary apparatus of representative species of two, quite distinct, families of the Heteroptera, namely Coreidae and Miridae. Chapter Four describes the results of tests for salivary enzymes. Thereafter, each species is treated separately. Chapter Five characterises one particular, sucrose-hydrolysing enzyme in the saliva of the coreid and Chapter Six describes experiments aimed at determining how the enzyme functions during the feeding process of this and other coreids. As a parallel to the coreid study, Chapter Seven establishes the involvement of salivary pectinase in the feeding of the mirid and Chapter Eight describes experiments aimed at elucidating the involvement and *rationale* of the salivary pectinase during the feeding process of this and other mirids. In Chapter Nine the superficial resemblances and functional differences between the two types of feeding process are discussed.

It may be noted that the major part of the work described here deals with the coreid. This was largely dictated by the current status of understanding (and lack of knowledge) concerning the salivary physiology of coreids. Thus, in relation to the coreid, the present study introduces an essentially new concept in Heteropteran feeding. In contrast, experimental work on the mirid is more confirmatory in nature; nevertheless for consistency of presentation, chapters relating to the mirid have been presented in a fashion symmetrical with that adopted for the coreid. Furthermore, for accessability, the reference section has been placed at the end of this work rather than, by convention, between the body of the text and the Appendices.

Appendices One to Three comprise recently published papers that describe, in part, the work embodied in this thesis. Appendix One, co-authored with V.V. Madhusudhan and Dr P.W. Miles, is a compilation of methods developed cooperatively by the three authors. It comprises largely of materials and methods as

described in Chapters Four and Seven. Appendices Two and Three are co-authored with Peter Miles. Appendix Two investigates the possible function of the salivary sucrase of a coreid, as described in Chapter Six. Appendix Three investigate the composition of coreid saliva, characterises the salivary sucrase and proposes an hypothesis (see Appendix Two) as to its function. This comprises part of Chapter Four and all of Chapter Five.

CHAPTER ONE

A REVIEW OF SALIVARY FUNCTION IN THE PHYTOPHAGOUS HETEROPTERA, WITH SPECIAL REFERENCE TO THE SALIVARY INVERTASE AND PECTINASE OF THE FAMILIES COREIDAE AND MIRIDAE, RESPECTIVELY

1.1. SALIVARY FUNCTION IN HETEROPTERA

1.1.1. Morphology of the salivary apparatus and mouthparts of Heteroptera

The salivary glands in Heteroptera typically consist of a pair of principal and accessory glands situated in the thorax on either side of the foregut. The principal gland usually consists of an anterior and a posterior lobe in the families Miridae (Cimicomorpha) and Pentatomidae (Pentatomorpha). In Reduvioidea other than the Cimicomorpha, the principal gland may appear as a single lobe or possess at most a faint division into anterior and posterior lobes, as in the genera *Cimex* and *Rhodnius*. In the Pentatomorpha, however, the families other than the Pentatomidae typically have principal glands with additional lobes, e.g. with a lateral lobe as in Lygaeidae or with both lateral and median lobes as in Pyrrhocoridae and Coreidae. According to Baptist (1941) the anterio-median lobe complex of the quadrilobed glands corresponds to the typical anterior lobe while the remaining lobes are derived from the posterior lobe. Whereas Miles (1972), on physiological and functional grounds, believed all lobes other than the posterior lobe were derived from an original anterior lobe.

Bronskill *et al.* (1958) provided an anatomical and histological description of the salivary glands of the lygaeid, *Oncopeltus fasciatus* (Dallas). The paired, trilobate glands of that insect are about 3 mm in length and 1 mm in width and lie dorsolaterally along the prothoracic alimentary canal in the region where the oesophagus merges with the first stomach. The three lobes, anterior, lateral and posterior are joined at the hilus, a junction from which the salivary ducts emerge. Each lobe is covered by a network of trachea and tracheoles these being branches of the visceral tracheae of the first thoracic spiracle. The anterior end of the anterior lobe is innervated from the ganglionic mass that lies centrally above the brain, whereas the rest of the principal gland including the lateral lobe is innervated by the suboesophageal ganglion (Bronskill *et al.* 1958).

In most Heteroptera the glandular epithelium consists of a single layer of narrow glandular cells surrounding a cavity in each lobe. The nuclei are centrally

placed and the cytoplasm has a fine granular nature (Baptist 1941), the latter were thought to be secretory products stored as zymogenic granules (Bronskill *et al.* 1958). In an histological examination of the salivary glands of *O. fasciatus* they described the glandular epithelium as consisting of simple cuboidal secretory cells mounted on a basement membrane the latter surrounded by a layer of squamous epithelial cells termed the membrana propria. The salivary duct consists of simple cuboidal uni- or bi-nucleated cells with a thickened inner cuticular, thickly striated lumen wall, which, in the accessory duct, is convoluted. A non cellular basement membrane forms the outer border of the duct.

The functional part of the hemipteran mouthparts is the stylet bundle, composed of the mandibular and maxillae stylets which fit closely together to form a double canal system (Miles 1968, Miles 1987b). Saliva is pumped down one of these canals and fluids are sucked up the other. Feeding activity typically begins with superficial test probes of the substrate involving tactile monitoring by mandibular and labial mechanoreceptors. Simultaneous involvement of external chemoreceptors is to some extent problematical. Probably most Heteroptera have chemoreceptors on the antennae and tarsi, but experiments on labial chemoreceptors have failed to take into account that saliva is dabbed onto surfaces and almost immediately sucked back into contact with the epipharyngeal organ within the head, anterior to the sucking pump (Miles 1958). A continued function for the saliva in gustatory monitoring of the food source is indicated by the mode of action of the stylets, which appear to engage in directed searching activity while penetrating substances as described below.

The stylets advance mainly by reciprocating movements of the mandibular stylets (Miles 1987b), while the maxillae typically move together in advance of or behind the tip of the protracted mandible (Pollard 1973). Directional control of the stylet has been described (Miles 1958) for the lygaeid *O. fasciatus*. The tips of the mandibular stylets are outwardly barbed and have an inward curve. When a mandible is stationary, either in advance of or behind the rest of the stylet bundle, the barbs of the tip anchor it in the medium. If one mandible is advanced alone, however, into a solid or semisolid medium, the curvature of the tip tends to deflect it contralaterally and the stylets that follow are forced along that path. Progress straight ahead is achieved by advance of the combined maxillae, either alone or with one mandible, its curved tip fitting neatly over the apex of the maxillae without influencing their direction. Retraction of the bundle is facilitated by retreat of the maxillae with the tip of the leading mandible again curving over the apex of the combined maxillae, thus disengaging the mandibular barbs from the medium. The whole bundle can thus be withdrawn for any required distance by a reciprocating withdrawal of the maxillae with first one then the other mandible (Miles 1958). By

these means, some (by inference, possibly all) Pentatomorpha can partially withdraw their stylets and commence a new pathway, resulting in a series of branched tracks (Miles 1958, Naito and Masaki 1967).

Such branching can be interpreted as searching activity for a suitable, definitive feeding site within the substrate. For such a search to be effective, however, some means of monitoring the substrate is required. At one time, it was postulated that neurones in the mandibular stylets of Homoptera and Heteroptera were gustatory, but later work showed that they were tactile. Later observations on various Homoptera and Heteroptera make it most likely that, as with gustatory monitoring of surfaces, ejection and sucking back of saliva subserves gustatory exploration of the interior of substrates (Miles 1987b).

1.1.2. The composition of the saliva of Heteroptera

During the course of feeding, the Homoptera and Heteroptera: Pentatomorpha typically discharge two types of saliva. One is a solidifying component termed "sheath material", and there is also a "watery saliva" that contains salivary enzymes (Miles 1972).

1.1.2.1. The stylet sheath

The solidifying component of the saliva typically forms a tubular stylet sheath, composed of disulphide and hydrogen bonded lipoprotein. The precursors of the stylet sheath are maintained in solution within the glands by accessory sulphydryl compounds which keep the sheath precursors in a reduced condition and by free amino acids which prevent hydrogen bonding. As the sheath precursors are ejected, they solidify rapidly as they are exposed to oxygen and the sulphydryl and amino acid solutes are allowed to diffuse away (Miles 1972). The sheath itself consists mostly of protein but contains about 10% phospholipid (Miles 1964b, 1967b) which enables it to bond to waxy plant surfaces (Saxena 1963). Kinsey and McLean (1967) showed that the stylet sheath once formed is impermeable; they demonstrated that dyes did not pass from within the sheath to the plant tissue and concluded that soluble plant material was obtainable only from the open tip of the stylet sheath, as demonstrated by Miles (1964b).

The function of the stylet sheath has remained "obstinately controversial" (Miles 1990). It has been implicated in the attachment of the labium to the host plant during feeding (Saxena 1963), in the prevention of the loss of watery saliva into intercellular spaces (Miles 1972), in providing a lubricating casing to the stylets (Smith 1985) and in sealing the stylets around the immediate feeding site, which, as in phloem, may be under pressure (Mittler 1954). Its precise function however remains uncertain as a complete stylet sheath is facultatively produced in some Homoptera and Heteroptera (Backus 1988). Pollard (1973) postulated that sheath

material seals off ruptured cells and prevents the influx of unwanted substances into the bore. It also may serve to bridge cells and intercellular spaces (Lowe 1967) and to protect the stylets from non nutritive or even highly toxic substances (Miles 1965). It may also seal off wounds made during plant penetration and minimise the mobilisation of plant compounds involved in defence against invading organisms or in hypersensitive reactions to wounding (Miles 1987b).

1.1.2.2. The watery saliva

Many of the reports on salivary composition of sucking insects, certainly those published before 1960, relied on analyses of the salivary glands rather than on the composition of the saliva actually ejected by the insects. Such studies, especially those that relied solely on extracts that contained contents of gland cells would not have been able to distinguish between the endoenzymes involved in the synthesis of salivary components and those actually secreted. In the introduction to work on salivary enzymes included below, the authors quoted are those who made such a distinction.

The watery saliva is alkaline with a pH of between 8.0 and 9.0 (Miles 1987b). It is ejected during substrate penetration and feeding. It may assist in the formation of the stylet sheath (see below). Some is reingested by the insect during feeding but a proportion may diffuse into the plant. The watery saliva of Homoptera and Heteroptera has been variously found to contain digestive enzymes (hydrolases) such as esterases (Feir and Beck 1961), cellulase (Adams and Drew 1963), amylase (Hori 1970a, Nuorteva and Laurema 1961), pectin polygalacturonase (Adams and McAllan 1958, Laurema and Nuorteva 1961, Strong and Kruitwagen 1968, Hori 1974), proteinases (Nuorteva 1958, Feir and Beck 1961), acid phosphatase and phosphorylase (Kloft 1960), proteinase (Nuorteva 1956, Nuorteva and Laurema 1961) and invertase (Miles 1987a); both the stylet sheath and the watery saliva have also been shown to contain oxidising enzymes with diphenolase (catechol oxidase) and peroxidase functions (Miles 1968, Miles and Slowiak 1970).

The salivary glands of individual species may contain more than one salivary enzyme. Miles (1960b) recorded an amylase (using starch) in the lumen of the posterior lobe of *O. fasciatus*, and an invertase and maltase from the gland as a whole. Proteinase, lipase, pectinase and cellulase were not detected in the salivary glands but the former two were found in the gut. In subsequent work Miles (1967a) reported an amylase and an esterase, both from the lumen of the posterior lobe, and a polyphenol oxidase from the accessory lobe and accessory duct.

Baptist (1941) found no more than two enzymes in the posterior lobes in a number of Heteroptera tested. Protease and lipase were detected in various predatory species representing several families, invertase and amylase in a lygaeid, an amylase

only in a coreid and an invertase only in a pentatomid, a pyrrhocorid and a lygaeid. In species that suck vertebrate blood, e.g. *Triatoma, Rhodnius and Cimex*, no digestive enzymes were detected but there was a positive reaction for an anticoagulating enzyme. He concluded that digestive enzymes in the saliva of Heteroptera were related to the type of food consumed and were concerned with the digestion of the particular component of the food which represented the greatest proportion.

As indicated above, the saliva also contains significant quantities of amino acids (Miles 1964b) which are thought to prevent the premature gelling of the stylet sheath precursors (Miles 1987b). The presence of phenolic compounds have also been detected (Miles 1969). The growth-inducing substance indole-3-acetic acid (IAA) has been found in the salivary glands of aphids and some heteropterans (Miles and Lloyd 1967, Miles 1987b) but was not concentrated in the salivary glands of *Creontiades dilutus* (Stål) when IAA was incorporated in artificial diets (Miles and Hori 1977, Hori and Miles 1977). Miles (1964a) found that distribution of oxidase activity within the glands was similar for related species but often varied between families: e.g. similarities existed between the three pentatomids tested and the closely related scutellerid; between the lygaeids and a pyrrhocorid; nevertheless, among the aphids tested, *Macrosiphum* differed somewhat from the two species of *Aphis*. Of the Heteroptera: Pentatomorpha tested, the diphenolase reaction with dihydroxy-phenylalanine (DOPA) occurred in the accessory gland and duct and in the principal salivary duct, whereas in the Cimicomorphic families (Miridae and Nabidae) phenolase activity was found only in the accessory apparatus.

1.1.2.3. Methods of collection of Hemipteran saliva

Saliva has been obtained for chemical analysis by the recovery of salivary deposits on membranes (Edwards 1961, Miles 1959), by collecting saliva directly from the mouthparts (Miles 1967b, Strong 1970) sometimes induced by high humidity or the application of pilocarpine (Miles and Slowiak 1970), by collecting saliva through the husk of its foodplant (Feir and Beck 1961) and through a membrane (Adams and McAllan 1958, Miles and Harrewijn 1991). Whole glands or separated lobes of larger Heteroptera have also been tested for their content of salivary enzymes (Bronskill *et al.* 1958, Miles 1967a, Hori 1968b).

1.1.3. A classification of the feeding strategies of Hemiptera

The modes of Hemipteran feeding were classified by Miles (1972) into five categories depending on the function of the salivary components and their host plants. These categories are scratch and suck feeding of Tingidae, stylet sheath feeding of Homoptera and the pentatomorphic Heteroptera, lacerate and flush feeding

of seed feeding Lygaeidae, Miridae, some Tingidae and Pyrrhocoridae, predation by some Miridae and Reduviidae and blood sucking in many of the Reduvioidea. Two of these feeding strategies are considered in further detail below.

1.1.3.1. Stylet sheath feeding

With stylet sheath feeding small amounts of sheath material are secreted onto the plant surface prior to stylet penetration. This has been termed the "flange" by Nault and Gyrisco (1966). The excretion of this solidifying component of saliva is continued whereever the stylets penetrate, thus forming a solid tube or lining. This is achieved by successive backward and forward movements of the stylets (Miles 1960a) with the excretion of a drop of solidifying saliva on each backward movement, the subsequent excretion of watery saliva to make it "balloon" out followed by forward movement of the stylets (McLean and Kinsey 1965, 1968). In this manner it is theoretically possible for the insect to secrete a stylet track without the stylets coming into direct contact with the substrate (Miles 1965). The stylet sheath assumes a beaded appearance in a soft agar medium (Miles 1965, 1968, 1987b) but in plant tissue the sheath may flow into irregular spaces surrounding the stylets. The watery saliva with its complement of amino acids and salivary enzymes may diffuse some distance from the tip of the stylets. Enzymes such as pectinase and cellulase have been thought to assist in the penetration of the stylets (Miles 1972); also the plant tissue is being continually sampled by the successive sucking back of small amounts of liquid (McLean and Kinsey 1967).

Experiments on the release of watery saliva into plants have mostly been done on aphids. Once their stylets have reached a source of readily ingestible fluids such as a phloem sieve tube, release of saliva into the plant may appear to cease (Kloft 1960), although electronic monitoring and analysis of feeding has indicated that watery saliva may be continually mixed with the ingested fluids and several authors report more or less continuous release of radio labelled substances from aphids while they are feeding (reviewed in Miles 1987b). In species which feed on mesophyll tissue, there seems little doubt that watery saliva is secreted throughout feeding (Kloft 1960): they presumably eject saliva into each cell as it is encountered and suck out the contents before secretion of the sheath is continued and feeding commences in another cell (Miles 1972).

1.1.3.2. Lacerate and flush feeding

Lacerate and flush feeding typically occurs in Lygaeidae, Pyrrhocoridae, and some Pentatomidae and Miridae (Miles 1972) which feed on the seeds or growing tips of plants. Those which feed on both mainly secrete a complete stylet sheath and feed as indicated above on growing tissues but produce only an abbreviated one

within seeds (Miles 1967a, Bongers 1969). The pentatomid, *Eurydema rugosa* Motchulsky, which is also a facultative seed feeder, appears to produce a shortened stylet sheath even in vegetative tissue (Hori 1968a). Mirids, however, which feed on growing tissue and fruiting bodies, appear not to produce a stylet sheath at all.

Once the stylets of the lygaeids *O. fasciatus* on milkweed and *Dysdercus koenigii* (F.) on cotton are within the seed they are moved about rapidly for periods of up to two hours, with the secretion and sucking back of watery saliva (Saxena 1963). During this time the entire contents of small seeds or areas of larger seeds are liquefied and removed (Miles 1959, Saxena 1963). The feeding behaviour of the seed-feeding milkweed bug, *O. fasciatus,* was reported in further detail by Feir and Beck (1963). They categorised feeding behaviour into (a) orientation and initiation of feeding, (b) duration and maintenance of feeding, and (c) cessation of feeding. Orientation to the food source was achieved over small distances by the detection of olfactory stimuli from the seed coat by chemosensory receptors on the antennae. The search for food was otherwise random. A suitable feed site on the seed is located by successive sampling of chemosensory cues after contact with the seed coat by the labium, after which stylet penetration occurred. The number of feeding attempts and the duration of feeding was greatly reduced when the seed coat was removed yet was close to normal when a relatively inert diet of powdered cellulose was covered by a seed coat. This may have indicated a continued chemosensory input from chemoreceptors on the labium, although the experimenters may not have prevented the possible diffusion of phagostimulants from the covering testa into the inert diet. Bugs invariably moved to a source of water directly after feeding on seeds. Cessation of feeding may be caused by dehydration or satiation (Feir and Beck 1963) or possibly by the depletion of salivary enzymes (Miles 1959).

Stylet sheath feeding typically causes little immediate phytotoxic effects but more long term disturbances to growth (Miles 1972) whereas lacerate and flush feeding results in an immediate lesion over an area greater than the reach of the stylets (Miles 1987a). Miles (1968) suggested that the small lesions made by stylet sheath feeders of the Pentatomorpha maximised the efficiency of feeding on individual parenchymal cells by preventing loss of contents into intercellular spaces, whereas lacerate and flush feeders, with an essentially vestigial stylet sheath, need to flush out the contents of ruptured tissues with large quantities of dilute saliva.

Hori (1968a) in a description of the feeding behaviour of the pentatomid, *E. rugosa* estimated the reach of the stylets to be the total length of the first and second segments of the rostrum. This species forms a stylet sheath only in the immediate vicinity of the stylet puncture and feeds for several hours at a time, during which the insect partially retracts its stylets and recommences feeding along a newly formed branch of the stylet track. In this manner a number of radiating arms extend

from the initial stylet puncture. The lesion becomes senescent, forming white blotches yet does not increase in size. He followed the terminology of Carter (1962) in classifying these symptoms as local lesions. Other categories in order of increasing severity are local lesions with development of secondary symptoms, tissue malformations, and symptoms indicating translocation of the causal entity, both limited and systemic. Hori (1968a) concluded that, as the lesion produced by *E. rugosa* did not extend beyond the possible reach of the stylets, the feeding damage was caused only by mechanical destruction of cells and not by any specific salivary secretion.

1.2. ETIOLOGY OF COREID AND MIRID LESIONS

The feeding mechanisms of two heteropterans, a mirid, *Helopeltus clavifer* (Walker) and a coreid, *Amblypelta* sp. which produce considerable damage to cocoa and cassava respectively in Papua New Guinea were compared by Miles (1987a). Both species were found to cause darkened lesions extending some considerable distance from both the feeding puncture and the limit of the extended stylets. Those of *H. clavifer* occurred in parenchymal tissue close to or immediately below the epidermis of cocoa pods and appeared rapidly as water soaked areas. The effects of the *Amblypelta* sp. were not always and immediately visible externally but when tissue in the region of a feeding puncture were dissected, lesions were observed below the vascular tissue of stems, extending up to 7 mm axially and about 3 mm laterally. Based on measurements of manipulated mouthparts, maximum exsertion of the stylets was determined to be 0.15 mm for *Amblypelta* sp. and 0.05 mm for *H. clavifer*. Lesions from each extended a further 3.5 mm beyond the possible penetration of the stylets.

When *Amblypelta* sp. fed on young sweet potato stems and cassava tips a few millimetres in diameter, the stylets typically penetrated 0.6 mm into the pith parenchyma yet the lesions extended a further 3-4 mm in both directions along the stem and often resulted in the death of a growing tip. The coreid stylet track was defined by the presence of a stylet sheath which filled punctured cells but had little effect on immediately adjacent, intact cells. The stylet track of the mirid, however, was defined by no more than a few solid deposits, possibly similar in nature and origin to the stylet sheath of Pentatomorpha. In addition, the lesions of the mirid were characterised by oxidative melanisation of both ruptured and adjacent cells.

All feeding tracks ended in parenchymal cells which either had their contents apparently removed or plasmolysed. In lesions caused by the coreid on young sweet potato tissues, many of the cells adjacent to the feed site collapsed and the intercellular spaces became enlarged. No such enlargement occurred in the more

robust cassava tissues but, after several days, necrotic cells shrank away from the feed site and a large necrotic cavity-like lesion was formed. Cells in the lesions caused by the mirid remained much the same size but darkened in time as oxidised substances diffused in. Prominent dark patches resulted on cocoa pods within one day of feeding of the mirid and the coherence of cell walls was markedly reduced in those several days old.

Miles (1987a) also detected the presence of invertase in the salivary glands of the coreid and a pectinase in those of the mirid and attributed the formation of their lesions in part to the presence of these enzymes. He postulated that the coreid invertase resulted in the release of osmotically active substances into the intercellular spaces causing an efflux of nutrients from surrounding cells which was then ingested by the insect.

1.2.1. Australian Coreidae and Miridae considered in this discussion

The present work will investigate other species belonging to these same families and the possible roles of their respective salivary enzymes in their feeding strategies, in particular, the effect of their salivary enzymes on the physiology of their hosts.

Mictis profana Fabricius (Coreidae) was selected for experiments for a number of reasons. Like *Amblypelta* sp. discussed above, it secretes a readily demonstrable sucrose-hydrolysing enzyme in its saliva. Locally, it is a commonly occurring species regularly encountered throughout the greater part of the year. Also, due to its size it was considered suitable for the procurement of large volumes of 'pure' saliva for observations on its enzyme activity. Whilst not a significant pest species in itself, it represents a family of considerable economic importance worldwide.

Preliminary experiments confirmed the presence of a powerful salivary pectinase in *C. dilutus* (Miridae). This species is smaller than *M. profana*, is somewhat more seasonal in occurrence, but periodically attains immense populations in stands of lucerne. It is an important pest species in numerous crops and is a representative of perhaps the most agriculturally destructive of all Heteropteran families.

Thus an understanding of the nature of the salivary secretions of these two species could add significantly to an understanding of the etiology of plant lesions. Moreover, with the growing importance of genetic transformation to provide crop cultivars with pest resistance, such understanding could arguably have significant implications in directing a search for resistance genes.

1.3. ENZYME FUNCTIONS

1.3.1 Invertase

1.3.1.1. Properties of sucrose-hydrolysing enzymes

Invertase was first extracted from yeasts in 1860 by Berthelot (Ohlenbusch and Vögele 1974) and has been known the longest of any of the carbohydrases (Lampen 1971). Invertase activity has been demonstrated in bacteria, moulds, higher plants, in the intestine of a number of animals, including humans (Ohlenbusch and Vögele 1974, Dalqvist 1974) and in insects (Miles 1987a).

Invertase (β-fructofuranosidase, β-D-fructofuranoside fructohydrolase, sucrase, saccharase, EC 3.2.1.26) from yeast is a glycoprotein with a molecular weight of about 270,000 (Bergmeyer *et al.* 1974). The enzyme has a pH optimum of 5.4, and catalyses the hydrolysis of sugars that have an unsubstituted end terminal β-D-fructofuranosyl residue. The nature of the remainder of the target molecule is of comparatively little importance in determining substrate affinity, although large sugar moieties decrease the rate of cleavage (Lampen 1971). The preferred substrate is sucrose which is essentially a molecule comprising a fructose (β-fructofuranoside) and a glucose (α-glucoside) moiety. Consequently there are two different types of sucrose hydrolysing enzymes, the β-fructofuranosidases (EC 3.2.1.26), and α-glucosidases (sucrose α-D-glucohydrolase, EC 3.2.1.48) which disassemble the sucrose molecule from the fructose and glucose end respectively. β-fructofuranosidase is characterised by its ability to hydrolyse raffinose and is typically found in yeasts, fungi and plants, while sucrose α-D-glucohydrolase is inactive towards raffinose (Stecher *et al.* 1968), but shows some maltose activity and is typically found in the intestinal mucosa of animals (Kolinská and Semenza 1967, Dalqvist 1984).

The catalytic properties of yeast invertase indicate that the specific active site of invertase combines with the glycosidic oxygen and the -OH groups at C-6 and C-3 of the fructose moiety whereby cleavage of the glycosidic linkage occurs on the fructose side of the glycosidic oxygen. A specific interaction with the -OH at C-2 of glucose is supported by the apparent preference for sucrose over other disaccharides (Lampen 1971). Purified yeast invertase has been determined to have a specific activity of 1,000- 3,000 units per milligram (U/mgm) protein at 25°C and extracts from bakers yeast have a specific activity of 30-40 U/mgm (Ohlenbusch and Vögele 1974). In comparison, bee 'invertase' which causes the hydrolysis of ingested sucrose (and maltose), was characterised as an α-D-glucoside glucohydrolase (Huber and Mathison 1976) and has a specific activity of only 2 U/mgm at 30°C (Ohlenbusch and Vögele 1974). Honey, in fact, is comprised almost entirely of

invert sugar, a 50% mixture of each of fructose and glucose, due primarily to hydrolysis of the sucrose molecule (Stecher *et al.* 1968).

Several methods can be utilised in the determination of the activity of disaccharases including invertase. One of these is by the estimation of reducing sugars by the method of Nelson (1944) with the appropriate disaccharide (sucrose, raffinose, maltose, trehalose) used as a substrate (Dahlqvist 1984). Activity of enzymatic hydrolysis to glucose and fructose may also be determined by estimating specifically the amount of glucose liberated: enzymic activity is stopped by heat inactivation and glucose oxidase, peroxidase and *o*-dianisidine are added after a standard interval; the resulting colour reaction is stopped with acid and colour development read spectrophotometrically (Goldstein and Lampen 1975). The amount of invertase activity is defined as the amount of enzyme at pH 4.9 which hydrolyses sucrose to produce 1 µmole of glucose per minute at 30°C, and specific activity is expressed as units per mgm of protein, the latter estimated by the method of Lowry *et al.* (1951).

The presence and characterisation of yeast and the fungal invertases have been examined in detail in the review of Lampen (1971). Due to its historical status and economic importance a great deal of the catalytic properties of yeast invertase are known. Purification of the invertase from yeast is, however, complicated by the heterogeneity of yeast extracts, which are known to possess a complex of closely related enzymes.

In yeasts, enzyme activity occurs within the outer edge of the yeast cell wall which is outside the permeability barrier of the plasma membrane (Lampen 1971); in the fungal pathogen, *Neurospora,* most of the enzyme is retained within the fungal cell but external to the cell membrane and thus freely accessible to external substrates. The isolated enzyme has a molecular weight of 210,000 with four disulphide bonded subunits each with a molecular weight of 51,500. Its pH optimum is broad, between 4.5 and 6.0. Sucrose and raffinose in order of preference are disassembled by the fungal invertase but trehalose is not (Lampen 1971).

The presence of invertase from micro-organisms (β-fructofuranosidase) and from the digestive systems of higher organisms (sucrose α-D-glucohydrolase) can be clearly explained in terms of direct nutritive benefits. The salivary invertases of the Heteroptera, however, are not so easy to rationalise. Little is known about them: their presence has been recorded (Miles 1987a) but their characterisation and properties have not. The nutritive significance of invertase in the salivary apparatus of certain phytophagous Hemiptera is problematical since sucrose is usually greatly in excess in the diet of phytophagous Heteroptera and only some of them possess the salivary enzyme. Thus the presence of a sucrose hydrolysing enzyme in the saliva may have a predigestive rather than a dietary role since the products cannot be

considered a limiting resource as are e.g. the amino-nitrogenous compounds. The possibility of an osmotic role has previously been alluded to (Miles 1987a), and indeed may be an important factor in the otherwise passive imbibement of nutrients. As stated above, the higher plants themselves possess a compliment of invertases which are implicated in phloem transport. It is thus conceivable that the insect enzymes mimic the functions of those of the plant, thereby modifying the plant's physiological status to the insect's advantage. An understanding of plant transport systems is therefore required in the investigation of such a strategy.

1.3.1.2. Role of sucrose and invertase in plant metabolism and phloem transport

The concept of phloem transport has been investigated by plant physiologists for many years and is discussed in terms of "sources" and "sinks", terms first coined by Mason and Maskell (1928). A source is defined as a site which supplies specific materials to the plant transport system and a sink is a site at which substances are being removed. Typical sources are mature leaves, tubers, rhizomes and cotyledons; sinks include shoot apices, roots and developing storage organs (Ho and Baker 1982, Dale and Sutcliffe 1986). An organ can be a designated sink for some compounds and at the same time be a source for others. Thus a shoot apex can be a sink for carbohydrates and a source of auxins (Dale and Sutcliffe 1986). Furthermore there may be temporal separation. Cotyledons of (pea) seeds may function as sinks during their development and as sources during germination, with a corresponding influx and efflux, respectively, of amino acids and other compounds (De Ruiter *et al.* 1984). The concepts of sources and sinks can be applied equally at the cellular or subcellular levels. That is, a cell may be a sink during its growth and a source, *i.e.* photosynthetic, at maturity; at the subcellular level, organelles such as chloroplasts may act as sources of carbohydrates while others such as mitochondria behave as sinks. The strength of a source may be measured in terms of its metabolic activity, while that of a sink is its ability to utilise or store transported materials.

Import of assimilates reduces rapidly with the development of photosynthetic activity. When expressed as carbon fixed per unit leaf area, photosynthetic activity reaches a maximum when the leaf is from one to two thirds expanded and may gradually decline once laminar expansion nears completion (Dale and Sutcliffe 1986). A significant correlation between photosynthetic activity and the amount of translocated carbon was found by Hofstra and Nelson (1969) and a linear relationship between rate of photosynthesis of a source leaf and the import of carbon by an adjacent sink leaf was found in sugar-cane over a range of light intensities (Serviates and Geiger 1974). These data may, however, be somewhat confounded by the partitioning of carbon between export and storage pools in the same tissue as

concluded by Ho (1976), since some of the assimilated carbon is stored as starch and only a percentage of stored products is mobilised. Partitioning between storage and phloem loading is a complex interaction but it appears that starch accumulation is controlled independently of photosynthesis and is correlated with and modified by the intensity of other processes utilising the same substrates, including sucrose synthesis and translocation (Silvius *et al.* 1979). The possibility exists that carbon translocation is assisted by increased activity of enzymes responsible for starch disassembly (starch phosphorylase) and sucrose synthesis (sucrose synthase, sucrose-phosphate synthase), providing further evidence for a biochemical or enzymatic process of translocation control (Dale and Sutcliffe 1986). Interestingly, Ho (1976) found a highly significant correlation between the levels of sucrose in fully expanded tomato leaves and the rate of export of carbon.

Photosynthetic assimilates are loaded into the phloem via the extensive network of minor veins which interconnect the mesophyll cells (Geiger and Cataldo 1969). The pathway of loading has been well documented for sugar beet (Geiger 1975, 1976). Phloem loading is the relatively rapid process by which photosynthates and other translocated molecules are accumulated and concentrated in the sieve elements. The actual pathway of assimilates from chloroplast to sieve element and the active site of phloem loading, however, remains uncertain. The loading of sucrose from the mesophyll into the sieve elements and the unloading from the sieve elements into sink cells may take place via the plasmodesmata (the symplastic pathway) or through cell walls into intercellular regions (the apoplastic pathway) (Ho and Baker 1982). A symplastic pathway requires a downhill concentration gradient of assimilates between the mesophyll and the sieve elements and to the sink cells; an apoplastic pathway, however, requires transport of sucrose across the plasma membrane and hence may require energy. In sugar-beet leaves, movement of sucrose through the parenchyma cells is symplastic, but movement from them is into the free space. Once in the apoplast, sucrose diffuses along a concentration gradient which can be enhanced by an efflux of sucrose or a rapid removal by the loading process (Dale and Sutcliffe 1986). Loading may also be stimulated by the addition of ATP to the leaf (Kursanov and Brovchenko 1961, Ho and Baker 1982, Sovonick *et al.* 1974). It has also been postulated that sugars and some amino acids are involved in a proton co-transport involving an ATP driven pump, which provides a proton efflux and a potassium influx (Malek and Baker 1977, 1978). Other correlates of active (meristematic) sinks are the high concentrations of the growth regulating substances such as auxins, gibberellins and cytokinins (Goodwin 1978). Whatever the precise mechanism, however, the importance of sucrose is certain. Sugars are readily transported across membranes yet are not readily broken down in the transport path. In addition Fondy and Geiger (1977) found that, from a mixture

of sugars, only sucrose was rapidly loaded and concentrated in the phloem. In summary, the supply of assimilate to a sink may thus involve movement in the apoplast or symplast or both, with both active and passive transport systems. The concentration gradient between phloem and sink is important in that rapid utilisation of metabolites at a sink causes a correspondingly large gradient between it and the phloem and hence increased phloem unloading (Gifford and Evans 1981).

Phloem unloading is the process by which assimilates are translocated from the sieve elements into sink cells and, like loading, may involve both the symplastic and apoplastic routes (Ho and Baker 1982). In growth sinks such as growing leaves and root tips there is a continuous symplastic connection between the sieve elements and surrounding tissue (Turgeon and Webb 1976). They are thought to involve the transport of all metabolites other than sucrose out of the sieve elements by passive diffusion (Dale and Sutcliffe 1986). The fate of sucrose has been investigated by several researchers, some of whom consider that sucrose too diffuses out of the sieve tube along a concentration gradient towards a sink (Keener *et al.* 1979, Gifford and Evans 1981) and that this symplastic unloading is maintained by the metabolic activities of the sink (Geiger and Fondy 1980). Other hypotheses involve the possibility of an ATP driven mechanism (Browning *et al.* 1980, Cronshaw 1980, Wareing 1978) or the active unloading of sucrose caused by its hydrolysis by invertase (Eschrich 1980). It is also postulated that invertase at the sieve element controls the release of sugars from the phloem only as glucose and fructose (Keener *et al.* 1979). In sugarcane stems, sucrose translocated in the phloem is hydrolysed by cell wall bound invertases (Glasziou and Gayler 1972, Hatch *et al.* 1963), the products of which are resynthesised into sucrose phosphate and stored as sucrose in the vacuoles. This is further supported by Giaquinta (1979) who found high levels of invertase in the actively growing roots of sugar-beet which disappeared at the commencement of the sucrose storage phase, at which time the reversible enzyme, sucrose synthase, increases its activity. Hence invertase is thought to be important in the partitioning between sucrose translocation and storage.

1.3.1.3. Intervention of Hemipteran saliva in nutrient transport in plants: formation of an hypothesis

Sinks are defined as regions of the plant where translocated metabolites are being received and typically occur as meristems and other actively growing, or storage organs. Speed of translocation of assimilates is proportional to sink size according to Wardlaw and Moncur (1976), who reported a reduction of translocates with the removal of grains from a cereal head. Peel and Ho (1970) varied the colony size of the willow aphid, *Tuberolachnus salignus* (Gmelin), to create artificial sinks of varying size and, by collecting aphid honeydew, determined that as sink size grew

so did the rate of translocation. More honeydew was secreted by the larger colony than on the basis of biomass alone and they concluded that a greater length of stem contributed to the maintenance of the larger colony and also that there must be a corresponding increase in the rate of translocation and/or number of sieve elements tapped. In general, for sinks of the same kind, sink strength is proportional to size such that when a large and a small sink are equidistant from a source the larger sink will be favoured (Dale and Sutcliffe 1986). Sink location in respect to its source is also of importance. Developing tomato fruit (Khan and Sagar 1967) and the bolls of cotton (Brown 1968, Horrocks *et al.* 1968) both receive assimilates from the leaves immediately surrounding them. A number of other compounds however are translocated over some distances in the plant such as nitrogen compounds which may originate from the roots or other distant parts of the plant.

Sucrose was the major carbohydrate in the phloem, the latter accounting for about 80% of translocates (Zimmerman 1957a). Phloem sap is typically slightly alkaline with a pH of 7.8-8.4 and of much higher dry matter content than xylem sap. Total sucrose concentration was found to vary from 10% in *Ricinus* phloem (Hall and Baker 1972) to 25% in different trees depending on the species, time of day and season (Zimmerman 1957a,b). Carbohydrate concentration varies from 5 to 30% and accounts for 80-90% of dry matter. Rabideau and Burr (1945) demonstrated that sucrose was translocated from the source in the leaf and at the junction of the leaf petiole moved both up and down to sinks in the shoot apex and root respectively. Very little was found in mature leaves. Glucose and fructose were not found in phloem exudates but were detected in a 1:1 ratio in the phloem cells, suggesting that they are not translocated and that they probably occur only in the non-conducting cells of the phloem. Alternatively they may be transported so efficiently that they do not accumulate in the phloem channels and appear only in the accumulating cells (Dale and Sutcliffe 1986). The concentration of nitrogenous compounds in the phloem of a number of plants is low in comparison with total carbohydrates and of the order of 0.001 M (Zimmerman 1957a) or up to 1% (Pate 1975). Mittler (1958) detected amino acids and amides in the exudate produced by stylectomy of aphids and found that the concentration of nitrogenous compounds varied throughout the year; it was highest during leaf expansion, decreased to a low value, then increased again as the leaves became senescent and nutrients were mobilised.

It is conceivable that bugs which utilise a salivary 'invertase' do so to create an artificial sink such that the plant is induced to mobilise stored assimilates and to translocate photosynthates to the feeding site. It is possible for the insect to imitate plants physiological cues to its own benefit by initiating an efflux of sucrose by hydrolysis. This hypothesis is supported by the location of the preferred feeding site of *M. profana*, which is the actively growing terminal shoots of its host plant. Here

the leaves are rapidly expanding and photosynthate from nearby sources is being translocated to them.

In the context of sources and storage it may be opportune to mention the role of certain plant enzymes in the reactivation or mobilisation of stored metabolites. For a number of sink organs, starch rather than sugars is the principal form of storage carbohydrate. In corn kernels, imported sucrose is enzymatically hydrolysed, the hexoses forming the precursors for starch synthesis (Shannon 1972, Shannon and Dougherty 1972). Interactions between sources and sinks in specialised storage organs such as tubers and seeds are known to be dependant on a biochemical signal which stimulates, at least in the latter, the activation of gibberellins (Jones 1971).

Interestingly amylase is one of a group of hydrolytic enzymes which are important in modulating the flow of metabolites from the endosperm to the growing tips of young seedlings (Baset and Sutcliffe 1975). Amylase has been detected in the salivary glands of Homoptera and several Heteroptera. Presumably it functions to solubilise starch deposits for digestion, but it is at least conceivable that when such insects are feeding on growing tissue, a salivary amylase may be involved in the mobilisation of stored source products by mimicking the plant's physiological cues, just as salivary 'invertase' serves to indicate a sink and divert phloem transport.

1.3.2. Pectinase

1.3.2.1. Properties of pectolytic enzymes

Laurema and Nuorteva (1961) tested for the presence of pectin polygalacturonase in the salivary glands of 17 species comprising 8 families of Homoptera: Auchenorrhyncha and 13 species of Heteroptera in the families Lygaeidae, Coreidae, Pentatomidae and Miridae. Only in the Miridae, and indeed in all six of the species tested, the presence of pectin polygalacturonase was detected. This enzyme had previously been detected in the saliva of a number of Aphididae and from only one species from each of Homoptera: Auchenorrhyncha and Heteroptera (Adams and McAllan 1956, 1958, McAllan and Cameron 1956). The heteropteran tested by Adams and McAllan (1958) was a lygaeid whereas that tested by Laurema and Nuorteva (1961) showed no pectinase.

Pectolytic enzymes are widely distributed in higher plants, microorganisms, and some protozoa, nematodes and, of course, insects. In higher plants they are involved in cell elongation, softening of some plant tissue during maturation, in the decomposition and recycling of plant materials (Whitaker 1990), and in the allelochemical responses of plants to invasion by pathogens (West 1981). At the same time, microbial pectolytic enzymes are involved in plant pathogenicity, vegetative spoilage and rotting.

Their substrate, the pectic substances are a heterogeneous group of insoluble polysaccharides of no definite molecular mass that are found principally in the middle lamella which serves to hold the cells together, and in the cell walls themselves of higher plants (Whitaker 1990). The main structure is a backbone of polygalacturonic acid units. Intermediate rhamnose units also occur and there is cross linkage to other polysaccharides. The insolubility of pectic substances within the plant is due to polymer size and to cross linkage. Soluble pectin (polymethylgalacturonate) molecules are composed of subunits with at least 75% of the carboxyl groups of the galacturonate units esterified with methanol. Pectic acid (polygalacturonic acid) is a soluble material in which the methyl groups have been removed. Derivatives with intermediate degrees of demethylation are termed pectinic acids. Methylation greatly enhances the viscosity of pectic solutions (Merrill and Weeks 1945, Walkinshaw and Arnott 1981). The pectolytic enzymes comprise an esterase (pectin methylesterase), polygalacturonases including endo- and exo-enzymes and lyases including endo- and exopectate lyases. Pectin methylesterase acts on pectin by removing methyl groups from the galacturonate unit by hydrolysis. The polygalacturonases split pectic acid to smaller chains. The lyases split cross linkages. The difference between endo- and exo-enzymes will be described below.

Pectin methylesterases have been isolated from various fruits (Lee and Macmillan 1970, Brady 1976), from bacteria (Miller and Macmillan 1971, Sheiman *et al.* 1976), and from fungi (Endo 1964, Schejter and Marcus 1988, Yoshihara *et al.* 1977, Miller and Macmillan 1971, Forster 1988). They have a relatively low molecular weight of from 26,000 to 45,000 from both higher plants and micro-organisms (Whitaker 1990). Pectin methylesterase is a very specific enzyme with exceedingly low activity on non-galacturonide esters. In plant tissue its pH optimum is near 7.0 and its activity is increased several fold in the presence of salts, maximal at 0.03 M (Kertesz 1955).

Endopolygalacturonases hydrolyse the internal glycosidic bonds of the pectic substrate in a more or less random manner resulting in a series of smaller polygalacturonate molecules, the size of which is determined by the specific enzyme (Whitaker 1990). The endopolygalacturonases have little or no activity on highly methylated pectins unless in the presence of a pectin methylesterase. Their activity can be determined by measuring the rate of formation of reducing groups by the 3,5-dinitrosalicylate method of Bernfield (1955) or the arsenomolybdate-copper method of Somogyi (1952), both give results which correlate with the number of glycosidic bonds hydrolysed. Endopolygalacturonase activity can also be determined by measuring the reduction of viscosity of the substrate. Viscosity falls off very rapidly with cleavage of the substrate; there is no direct correlation between viscosity

reduction and the number of glycosidic bonds hydrolysed, however, as viscosity reduction is reliant on the position of the glycoside bond within the polygalacturonate chain. The viscosity method cannot be used at low pH values where polygalacturonates form gels. The endopolygalacturonases often occur in multiple molecular forms whereby the size and position of the binding locus of the active sites determines the relative affinity to size of polygalacturonate substrate. Thus there is an enzyme specificity to an optimum substrate unit size beyond which enzyme activity decreases (Whitaker 1990). The estimated molecular weights of the endopolygalacturonases are somewhat higher than for pectin methylesterases and vary from 30,000 in some yeasts (Lim *et al.* 1980) and bacteria (Wang and Keen 1970) to 84,000 for tomato (Pressey and Avants 1973) and 85,000 for the mould, *Aspergillus niger* (Cooke *et al.* 1976).

Exopolygalacturonases act on the ends of high molecular weight pectate chains. The usual product is galacturonate. Specificity is best determined by showing activity against pectic acid but not on fully methylated pectins in the absence of pectin methylesterase, by measuring the amount of galacturonic acid and digalacturonate produced using pectate and fully methylated pectin in the absence of pectin methylesterases. Exopolygalacturonases have been found in the fruits of various plants, in oat seedlings, and from fungi, bacteria and insects (Whitaker 1990).

Other pectic enzymes include the oligogalacturonate hydrolases, which act on low molecular weight oligogalacturonates, and endopolymethylgalacturonases. Also the pectic lyases, namely endopolygalacturonate, exopolygalacturonate, oligo-galacturonate and endopolymethylgalacturonate lyases all of which are found only from pathogenic fungi and bacteria.

Extensive research effort has been concentrated on the pectolytic enzymes of fungal and bacterial pathogens (Whitaker 1990, Cruickshank and Wade 1980). In the bacterial genus *Erwinia* (Novacky *et al.* 1990) pectolytic enzymes occur with a number of other extracellular enzymes which have the ability to degrade plant cell saccharides, protein and lipids and which presumably act in sequence on the cell wall (Novacky *et al.* 1990). Pectinases are thought to be the first of these as pectic substances present the first barrier to pathogen attack (Darvill *et al.* 1980, Keegstra *et al.* 1973). In other bacteria such as *Clostridium multifermentans* there is a complex of the pectin methylesterase and pectate lyase which operates on the cell wall. Thus demethylation and cleavage of the glycosidic linkages of the galacturonate residues occur simultaneously in the disassembly of the pectate molecules (Sheiman *et al.* 1976). Bateman (1972) found that two pectinases from the bacterium, *Sclerotium rolfsii* act sequentially, one having an endo-, the other an exo- mode of disassembly. Pectic enzymes diffuse ahead of pathogenic growth (Novacky *et al.* 1990) thus aiding pathogenic colonisation. Endopectic enzymes

were found to cause 50% decrease in viscosity of a pectin substrate with an estimated 10% cleavage of bonds (Cooper 1983) and an endopectate lyase from *Erwinia carotorova* caused loss of linkage and solubilisation of all the polygalacturonate, comprising a total of 30% of cell wall content in carrot (Konno and Yamasaki 1982). Action of pectic enzymes within plant tissue can be determined by measuring the changes in the permeability of plant tissue, by means of the conductivity of a bathing solution (Novacky *et al.* 1990), by observing cell death histologically or estimating tissue coherence following maceration by agitation or sonication (Mount *et al.* 1970).

Verticillium albo-atrum, a fungal pathogen, which causes severe wilt symptoms in lucerne, produces the pectolytic enzymes endopolygalacturonase, exopectate lyase and pectin methylesterase. According to Heale and Gupta (1972), the endopolygalacturonases could not be implicated in wilting since they were detected only after advanced symptoms had developed. On the other hand, high concentrations of exopectate lyase were found to coincide with the onset of wilting and they suggested that this enzyme led to gel blockage of the xylem vessels. An unidentified protein with a molecular weight of about 40,000 promoted an increase in the viscosity of pectin solutions and could be important in catalysing gel formation. They suggested that these gels originate as a result of pectic enzyme activity which releases polymer fragments, their presence inhibiting the upward flow of water thus contributing to the wilting symptoms of diseased plants.

Some authors have suggested that wilting is primarily due to the effects of non enzymatic substances (toxins) disrupting cell metabolism (Gaumann 1958). Others have implicated the role of fungal pectolytic enzymes (Scheffer *et al.* 1956). Other lines of evidence such as reduced vascular flow of the xylem of infected plants (Dimond and Waggoner 1953), the presence of pectinaceous gels in the vessels of diseased plants (Beckman and Zaroogian 1967), detection of pectic enzymes in wilted plants (Blackhurst and Wood 1963) and the presence of a midlamella containing degraded pectate in infected xylem vessels (Pierson *et al.* 1955) support the hypothesis that wilting due to many phytopathogenic fungi is primarily caused by pectolytic enzymes.

No discussion of the role of pectolytic enzymes secreted by pathogens within host plants is complete without mention of the seeming anomaly that the plants' own pectinases are activated as a result of challenge by incompatible pathogens (West 1981) and mechanical wounding (Ryan *et al.* 1981). Although the precise trigger for these reactions is still obscure, pectinases in the cell wall liberate oligosaccharide fragments from the cell wall and midlamellar pectins. These products act as messenger substances and travel by apoplastic and symplastic routes to surrounding cells, switching on production of phytoallexins (West 1981). In sycamore and tobacco leaves, mechanical wounding causes liberation of pectic fragments termed

proteinase inhibitor induction factors. These cause the synthesis of polypeptides which, when ingested, are active against the proteinases of herbivores (Ryan *et al.* 1981). How the involvement of plant pectinases in allelochemical responses to pathogens (and possibly to sucking insects) interacts with the activity of the pathogens' (and insects') own pectolytic enzymes is currently unresolved.

1.3.2.2. The role of pectolytic enzymes in the saliva of Hemiptera: corroboration of an hypothesis

When feeding on lucerne at high densities, *C. dilutus* has been observed to cause wilt symptoms reminiscent of the wilting, cited above, caused by a fungal pathogen. Also, leaf abscission similar to that caused by the insects when feeding on lucerne seed heads has been observed in some non-host plants stood in pectinase solutions. It is likely that salivary pectinases in the Miridae are able to diffuse some distance from the feed site, causing progressive degradation of the cell walls. To date the role of the salivary pectinase to liberate available cell constituents, thus making them available as a food source (Strong and Kruitwagen 1968, Miles 1987a), has been speculative. So too is the action of the stylets in lacerating cell walls (Strong 1970, Miles 1972). In the present work, the release of plant compounds from stem sections during incubation in pectinase solutions and salivary preparations is investigated, as is the effect of their elution with artificial laceration.

1.3.3. A comparison of the salivary physiology of coreids and mirids: aim of the present work

The presence of particular salivary enzymes in some Heteroptera have seemingly logical explanations in terms of the insects' nutritional requirements (see section 1.1.2.2). These have often been alluded to but frequently remain speculative or unknown. In this context, the occurrence, source, secretion and possible function of the salivary sucrase of a coreid, *M. profana* and of the salivary pectinase of a mirid, *C. dilutus*, is investigated.

CHAPTER TWO

THE BIOLOGY AND BEHAVIOUR OF *Mictis profana* Fabricius (COREIDAE) WITH OBSERVATIONS ON THE DEVELOPMENT OF FEEDING LESIONS

2.1. ABSTRACT

Males of *M. profana* are slightly larger than females and can be distinguished by the shape of the abdomen and the presence of a metatibial spur. In South Australia host range includes *Acacia iteaphylla* F. Muell. ex Benth., *A. pycnantha* Benth., *Cassia corymbosa,* lemon and rose. Feeding by *M. profana* causes terminal wilting acropetal to the feed site on young, small diameter shoots of indigenous hosts, an effect similar to that noted for other coreids. When the insects are confined in the laboratory with broad bean seedlings, laterally branched stylet tracks terminate around the vascular bundles causing mainly acropetal oxidation of vascular tissue and lateral darkening of adjacent bundles. When confined with bean pods, water soaked lesions several mm in diameter occur around feeding punctures. On stems of a natural host, *A. iteaphylla,* feeding punctures, indicated externally by stylet sheath flanges, occur as linear chains. Successive punctures are invariably made in a basipetal direction. An initial increase in water content at the feed site precedes their progressive dehydration thereafter causing progressive senescence down the shoot tip. Artificial diets incorporating phenolic compounds indicate the secretion of a salivary oxidase.

2.2. INTRODUCTION

The family Coreidae contains many economically important species world wide. They are generally large, sometimes colourful bugs with characteristic defensive, repellent odours. In Australia they include *Leptoglossus australis* (Fabricius), which damages cucurbits, paw-paw, passionfruit and citrus, several species of fruit-spotting bugs, *i.e. Amblypelta nitida* Stål which cause kernel malformation in macadamia and damage to a wide range of fruit crops including avocado, stone fruits, citrus, mango, custard apple, pineapple, cotton and beans, and *Aulacosternum nigrorubrum* Dallas, the false cotton stainer.

M. profana has been recorded from Indonesia, Fiji and Papua New Guinea. In Australia it has a wide distribution, exclusive of Tasmania (Flanagan 1994). In

Australia its food plants have been recorded from nine families comprising 15 indigenous plant species and a further 13 exotic species. Most, however, belong to the Mimosaceae (Flanagan 1994). Flanagan (1994) also described the life history of *M. profana* in northern Australia, and indeed, suggested it as a possible biological control species for the exotic invasive shrub, *Mimosa pigra* L.

Other Australian coreids include a complex of a dozen or so species of *Amorbus* (Subfamily Coreinae, Tribe Amorbini). They, too, are large, from 20-25 mm in length, and possess a number of general similarities with *Mictis*. These include the morphology of the egg burster, their feeding habits, and a number of nymphal morphological characters, one of which is the apparently generically unique development of the wing buds in the second instar (Kumar 1965). Species of *Amorbus*, apparently feeding specifically on *Eucalyptus* spp. produce rapid wilting, formation of necrotic lesions and abscission of the young, growing tips of their hosts, symptoms similar to those reported above for *M. profana*. Feeding lesions of another coreid, *Amblypelta* sp. from cassava in New Guinea were found to extend some considerable distance from feeding punctures (Miles 1987a) and, like those of *M. profana* and *Amorbus* spp., often resulted in the death of the growing tip. Stem wilting and tip senescence caused by a coreid in cucurbits was thought by Neal (1993) to be caused simply by a mechanical interruption to xylem vessels rather than to any salivary enzyme or toxin.

2.2.1. General biology

Eggs of *M. profana* are laid in linear chains of up to 14 at a time and are lightly covered by a powdery substance (Kumar 1965). Flanagan (1994) reported a mean total fecundity of 196 (range 121-320) with a mean number of eggs of 11 per chain (with up to 23 in one chain) laid with a mean interval of 5.5 days. Hatching occurs in about 10 days (Kumar 1965) and the first moult about 6 days thereafter. Feeding by first instar nymphs was not observed; they aggregate at the oviposition site and disperse to the tips of young shoots only after moulting. Attempts at rearing in the laboratory failed beyond the second instar (Kumar 1965), an observation corroborated here. Even when the insects were supplied with regular replacement of foliage, Flanagan (1994) reported greater than 50% mortality in first and second instars. Descriptions and measurements of the egg and of each of the nymphal instars is presented by Kumar (1965). He described the nymphs as generally being light brown with yellow or orange markings. Specimens from the Adelaide region, however, appear somewhat darker, being dark grey to black with orange or yellow markings becoming paler to light grey brown in later instars. Adults are generally light grey, distinctively marked with a prominent pale cross on the elytra and a dark grey hemelytron. The distal antennal segment and the orifice of the scent gland are

orange, and the abdominal tergites are a bright orange-red which is only visible while the insect is in flight. Males are slightly larger than females: ♂, 21.2 mm (±1.60 SD; n=5), ♀, 20.0 mm (±1.00 SD; n=5) and are somewhat greater in weight, ♂ 0.3009 gm (±0.064 SD; n=7), ♀ 0.2445 gm (±0.049 SD; n=7). They have a shallower abdomen, but possess both an enlarged hind femur and a flanged metatibia bearing a prominent metatibial spur.

In southern Australia there appear to be two broadly overlapping generations per year corresponding to a spring to early summer and a late summer to early autumn generation respectively. Adults and (rarely) final instar nymphs hibernate in dense cover towards the centre of the plant during winter. They do not diapause, however, and will resume feeding during warm weather or when brought into the laboratory and placed on fresh plant tissue in warm conditions (e.g. about 20°C or higher).

The genus *Amorbus* is represented by a complex of about 15 robust, brown, similar looking species producing symptoms as described above on various species of *Eucalyptus* and, like *M. profana* is regularly encountered from early December to late April.

2.2.2. Host plant association

Kumar (1965) recorded *M. profana* from *Cassia fistula* L. (Indian Laburnum), *C. coluteoides* Collard (Easter Cassia), *Acacia cunninghami* Hook., and also from tomatoes and beans. Flanagan (1994) recorded it on 10 species of *Acacia* and, amongst other exotic species, on *Wistaria* and *Hibiscus*. It is hereby recorded in South Australia on *A. iteaphylla* F. Muell. ex Benth. (Flinders Range Wattle), *A. pycnantha* Benth. (Golden Wattle), *C. corymbosa* (Buttercup tree), and on lemon and roses. The former three are commonly grown as ornamentals in southern Australia; on citrus it is considered a pest of minor commercial importance.

2.3. MATERIALS AND METHODS

2.3.1. Feeding behaviour

Field observations on the feeding behaviour and feeding damage by *M. profana* were made mostly on *A. iteaphylla*. Observations on other host species were made incidentally. In the laboratory, juvenile and adult *M. profana* were fed on cut stems of *A. iteaphylla* in water, on broad bean (*Vicia faba* L.) seedlings or directly on the pods of green runner beans (*Phaseolus vulgaris* L.) purchased from a local grocer.

2.3.2. Observations on feeding lesions of *M. profana*

2.3.2.1. Observations on the rate of stem abscission by *M. profana*

Prior to diurnal commencement of feeding, adult *M. profana* were placed individually on young *A. iteaphylla* shoots in the field and evidence of damage was assessed at 2, 4, 24 and 48 hr.

2.3.2.2. Moisture content of stems of *A. iteaphylla* fed upon by *M. profana*

Stem section about 6 mm in length of *A. iteaphylla* on which *M. profana* had just commenced diurnal feeding but which were terminally wilted from previous feeding were cut, weighed, dried at 40°C for 2 days, re-weighed and percentage moisture calculated. Stem sections which were undamaged and showed no evidence of feeding by *M. profana* were similarly sampled as controls.

2.3.3. Histological examination of the feeding lesions of *M. profana*

In the laboratory, adult *M. profana* were placed on broad bean seedlings, and transverse stem sections, several complete cells in thickness were cut, either incorporating single feed sites or comprising a series of adjacent sections both apical and basipetal to the feed site. Sections were placed in Carnoy's fixative, rinsed in 100% ethanol, transferred to 95% ethanol, stained in 1% light green in 95% ethanol, washed in 100% ethanol with several changes, placed in 50% xylol in ethanol then 100% xylol, mounted in Canada balsam and dried at 40°C for 7 days.

2.3.4. Observations on the structure of the stylet sheath

A 3% agar diet was prepared incorporating 10% sucrose, and, prior to gelling, 0.5 mg/ml of either chlorogenic acid, catechol or DL-DOPA were added. The diets were poured into 35 mm glass petri dishes, allowed to cool and covered with Parafilm. As a phagostimulant, an extract of rose leaves, made by bathing them in ethanol until the solution was tinged with green was added dropwise to the Parafilm surface and allowed to evaporate. Diets were offered to 8 adult and 2 juvenile *M. profana* collected from rose, and left for 5 days caged in a plastic canister at room temperature before observations of the structure of the stylet sheath were made.

A similar process was used to present a 10% sucrose in 3% agar diet with an extract of young *A. iteaphylla* leaves on the Parafilm surface, to insects collected from *A. iteaphylla*. Following removal of the Parafilm, these gels were stained in 0.02% ruthenium red for 3 hr then destained in water.

2.3.5. Measurements of stylet penetration

2.3.5.1. Maximum exsertion of the stylets based on measurements of manipulated mouthparts

The mouthparts of adult *M. profana* were manipulated to the extent that the labium was sufficiently elbowed so as to equal the length of the labrum, at which point the exsertion of the stylets were measured (Fig. 2.1).

2.3.5.2. Measurements of stylet penetration in plant tissue

The lengths of elbowed labia of adult *M. profana* were measured during feeding on broad bean stems in the laboratory and the exsertion of the stylets was calculated.

2.4. RESULTS

2.4.1. Feeding behaviour and rate of lesion formation by *M. profana*

2.4.1.1. Field observations

M. profana causes wilted to abscissed young or meristematic shoot tips on their host plants. Feeding punctures are indicated by the presence of conical stylet sheath flanges on the plant surface and occur singly or, with subsequent feeding, in a longitudinal (axial) row along the plant stem (Fig. 2.2). When feeding is uninterrupted, they typically occur at a distance of about 0.3 mm. The duration of each feeding event can be for 20 or more minutes, although individuals in the field have been observed to feed at a single puncture for a number of hours. Progression of successive feeding punctures invariably occurs in a basipetal direction. Bugs feeding in this way will continue for periods in excess of several weeks, often on the same stem but sometimes moving to adjacent stems to recommence feeding. In excessively hot or windy conditions, or during cold spells or rain, individuals may move down the stem for shelter. They do not necessarily return to the same stem on return but they do select one of a similar diameter as that recently vacated. Low instar juveniles commence feeding close to the meristematic shoot tip while larger individuals and adults select stem sections of a greater diameter somewhat more basally.

On *A. iteaphylla* in the field, stems apical to a single feeding puncture begin to wilt within two hours (presumably dependant upon ambient temperature, humidity, stem turgidity and other parameters) from the commencement of feeding (Figs 2.3 & 2.4). The stem assumes a turgid, water-soaked appearance (Fig. 2.2) at the feeding puncture; indeed water content of stem sections in the immediate vicinity

Fig. 2.1. Exsertion of the stylets of *M. profana*. A, exsertion at which feeding typically occurs, B, maximum exsertion estimated at the point where the labrum is sufficiently elbowed so that the labrum contacts the 'substrate'. In B the stylets and the labrum have been separated from the remainder of the rostrum in order to clarify their linear relationship.

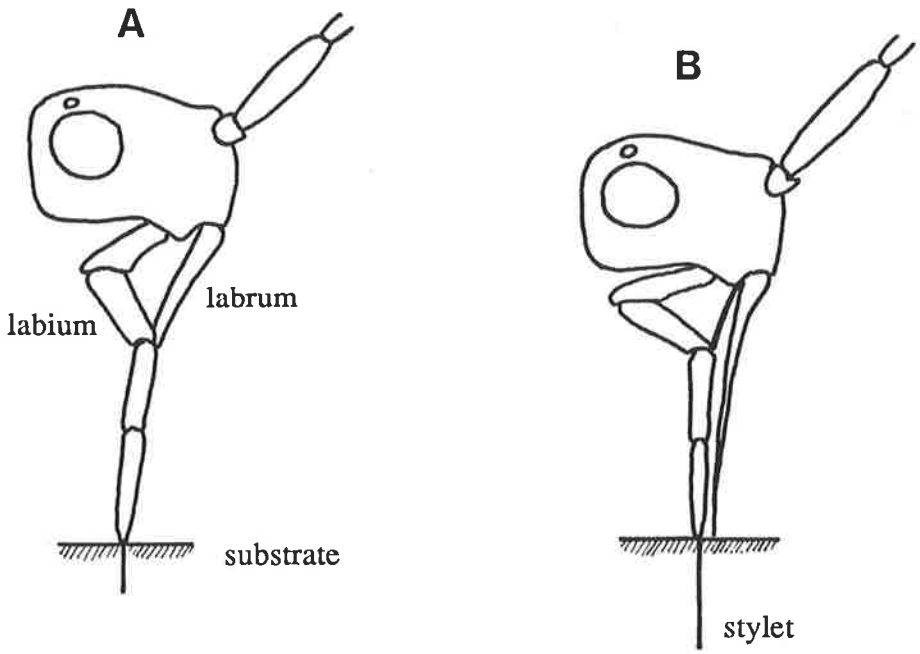


Fig. 2.2. Top left, axial row of stylet sheath flanges on stem of *A. iteaphylla*. Note water soaked appearance around feed sites and progressive collapse of stem apically.

Fig. 2.3. Top right, stem of *A. iteaphylla* 2 hr from commencement of feeding by an adult *M. profana*.

Fig. 2.4. Bottom left, terminal wilting on stem of *A. iteaphylla* 4 hr from commencement of feeding by an adult *M. profana*.

Fig. 2.5. Bottom right, terminal wilting and leaf senescence on stem of *A. iteaphylla* 8 hr from commencement of feeding by an adult *M. profana*.

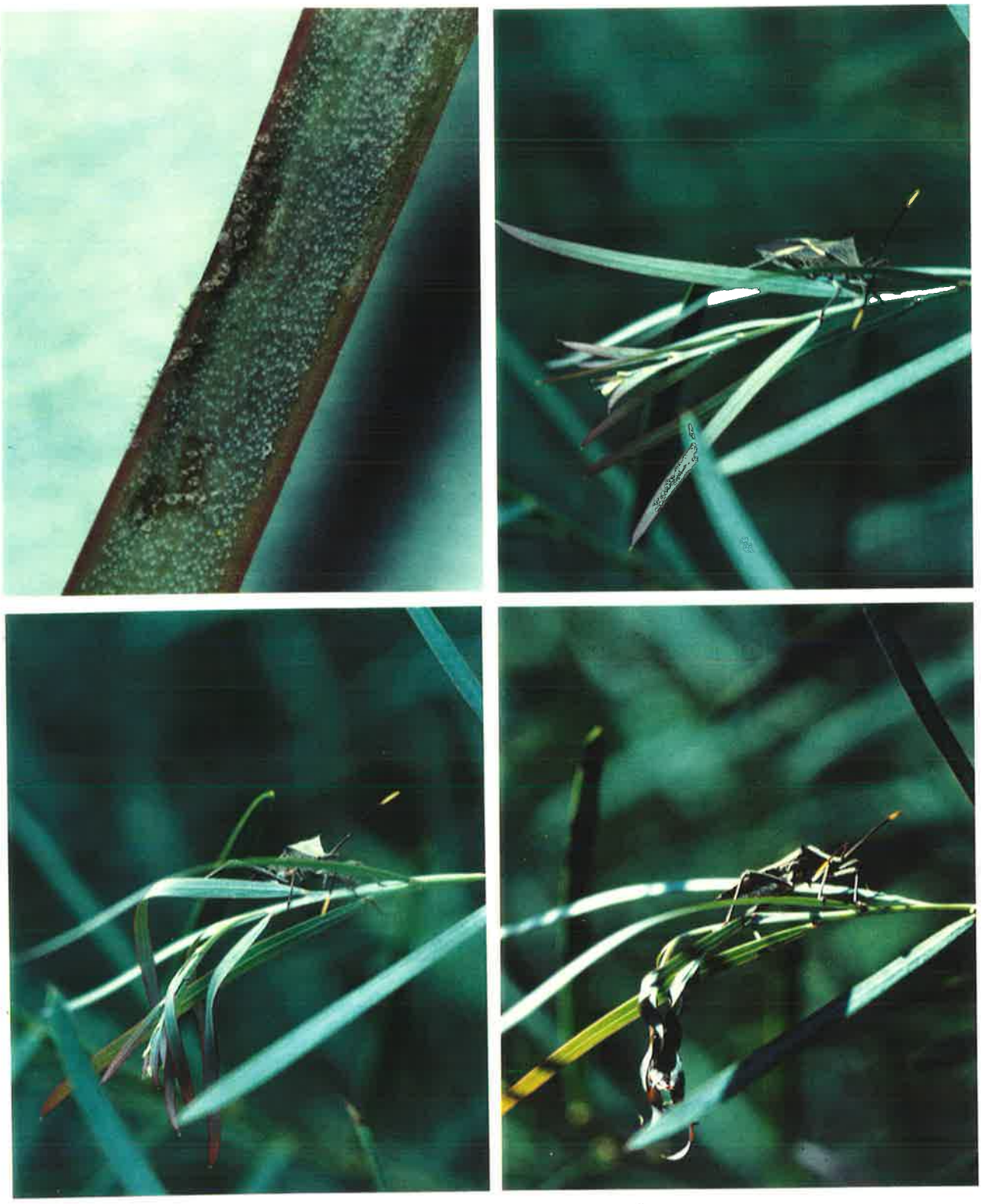


Table 2.1. Progressive feeding damage to stems of *A. iteaphylla* by *M. profana*.

Time from commencement of feeding (hr)	Symptoms
0	Nil
2	Leaves slightly limp
4	Leaves limp and slightly twisted
24	Leaves twisted, wilted and darkened; stem constricted
48	Leaves twisted, dead and blackened; stem collapsed

of recent feeding activity was significantly higher: 48.5% (± 7.44 SD; n=5) than that of "unfed" stem sections: 35.4% (± 4.40 SD; n=5) (P = 0.10). Apical to this, leaves become twisted and progressively darken (Fig. 2.5), from olive-green to grey green and finally to greenish black, with simultaneous drying out and collapse of the stem. This progressive senescence and collapse of the shoot tip is indicated in Table 2.1. On rose, feeding on bud petioles causes a linear, darkened and somewhat collapsed area with subsequent wilting and bud abscission. On broad bean seedlings in the laboratory, feeding sites were indicated by local "water-soaked" lesions which turned brown but without observable wilting. On runner beans, lesions appear simply as water-soaked areas of several mm in diameter.

2.4.2. Histological examination of feeding lesions of *M. profana*

Sections of the shoots of *A. iteaphylla* showed them to be heavily structured phyllodes, with thick cell walls, attributes which made observations of feeding punctures difficult. Histological observations undertaken using broad bean seedlings revealed that *M. profana* inserts its stylets and feeds mainly in or near the parenchyma of the vascular bundles. In the course of a single feeding event it often partially retracts its stylets before commencing another branch of the stylet track resulting in a series of branched tracks around the vascular bundles (Fig. 2.6). These branches are aligned transversely with respect to the plant section and result from lateral deflections of the stylets.

Oxidation as indicated by melanisation spreads gradually and mainly acropetally a few mm from a single, (*i.e.* experimentally interrupted) feeding puncture (Fig. 2.7). Lateral oxidation of adjacent vascular bundles were also observed. Otherwise, little could be seen other than an emptying of cells in the close vicinity of the feeding puncture. Attempts to obtain satisfactory sections of *A. iteaphylla* apical to the feed site failed due to their collapse and loss of integrity.

2.4.3. Structure of the stylet sheath and feeding behaviour on agar gels

Feeding sites were indicated by the presence of stylet flanges on the Parafilm surface. In control diets in the absence of phenolic compounds feed sites occurred as single to multi-branched tracks often with a beaded appearance. If left undisturbed, individual bugs after withdrawal from one puncture commenced another ± 0.5 mm from the previous one, such that, over a period of several days, linear chains of up to 26 individual feed sites were recorded. It was observed, too, that branching of stylet tracks occurred normal to the direction of the feeding chains indicating that stylet movement or probing by *M. profana* is more or less restricted to the lateral plane.

With diets containing chlorogenic acid, the feed sites were multi-branched and blackened in proximity to the feeding punctures; and each branch comprised solidified stylet sheath material incorporating black granules.

With catechol, some feed sites occurred singly as prominent, blackened multi-branched tracks also containing black granules. In four observations, however, feeding punctures occurred in linear chains of 4, 8, 11 and 13, respectively; in these the stylet sheaths were unbranched, blackened at the base and surrounded by a grey diffusate.

The feed sites in DOPA were similar to those in catechol except that they occurred in groups of 5, 7, 7 and 11, in four observations respectively. In ruthenium red, stylet sheaths stained dark red basipetally but were somewhat less stained apically.

2.4.4. Measurements of stylet penetration

Adult *M. profana* were observed to feed with stylet penetration of 0.95-1.20 mm (1.07 ± 0.13 SD, n=3). Maximum stylet exsertion was 1.50-1.70 mm (1.62 ± 0.11 SD, n=3).

2.5. DISCUSSION

Similar to the feeding lesions of *M. profana*, shoots with withered tips were also found on *Eucalyptus camaldulensis* Dehnh. on which an undetermined species of *Amorbus* was feeding. Withering was also observed on cut shoots of *A. iteaphylla* in the laboratory following feeding by *M. profana*. Terminal wilting of shoots has been reported on cassava and sweet potato stems by *Amblypelta* sp. (Miles 1987a), on cucurbits by *Anasa tristis* (DeGeer) (Neal 1993) and on *E. regnans* F. Muell. and *E. obliqua* L'Herit. by *A. angustior* (Westwood) but not by *Gelonus tasmanicus* Le Guillou (M. Steinbauer, pers. comm.).

The tissues of *A. iteaphylla* are heavily sclerified and darken rapidly when damaged. Cross sections of feeding sites revealed little detail other than the stylet tracks running to the vicinity of major vascular tissue, an observation consistent with that of Neal (1993) for *A. tristis* feeding on cucurbits. In *A. iteaphylla,* the stylet sheaths ran to the main vascular bundles on either side of the narrow (2-3 mm) ovate stem, and there was an overall darkening of tissues which appeared to become somewhat shrunken throughout the section. Rose stems are also well sclerified and have a high phenolic content (Peng and Miles 1991). On rose, externally visible darkening occurred with hours of attack by *M. profana,* and cross sections also showed heavily oxidised, shrunken lesions. Clearer information on the effects of the

Fig. 2.6. Branching of stylet track of *M. profana* near vascular bundles of broad bean stem section. Outer limits of stylet track are indicated by arrows.

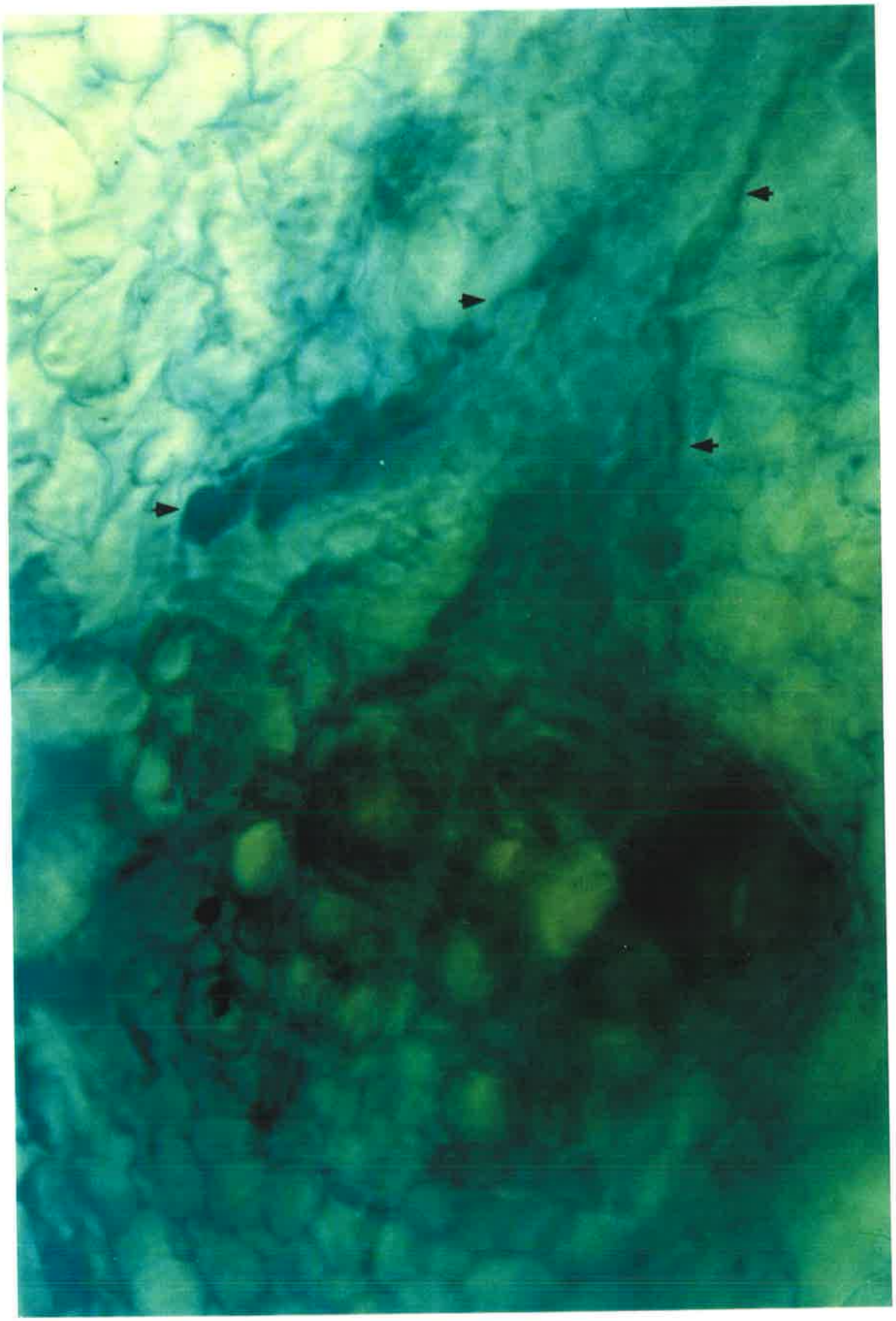
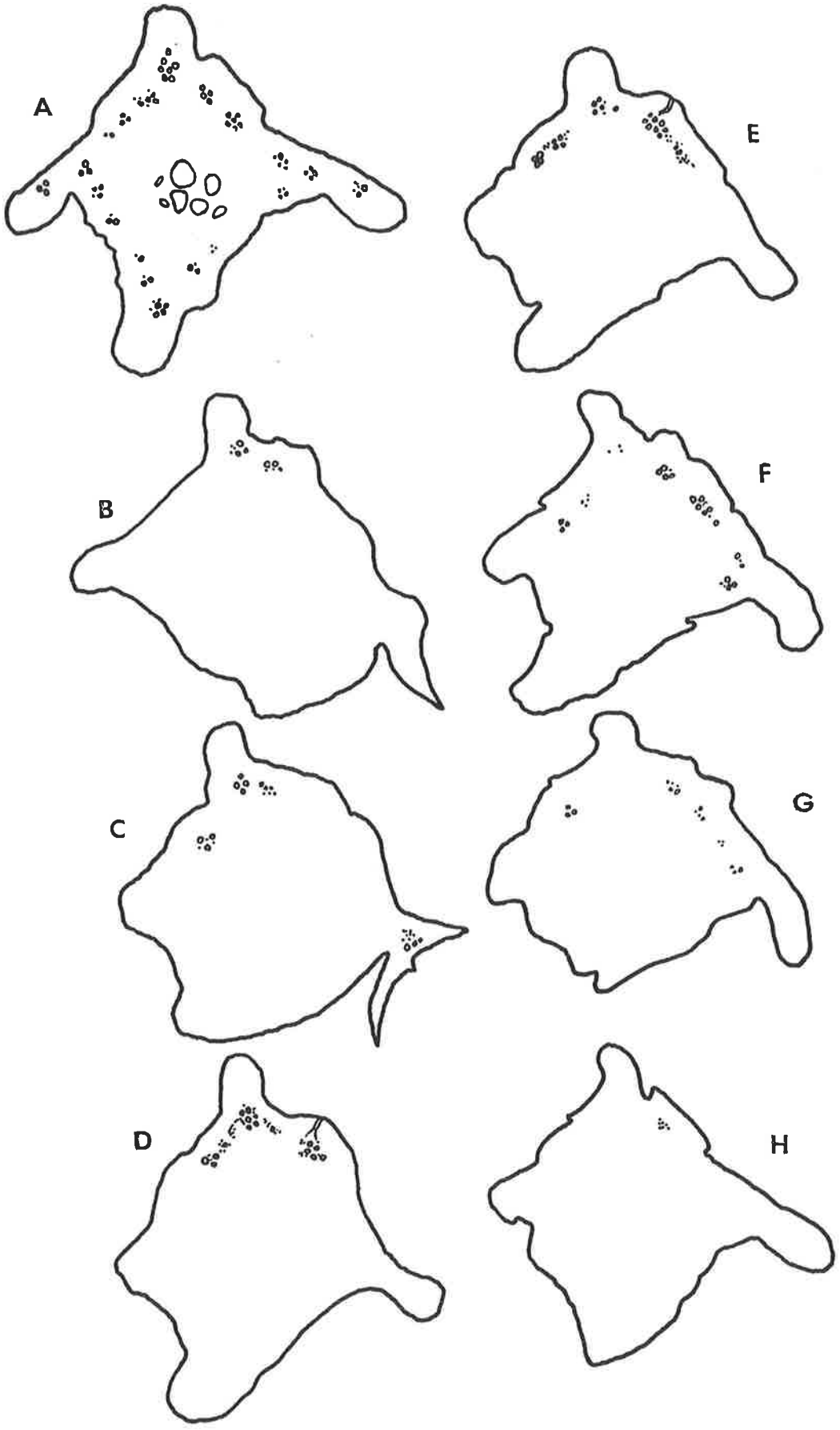


Fig. 2.7. Transverse sections of broad bean stem showing oxidative melanisation of vascular bundles following 2 hr feeding by *M. profana*: A, a typical distribution of all vascular bundles; B-C, successive sections apical to the feed site, D-E, sections containing the feed site, F-H, successive sections basipetal to the feed site.



insect on tissues was obtained from the less pigmented lesions produced on beans.

On broad bean stems, the insects produced externally observable, clearly demarked 'water soaking' at the feeding site within 30 min, an observation consistent with that of Miles (1987a). On runner bean pods, prolonged feeding at a single site produced a circular, water soaked area of approximately 5 mm diameter. Within 2 hr of feeding, the lesions had a sunken appearance. Histological examination of unstained lesions more than a few hours old showed some degree of oxidation of damaged tissues. The hyaline sheath material, bordered within the plant by slightly darkened cell contents, can be readily recognised with experience and in all tissues usually ended close to or within vascular parenchyma. A noticeable feature of lesions in bean stems was the particularly dark staining of parts of vascular bundles, especially the xylem vessels, for several mm acropetally and a mm or so basipetally. Indeed, similar occluded lesions were reported in the thicker stems of cassava (Miles 1987a) and isolated necrotic lesions on leaves of *Cucurbito pepo* L. (Neal 1993). In the latter, stem wilting did not occur unless a sufficient number of vascular bundles were affected, that is, only when the stem itself had effectively become 'girdled' by feeding damage.

Heteroptera normally seal their stylets into plant tissues with a flange of stylet sheath that is continued into growing plant tissues to the definitive feeding site. Further solidifying material is apparently secreted into the sheath on withdrawal, effectively sealing off the internal lesion from the exterior (Miles 1959). On one occasion, however, rhythmic welling up and disappearance of fluid every few seconds from the immediately preceding puncture indicated an imperfect seal, the apparent injection and sucking back of fluid from the next insertion point and, fortuitously, the continuity of successive feeding sites.

From these observations it seemed likely that the insect injected saliva that in some way infiltrated intercellular spaces (causing the water soaked appearance in beans) and at the same time initiated the local senescence of plant tissues. Prior to this loss of turgor and consequent acropetal wilting, however, there was an initial and local increase in moisture content of 'fed' sections. As indicated in a later section, this increase was much more than could be ascribed to the insects' injected saliva. Thus the effects of coreid feeding would seem to include a transient offloading of transported fluids from the plants' vascular tissues, followed by a local loss of vascular function so that, in order to continue feeding the insect has to retract it stylets and successively move its feed site marginally downward to where the vascular tissues are still unaffected by previous feeding activity.

CHAPTER THREE

DESCRIPTIONS OF THE SALIVARY GLANDS AND DIGESTIVE SYSTEM OF *Mictis profana* Fabricius (COREIDAE) AND SALIVARY GLANDS OF *Creontiades dilutus* (Stål) (MIRIDAE)

3.1. ABSTRACT

The principal salivary glands of *M. profana* are pentalobate and consist anteriorly of an anterior, median and a lateral lobe of fundamental Coreoid form and a two-lobed lateroposterior and posterior lobe complex each comprising multiple, compact and confluent digitations. The accessory gland is a long tubular duct-like process with a slightly swollen but otherwise undifferentiated terminal section. The principal salivary glands of *C. dilutus* are simply bilobed and comprise an anterior and posterior lobe characteristic of mirids and pentatomids. The accessory gland of *C. dilutus*, like that of mirids generally terminates in an enlarged vesicular, bulbous process which lies proximal to the posterior lobe.

3.2. INTRODUCTION

The paired salivary glands of Heteroptera are situated in the thoracic cavity on either side of the foregut. They occur primitively as a single lobe in the genera *Cimex* (Cimicidae) and *Rhodnius* (Reduviidae) (Baptist 1941). In the families Miridae and Pentatomidae (Pentatomorpha) they occur typically as a principal gland comprising an anterior and a posterior lobe, with an accessory gland connected via an accessory duct. In Pentatomorpha the principal glands have distinct, and often additional lobes. Thus, as well as possessing an anterior and posterior lobe, lateral lobes also occur in Lygaeidae, and both lateral and median lobes in Pyrrhocoridae and Coreidae. The derivation of the lobes, as mentioned previously, is unclear although Miles (1972) suggested, on physiological and functional grounds, that all lobes other than the posterior lobe originated from an ancestral anterior lobe.

Baptist (1941), in addition to reviewing literature dating to as early as the investigations of Dufour in 1833 on the morphology of the salivary glands of Heteroptera, provided anatomical and histological descriptions of a number of Heteropterans representing 16 families. They included the aquatic, predatory Notonectidae, Nepidae and Gerridae, the terrestrial, predatory Reduviidae, the vertebrate blood feeding Cimicidae and the principally or exclusively phytophagous

Capsidae (=Miridae), Pentatomidae, Lygaeoidea and Coreoidea. Nuorteva (1956) described the salivary glands of 15 species of Heteroptera, then included within the four families, Miridae, Lygaeidae, Coreidae and Pentatomidae, and compared them anatomically with a number of descriptions of salivary glands presented elsewhere in the literature, all in connection with physiological and phytopathological studies. Since then, Bronskill *et al.* (1958) provided an anatomical and histological description of the trilobate salivary glands of the lygaeid, *O. fasciatus* and Hori (1968a,b) described the bilobed glands of *E. rugosa* (Pentatomidae) and *Lygus disponsi* Linnavuori (Miridae).

3.3. MATERIALS AND METHODS

3.3.1. Dissection of salivary glands of *M. profana* and *C. dilutus*

M. profana was collected from *A. iteaphylla,* and *C. dilutus* was swept from lucerne, *Medicago sativa* L. Both species were from the grounds of the Waite Agricultural Research Institute. For the dissection of salivary glands, the insects were held ventral side uppermost under isotonic saline, the anterior pair of legs were removed, then each side of the prothoracic sclerite was cut dorsolaterally from its anterior margin using micro-scissors, enabling the removal of the sclerite with forceps. By grasping the head with forceps and with simultaneous pressure applied to the abdomen, the head and dorsal prothoracic sclerite were gently pulled away thus drawing the anterior portion of the midgut and salivary glands from the thoracic cavity. The salivary glands could then be segregated by grasping either the accessory glands or the hilus of the principal gland and gently pulling or teasing away from the attached tracheoles.

3.3.2. Dissection of digestive tract of *M. profana*

For the dissection of the digestive tract, an adult female *M. profana* was pinned upside down on wax, the venter (thoracic and abdominal segments) was carefully removed by cutting around with micro-scissors, and after gently teasing away white fat body, the internal organs were exposed.

The salivary glands and digestive tract were drawn with the aid of a microscope with a squared eyepiece graticule.

Fig. 3.1. Digestive tract of *M. profana*.

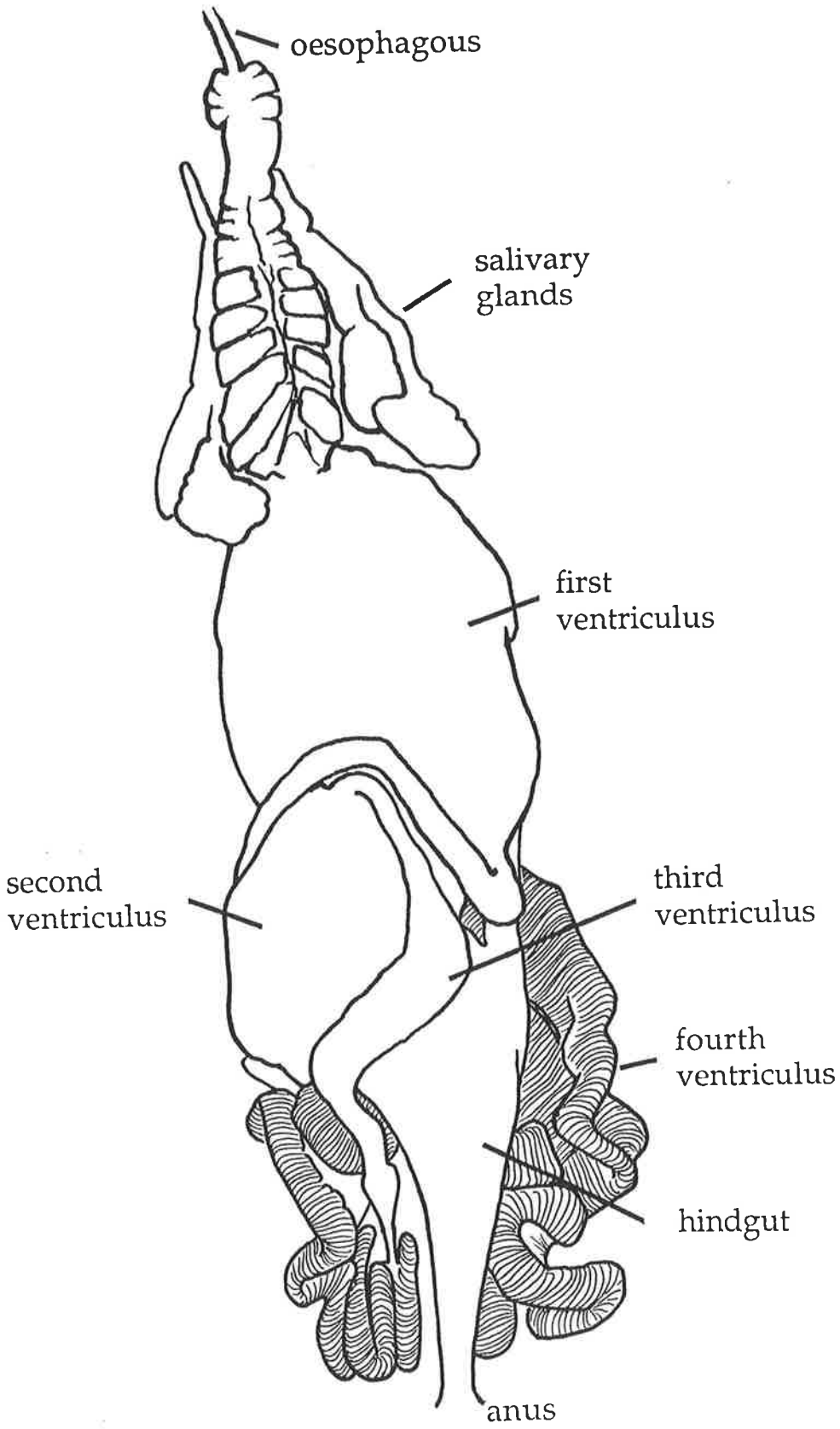
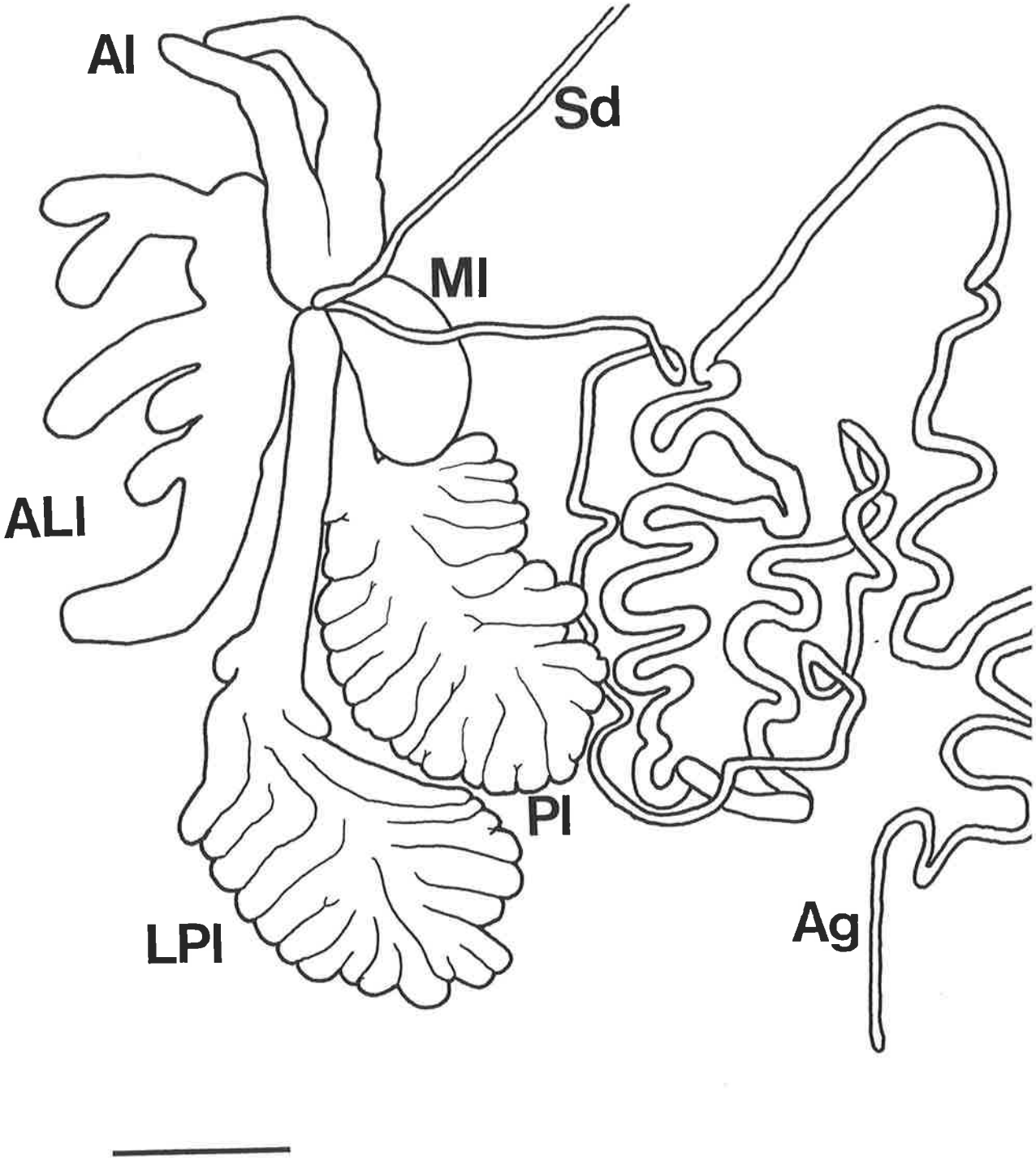


Fig. 3.2. Salivary glands of *M. profana*: Ad, accessory duct; Ag, accessory gland; Al, anterior lobe; ALl, anterolateral lobe; LPl, lateroposterior lobe; Ml, median lobe; Pl, posterior lobe; Sd, salivary duct. Scale = 1 mm.



3.4. RESULTS

3.4.1. Salivary glands of *M. profana*

The paired multilobate vesicular glands of *M. profana* are about 6 mm in length and lie dorsolaterally along the prothoracic alimentary canal in the region where the oesophagus merges with the first stomach (Fig. 3.1). The anterior lobe (Fig. 3.2) comprises two digitations with one or both often angled anteriorly. In Taylor and Miles (1994), the illustration of the anterior lobe included digitations that on subsequent morphological examination appeared to be distinctly separated. The existence of an additional lobe, here termed the anterolateral has therefore been recognised. The anterolateral lobe is always multidigitate but is otherwise somewhat variable in shape and number of digitations between and within individuals. The median lobe is a thin walled, ovoid structure. The posterior lobe appears superficially as a large, broad and flattened, somewhat globular, vesicular lobe which on closer examination comprises a number of closely packed digitations which merge to form a common, central duct. Another lobe, here termed the lateroposterior lobe, is similar in structure to the posterior lobe except that it is joined to the hilus by an elongate tubular process. All major lobes, anterior, anterolateral, median, posterior and lateroposterior are joined at the hilus, a junction from which the accessory and salivary ducts emerge. In the intact individual, the tubular duct of the accessory gland passes posteriorly from the hilus, thickens slightly and becomes convoluted henceforth; it returns to the vicinity of the hilus, passes back to the posterior lobe, then forward again into the insects head where it loops around the muscles of the sucking pump before returning to become an undifferentiated glandular terminus at the posterior end of the salivary gland complex. The salivary glands and prothoracic alimentary canal are closely associated with tracheae and tracheoles from the first thoracic spiracle. A prominent, longitudinal trachea which services the hilus, accessory ducts, the prothoracic alimentary canal and the medial (inner) face of the principal salivary glands passes between the alimentary canal and the salivary glands. A secondary branch from this tracheal trunk divides off in proximity to the hilus and its tracheoles service the anterior, lateral and median lobes. Three further branches service both the posterior and lateroposterior lobes. The accessory glands and posterior lobes are strongly supplied with tracheae, all of which are held together by a network of tracheoles around which the convolutions of the accessory ducts intertwine.

The descriptions of lobe contents given here are essentially those already reported in Taylor and Miles (1994). The names of the lobes adopted in that paper followed logically from differences in the contents of various parts of the gland, but

differed in one important respect from the nomenclature originally used for coreid glands by Baptist (1941) and followed by Nuorteva (1956). These authors did not distinguish between lobes clearly homologous to those differentiated in Taylor and Miles (1994) as the lateral and posterior; instead, the lobe that Baptist and later Nuorteva called "lateral" appears more likely to be the morphological equivalent of part of the anterior lobe illustrated in Taylor and Miles (1994). It should be noted, however, that present research revealed no functional difference between the lobes that Baptist and Nuorteva designated as anterior and lateral but indicated clear differences between the contents of the lobes considered by them to form part of the same, albeit morphologically complex posterior lobe. As a compromise between past and present terminology and in order to avoid switching a name from one part of the gland to another, functionally different part, the following nomenclature has been adopted here: for Baptist's and Nuorteva's lateral, the term anterolateral is used; for the part of Baptist's and Nuorteva's posterior complex that was termed the lateral lobe in Taylor and Miles (1994), the term lateroposterior is now used.

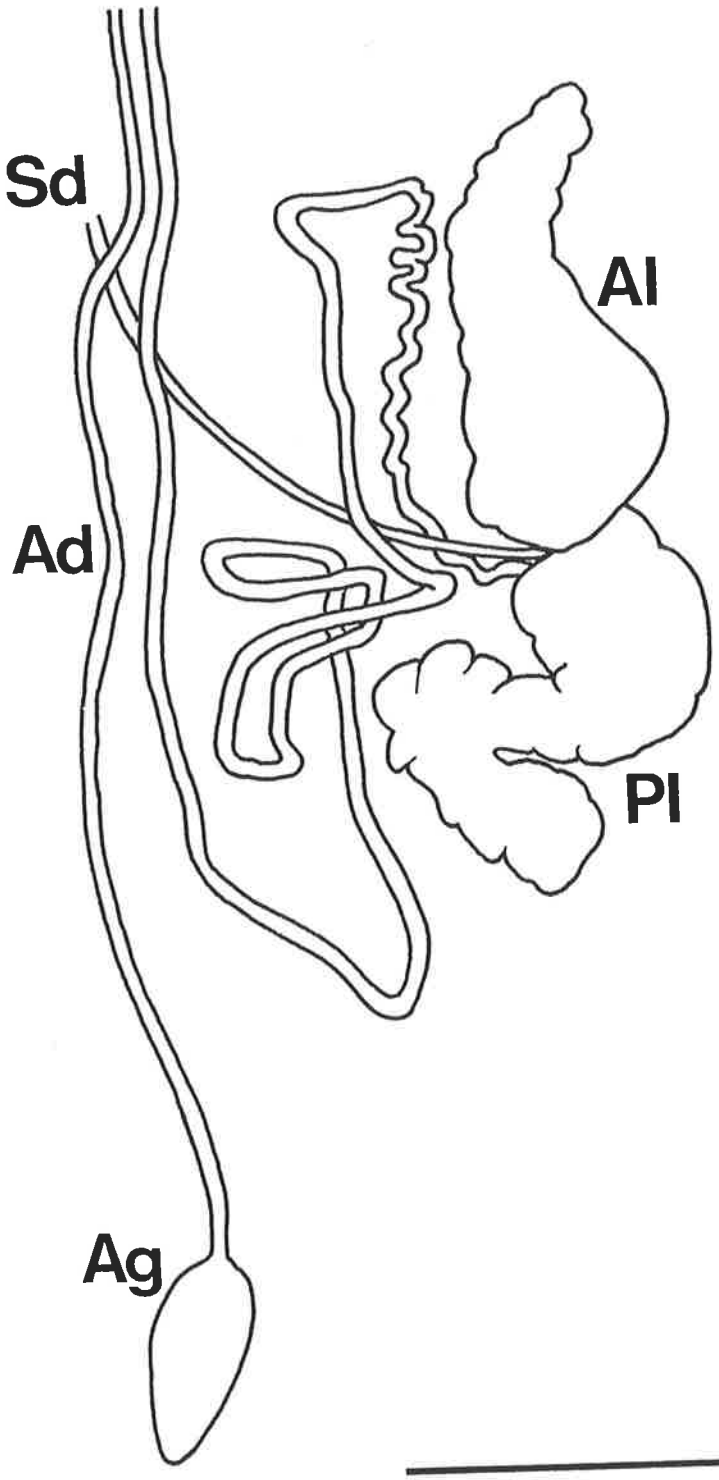
3.4.2. Salivary glands of *C. dilutus*

The paired bilobate glands of *C. dilutus* (Fig. 3.3) are about 2 mm in length and lie similarly along the prothoracic alimentary canal. The anterior and posterior lobes are thin walled and vesicular, the latter distinctively angled. Both lobes join at the hilus, a junction from which the accessory and salivary ducts emerge. From the hilus the tubular duct of the accessory gland passes anteriorly with slight convolutions to near the apex of the anterior lobe, returns to the vicinity of the hilus where the duct folds back on itself several times, passes back to the posterior lobe, then forward again into the insects head where it loops round the cibarial muscles before returning to an ovoid, bulbous, terminal accessory gland towards the posterior end of the salivary gland complex. The anterior lobe, hilus and accessory duct adjacent to the anterior lobe appear to be serviced by one tracheal branch while another radiates from a central position on the median face of the lateral lobe and services both the lateral lobe and the proximal convolutions of the accessory duct. The salivary duct and both the outgoing and returning ducts of the accessory gland run confluently from the principal salivary gland to the insects head.

3.4.3. The digestive tract of *M. profana*

The digestive tract is depicted in Fig. 3.1. As it was not the primary purpose of this study to investigate the digestive tract in detail, its inclusion here is to indicate the position and nomenclature of the various portions of the tract used in several

Fig. 3.3. Salivary glands of *C. dilutus*: Ad, accessory duct; Ag, accessory gland; Al, anterior lobe; Pl, posterior lobe; Sd, salivary duct. Scale = 1 mm.



small, yet important experiments, which were undertaken to clarify the role of the salivary glands in the function of the digestive system as a whole (see subsequent chapters and conclusion).

3.5. DISCUSSION

The principal salivary glands, as in all Heteroptera excepting Notonectidae, are vesicular, having a one-layered glandular epithelium made up of small cells which discharge their secretion into a relatively large storage cavity (Baptist 1941). The accessory gland is tubular or duct-like and is considered homologous with the reservoir of the salivary glands of other orders (Baptist 1941). The principal glands vary from single lobed to multilobate (Baptist 1941, Nuorteva 1956). Anatomically, they are considered to be important in the higher classification of the Heteroptera (Leston *et al.* 1954). Indeed Nuorteva (1956) suggested homologies for the various lobes for four major superfamilies of the Heteroptera.

The structure of the bilobed salivary glands for *C. dilutus* is consistent with previous descriptions of those of other Miridae (Baptist 1941, Nuorteva 1956), a cosmopolitan family with an apparent remarkably similar gland anatomy throughout. Bilobate glands, comprising an anterior and posterior lobe, are found also in Tingidae, Nabidae and Pentatomidae (Baptist 1941).

Baptist (1941) described and figured the quadrilobed salivary glands of *Corizus parumpunctatus* Schill. (Corizidae) (Superfamily Coreoidea), then placed in Coreidae. Similarly, Nuorteva (1956) described those of a further nine species of Coreoidea now placed in Alydidae, Rhopalidae and Coreidae. He considered quadrilobed principal glands to represent the fundamental coreoid type and supernumerary lobes to be derived from the development of 'digitations' of the posterior lobe. According to this interpretation the [apparently] hexalobed principal glands of *A. tristis* (Coreidae) consist of anterior, median and lateral lobes and a trilobed posterior lobe.

On close examination and by carefully tracing the origins of each lobe to its junction at the hilus, the principal salivary glands of *M. profana*, and indeed those of two other coreids, *A. angustior* and *G. tasmanicus* are here considered to possess a total of five lobes. Although the salivary glands of these three species vary greatly in complexity, the homologies of the individual lobes and digitations are quite clear. This interpretation (of pentalobate glands) is inconsistent, however, with that proposed by Nuorteva (1956) of *A. tristis* but it is not possible from his diagrams and descriptions to determine the precise derivation of each of the posterior lobes depicted therein. A superficial morphological similarity of the lateroposterior and

posterior lobes as named herein is consistent with the observations of Baptist and Nuorteva that both lobes are derived from an ancestral posterior lobe. Nevertheless, as stated above (Section 3.4.1), a functional difference between the lobes has already been presented in Taylor and Miles (1994) and is elaborated in later sections of the present work.

Comparing the salivary glands of coreids and mirids, the latter possesses the more fundamental configuration of a functionally discrete anterior and posterior lobe and an accessory duct terminating in a bulbous vesicle. The vesicular nature of the mirid accessory gland is consistent with its function as a reservoir for the salivary pump and is indicative of the volume of watery saliva presumably secreted during feeding (see subsequent chapters). The principle glands of *M. profana* possess supernumerary lobes and/or digitations from the primitively bilobed configuration but there is a simple ductiform accessory gland. This is thought to be consistent with the hypothesis that rather smaller quantities of saliva are required when salivation is localised due to the formation and function of a stylet sheath.

CHAPTER FOUR

THE DETECTION OF ENZYMES, AMINO ACIDS AND OTHER COMPOUNDS IN THE SALIVARY GLANDS AND DIGESTIVE TRACT OF *Mictis profana* Fabricius (COREIDAE), *Creontiades dilutus* (Stål) (MIRIDAE) AND OTHER HETEROPTERA

4.1. ABSTRACT

The contents of all lobes other than the posterior and lateroposterior in the principal salivary glands of *M. profana* can form substantial gels insoluble in water, consistent with their contributing to the solidifying saliva (stylet sheath); the posterior lobe secretes most if not all the sucrose-hydrolysing enzyme that occurs in the non-gelling (watery) saliva. A similar enzyme was found in the salivary glands of four other coreid species. No other carbohydrases and neither proteinase nor lipase were detected. Acid and alkaline phosphatases were found in gland extracts but not in secreted saliva. The saliva also contains catechol oxidase from the accessory gland and ducts. Watery saliva contained a number of free amino acids including phenyl alanine and tyrosine, but no DOPA nor other diphenolic substrates of the catechol oxidase. Total free amino acid concentration varied up to 1.8 µg/µl leucine equivalents. The watery saliva has marked surfactant properties, possibly relevant to its function within food plants.

4.2. INTRODUCTION

Although general patterns of salivary secretion by Heteroptera: Pentatomorpha and Homoptera have emerged (Miles 1972), the degree of phytotoxicity associated with their feeding varies widely between taxa. It has been customary to ascribe such differences to "salivary toxins", although usually without recognition and/or substantiation of any specific toxin. Yet where specific salivary components have been implicated in the development of feeding lesions, they may not appear to be intrinsically toxic, but to cause specific symptoms only if deployed in different ways (Miles 1989, 1990). Whilst few species of Hemiptera have been subject to comprehensive salivary analyses, there appears to be considerable familial uniformity in the known composition of the saliva with respect to specific components. Nevertheless, not all members of any one taxa affect their food sources similarly. Coreids, in particular, cause a variety of symptoms, including death of entire shoots acropetal to the feed site (Neal 1993), and large necrotic lesions in

cocoa pods and in stems of sweet potato and cassava (Miles 1987a). The latter resemble the feeding lesions of some tropical Miridae, yet, unlike mirids, which secrete a powerful salivary pectinase (Strong and Kruitwagen 1968), no apparently toxic component has hitherto been identified in their saliva (Hori 1975, Miles 1987a, Neal 1993). Neal (1993) postulated instead that death of shoot tips caused by the squash bug, *A. tristis* on *C. pepo*, resulted from an interruption of xylem flow. He thought that the vascular tissue became blocked with stylet sheath material, a phenomenon which would not be possible in lesions caused by mirids.

This chapter describes attempts to provide a definitive analysis of the chemical and physical nature of the salivary secretions of the coreid, *M. profana,* in order to provide a basis for investigation of the etiology of the characteristic symptoms caused by coreids generally, including local necrosis at the feed site and subsequent acropetal senescence of shoot tips.

4.3. MATERIALS AND METHODS

4.3.1. Live materials and their use

M. profana was collected from *A. iteaphylla,* and *Amorbus* sp. (Coreidae) from *E. camaldulensis,* in the grounds of the Waite Agricultural Research Institute. *Au. nigrorubrum* (Coreidae), which is an occasional pest of cotton, was sent from the Kimberley Research Station in Western Australia, and *A. angustior* and *G. tasmanicus* (Coreidae) from *E. regnans* and *E. obliqua* were sent from CRC Temperate Hardwood Forestry, Tasmania. *Poecilometis apicalis* (Westwood) (Pentatomidae) was collected from *E. cladocalyx* F. Muell. from the grounds of the Waite Agricultural Research Institute, and *C. dilutus* from lucerne (*M. sativa*) both from Waite Institute and from roadside verges near Callington, South Australia.

M. profana was subjected to all the tests detailed below. *Amorbus* sp., *A. angustior, Au. nigrorubrum, G. tasmanicus* and *P. apicalis* were subject only to tests for sucrase in salivary gland extracts. *C. dilutus* salivary glands were tested for sucrase, amylase and pectinase.

4.3.2. Collection of salivary preparations

4.3.2.1. Collection of watery saliva

Larger Heteroptera (>15 mm: *M. profana, Amorbus* spp., *P. apicalis*) were secured undamaged and upside down on polystyrene foam by pins crossing the abdomen and thorax, and 20 µl 200 gm/litre free pilocarpine base in acetone (Binnington and Schotz 1973) was applied to the abdomen. A 20 µl Drummond Microcaps™ tube was placed with its tip in contact with the end of the rostrum to

collect the saliva. Salivation could be induced and/or enhanced by placing the insect in a stream of warm air (40-44°C).

4.3.2.2. Preparation of salivary gland extract

The salivary glands of 4-10 individuals of the small Heteroptera (6-15 mm) (*C. dilutus*, *G. tasmanicus*, *Au. nigrorubrum*) were dissected under isotonic saline, placed in 20 µl water in a 1.5 ml capped Eppendorf™ tube, ground with a glass rod, centrifuged at 13,000 rpm for 5 min and the supernatant (= salivary gland extract) transferred to a new tube.

4.3.3. Examination and separation of gland contents

4.3.3.1. Detection of precursors of sheath material and sulphydryl groups

Glands were dissected into 0.9% NaCl, transferred to glycerol for ≥24 hr, allowed to swell in distilled water and the cellular investment was then punctured or teased away, while noting the disposition of contents that had gelled in glycerol and their subsequent solubility in water or 8 M urea. Sulphydryl content was determined by immersing whole glands or constituents in 5% sodium nitroprusside and adding 1 M ammonium hydroxide dropwise: a transient pink colouration indicates free -SH groups.

4.3.3.2. Determination of pH of saliva and contents of lobes of the salivary glands

Individual lobes of the salivary glands were ruptured onto pH paper (Merck's Spezial-Indikator strips # 9542 for pH 4.0-7.0).

4.3.3.3. Observations on turbidity and surfactant properties of saliva

Observations on turbidity and surfactant properties of saliva were made during its collection and subsequent discharge from microcapillary tubes and the plastic tips of micropipettes.

4.3.4. Preparation and use of gland extracts for qualitative enzyme tests

4.3.4.1. Preparation of buffered salivary gland extracts

Whole salivary glands of adult insects were dissected in saline, rinsed in distilled water and ground in 1.5 ml Eppendorf tubes in 25 µl per gland of 0.01% dithiothreitol (to inhibit possible coprecipitation of enzymes with sheath precursors). Homogenates of individual lobes were prepared similarly. 'Unbuffered extract' was the supernatant from homogenates centrifuged at 0°C. 'Alkaline extract' was made by adding 1 volume 1 M sodium acetate to 5 volumes of unbuffered extract. 'Buffered extracts' were made by adding 1 volume 0.04% indicator (bromocresol green for pH 5, bromocresol purple for pH 6, bromothymol blue for pH 7, phenol red

for pH 8) to 6 volumes of alkaline extract, followed by small quantities of 0.1 M HCl and/or NaOH to give colours corresponding to the required pH; the solutions were finally made up to 12 volumes with CO_2-free distilled water. Tests on extracts without dithiothreitol or adjustment of pH confirmed that neither had produced spurious results for either presence or absence of the enzymes. Unless otherwise stated, incubation of extracts with enzyme substrates was for 2 hr in 1.5 ml Eppendorf tubes in a 37°C water bath.

4.3.4.2. Phosphatase tests

The substrate was 0.004% disodium *p*-nitrophenyl phosphate, added to an equal volume of either McIlvaine citrate buffer pH 5.0 (Pearse 1968) for acid phosphatase or 1.5 M 2-amino-2-methyl-1-propanol pH 10 for alkaline phosphatase. Aliquots of 10 µl unbuffered extract and alkaline extract were incubated with equal volumes of the acid and alkaline phosphatase substrates respectively. The mixture was then made up to 70 µl with 0.1 M NaOH and the colours compared with controls in which water and 0.16 M sodium acetate took the place of the unbuffered and alkaline extracts respectively. Appearance of a yellow colouration, intensified on addition of NaOH to unbuffered mixtures, indicates phosphatase action.

4.3.4.3. Colorimetric tests for carbohydrases

Substrates were 2% w/v aqueous maltose, cellobiose, melibiose and lactose (for EC 3.1.20-23 inclusive); sucrose (for EC 3.2.1.26 and EC 3.2.1.48), raffinose (for EC 3.2.1.26) and 1% hydrolysed starch (Connaught Medical Research Laboratories potato starch, hydrolysed for gel electrophoresis) in 0.2% NaCl (for EC 3.2.1.1). Aliquots of 20 µl buffered extracts were incubated with 5 µl substrate. After addition of 100 µl Tauber and Kleiner (1932) copper lactate reagent, the tubes were capped and lids punctured using a fine pin, placed in a boiling water bath for 10 min, and cooled; 200 µl of Nelson (1944) reagent was added with shaking and 300 µl of the contents mixed with 4 ml distilled water in a spectrophotometer cuvette. Release of reducing monosaccharide from substrates is indicated by increased absorbance at 500 nm compared with a substrate control in which water replaced the extract. Extract blanks without substrate gave negligible absorbances.

In an alternative amylase test, to buffered extracts and starch substrate, incubated as indicated above, was added 10 µl of a solution containing 0.05% iodine, 1% potassium iodide and 1% hydrogen peroxide. Digestion of starch is indicated by differences from the blue-black colour of the starch-iodine complex formed by a substrate control. The peroxide is included to prevent reduction of the iodine by reducing substances in the gland extracts (Miles 1967a, Miles and Harrewijn 1991).

For experiments specific to *C. dilutus*, 5 µl salivary gland extract (the salivary glands of 4 adults dissected under isotonic saline and ground in 20 µl water) was incubated at 25°C for 3 hr in an equal volume of 1% hydrolysed starch in 2% NaCl then 10 µl 0.1% iodine in 2% potassium iodide: 30% H_2O_2 (2:1) was added, shaken and colours compared for differences from appropriate controls as described above.

4.3.4.4. Microviscosimetric tests for pectinase and cellulase

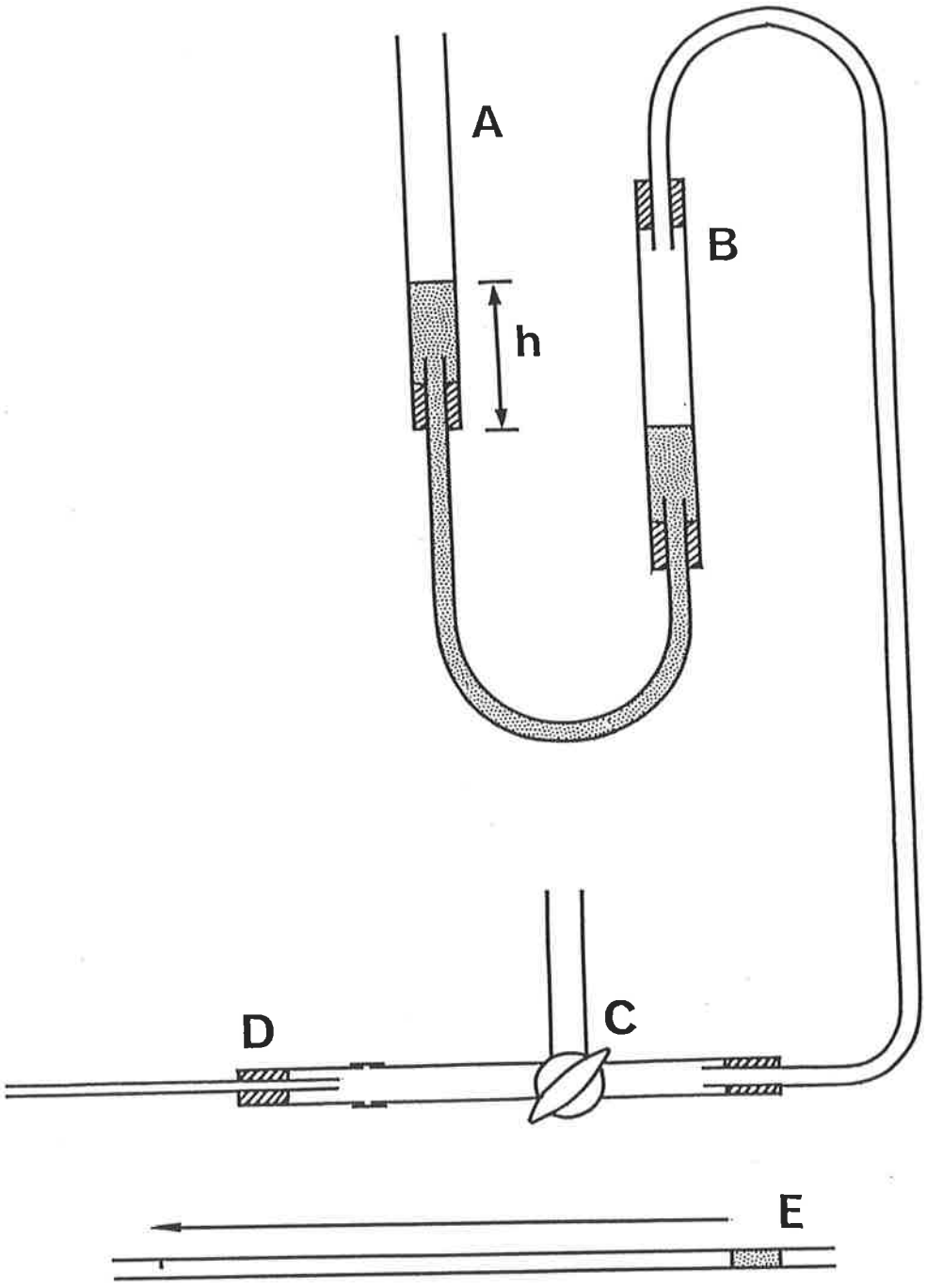
Substrates before pH adjustment were 3% polyglacturonic acid, ex-citrus and with 9.1% methoxy content (for EC 3.2.1.15), and 2.5% methyl cellulose (for EC 3.2.1.4), both made up in 1 M sodium acetate. To produce buffered substrates, pH was monitored with a meter and adjusted with 0.1 M HCl and NaOH while stirring to give values of 5, 6, 7 and 8. The final volumes were adjusted to give 1% substrate. The apparatus used is illustrated in Fig. 4.1. The graduated capillary tubes were thoroughly cleaned by prior washing in 5 M nitric acid, rinsing in distilled water and drying at 100°C. Aliquots of 2 µl of the buffered extracts were incubated with 10 µl of the appropriately buffered substrates, the mixtures were allowed to equilibrate to room temperature for another 30 min and their viscosity assessed by allowing a 2 µl aliquot to travel between two marks on the capillary tube. A head height of 20-30 mm water gave run times of 20-30 sec for controls consisting of the substrates incubated with water in place of the extracts. The action of standard preparations of 1 mg/ml Sigma™ pectinase or cellulase reduced run times of appropriate substrates to 1-2 sec, approximately the same as those given by gland extracts to which water was added in place of substrate.

4.3.4.5. Test for proteinase

Buffered substrates were produced by adding 0.1 M HCl and NaOH to 2% azoalbumin using a pH meter to bring solutions to pH 5, 6, 7, and 8. Aliquots of 10 µl of buffered extracts were incubated with an equal volume of buffered substrate; 20 µl of 10% trichloroacetic acid was added, the mixture shaken and centrifuged and the colours of the supernatant compared with two sets of controls in which water replaced extract and substrate respectively. The appearance of a yellow supernatant indicates proteinase activity.

For *C. dilutus* the salivary glands of 5 adults were dissected in isotonic saline, placed in 20 µl water and ground with a glass rod (total volume about 30 µl). Volumes of 15 µl salivary gland extract was incubated at 35°C for 3 hr with an equal volume of substrate and treated as per above.

Fig. 4.1. Microviscometer for pectinase and cellulase tests. A, water head tube of adjustable height; B, fixed water head tube; C, three way stopcock; D, Microcap™ holder and 25 µl capillary tube; E, detail of capillary showing solution in place to run between two marks. The water levels are equalised with the stopcock turned air–water head, then the head height is set with the stopcock turned air-capillary. The sample is brought to the first mark using another Microcap assembly, and the movement of the sample to the next mark is timed with a stopwatch when the stopcock is turned capillary–water head.



4.3.4.6. Test for lipase

Buffered substrates were prepared by mixing Sigma™ lipase substrate (stabilised olive oil emulsion) 10:1:1 with 1 M sodium acetate and 0.04% indicator solutions, adjusting the pH to match the colours of buffered extracts using 0.1 M HCl and NaOH, and finally bringing the volume to 1.5 times the original volume of the Sigma substrate. Mixtures of 10 µl each of buffered extract and buffered substrate were incubated and their colours compared with those of unreacted extracts and substrates. Lipase action is indicated by a colour change indicating a more acid pH in the incubated mixtures.

4.3.4.7. Oxidase reactions of salivary glands and saliva

Catechol oxidase (EC 1.10.3.1) activity in glands or lobes was determined by transferring them rapidly to 1 mg/ml DOPA in buffered saline pH 7.4 (8.0 gm NaCl, 0.2 gm KCl, 0.2 gm potassium dihydrogen phosphate and 1.15 gm dipotassium hydrogen phosphate in 1 litre CO_2-free distilled water) and incubating for ≥1 hr at 25°C. Sites of oxidase activity turn dark brown.

To detect catechol oxidase and/or peroxidase (EC 1.11.1.7) in watery saliva, 10 µl was mixed with 100 µl 10% catechol (unbuffered), either with or without 1 µl 3% hydrogen peroxide. Two sets of control solutions contained water in place of catechol and saliva respectively. After incubation over night at 25°C, 500 µl distilled water was added to all reaction mixtures and controls, and their spectra were recorded between 350 and 900 nm against distilled water in narrow width cuvettes, using a Varian™ DM100S recording spectrophotometer with a DS-15 data station. Results were obtained by subtracting the corresponding control spectra for saliva and substrate in the presence or absence of H_2O_2.

4.3.5. Determination of free amino acids

Total soluble amino acids in the watery saliva were estimated by reacting 5 µl with 200 µl of a 1:1 mixture of the ninhydrin reagent and acetate cyanide buffer of Rosen (1956) in a boiling water bath for 10 min, cooling and diluting with 1 ml 50% aqueous propan-2-ol; absorbance at 570 nm was compared with a standard curve for freshly prepared DL-leucine.

To determine which free amino acids were present, lobes were separated in distilled water and transferred to Eppendorf tubes without addition of solvent other than any water adhering to them, ground with a glass rod, centrifuged, and 2-4 µl quantities of supernatant subjected to thin layer chromatography (TLC) using Sigmacell™ type 100 plates activated for 30 min at 80°C. For two dimensional chromatography, development was in methyl ethyl ketone, propan-2-ol, 1 M HCl

(15:60:5) followed by 65% aqueous propan-2-ol; also in *tert*-butanol, methyl ethyl ketone, 25% ammonia, water (50:30:10:10), followed by *n*-butanol, acetone, acetic acid, water (70:70:20:40). For unidimensional TLC of extracts and watery saliva, the first solvent system was used. Most amino acids were identified by spraying chromatograms with 0.1% ninhydrin in ethanol, glacial acetic acid, 2,4,6-collidine (50:15:2), heating at 100°C for 10 min, and comparing the position and colour of spots with chromatograms of mixtures of 0.1% amino acid standards dissolved in the solvent mixture used for first dimension or unidimensional development. Some compounds were confirmed using reagents described in Block *et al.* (1958).

4.3.6. Detection of enzymes in the digestive tract of *M. profana*

4.3.6.1. Test for proteinase

The procedure for proteinase under section 4.3.4.5 was used except that buffered extracts of portions of the digestive tract were used. These were the gut wall and contents of: entire paired salivary glands, first ventriculus, tubule between first and second ventriculus, second ventriculus, third ventriculus and fourth ventriculus, all as described in Chapter Three.

4.3.6.2. Test for additional invertase/sucrase input from digestive tract

The salivary glands and digestive tracts of four adult *M. profana*, one of which had been deprived of food for 16 days were dissected out under isotonic saline, then portions of the digestive tracts were taken, and where possible (for one individual) the contents only and the respective gut walls of the various portions were taken (the latter rinsed as thoroughly as possible and ground with a glass rod) and 10 µl of each incubated in an equal volume of either 10% sucrose or water at 37°C for 2 hr and reacted with Tauber and Kleiner's reagent for the detection of reducing sugars as described in section 4.3.4.3.

4.4. RESULTS

4.4.1. Properties of watery saliva

4.4.1.1. Collection of watery saliva

Under the influence of pilocarpine, individual *M. profana* over the summer months usually secreted 20-30 µl over 3-4 hr. Exceptionally, one individual secreted 58 µl over a period of 5.5 hr. In comparison with a similar test carried out in autumn, a month or so before the insects entered a period of winter quiescence, four insects yielded one, two, three, and four 2 µl aliquots of saliva respectively, considerably less than that provided by specimens in mid summer. Similarly, insects caged indoors for only short periods (within 24 hr), even when offered an unlimited supply

of fresh host tissue, yield progressively smaller volumes of watery saliva. It was observed that the rate of secretion was not uniform nor did the saliva show constant properties. A description of these properties will follow later.

4.4.1.2. Determination of pH of contents of lobes of the salivary glands

The pH of all parts of freshly dissected glands from *M. profana* appeared to be in the range 5.0-5.4, becoming more alkaline on standing overnight.

4.4.1.3. Observations on turbidity and surfactant properties of saliva

It was noticed on many occasions during collection of saliva that the secretion made a very low contact angle with the walls of the capillary tubes into which it was collected. Discharge of aliquots of the secretion was complicated by its capacity to wet the plastic tips of micropipettes and by the appearance of persistent bubbles. Although no *ad hoc* tests of surfactant properties were conducted, these observations alone point to the wetting properties of the saliva.

4.4.2. Examination and separation of gland contents

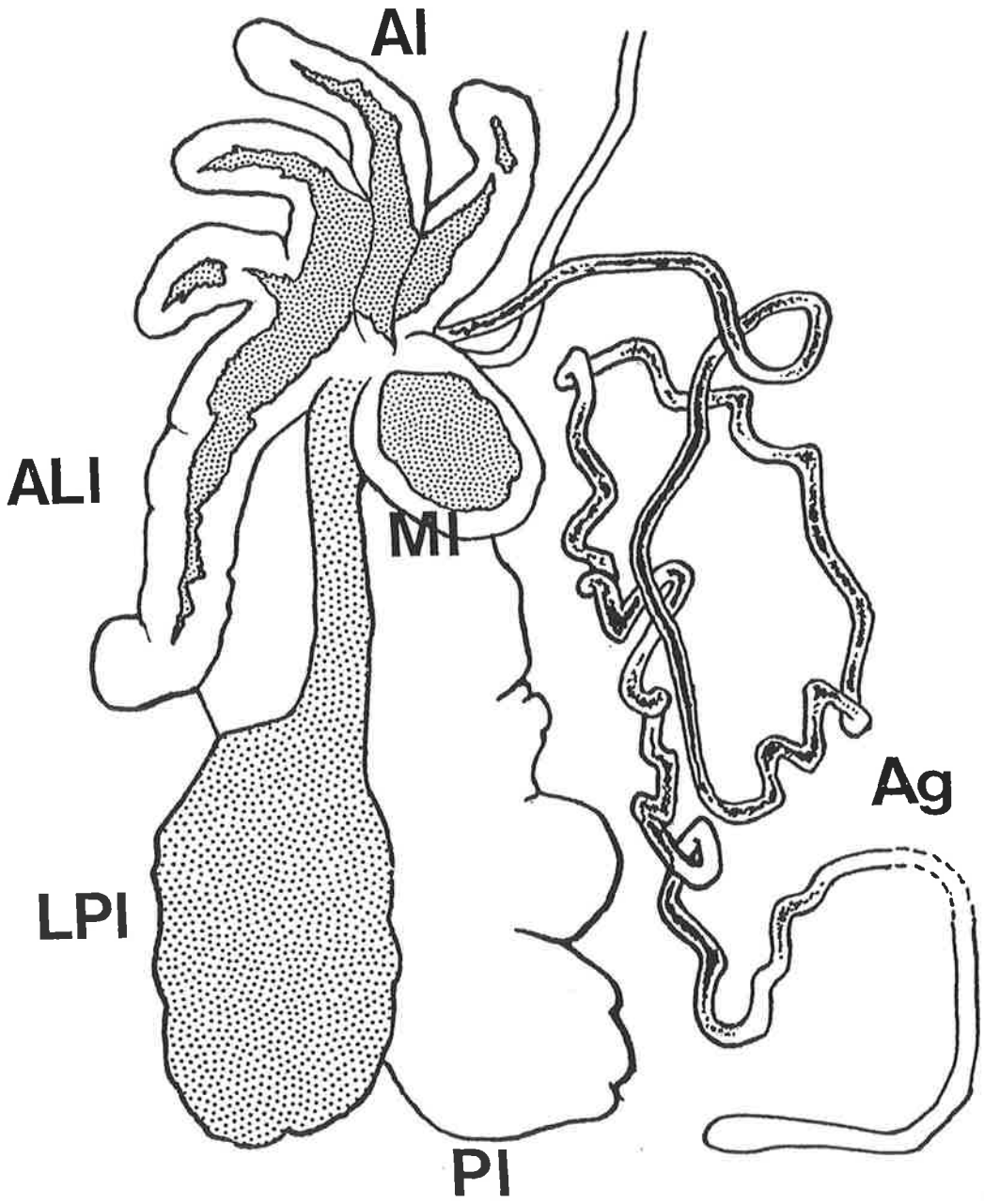
4.4.2.1. Origin of the stylet sheath: gelling and free sulphydryl content of gland lobes

After immersion in glycerol and subsequent transfer to water, all parts of the salivary glands of *M. profana* (Fig. 4.2) initially showed solid content, although that in the posterior and in most of the lateroposterior lobe soon dissolved. In 8 M urea, the contents of the lateroposterior, and posterior parts of the anterolateral and anterior lobes also dissolved, whereas opaque, solid material remained in the anterolateral and anterior lobes and a hyaline mass in the median lobe. The nitroprusside reaction for free sulphydryl groups was given by most of the contents of the freshly dissected principal gland and also by the cells of the accessory gland and ducts; less reactivity was found in glands that had been stored in the refrigerator overnight, especially in the median and anterior lobes.

4.4.3. Enzyme activity of gland extracts

No β-D-glucosidase, α-D-galactosidase, β-D-galactosidase, amylase, pectinase, cellulase or lipase activity was detected at pH 5, 6, 7 or 8 in extracts of the whole glands or saliva of *M. profana* (Table 4.1). Both acid and alkaline phosphatase were found in extracts of the whole glands but not in watery saliva. Sucrose was hydrolysed both by gland extracts and by watery saliva at all pH values tested, with highest activity at and above pH 7, but the preparations had no effect on raffinose, which is also a substrate for β-D-fructofuranosidase (EC 3.2.1.26, e.g.,

Fig. 4.2. Salivary glands of *M. profana* showing distribution of catechol oxidase activity and distribution of gelled contents after immersion in glycerol. Ag, accessory gland showing reaction of duct contents after incubation in DOPA; Al, ALl, Ml, anterior, anterolateral and median lobes showing insoluble, hyaline gels that form on immersion in glycerol; LPl, lateroposterior lobe which contains flocculent material that does not redissolve in water; Pl, posterior lobe, the contents of which redissolve in water after gelling in glycerol.



yeast invertase) but not for sucrose α-D-glucohydrolase (EC 3.2.1.48). Relatively weak maltase activity, ≤ 20% that of sucrose-hydrolysing activity, was found in salivary glands, which would be consistent with the presence of sucrose α-D-glucohydrolase. Because of the variable enzyme activity of the watery saliva in relation to substrate blanks for maltose, unequivocal demonstration of maltase activity in the secreted saliva was not obtained. Sucrose was hydrolysed by all coreids tested (Table 4.2). In contrast, preparations of the glands of the pentatomid, *P. apicalis*, and the mirid, *C. dilutus*, had no sucrose-hydrolysing activity. In order to simplify descriptions of the activity of the sucrose-hydrolysing enzyme of coreids, and to distinguish it from the activity of plant invertase, the insect enzyme will henceforth be referred to as sucrase.

Salivary preparations from *C. dilutus* gave unequivocally positive results for pectinase, as did those given by a standard of Sigma™ pectinase. The salivary glands of *C. dilutus* was also shown to contain an amylase but yielded no evidence of a salivary proteinase.

Catechol oxidase was found in the accessory gland and the accessory and common ducts of *M. profana*, although never uniformly (Fig. 4.2). Whether or not the parts of the system that stained in DOPA were always the same or could vary with time was not determined. The presence of catechol oxidase in the watery saliva was confirmed spectrophotometrically (Fig. 4.3). There was no evidence of a salivary peroxidase, however, since catechol oxidase activity was depressed in the presence of H_2O_2.

4.4.4. Amino acid content of saliva

Estimates of total amino acid content in pilocarpine-induced saliva from four insects revealed variable amino acid concentrations from 2.42 to 13.47 mM (0.32-1.77 μg/μl) leucine equivalents. Results of TLC separation are indicated in Table 4.3. Consistent with the variable total amino acid content, chromatograms of some samples of watery saliva gave very much clearer spots and separations than others. Although cysteine/cystine could not be demonstrated in the watery saliva, a distinct reaction for sulphydryl compounds was often given by a streak below cystine and/or at the origin.

4.4.5. Detection of enzymes in the digestive tract of *M. profana*

Proteinase was detected only in the third ventriculus (see Fig. 3.1), the connective tubule between the second ventriculus, essentially a storage vesicle (stomach), and the absorptive fourth ventriculus. Reducing sugars were present in

Table 4.1. The detection of enzymes in the salivary gland extracts and watery saliva of *M. profana*. Strength of reaction indicated by number of crosses. (-indicates absence of an identifiable quantity).

Enzyme	Salivary gland extract	Saliva
Phosphatases		
acid phosphatase	+	-
alkaline phosphatase	+	-
Carbohydrases		
sucrase/invertase	+++	+++
raffinase	-	-
maltase	+	+
amylase	-	-
Pectinase	-	-
Cellulase	-	-
Proteinase	-	-
Lipase	-	-
Oxidases		
catechol oxidase	+	+
peroxidase	-	-

Table 4.2. The detection of enzymes in the salivary gland extracts of 5 species of coreids (*M. profana, Amorbus* sp., *A. angustior, Au. nigrorubrum and G. tasmanicus*) a pentatomid, *P. apicalis* and a mirid, *C. dilutus*.

Enzyme	Coreidae					Pentatomidae	Miridae
	M. profana	*Amorbus* sp.	*A. angustior*	*Au. nigrorubrum*	*G. tasmanicus*	*P. apicalis*	*C. dilutus*
invertase/sucrase	+++	++	++	++	+++	-	-
amylase	-						++
pectinase	-	-					+++
cellulase	-						
proteinase	-						-
lipase	-						-

Fig. 4.3. Spectrophotometric detection of catechol oxidase and peroxidase in the watery saliva of *M. profana*: Top, absorbance spectrum of watery saliva incubated overnight in catechol against a catechol blank. A strong peak at 297.8 nm indicates the presence of a salivary oxidase. Bottom, absorbance spectrum of watery saliva incubated overnight in catechol in the presence of H_2O_2 against a catechol H_2O_2 blank. A suppressed peak at 292.8 indicates the absence of a salivary peroxidase.

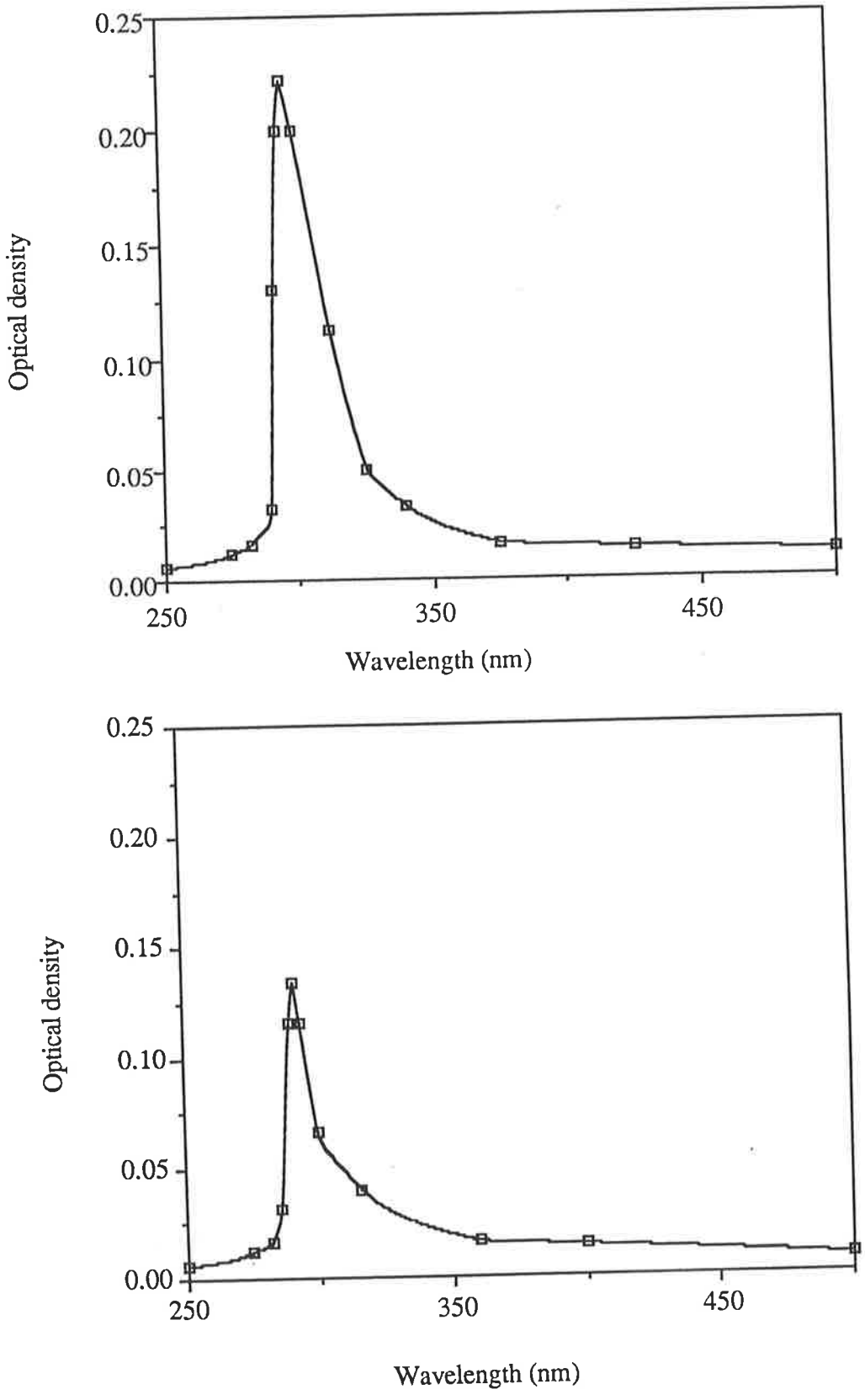


Table 4.3. Amino acids identified by thin layer chromatography of salivary gland extracts and watery saliva of *M. profana*. Strength of reaction indicated by number of crosses (? indicates uncertain separation; –, absence of an identifiable quantity). Compounds were recognised by revelation with ninhydrin-collidine, and/or confirmed using reagents described in Block *et al.* (1958).

Amino compound	Source		Confirmation reagent
	Extract	Saliva	
Leucine/isoleucine	+	+	
phenylalanine	+		
methionine	+	±	nitroprusside
valine	+	+	
tryptophan	+	?	
tyrosine	+	+	
proline	+	+	isatin
glutamic acid	+	+	
alanine	+	+	
DOPA	–	–	
threonine	+	?	
aspartic acid	+	+	
serine	+	+	
taurine	+		
glycine	+	+	
glutamine	+	+	
asparagine	+	+	
arginine	++	+	α-naphthol-Br_2
lysine	+	+	isatin
histidine	+	+	sulphanilamide
cysteine	–	–	nitroprusside
cystine	++	–	platinic iodide
subcystine	+	+	

Table 4.4. Analysis for sucrase and detection of reducing sugars in the salivary glands, and in the gut walls and contents of the digestive tract of *M. profana*. Intensity of blue colouration indicates amount of reducing sugar detected. Differences of intensity within treatments, between incubation mixtures with and without sucrose, indicate sucrase activity *vs* the intrinsic presence of reducing sugars. Light green indicates no reaction; "-" indicates not tested; * pale blue for fasted bugs.

portion of digestive system	**gut wall and contents**		**gut wall only**		**contents only**		**control**
	incubated in sucrose	incubated in water	incubated in sucrose	incubated in water	incubated in sucrose	incubated in water	
salivary glands	dark blue	blue	-	-	-	-	lt. green
first ventriculus	dark blue	pale blue	-	-	-	-	lt. green
tubule between first and second ventriculus	dark blue	pale blue	blue	lt. green	dark blue	pale blue	lt. green
second ventriculus	dark blue	dark blue	light blue	lt. green	dark blue	blue	lt. green
third ventriculus	dark blue	pale blue	pale blue	lt. green	dark blue	pale blue	lt. green
fourth ventriculus	dark blue	dark blue*	-	-	-	-	lt. green
hindgut	blue	pale blue	pale blue	lt. green	blue	pale blue	lt. green

the lumena of all portions of the digestive tract with the least amounts found in the hindgut. There was no evidence of *additional* sucrase being secreted into the gut lumen since the weak hydrolysing power found within the tract itself was consistent with the ingestion and conservation of salivary enzymes in the digestive tract (Table 4.4). Any trace of reducing sugars in the gut wall extracts was thought to result from residual amounts of enzyme adhering to the gut wall (due to insufficient rinsing of the gut wall during the preparation of the extract) and not to the presence of endogenous enzyme.

4.5. DISCUSSION

While an understanding of the activity and composition of the saliva of Hemipterans has often been considered of paramount importance in the study of insect-plant interactions, many studies on the etiology of plant damage by salivary enzymes have previously relied on homogenates of the entire insect body, of the head only, or of dissected salivary glands, rather than the procurement of pure saliva. The problem of distinguishing body or cell contents from the secretions actually ejected renders the analysis of whole tissues, even of the salivary glands themselves, of uncertain relevance. Yet, the dilute nature of the salivary secretions and the minute quantities in which they are often obtainable have made analysis and detection of the definitive salivary components very difficult. Major advances became possible only when "pure" saliva could be collected in sufficient quantities for analysis, but often required modification of conventional methods of analysis to enable the detection of minute activities in very dilute preparations. In this context the selection of *M. profana* for salivary analysis was advantageous. Not only was the test insect large, but the salivary glands and individual lobes were easily dissected and separated respectively and, most importantly, significantly large volumes of saliva could be collected directly from the mouthparts of individual bugs.

4.5.1. Origins of the stylet sheath

Investigation of the origins of the sheath material and watery saliva are relevant to the evolutionary relationship within the Pentatomorpha from simple, bilobed principal salivary glands of pentatomids and mirids to complex, multilobed glands of such forms as coreids. Baptist (1941), on morphological grounds, considered that additional lobes were subdivisions of the posterior lobe, whereas Miles (1972) suggested that all lobes other than the posterior lobe were concerned with elaboration of the sheath material and might therefore be derived from the anterior lobe, which is the source of the stylet sheath of pentatomids. In the present experiments, the formation of water-insoluble gels in the anterior, anterolateral and

median lobes tends to support the suggestion that they contribute to the sheath material, but there was also evidence from electrophoretic patterns that they also contributes to the watery saliva. The gelled contents of the lateroposterior lobe also provided some insoluble material, but its morphological similarity to the posterior lobe may indicate that its affinities are with the posterior lobe rather than any of the other lobes. The origins of these lobes, as well as the total functions of their products (see below) thus remain in doubt.

The distribution of free sulphydryl groups throughout the lobes in *M. profana* is consistent with the need to prevent premature formation of sulphydryl linkages in the sheath precursors before they are discharged (Miles 1972). The insolubility in water and urea of the gelled contents of the median and anterior lobes point to them as the most likely origin of precursors that contribute structural sulphydryl groups. Disappearance of a sulphydryl reaction from these lobes on standing could well be due to the capacity of the active groups in the sheath precursors to interact. The persistence of the sulphydryl reaction in the other lobes could be due to smaller molecular species that serve mainly to block structural sulphydryls during mixing of the salivary secretions within the glands. The identification of cystine in extracts of the glands indicates that cysteine/cystine could be responsible. Although these amino acids were not themselves detected in chromatograms of watery saliva, compounds that gave sulphydryl reactions and caused streaks below cystine and/or remained at the origin could well have contained cysteine. This would be consistent with a previous study on a pentatomid bug that indicated the sequestering of cysteine by the salivary glands, where it appears both as the free amino acid and incorporated into the sheath precursors (Miles 1969).

Protein gels that are insoluble in water but soluble in urea indicate hydrogen bonding. Hence the secretions of the lateroposterior lobe could provide some of the bulk of the sheath material and contribute to its rapid gelling once secreted. The contribution of the lateroposterior lobe to the watery saliva, apparently shown in electrophoretic gels (see later), is problematical, however, and will require further study. One possibility is that the sheath material has the capacity to separate out as a discrete mass from mixtures of contents of all the lobes as they are discharged.

4.5.2. Detection of salivary enzymes

The occurrence of phosphatase in the salivary glands of Heteroptera has been noted previously (Bronskill *et al.* 1958) and its presence, apparently as an endoenzyme of the salivary glands, is consistent with a possible role in elaboration of the phospholipid of the sheath precursors of Heteroptera (Miles 1961, 1967b).

Sucrose hydrolysing activity has previously been shown in the salivary glands of two coreid spp. (Hori 1975, Miles 1987a). *M. profana*, *Amorbus* sp., *A. angustior*,

Au. nigrorubrum and *G. tasmanicus* (all coreids) all exhibited salivary sucrose hydrolysing activity whereas the pentatomid, *P. apicalis* and the mirid, *C. dilutus* did not. As discussed in section 1.3, two enzymes, both of which have been termed invertase and/or sucrase, are capable of hydrolysing sucrose: yeast and plant invertase (EC 3.2.1.26) is also capable of hydrolysing raffinose (Ohlenbusch and Vögele 1974); the other, here termed sucrase, (sucrose α-D-glucohydrolase, EC 3.2.1.48) is not capable of hydrolysing raffinose but shows some activity against maltose (Dahlqvist 1974). The carbohydrase activity in *M. profana* saliva and/or salivary glands hydrolyses sucrose but not raffinose and has weak activity against maltose. The presence of a specific maltase (EC 3.2.1.20) could not be completely eliminated but the observed maltase activity was no more than could have been due to a sucrose α-D-glucohydrolase. The only carbohydrase activity unequivocally established by Hori (1975) in the salivary glands of another coreid was against sucrose and maltose and hence also consistent with sucrose α-D-glucohydrolase activity. The identity and characterisation of this enzyme will be further investigated in the following chapter.

Pectinase activity in the saliva of mirids has previously been reported (Laurema and Nuorteva 1961, Hori and Miles 1993). Indeed, the activity of a "violently toxic principle" which "dissolved" plant cell walls and was attributable to the action of a pectinase was described from mirids by Smith (as early as 1920) and Leach and Smee (1933). Amylase, too, was reported in the salivary glands of a pentatomid (Nuorteva and Laurema 1961) and in Lygaeidae (Hori 1970a). Thus it is not surprising to detect it in *C. dilutus*, the juveniles of which are apparently obligate pod feeders (Hori and Miles 1993).

4.5.3. Detection of salivary amino nitrogen compounds

Other constituents of the saliva of plant bugs that have previously excited interest are the free amino acids, which at one time were considered candidates for specific cecidogenic activities (Anders 1961, Schäller 1968). Previous work with radio tracers on the composition of the salivary secretions of a pentatomid indicated that any small molecular weight solute present in the haemolymph is likely to appear also in the watery saliva, even if in trace amounts (Miles 1967b), also that cysteine was probably necessarily sequestered in the salivary glands and incorporated into the precursors of the sheath material (Miles 1969). The present study supports the conclusion that the saliva contains at least most of the amino acids normally present in the tissue fluids of the insect, although the variable free amino acid content of saliva secreted under the influence of pilocarpine, down to just over 0.3 μg/μl in leucine equivalents, meant that some individual compounds were difficult to detect with complete certainty. Points of potential interest are the occurrence of histidine, a

particularly strong reaction for arginine, and failure to find DOPA. Histidine was claimed by Anders (1961) to be a cecidogenic agent secreted by the grape phylloxera (*Dactylosphaera vitifolii* Shimer); this amino acid was not among those found by Schäller (1968) in the saliva of phylloxera, however, and its secretion by coreids is not associated with any tendency to hypertrophic growth in tissues adjacent to the feeding site. Arginine is a particularly alkaline amino acid and could perhaps contribute to the control of the pH of watery saliva. DOPA was previously postulated to be an *in situ* substrate for the salivary oxidase (Miles 1964a), but recent papers on oxidative salivary functions in aphids (Miles and Oertli 1993, Jiang and Miles 1993) suggest that the primary substrates for oxidising enzymes are likely to be substances occurring within food plants.

The main contribution of this chapter indicates that sucrase is a major constituent of the secreted saliva of some coreids; that it is perhaps a characteristic of the family and could well be causally related to another, namely the production of terminal wilting in actively growing shoots. This enzyme is further characterised in the following chapter, and a possible role for the enzyme based on its intervention in the vascular function of the food plants is explored later.

CHAPTER FIVE

THE CHARACTERISATION, SOURCE AND SECRETORY ACTIVITY OF THE SALIVARY SUCRASE OF *Mictis profana* Fabricius (COREIDAE)

5.1. ABSTRACT

The posterior lobe of the salivary glands of *M. profana* secretes most if not all the sucrose-hydrolysing enzyme that occurs in the watery saliva. The enzyme has a pH optimum of 7.25 and a substrate specificity consistent with sucrose α-D-glucohydrolase (EC 3.2.1.48). Topical application of pilocarpine caused individual *M. profana* to secrete up to 58 µl watery saliva (at up to 0.4 µl/min) which showed continuous and independent variation of sucrase activity (up to 0.01 Units/µl) and of pH between 6 and 8; high sucrase content tended to coincide with high pH. Of the many proteins/subunits separable by electrophoresis of gland contents and saliva, four had sucrase activity, the most mobile with MW ca 66,000.

5.2. INTRODUCTION

The number of studies on the occurrence of salivary enzymes far exceed those of their properties, characteristics and variability. Nuorteva (1954) attributed the injury caused to wheat kernels to the presence of salivary amylases and proteinases in the nymphs but not the adults of *Lygus rugulipennis* Poppius. He recorded an optimum pH range of between 5.4 and 8.0 for the salivary amylase of *Capsus ater* L. and noted that proteinases in this species occurred in individuals collected from oats but not from grasses. The salivary proteinases of *Miris dolabratus* L. had pH optima between 6.4 and 7.6 while that of *Dolycoris baccarum* L. was active in neutral or slightly acidic pH. In the latter species, Nuorteva and Laurema (1961) found that an increase in the protein content of an artificial diet stimulated the production of salivary proteinases. Hori (1970b) noted seasonal variability in the amylase and proteinase contents of the salivary glands of *L. disponsi* Linnavuori, also from instar to instar, between individuals of the same developmental stage and, most surprisingly, between pairs of salivary glands of the same insect. He found that the variability of amylase was much smaller between the salivary glands of one individual than between those of separate individuals, indicating that both glands respond simultaneously to the stimulus for salivation. Inconsistently however, this relationship was reversed for salivary proteinase.

Hori (1970c) compared the salivary proteinase with those of the gut. The activity of both varied with temperature in the same way. No activity was detectable below 7°C or above 60°C. A weak pH optimum occurred at pH 5 and a stronger but broad activity peak occurred between pH 6 and 8, centred slightly acid of neutral, the latter consistent with the findings of Nuorteva mentioned above.

Salivary sucrase in the Heteroptera, unlike that from the head of the honey bee (Huber and Mathison 1975), has apparently not been previously characterised. The bee enzyme was characterised as an α-D-glucoside glucohydrolase (EC 3.2.1.20) and was found to have a MW (based on three methods of estimation) between 51,000 and 82,000. Its pH optima, of 5.5 and 6.5, were dependant upon substrates of sucrose and *p*-nitrophenyl α-D-glucoside, respectively.

5.3. MATERIALS AND METHODS

5.3.1. Quantitative determinations of salivary sucrase

Salivary glands were placed in Eppendorf tubes with 20 µl/gland distilled water and disintegrated using a Branson Sonifier™. After centrifugation, 5 µl of the supernatant were mixed with 50 µl Michaelis buffer (Pearse 1968)(of varying pH in order to determine the optimum, then at pH 7.25 in tests thereafter) and 25 µl 10% sucrose in Eppendorf tubes. Following incubation, 100 µl Tauber and Kleiner (1932) reagent was added, the tubes were sealed, shaken, placed in a boiling water bath for 10 min and cooled; 200 µl Nelson (1944) reagent was added with shaking, and 200 µl of the contents mixed with 3 ml distilled water in a spectrophotometer cuvette. The amount of monosaccharide liberated was estimated from the absorbance at 500 nm against water compared with a standard curve for freshly prepared solutions of glucose, after correcting for a substrate and reagent blank in which water replaced the extract, and an extract and reagent blank in which water replaced the substrate. Extract blanks gave negligible readings. Estimates of sucrase in watery saliva were made as described above but using 1 µl aliquots of saliva in place of 5 µl extract.

5.3.2. Sucrase activity and pH of secreted saliva

Adult *M. profana* were secured upside down on polystyrene foam using pins, 20 µl pilocarpine base in acetone applied to their abdomen and successive volumes of saliva were collected in 2 µl Microcaps capillaries until salivation ceased. The time taken to secrete each 2 µl volume was also recorded. To each aliquot an equal volume of 0.04% indicator solutions (bromothymol blue for pH 7, phenol red for pH 8) was added and pH compared to BDH™ Capillator standards. All were adjusted to a common pH of 7 using 0.1 M HCl, 5 µl taken, reacted with 5 µl 2%

sucrose, incubated at 37°C for 3 hr and reacted with Tauber and Kleiner reagents for the determination of reducing sugars as described above.

5.3.3. Determination of soluble protein and specific activity of sucrase

Posterior lobes and rest of glands were sonicated separately in 25 µl distilled water per pair of glands in Eppendorf tubes. Units of enzyme activity were estimated as µmoles of sucrose hydrolysed per min over 30 min at 25°C in 80 µl of a solution containing 3.125% sucrose (Bergmeyer and Graßl 1983). Soluble protein content was determined by diluting 10 µl of extract to 800 µl with distilled water and mixing with 200 µl Biorad™ protein dye reagent in a 1.6 ml narrow width cuvette; the absorbance at 595 nm against a reagent blank was compared with a standard curve for bovine serum albumin (BSA).

For analysis of variations between successive 2 µl volumes of watery saliva secreted by any one insect, aliquots of 0.80 µl were analysed for enzyme activity and protein content in place of the quantities of gland extracts indicated in the procedures described above.

5.3.4. Electrophoretic separation of denatured proteins

A Biorad Protean II™ vertical gel apparatus was used with a Biorad Model 500™ power pack. Stacking gels were of 3.08% acrylamide with 2.6% crosslinking; separating gels were of 9.24% acrylamide with 2.6% crosslinking. For separation of denatured proteins, the samples were of watery saliva, or obtained by discharging the contents of separated lobes in 10 µl water per lobe, or by sonicating whole glands or separated lobes in 10 µl distilled water per gland or lobe and centrifuging. Molecular weight standards contained 1 mg/ml each protein. Samples and standards were diluted 1:5 with a sample buffer containing 0.0625 M Tris buffer pH 6.8, 0.125% glycerol, 1.25% dodecyl sulphate, 0.0125% 2-mercaptoethanol and 0.005% bromothymol blue; the mixtures were heated on a water bath at 100°C for 2 min before loading 50 µl per well. The electrode buffer was 0.6% Tris, 2.88% glycine and 0.1% dodecyl sulphate. Gels were fixed in 50% trichloroacetic acid (TCA), stained in 0.1% Coomassie Blue R250 in 50% TCA, and destained in 0.7% acetic acid.

5.3.5. Identification of sucrase and invertase in electrophoretically separated proteins

Acrylamide electrophoresis was used to determine the approximate molecular weight of the salivary sucrase but with the omission of dodecyl sulphate and mercaptoethanol and the inclusion of 6% sucrose in the separating gel. Channels containing salivary gland extracts were run in triplicate and treated as follows: one

channel was stained in Coomassie blue. One channel was separated into 10 mm sections, each of which was incubated in separate Eppendorf tubes with 200 µl 6% sucrose for 3 hr at 30°C; the tubes were then heated for 10 min with 200 µl Tauber and Kleiner reagent in a boiling water, bath cooled and 400 µl Nelson reagent was added. The remaining channel was sprayed with the silver nitrate reagent of Trevelyan *et al.* (1950), left 10 min in the dark, sprayed with ethanolic sodium hydroxide (1.8 ml saturated aqueous NaOH to 100 ml ethanol) and left overnight.

For more sensitive separation and visualisation of proteins/subunits with sucrase activity, cellogel acetate electrophoresis of 0.5-1 µl samples of watery saliva, and of invertase (Sigma™ EC 3.2.1.26 ex *Candida utilis*) at various dilutions, was carried out as described in Richardson *et al.* (1986) on strips presoaked for 2 hr in 0.02 M phosphate buffer and run in the same buffer at 200 V for 35 min. A blot of the gel was obtained by laying it for 25 min on blotting paper partially dried after pre-soaking in 0.5 M sucrose. The blot was dipped in a minimal amount of 0.1% silver nitrate, blow dried, and placed in a minimal amount of ethanolic NaOH until bands began to show. Preparations were made permanent by treatment with thiosulphate.

5.4. RESULTS

5.4.1. Enzyme activity and pH of gland contents

Sucrose was hydrolysed both by gland extracts and by watery saliva at all pH values tested, with highest activity at and above pH 7, but the preparations had no effect on raffinose, which is also a substrate for β-D-fructofuranosidase (EC 3.2.1.26, e.g., yeast invertase) but not sucrose α-D-glucohydrolase (EC 3.2.1.48). Relatively weak maltase activity, ≤ 20% that of sucrase, was found in salivary glands, which would be consistent with the presence of sucrose α-D-glucohydrolase (see Table 4.1). Because of the variable enzyme activity of the watery saliva in relation to substrate blanks for maltose, unequivocal demonstration of maltase activity in the secreted saliva was not obtained.

The lobes of the principal salivary glands of Heteroptera are joined at a relatively simple hilus (Baptist 1941). Because of their complex morphology, perfect separation of the contents of those of coreids was found to be difficult, but preparations of separated posterior lobes of *M. profana* regularly contained 83-86%, and after particularly careful separation up to 97% of the sucrase in the whole gland.

The pH optimum of sucrase in the salivary gland extracts of *M. profana* was found to be close to 7.25 (Fig. 5.1). During this determination it was found that Tris buffer strongly inhibited the sucrase activity, and phosphate interfered with the

Fig. 5.1. pH optimum of sucrase in the watery saliva of *M. profana*.

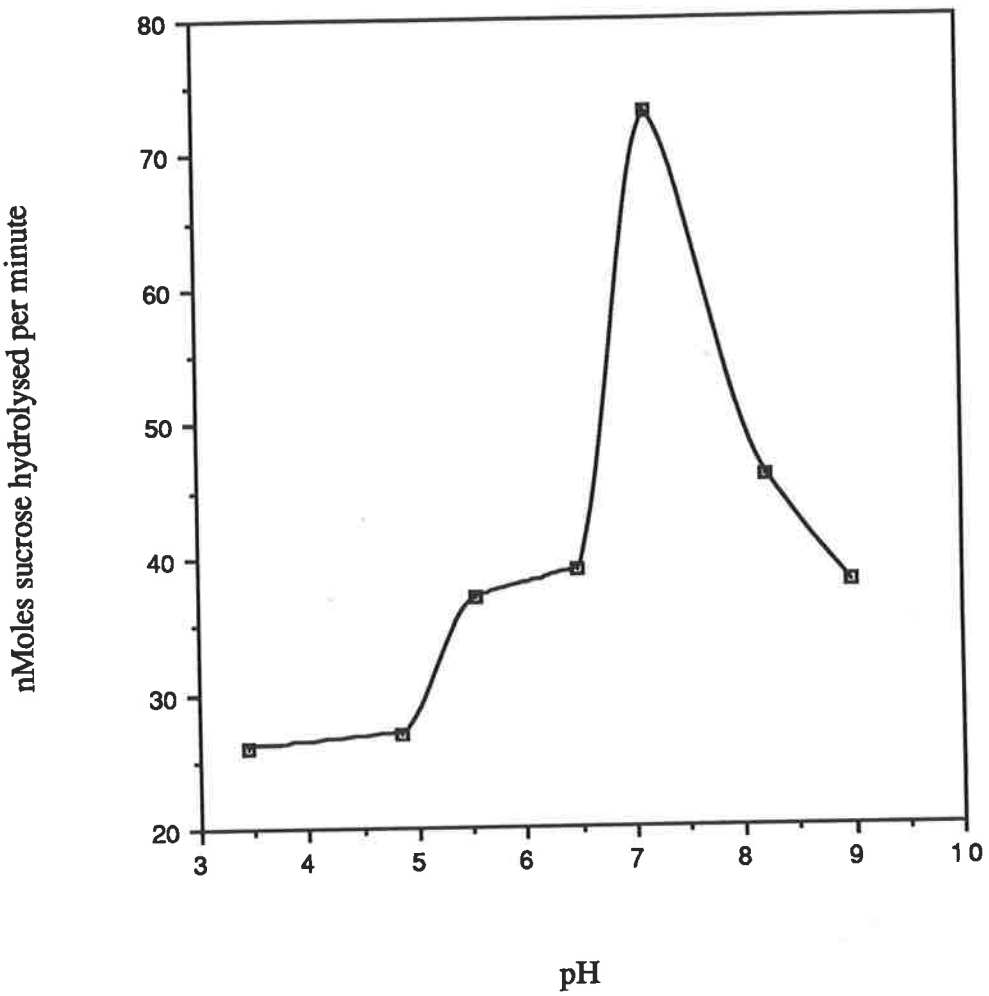


Fig. 5.2. Rate of salivation (µl/min.) of successive 2 µl volumes of secreted saliva of *M. profana*.

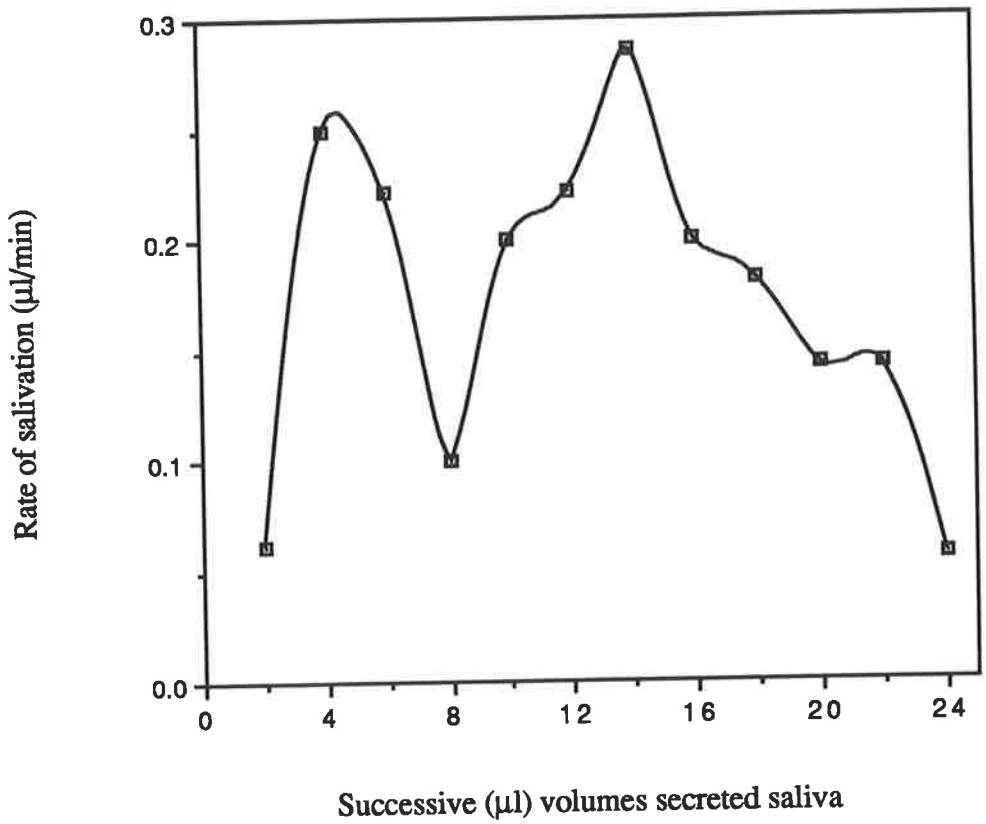


Fig. 5.3. pH of successive 2 µl volumes of secreted saliva of *M. profana*.

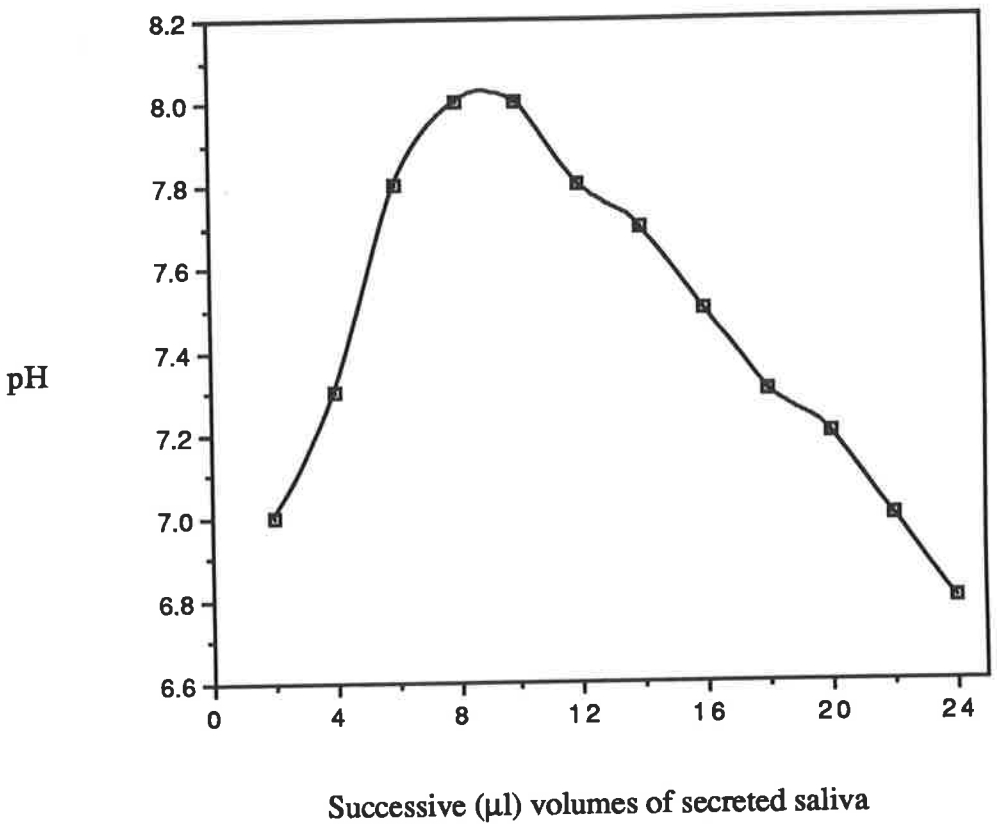
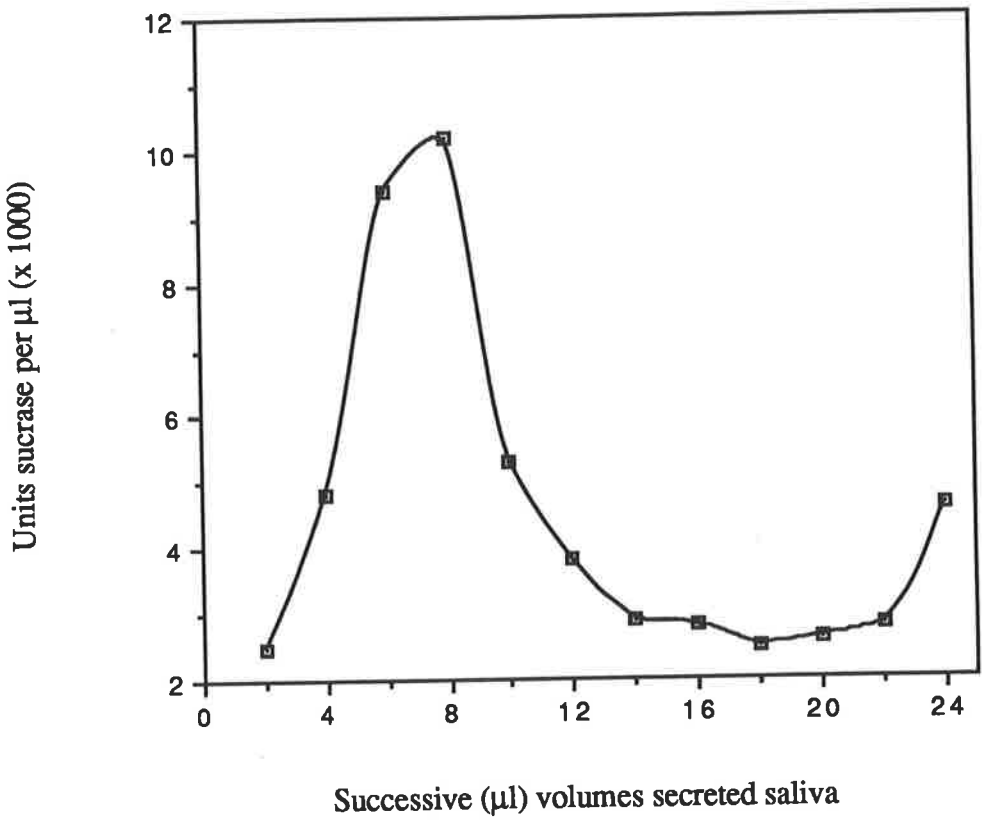


Fig. 5.4. Sucrase activity of successive 2 µl volumes of secreted saliva from *M. profana*.



reducing sugar test, but Michaelis (barbiturate) buffer gave satisfactory results.

5.4.2. Sucrase activity and pH of saliva of *M. profana*

Under the influence of pilocarpine, individual insects over the summer months usually secreted 20-30 µl over 3-4 hr. Exceptionally, one individual secreted 58 µl over a period of 5.5 hr period. The rate of secretion (Fig. 5.2) was not uniform, however, and typically began at 0.2 µl/min, increasing to up to 0.4 µl/min and dropping to less than 0.1 µl/min about 3 hr later. An increase in the secretion of the sucrase component during salivation was often preceded by a marked reduction in the rate of salivation. The saliva also showed varying properties (Figs 5.3-5.4). There appeared to be a clear relation between the pH of the saliva and the amount of sucrase activity present, although the pH at peak activity appeared to be higher than the pH optimum of the enzyme (see Discussion).

5.4.3. Specific activity of the salivary sucrase

A comparison between sucrase activity and soluble protein content (as BSA equivalents in extracts of glands and watery saliva of adults) was made in autumn, a month or so before the insects entered a period of winter quiescence. Both parameters varied between the glands of different specimens, but the posterior lobe routinely contained 65-70% of the water soluble protein of the glands ($n = 11$) and, allowing for imperfect separation from the rest of the gland, possibly all the sucrase (see above). Since the protein estimated in extracts and saliva was total soluble protein, the specific activity of the enzyme itself was not measured, but estimates were obtained of sucrase units relative to total protein. For tests on watery saliva, four insects yielded one, four, three, and two 2 µl aliquots of saliva respectively, considerably less than that provided by specimens in mid summer (see above).

In gland extracts and watery saliva, units of sucrase both per sample and per mg protein varied widely between the several individuals tested (Table 5.1). Total units in individual posterior lobes were only roughly correlated with soluble protein content ($r = 0.76$) whereas, in the watery saliva, the measured activity relative to protein content was more consistent (1.35 ± 0.07 U/mg; $r = 0.85$).

5.4.4. Electrophoretic separation of salivary proteins

Denaturing gels revealed a highly complex mixture of soluble proteins and subunits in the salivary glands (Fig. 5.5). The sonicated whole lobes yielded only slightly more complex mixtures (not shown) than their contents alone. The combined anterior, anterolateral and median lobes provided a much simpler pattern

Table 5.1. Sucrase (sucrose α-D-glucohydrolase) and soluble protein (as bovine serum albumin equivalents) in the salivary glands and watery saliva of *M. profana*.

Value sought	Whole gland (n = 10)		Posterior lobe (n = 13)		Per µl saliva (n = 6)	
	minimum	maximum	minimum	maximum	minimum	maximum
Units of sucrase (U)	0.009	0.094	0.004	0.081	0.0023	0.0123
Soluble protein (µg)	33.1	187.6	23.0	117.8	2.03	10.12
U/mg soluble protein	0.17	1.42	0.19	1.80	1.21	1.58

Fig. 5.5. SDS-PAGE electrophoresis of non-gelling proteins in the lumena of the principal salivary glands and in the pilocarpine-stimulated saliva of *M. profana*, compared with molecular weight markers and with the salivary gland proteins of a pentatomid. A, anterior, anterolateral and median lobes, L, lateroposterior lobe, P, posterior lobe, W, whole gland, S, secreted saliva of *M. profana*, I, yeast invertase, M, marker proteins, E, macerate of whole glands of *P. apicalis*. Gel stained in Coomassie blue; numbers indicate molecular weights of markers.

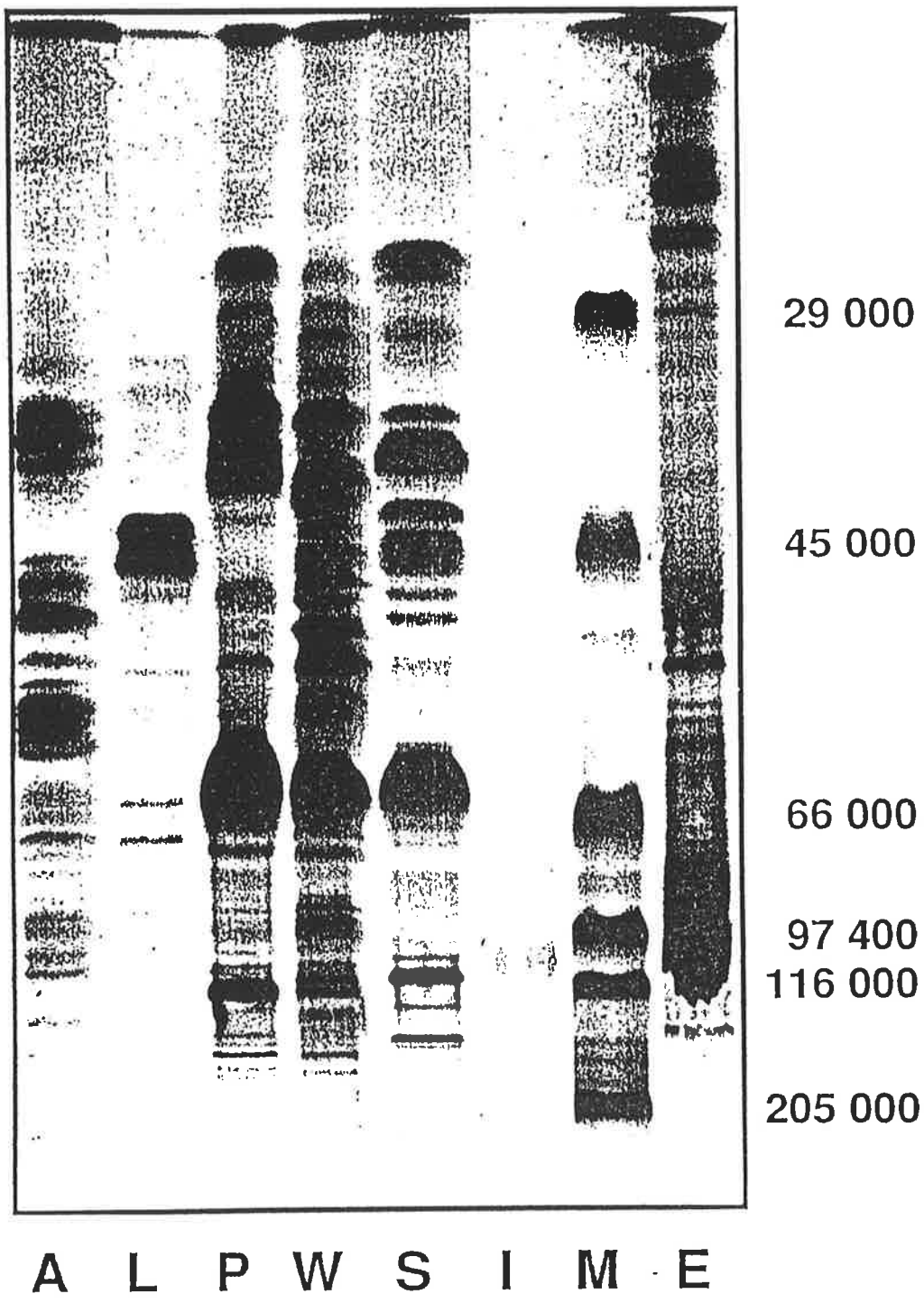
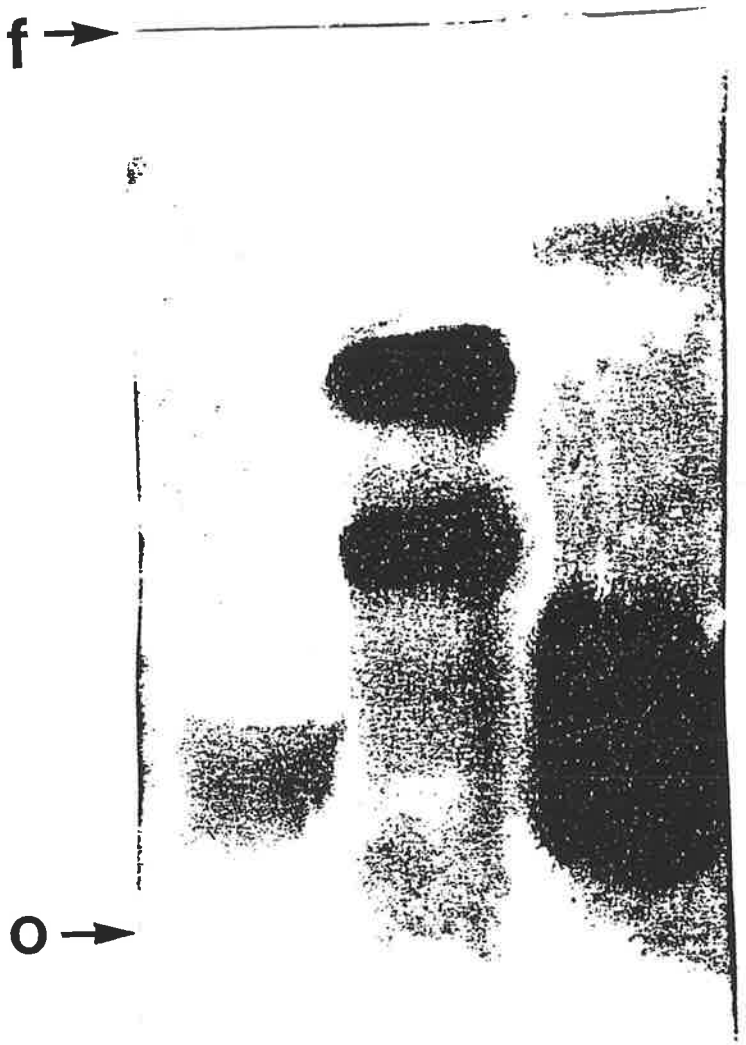


Fig. 5.6. Non-denaturing (native) cellogel electrophoresis of saliva of *M. profana* (centre) and of yeast invertase at 5 µg/ml (left) and 1 mg/ml (right); sucrose blot stained with silver reagent for reducing sugars; o = origin, f = front.



of soluble proteins. There were at least 14, clearly identifiable bands in the watery saliva, together with a similar number that were only faintly discernible; the clearest correspondence was with those in the contents of the posterior lobe, although at least two proteins appeared to be contributed by the lateroposterior lobe.

Nondenaturing gels, although they provided no clear separation of individual units when stained with Coomassie blue, produced one major locus for sucrase activity when segments were reacted with Tauber and Kleiner and Nelson reagents.

Although the Trevelyan *et al.* (1950) spray reagent for sugars was meant for paper chromatograms, it produced a distinct brown band in the same region of the gel, much the same as that occupied by BSA in gels stained Coomassie blue.

On cellogel strips, *M. profana* sucrase separated into four bands (Fig. 5.6), of which the two most mobile showed the most activity and ran well ahead of the *C. utilis* invertase. The latter showed subunits that, as had been expected, had much less activity and ran ahead of the main and presumably entire enzyme.

5.5. DISCUSSION

Sucrose α-D-glucohydrolase (EC 3.2.1.48) of mammalian intestine appears to have a molecular weight ≥ 200,000 (Kolinská and Semenza 1967), whereas the α-D-glucoside glucohydrolase (EC 3.2.1.20) from the honey bee was said by Huber and Mathison (1976) to be a homogeneous protein with MW estimated from SDS polyacrylamide gels of between 51,000 and 78,000. A single band of sucrase activity from the saliva of *M. profana* on non-denaturing polyacrylamide gels was found with MW similar to BSA, *i.e.* 66,000, which is therefore roughly consistent with the bee enzyme. Cellogel electrophoresis of the secreted saliva, however, revealed at least four bands with sucrase activity, the two most mobile giving the greatest activity. This could indicate that the original protein has four subunits which readily separate, that both dimer and monomer are highly active, and that the monomer has the MW indicated by acrylamide electrophoresis. It must be stressed, however, that these experiments dealt with very small quantities of unpurified preparations and that these conclusions are necessarily speculative.

The variability of content of the salivary glands of different individuals of *M. profana* with respect to sucrase activity and the capacity for continuous variation of the watery saliva with respect to pH, enzyme, total protein and amino acid content are consistent with previous reports of variability in the enzyme content of the glands of Heteroptera (Nuorteva and Laurema 1961) and even between the glands of a single individual (Hori 1970b). In the present study, enzyme activity of the saliva was measured in a way that would have avoided direct influence of the original pH

of the secretion, hence the apparent link between variation in sucrase and pH is unlikely to have been an artefact; indeed the indication that the two could vary independently indicated active coordination rather than an identical source for the two properties. The contents of the lobes of the principal gland are acidic whereas the watery saliva is usually neutral to alkaline and, since the accessory gland appears to contribute most of the volume of watery saliva (Miles and Slowiak 1976), it has also has been suggested to control its pH (Miles 1972). How it might do so has not been investigated. The secretion by the accessory gland of fluids from the haemolymph could well involve use of a cation pump, which could be expected to produce an alkaline secretion, but the sucrase of the watery saliva is clearly secreted mostly if not entirely by the posterior lobe, the contents of which have a low pH and should therefore cause a decrease in salivary pH. Since this does not occur, clearly the control of salivary pH is in need of elucidation. The highest pH of the saliva was actually greater than the optimum for the enzyme, but this may be related to a functional requirement to compensate for the low pH of plant sap, which presumably provides the milieu within which the enzyme has to function.

CHAPTER SIX

THE POSSIBLE FUNCTION OF A SALIVARY SUCRASE IN THE NUTRITION OF *Mictis profana* Fabricius (COREIDAE) IN RELATION TO THE ROLE OF SUGARS IN PLANT PHLOEM TRANSPORT

6.1. ABSTRACT

Feeding by *M. profana* on shoots causes a concurrent, localised increase in both water content and free amino acid concentration, consistent with phloem unloading. Coreids, unlike other groups of phytophagous Heteroptera, have been found to secrete a salivary sucrase, probably as the sole salivary carbohydrase, and attacked tissues had more sucrase activity than unattacked. The salivary sucrase of *M. profana* is postulated to cause unloading of solutes into the apoplast due to the osmotic effects of conversion of endogenous sucrose to glucose and fructose, allowing the insect to suck out the leaked contents of many cells from a single locus. The term "osmotic pump feeding" is proposed for such a process.

In attempts to simulate it, it was found that plasmolysis of bean cells was observable in 0.8 M solutions of sucrose, glucose or fructose, and in the hydrolysed products of 0.4 M sucrose, but not in 0.4 M concentrations of any one sugar. Similarly, in pressure bomb experiments, infiltration of 0.5 M sucrose *via* a cut end of a lucerne, (*M. sativa*) shoot had no effect on the amount of sap that could be extracted subsequently by a pressure differential equivalent to 690 kPa of suction on the cut end, whereas a mixture of glucose and fructose, stoichiometrically equivalent to 0.5 M sucrose caused an increase in both the quantity and the amino acid content of tissue sap.

6.2. INTRODUCTION

Zweigelt (1931) hypothesised that the salivary stylet sheath of aphids might act as a concentrated protein sol, drawing in solutes osmotically from surrounding plant cells. He thought that hydrolysis of starch into sugars by salivary amylase might also have osmotic effects. Kunkel (1967) suggested that parenchyma-feeding Homoptera might tap the contents of several cells by sucking from only one cell vacuole, thereby stimulating a flow of nutrients, presumably via the symplast, from surrounding tissues.

The possibility of an osmotic pump created by salivary action was again raised in relation to the phytotoxic effects of an *Amblypelta* sp. (Hemiptera: Coreidae) in Papua New Guinea (Miles 1987a). He described the lesions in young, succulent stems of sweet potato as an initial shrinkage of parenchymal cells followed by a 'ballooning' of surrounding intercellular spaces, leading to the total collapse of the affected tissues into melanised scars. In the harder stems of cassava (P.W. Miles, pers. comm.), the insect caused a similar collapse of parenchymal cells, which then pulled away from the surrounding cells leaving an empty cavity (see also section 1.2).

Similar to *Amblypelta* sp., *M. profana* also causes noticeable lesions in its food plants. These species and all other coreids so far tested (see Chapter Four), secrete a sucrose-hydrolysing enzyme in their saliva probably as the only salivary carbohydrase.

This enzyme has an action similar to that of the invertase normally found in plants and yeasts[1]. The presence of invertase from yeast and bacterial micro-organisms, and from the digestive systems of higher organisms, is usually clearly explained in terms of direct nutritive benefits. Similarly, it is usually assumed that the hydrolytic enzymes associated with the alimentary systems of insects, and specifically the predigestive role of the salivary enzymes of certain Hemiptera, are also related to nutritional needs. If this is so, the involvement of the salivary invertase of coreids is problematical. Sucrase would seem to be an unlikely salivary enzyme for plant sucking insects on a purely dietary basis. It is commonly reasoned that carbohydrates are in excess in the diets of those phytophagous Hemiptera in which phloem sap is the main contributor, and must endure a surfeit in order to obtain sufficient nitrogenous compounds for development, especially so when feeding on non-crop plants (White 1978). Sucrose, in particular, and its breakdown products glucose and fructose are major components of the soluble carbohydrate of tissue sap, and many sedentary phytophagous Hemiptera adopt a variety of biochemical strategies, such as transglycosylation reactions (or the synthesis of complex, non-assimilable carbohydrates) in the gut, to assist excretion of sugars and prevent assimilation of too much monosaccharide (Chippendale 1978). The action of a sucrase, producing readily assimilable monosaccharides, would therefore seem to run counter to this physiological trend. Why, then, have a salivary enzyme that

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[1] For the purpose of the present study, the distinction between the coreid and the plant/microbial enzymes would seem to have no practical significance. As indicated previously, the trivial names 'sucrase' and 'invertase' are here used where necessary to distinguish between the insect and plant enzymes respectively.

breaks down sucrose to readily assimilable products? Could it have other than a dietary function? Since invertase plays a vital role in plants in controlling the loading and offloading of phloem sap and hence the supply of nutrients to surrounding tissues, it would seem possible that the salivary enzyme of coreids could cause offloading of nutrients from the vascular bundles into the tissues on which the insect feeds. The continuous pumping of saliva and sucking back of fluids, together with the surfactant properties of the saliva noted previously, would presumably assist in water soaking of tissues and hence in the spreading and exploiting action of the saliva within them.

6.3. MATERIALS AND METHODS

6.3.1. Insect and plant material

M. profana was obtained from populations resident on *A. iteaphylla* and, in 1993, on cultivated roses in the grounds at the Waite Agricultural Research Institute. Broad bean plants (*V. faba*) were grown in 100 mm pots and used when about 200 mm tall. For some feeding experiments, runner beans (*P. vulgaris*) were purchased from a local green grocer. Lucerne (*M. sativa*, cv. Hunter River) used in simulation experiments were selected for uniformity of height and appearance from one year old plants grown in 140 mm diameter pots in a standard potting mix and cut back to promote new growth every few months.

6.3.2. Plasmolysis experiments

Transverse stem sections of broad bean seedlings were cut by hand under a dissecting microscope, using a new scalpel blade on each occasion. Uniform sections containing one or two layers of entire cells were found to give the most information. For plasmolysis experiments transverse sections of stem or leaf tissue were cut into water and rapidly transferred to several drops on a microscope slide of one of 0.1, 0.2, 0.3, 0.4, 0.6 and 0.8 M sucrose, or 0.4 M with respect to both glucose and fructose, and covered with a 22 mm diameter coverslip. They were periodically observed for evidence of plasmolysis for 2 hr, then placed in an airtight container with wetted filter paper and observed again at 24 hr.

6.3.3. Histological examination of plant material

For temporary mounts, plant tissue was prepared in aqueous bathing solutions for microscopic examination as described above. For the preparation of permanent slides, sections of stem and leaf tissues were cut into lactophenol (50% phenol in 50% lactic acid) (Gatenby and Beams 1950), passed through an ethanolic series to

100%, transferred to 50% xylene in ethanol then 100% xylene, and mounted in a standard xylene-based medium.

6.3.4. Detection of salivary sucrase in stem sections

To determine sucrase activity in sections of *A. iteaphylla* stem, 6 mm sections were cut from portions of stems on which the insects were observed feeding, also from similar but unattacked shoots. Each section as cut was placed into 100 µl of distilled water in a glass 'fusion tube' surrounded by ice. The sections were worked with a glass rod until a suspension of cells and fibres was obtained, and the mixture was centrifuged at 0°C. For estimation of sucrase, 10 µl aliquots of supernatant were incubated with 20 µl 10% sucrose (unbuffered) at 35°C for 3 hr. Reducing monosaccharide was estimated using Tauber and Kleiner reagent and Nelson reagent as described in Chapter Four.

6.3.5. Enzyme source and amino acid estimates

Commercial enzymes used were yeast invertase (EC 3.2.1.26), 400 U/mg and *Aspergillus niger* pectinase (EC 3.2.1.15), 1.1 U/mg, both from Sigma Chemical Company.

Concentration of total soluble free amino acids was estimated using the ninhydrin method of Rosen (1956). Aliquots of 10 µl were mixed in 200 µl water, 100 µl ninhydrin solution (3% ninhydrin in 2-methoxyethanol), 100 µl acetate cyanide buffer (0.001% NaCN in acetate buffer (360 gm sodium acetate trihydrate dissolved in 266 ml water, 66 ml glacial acetic acid and made up to 1l)), placed on a boiling water bath for 15 min, diluted when appropriate with 50% isopropanol and read spectrophotometrically at 570 nm.

6.3.6. Pressure bomb experiments

The apparatus used was from Soilmoisture Equipment Corporation, Santa Barbara, U.S.A. Lucerne shoots were selected for uniformity of height (about 200 mm), diameter and general appearance and placed overnight in distilled water or solutions of sugars or enzymes and, the following day, the cut ends were rinsed in distilled water and 150 mm of the shoot was placed in the pressure bomb and the pressure steadily increased over 3 min to 690 kPa using highly purified nitrogen. The volume of sap obtained was measured by taking up successive amounts as they exuded into a 20 µl Microcaps capillary which was then discharged into a 1.5 ml Eppendorf tube for later estimation of amino acid content.

6.4. RESULTS

6.4.1. Plasmolysis of plant tissue

Sections of *A. iteaphylla* stems were unsatisfactory for many histological observations due to the smallness of their heavily walled cells and their tendency to darken rapidly when cut. Plasmolysis of bean cells was most clearly observable in the palisade mesophyll where chloroplasts, bounded by the cell membrane were seen to shrink from the cell wall. Plasmolysis at 0.8 M was rapid and observable within 3 min. Evidence for plasmolysis at 0.6 M was not observable until 12-20 min and then only by partial separation of the cell membrane from the cell wall. No additional plasmolysis occurred beyond 30 min nor was there any evidence at 0.4 M or in lower sucrose concentrations. Plasmolysis was however observable between 5 and 7 min in a 1:1 mixture of 0.4 M of each of glucose and fructose. Thus the glucose plus fructose equivalent of a non plasmolysing concentration of sucrose was shown to plasmolyse bean cells.

6.4.2. Incubation of plant sections in yeast invertase

Incubation of sections in 0.1% yeast invertase alone apparently had little effect, but the dilute aqueous medium in which the observations were carried out would almost certainly have prevented creation of plasmolysing conditions even had endogenous sucrose been hydrolysed. Amino acid content of solutions in which sections had been incubated in invertase were highly variable and difficult to interpret because of the natural leakage of contents from partially damaged cells in the sections. Moreover, the experiments provided only a measure of simple diffusion of solutes from the sections and did not simulate the sucking action applied by the insects. In comparison, sections incubated over night in pectinase became darkened and showed clear signs of maceration.

6.4.3. Detection of salivary sucrase in stem sections

The attacked stems also contained significantly more sucrase activity and amino acids whether on a per mm or per mg fresh weight basis.

The saliva of *M. profana* itself contains up to 1.77 µg/µl leucine equivalents of free amino acids, but the smallest quantity of saliva that would be required to account for the increase in attacked shoots would be equivalent to over 3 µl saliva per mg fresh weight, whereas the highest possible estimate of difference in water content of the two types of tissue is only about 0.13 µl. That is, the chemical differences between attacked and unattacked tissues must have been due almost entirely to a change of endogenous constituents.

Results for reducing monosaccharide in extracts of stems were highly variable (Fig. 6.1), especially in the attacked tissues, and no significant difference from the unattacked stems was found. Invertase/sucrase activity was also variable but was detected in only two out of five replicates of unattacked sections, whereas all the attacked sections showed some activity.

6.4.4. Development and results of infiltration and pressure bomb experiments

Attempts to introduce solutions of yeast invertase or sugars into stems of *A. iteaphylla* or broad bean by injection with fine steel or glass needles were unsuccessful in that no water soaking of the injected tissues, such as caused by coreids, was observable and nearly all the solution that was to be introduced appeared to spurt out around the needles. The plants showed no more damage than that caused by the mechanical injury alone.

Furthermore, although necrosis of the cut end and wilting of *A. iteaphylla* shoots occurred after 24 hr of standing in 0.1% invertase or pectinase, and wilting also occurred in 1 M sugars, this was not considered a particularly convincing simulation of the more rapidly apparent damage caused by the insects. Even if substantial infiltration of the shoots *via* the cut end of stems had occurred, again there was neither simulation of the sucking action of the insects nor any indication of whether, by inducing plasmolysis with sugars, the insect would be able to increase the amounts of essential nutrients such as nitrogenous compounds that it could obtain from surrounding cells.

Application of suction to the cut end of the stems, although technically possible, proved to be practically difficult. Suction is, in effect, the application of a pressure at one point lower than that elsewhere, hence it was decided to simulate suction by increasing the ambient pressure on the rest of the shoot. This was achieved with the use of a pressure bomb. For this purpose, *A. iteaphylla* proved refractory: very little sap was obtainable from any shoots, however treated, and then only at high pressure (>1,400 kPa). Lucerne stems, however, readily provided contrasts (Table 6.2). In particular, despite large variances in the data, replacement of a non-plasmolysing concentration of sucrose with the appropriate concentrations of its hydrolysis products caused significant increases in both the volume of sap obtainable and its amino acid content ($P = 0.05$). When combined with the increased sap removal, the increased amino acid concentration provided an even more significant increase in the total amount of amino acids that could be obtained from the treated stems ($P = <0.001$). The 0.1% enzyme solutions did not have any effect, not even pectinase at a concentration that caused maceration of sections of plant

Table 6.1. Composition of sections of *A. iteaphylla* stems in relation to attack by *M. profana*. Values ± SE (n = 5), and t-tests of difference between unattacked and attacked sections (NS = non significant, * $P = 0.05$, ** $P = 0.10$).

Stem status	Fresh weight mg per mm	Moisture content %	Free amino acids as µg leucine eq./mg.	Reducing monosaccharides as µg glucose eq./mg.	nMoles sucrose hydrolysed/mg in 3 hr at 35°C.
Unattacked	1.29 ± 0.15	35.4 ± 2.0	12.64 ± 1.90	16.52 ± 0.76	6.6 ± 4.5
Attacked	0.99 ± 0.05 NS	48.5 ± 3.3**	18.31 ± 1.14**	19.56 ± 3.91 NS	29.0 ± 11.0*

Table 6.2. Sap obtained from 150 mm lucerne shoots under a pressure deficit of 960 kPa at the cut end, after standing overnight in various solutions. Means ± SE (n = 3), values in columns followed by the same letter do not differ at the 5% level (multiple range test of Duncan, 1955).

Solutions	µl sap obtained	meq. leucine in sap	Total µeq. amino acids
water	92.5 ± 21.5 a	0.34 ± 0.09 a	0.29 ± 0.04 a
0.1% enzymes:			
invertase	56.7 ± 12.4 a	0.53 ± 0.33 a	0.45 ± 0.23 a
pectinase	59.7 ± 23.2 a	0.73 ± 0.38 a	0.40 ± 0.18 a
other non-plasmolysing:			
0.5 M glucose	152.0 ± 18.8 a	2.81 ± 1.17 ab	4.61 ± 2.01 a
0.5M fructose	82.3 ± 3.8 a	4.47 ± 1.70 ab	3.55 ± 1.29 a
0.5 M sucrose	62.0 ± 8.1 a	4.00 ± 3.68 ab	3.27 ± 1.74 a
plasmolysing:			
1.0 M glucose	354.3 ± 94.6 b	8.55 ± 3.06 ab	35.89 ± 17.19 b
1.0 M fructose	255.3 ± 29.4 b	9.03 ± 2.38 ab	23.81 ± 7.22 ab
1.0 M sucrose	106.3 ± 11.1 a	12.65 ± 4.49 bc	13.89 ± 5.50 ab
0.5 M glucose + 0.5 M fructose	317.0 ± 6.2 b	19.77 ± 7.03 c	63.00 ± 22.74 c

tissue but, as discussed further below, this could have been due to strictly localised action of the enzymes at the cut end of the shoots, where they produced noticeable necrosis and, presumably, consequent disruption of vascular transport.

6.5. DISCUSSION

The classification of feeding of phytophagous Heteroptera as either "stylet sheath" or "lacerate and flush" feeding (Miles 1972) appeared inadequate once it was shown that a mirid, *H. clavifer* neither secreted a stylet sheath nor engaged in extensive laceration of the plant tissues on which it fed. Both the mirid and *Amblypelta*, a coreid, were found to feed for periods of several minutes, removing the contents of many cells without penetrating them with the stylets. Since neither species feeds directly on phloem sap, the problem arose of how they were removing nutrients from the plants. The mirid secreted a salivary pectinase and it seemed possible that this enzyme caused a purely biochemical maceration of a pocket of tissues, which were then sucked out. Strong (1970) was also of the opinion that lesions caused by *Lygus hesperus* Knight, another mirid, were due to the macerating action of its salivary pectinase rather than to any lacerating activity of the stylets. "Macerate and flush" would be a better appellation than "lacerate and flush" for such feeding.

For the coreid, however, no such resolution of the problem appeared possible, since the insect secreted no macerating enzymes in its saliva, yet caused a remarkable phenomenon in sweet potato, whereby the cytosol and dissolved contents of parenchymal cells were apparently discharged almost entirely into the apoplast, a process that was indicated by the engorgement of intercellular spaces in and between the collapsed remains of the cells (Miles 1987a). The ability of the insects to cause such massive unloading of cytoplasmic solutes into the apoplast and to suck them into stationary stylets represents a new category of feeding and one that requires a novel explanation.

The original osmotic pump hypothesis of Zweigelt (1931) is difficult to evaluate because it appeared to ascribe an osmotic, plasmolysing role to the stylet sheath of aphids, albeit possibly aided by a salivary amylase. It is now generally recognised that many, if not all the Aphididae feed by plugging into the positive pressure of sap in the phloem sieve tubes and it has been suggested that the sheath, far from provoking a response from the plant, may actually serve to minimise it (Miles 1990). Nevertheless, the ability of *Amblypelta* and apparently other coreids to remove nutrients from cells without mechanically rupturing them, and the consequent collapse of cells seen in the sweet potato, would seem to require enforced

discharge of solutes from plant cells into the apoplast, and arguably the most obvious cause would be the creation of an osmotically driven flow.

The discovery of sucrase in the saliva would seem to provide a ready explanation of how this could be achieved. It is known that the endogenous cytoplasmic and membrane-bound invertases of plants play a major role in the control of phloem transport and unloading. Such transport of sugar into the apoplast has been particularly well documented with respect to sugar cane (Thorne 1986) but the fundamental aspects of this process apply to plants generally. Moreover, a salivary sucrase would not seem to be directly mandated by the nutritional needs of plant-sucking insects, many of which are without it.

However hypothetically attractive the notion that the salivary sucrase of coreids may mimic the activity of endogenous invertases in its food plants, its experimental demonstration proved technically difficult. The insect is able to seal an exceedingly fine microsyringe into the plant and, by successive secretion and suction, aided by the strong wetting properties of the saliva (see Chapter Four), it is able to infiltrate a pocket of cells in a manner that could not be experimentally achieved. Infiltration *via* the transpiration stream in cut shoots offered one, albeit crude, alternative.

The 0.1% pectinase used as one treatment seemingly had little effect on stems placed in it other than to cause necrosis of the cut end and, judging from the wilting that was induced, consequent blockage of transpiration. Invertase used likewise had a similar effect. Since the activity of invertase is an integral part of the way plants control transport function, it is likely that it too would cause severe local trauma, whereas the effectiveness of this particular experiment depended on the ability of exogenous materials to spread extensively through the shoots.

Instead of attempting to infiltrate shoots with enzymes, therefore, the final aim of the experiments with cut shoots was to simulate the effects of sucrase by comparing the effects of introduction of its substrate and its reaction products. Even then, it was necessary to simulate somehow the sucking activity of the insects. Once this was done, even high variances in the data could not mask the powerful effect of the replacement of a non-plasmolysing concentration of sucrose with its products of hydrolysis. Further evidence that the saliva does indeed interfere with transport in the plant was the observation in sections of stems fed on by the insects of a systemic effect, evidenced by melanisation, on phloem and more especially xylem vessels adjacent to but apparently not directly contiguous with the end of the stylet tracks.

In the natural unloading of amino compounds into the apoplast, processes other than those mediated by invertase are involved (Bennett *at al.* 1986). Nevertheless, a strong osmotic gradient, such as postulated here, could be expected to cause a general outflow of cell solutes along with movement of sugars and water.

There are other components of the saliva that could have contributory effects in the production of plant lesions, particularly by action on the cell membranes. The surfactant properties of the saliva, apart from facilitating infiltration of saliva into the largely air-filled apoplast and causing the observed water soaking of tissues, would also be likely to have a destabilising effect on the lipoprotein of cell membranes. The catechol oxidase that occurs in the saliva, and that probably contributes to the characteristic darkening observed in lesions, is also known to generate peroxide and free radicals (Jiang and Miles 1993); it seems possible that such general oxidative activity could significantly increase the general leakiness of cell membranes by attacking the sulphide-zinc linkages that appear essential to membrane stability (Bettger and O'Dell 1981, O'Dell 1981). Even before necrosis became visible, therefore, oxidative activity initiated by the salivary enzyme could facilitate any osmotically driven outflow of nutrients into an initially dilute salivary fluid permeating the apoplast. It should be stressed, however, that surfactant properties and the presence of oxidases are likely to be features of the saliva of phytophagous Heteroptera generally, whereas salivary sucrase seems to be a characteristic of the coreids and is therefore more likely to be a key factor associated with the characteristic lesions they produce.

It may be noted that since the experiments reported here were completed a further simulation of the osmotic effects of coreid saliva, carried out by Dr Peter Miles, this time employing interrupted bursts of suction to infiltrate small sections of *A. iteaphylla* stem with invertase, confirmed that the enzyme caused release of free amino acids into solutions bathing the plant cells (Miles and Taylor 1994).

CHAPTER SEVEN

OBSERVATIONS ON FEEDING BEHAVIOUR AND THE SALIVARY PECTINASE OF *Creontiades dilutus* (Stål) (MIRIDAE)

7.1. ABSTRACT

Consistent with the findings of previous workers, a pectic enzyme, endo-polygalacturonase (EC 3.2.1.15) was detected electrophoretically from salivary gland extracts of *C. dilutus*. Adults on lucerne (*M. sativa*) produced rapid abscission of flower racemes and seed pods, and collapse of cell wall integrity of lucerne stems at high feeding densities. Incubation of stems caused leaf senescence in lucerne and leaf abscission in the non-host *A. iteaphylla*. Unlike *M. profana*, *C. dilutus* does not produce a coherent stylet sheath. Instead, stylet tracks in artificial agar media incorporating DOPA contain discrete black granules and are surrounded by a grey diffusate, indicative of a salivary oxidase.

7.2. INTRODUCTION

7.2.1. General biology

The Miridae is the largest family of Australian Heteroptera and contains many undescribed species (Carver *et al.* 1991). They are predominantly phytophagous although some species are predators of other soft bodied insects, or are known to be facultative haemolymph feeders, or to feed on the eggs of other insects.

The green mirid, *C. dilutus* (junior synonym: *Megacoelum modestum* Distant) is an elongate green to yellow or brownish green heteropteran of about 6 mm in length. Eggs are deposited in slits in the stem of its host plant with only the operculum visible. Growth and development were investigated by Foley and Pyke (1985), who swept adults from flowering lucerne and reared them on green beans sprinkled with cut lucerne clippings in gauze covered plastic containers. They measured the duration of each stage, from egg to adult at five constant temperatures (19-31°C), maintained at a constant relative humidity of 75%. Development time for males and females were similar, with a 59% survival rate. Total development time was 42.35 days at 19°C, 28.86 days at 22°C, 22.95 days at 25°C, 18.13 days at 28°C and 14.93 days at 31°C. At 25°C the duration from oviposition to eclosion was 8.32 days, and nymphal instars from one to five was 2.97, 2.20, 2.63, 2.48 and 4.33 days respectively. From these data they estimated total development time to be

280 day-degrees, a relatively low figure indicating the potential for rapid population increase under favourable conditions.

The species is considered of economic importance in a number of commercial crops (Carver *et al.* 1970) including lucerne (Wallace 1941), grapes, potatoes and cotton (Foley and Pyke 1985). They have also been recorded from beans, passion vine, the seed crops of carrot and parsnip, on some cucurbits, on peach, nectarine and prune fruits and on many vegetable plants (Hely *et al.* 1982). On passion vine and cucurbits the mirid feeds on the terminal shoots causing the growing tips to turn yellow and become senescent. Growth resumes from lateral buds and although growth and crop production is somewhat delayed on these crops it is not considered very destructive. On peach, nectarine and prune fruits the bugs occasionally cause injury by attacking the ripening fruit thus pitting the skin and causing discolouration of the flesh. If unripe fruit is attacked, long and persistent columns of gum exude from the puncture (Hely *et al.* 1982). In potatoes the insects appear in the crop just prior to flowering thus suppressing it, but their presence appears not to affect the production of tubers. In beans they attack the immature bud tissue in the axils of the stems that are destined to become flower racemes. These then wither and fall off leaving only a pair of bracts. This abscission of vegetative and flower buds by mirids is recorded also for cotton (Foley and Pyke 1985, Bishop 1980) and by feeding on the umbels of the seed crops of lucerne, carrots and parsnip seed heads fail to produce. The presence of small numbers of these insects can be responsible for heavy reductions of yield (Hely *et al.* 1982). Hori and Miles (1993) found that *C. dilutus* fed preferentially on flower sepals, ovules and pods. On foliage alone development failed beyond the second instar but they recorded 65% survival to adult on flowers and, from second instar onwards, high survival on pods.

7.2.2. Pectinase in the saliva of Hemiptera

The occurrence of a "violently toxic principle" in the saliva of mirids which assisted in "dissolving" plant cell walls thus aiding in the penetration of the stylets was recorded by Smith (1920, 1926). This was termed a diffusible "irritant" by Leach and Smee (1933) and was found to remain active for some time. An enzyme, pectin polygalacturonase, capable of hydrolysing the plant mid lamella was detected in extracts of whole aphids by Adams and McAllan (1956) and was subsequently found to originate in their salivary secretions (McAllan and Cameron 1956). Pectin polygalacturonase was apparently first recorded in the Heteroptera (six species of Miridae) by Laurema and Nuorteva (1961), was isolated from the posterior lobe (Strong and Kruitwagen 1968), and detected in the salivary glands of the Papua New Guinea mirid, *H. clavifer* investigated by Miles (1987a) and most recently in *C. dilutus* (Hori and Miles 1993).

7.2.3. Feeding lesions of mirids

The mirid, *H. bergrothi* Reut. was reported to feed for up to 20 min at a time on the stems of tea initially producing a faint water soaked spot (Leach and Smee 1933). The cell walls subsequently became light brown after 1 hr and began to collapse rapidly thereafter to form a dark purple brown lesion within 24 hr. They ruled out the possibility of it being caused by mechanical injury due to the extent of the lesion, and by fungal or bacterial pathogens due to the rapidity of the appearance of symptoms.

Lesions caused by the feeding of *L. disponsi* were examined histologically by Hori (1971) which, as in *L. hesperus*, possesses a polygalacturonase (Hori 1973). Similar lesions were described by Miles (1987a) for *H. clavifer* on cocoa pods and sweet potato stems. He estimated the maximum insertion of its stylets to be about 0.3 mm yet lesions with dimensions of up to 4 mm in diameter were observed on the surface of the pod or about 3 mm wide and up to 7 mm axial to the feed site on stems. He observed the appearance of a water soaked area within a few minutes from the commencement of feeding, the edges of which assumed a brown colouration within an hour, and which by 24 hr had become intensely oxidised. After about an hour of feeding the lesions formed contained apparently empty or plasmolysed cells into which oxidised substances had begun to diffuse. At 24 hr the cell walls had maintained their shape but thereafter they began to collapse.

7.3. MATERIALS AND METHODS

7.3.1. Detection and characterisation of salivary proteins

7.3.1.1. Electrophoretic separation of proteins

Either 2 or 5 µl aliquots of salivary gland extract was applied, using 8/1 or 6/4 Phastgel™ applicators respectively and run as per Phastsystem™ Text File 120 on a native gradient 8-25 gel for 225 volt hr in a Pharmacia LKB™ Phastsystem separation unit. Protein bands were either developed with Phastgel silver staining method (as per Phastsystem Text File 210) in a Pharmacia LKB Phastsystem development unit or stained with Coomassie blue (as per Phastsystem Text File 200). Molecular weights of the salivary proteins were estimated using a Pharmacia high molecular weight marker kit.

7.3.1.2. Electrophoretic separation of proteins from individual lobes

The accessory glands of two adult *C. dilutus* and three individual anterior lobes and three posterior lobes were placed in each of 3 wells in a Phastgel 6/4

applicator, run under native conditions on a Phastgel 8-25 gradient gel for 225 volt hr and developed using Phastgel silver staining method.

7.3.1.3. Identification of pectinase in electrophoretically separated proteins

The salivary glands of 5 adult *C. dilutus* were placed in 5 µl water, ground with a glass rod, 2 µl applied to each of two wells in a Phastgel applicator and run electrophoretically on a 8-25 gradient gel, under native conditions as described above. The gel was cut in half, one side was incubated in 1% pectin in phosphate buffer (pH 7.0) for 30 min and soaked in 0.5% ruthenium red for 20 min, the other incubated in 1% polygalacturonic acid for 30 min, transferred to 0.1 M malic acid for 15 min and stained in ruthenium red for 20 min. Pectin methylesterase bands appear dark on a red background while polygalacturonase produce colourless bands. Pectin lyases are evident as yellow zones or clear zones with yellow margins (Cruickshank and Wade 1980).

7.3.2. Observations on the structure of the stylet sheath

A 3% agar diet was prepared incorporating 10% sucrose, and prior to gelling 0.5 mg/ml of one of chlorogenic acid, catechol or DL-DOPA was dissolved and poured into 35 mm glass petri dishes. Diets were allowed to cool and covered with Parafilm. As a phagostimulant, an extract of lucerne leaves and flower racemes washed in ethanol until tinged with green was added dropwise to the Parafilm surface and allowed to evaporate. Diets were offered to about 100 freshly caught adult *C. dilutus* and left for 5 days caged in a plastic canister at room temperature after which observations of the structure of the stylet sheath were made. Alternatively, gels similarly prepared were offered to about 100 *C. dilutus* for 24 hr, fixed in Baker's formaldehyde (10% formaldehyde with a little $CaCO_3$ added) and stained with a reagent made by adding a few crystals of benzoquinone to water.

7.3.3. Observations on feeding damage by *C. dilutus* on lucerne

7.3.3.1. Feeding lesions of *C. dilutus* on plant tissue

About 100 adult *C. dilutus* were caged by means of an unventilated plastic canister over a glasshouse-grown, potted lucerne plant which was cut back and allowed to regrow, with 4-5 stems, to a height of about 30 cm.

7.3.3.2. Simulation of plant damage by pectinase

Freshly cut stems of lucerne and *A. iteaphylla* were placed in water or 0.1% pectinase at 25°C for 24 hr and observed for the development of necrotic or other symptoms.

7.4. RESULTS

7.4.1. Electrophoretic separation of salivary proteins of *C. dilutus*

7.4.1.1. Identification of pectinase in electrophoretically separated proteins

The presence of a salivary endopolygalacturonase is confirmed for *C. dilutus* by the presence of white bands on a pink-purple background using a substrate of polygalacturonic acid stained with ruthenium red (Fig. 7.1). Two prominent pale bands were observed, one with Rf value of about 0.55, (MW estimated at 105,000), the other, possibly two bands, migrated in the opposite direction and occurred below the origin. There was no evidence for the presence of a pectin methylesterase or pectic lyases.

About 26 protein bands were detected by silver staining in whole gland homogenates of accessory glands and 24 in each of the anterior and posterior lobe (Fig. 7.2), although in the latter two there was a significant amount of brown streaking, probably obscuring a number of bands. A strong protein band with Rf value of 0.55 consistent with the position of endopolygalacturonase activity was observable from the posterior lobe whole gland homogenate. Furthermore a single band below the origin of the anterior lobe and two bands below the posterior lobe corresponded to the reverse polar bands stained with ruthenium red (Fig 7.1).

7.4.2. Development of feeding lesions by *C. dilutus*

On runner beans, feeding sites of *C. dilutus* appeared as water soaked areas several mm in diameter which turned pale brown and softened on standing. Any such areas were vulnerable to subsequent phytopathogenic colonisation and decomposed rapidly. On mature lucerne with population densities similar to those encountered in the field the insects caused abscission of flower racemes and seed pods (Fig. 7.3). Excessive numbers caged in a humid environment on fully grown, yet young lucerne stems caused an initial wilting of leaf petioles (Fig. 7.4) followed by a total collapse of normally rigid stem tissue within 3 days of introduction (Fig. 7.5). This is consistent with the hypothesis that the activity of pectinase results in the destruction of the integrity of the cell walls and eventual total collapse of the stem.

7.4.3. Simulation of feeding damage by pectinase

Attempts at artificially inducing wilt symptoms by incubating stem sections in commercially available pectinase failed with lucerne other than to cause leaf senescence. For the non-host, *A. iteaphylla,* incubation in pectinase brought about

Fig. 7.1. Left, endopolygalacturonase in the salivary gland extract of *C. dilutus* following electrophoretic separation, incubation in malic acid and development using ruthenium red.

Fig. 7.2. Right, electrophoretic separation of proteins in salivary gland macerate of *C. dilutus* using silver staining. Left, accessory gland, middle, anterior lobe, right, posterior lobe. Position of endopolygalacturonases are indicated by arrows; 'o' = position of origin.

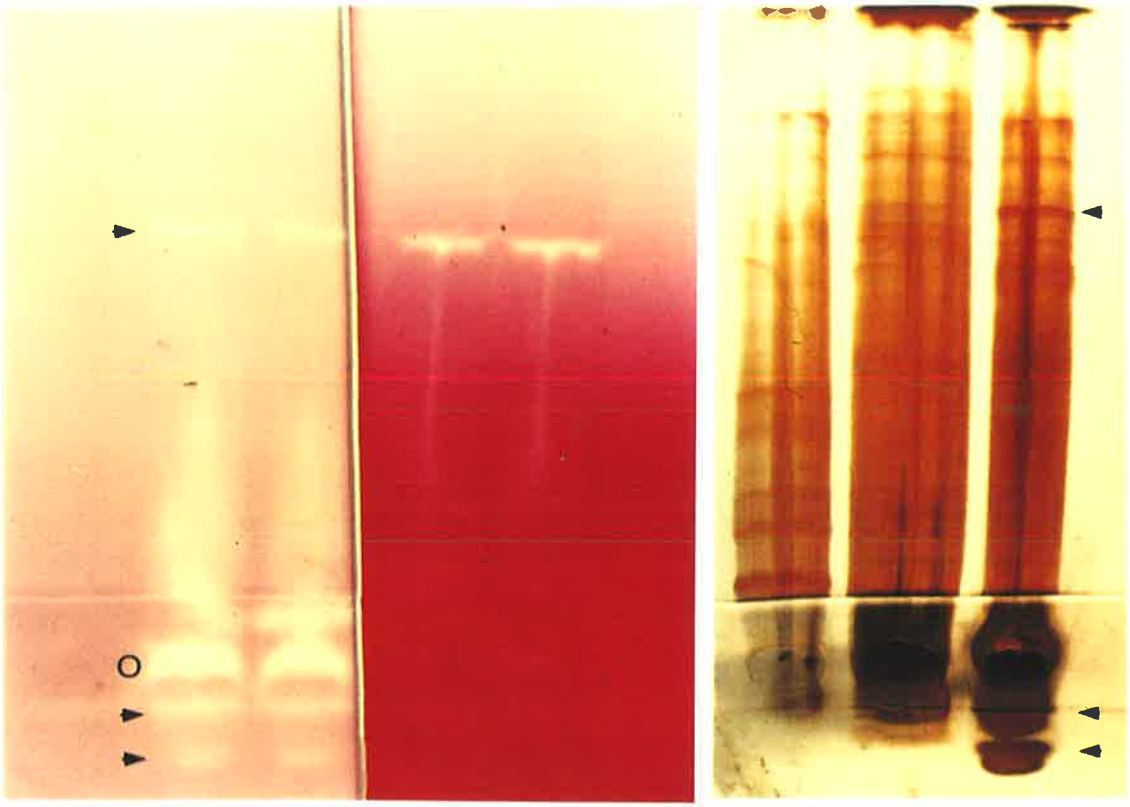


Fig. 7.3. Top, abscission of flower racemes and seed pods by *C. dilutus* feeding (left), and normal lucerne shoots in the absence of bug feeding (right).

Fig. 7.4. Middle left, wilting of terminal leaves and leaf petioles of lucerne in the presence of artificially high densities of *C. dilutus*.

Fig. 7.5. Middle right, collapse of lucerne stems in the presence of artificially high densities of *C. dilutus*.

Fig. 7.6. Bottom, artificial simulation of feeding damage on lucerne and *A. iteaphylla*: lucerne in water and pectinase, respectively (left), *A. iteaphylla* in water and pectinase (right). Note abscission of leaves in the latter.

rapid abscission of phyllodes (Fig. 7.6) which, as described above, is consistent with the characteristic symptoms that occur with flower racemes and seed heads of lucerne.

7.4.4. Structure of the stylet sheath and feeding behaviour on agar gels

Bugs were observed feeding on all 3% agar gels containing sucrose irrespective of other additives. Feeding sites in control diets appeared as faint depressions on the surface of the agar. The paths taken by the stylets were barely visible. Any eggs laid had a bluish green tinge and a green operculum which was ringed with a light brown colour. In chlorogenic acid, the paths taken by the stylets were slightly more visible with a light grey colouration and, rarely, contained minute darkened "granules". Eggs were bluish green but darkened in the vicinity of the operculum. In catechol, feed sites appeared as darkened punctures on the gel surface; the paths taken by the stylets were lightly suffused with grey and contained numerous black granules. Stylet tracks were often linear and unbranched, but sometimes had five or more branches indicating partial withdrawal, then reinsertion, of the stylets. Eggs appeared dark grey and were blackened in the vicinity of the operculum. In DOPA, feed sites were surrounded by a grey diffusate with the occurrence of similarly darkened granules. Eggs were a grey green colour with a darkened operculum.

Diets fixed in Baker's formaldehyde appeared as follows: feeding sites in DOPA appeared as black punctures at the Parafilm-agar interface with a dark, linear structure, either straight or slightly curved, immediately surrounded by an "aura" of grey pigmented oxidised DOPA. Some feed sites appeared without a distinct "core" but instead as a shallow grey diffusion. Feeding sites in the control gel and those containing chlorogenic acid or catechol were not visible after fixing in formaldehyde.

7.5. DISCUSSION

The violently destructive nature of the saliva of mirids have a long and considerable history in the entomological literature. While the symptomatology of the salivary 'toxins' has been extensively researched (Smith 1920, Smith 1926, Leach and Smee 1933, Shull *et al.* 1934, Jeppson and MacLeod 1946, Strong 1970), the identity of the causative entity was not proposed until considerably later (McAllan and Cameron 1956, Laurema and Nuorteva 1961, Strong and Kruitwagen 1968, Takanona and Hori 1974, Laurema *et al.* 1985). Not surprisingly, a salivary pectinase was also reported to be associated with the particularly strong phytotoxic symptoms caused by *C. dilutus* (Miles and Hori 1977, Hori and Miles 1993, and here-in). Remarkably however, *C. dilutus* was, for many years, not considered

particularly deleterious as a pest species (Gross, pers. comm., in Miles and Hori 1977); nor was it even mentioned as a potential pest in seed crops of lucerne (Doull 1961). Indeed its presence in lucerne, even during the height of summer infestations, is still all but ignored in dry land fodder crops, where seed production is of little importance.

Bishop (1980) investigated its potential for damage on cotton and recorded a 54% reduction in the number of squares produced. Similarly, Hori and Miles (1993) found that the magnitude of damage to flowers and pods, even at low insect numbers, was potentially considerable. This is consistent with previous reports of other mirids on lucerne and other crops. Thus *L. hesperus* and *L. elisus* Van Duzee instigated flower drop and injury to lucerne seed pods (Sorenson 1936, Shull *et al.* 1934) and abscission of cotton squares (McGregor 1927, Ewing 1929). Carlson (1940) and Jeppson and MacLeod (1946) reported that mirids feeding on the growing tips of lucerne not only caused their abscission but instigated a rapid development of lateral bud primordia. Carlson (1940) found a consequential increased number of (shorter) stems due to mirid feeding, leading Strong (1968) to hypothesise that this increased the number of flower buds produced, and indeed maintained conditions conducive to the reproduction and development of the insect: it may be recalled that the presence of flower racemes and developing seed pods were obligatory to the development of juvenile *C. dilutus* (Hori and Miles 1993).

The macerating action of mirid saliva has also been consistently documented since Smith (1920) used dissected salivary glands to demonstrate the action of salivary pectinase on potato cubes. The salivary pectinase (endopolygalacturonase EC 3.2.1.15) of the mirid *L. rugulipennis* was characterised by Laurema *et al.* (1985) as a basic protein with a pH optimum of 5.0 and peak activity at 37°C. Degradation of pectic acid was thought to be due to breaking of the pectic chains, with a 50% loss in viscosity when only a small percentage of glycosidic bonds were hydrolysed. The main products were glycosides comprising three to five galacturonic acid units. Substrate specificity indicated the possibility that up to three separate pectic glycosidases were present.

Electrophoretic separation of salivary proteins of *C. dilutus* using acrylamide gels and staining with ruthenium red also indicated the presence of three prominent bands/subunits (Fig. 7.1), one a basic protein, the other two migrating in the reverse direction. The presence of pectin methylesterase and pectic lyases was not detected. The molecular weight of the pectinase band was estimated to be about 105,000, somewhat higher than that (80,000) of the aphid, *Therioaphis trifolii* (Monell) (Madhusudhan 1994).

Although not further investigated, oxidative reaction to phenolic compounds in artificial diets has here been noted. The apparently universal presence of oxidative

enzymes in the saliva of phytophagous Hemiptera has been implicated in the detoxification of defensive phytochemicals to inactive polymers (Jiang and Miles 1993) or even to products with seemingly phagostimulant properties (Peng and Miles 1988).

One point to emerge from this study is that although *C. dilutus* did not secrete a coherent stylet sheath, it did secrete a granular material with stain reactions similar to those of the stylet sheath of *M. profana* (Chapter Two). Such a finding could well have considerable implications for the debate on the function of the sheath, to which allusion was made in Chapter One. Although no further investigation was made here of the oxidative granules secreted by *C. dilutus*, clearly they could be considered as homologous with the sheath material of other plant-sucking insects and point to an underlying function that does not necessitate a complete, tubular stylet sheath.

The powerful nature of the salivary pectinases on plant material, as discussed above, is investigated in the following chapter.

CHAPTER EIGHT

THE FUNCTION OF SALIVARY PECTOLYTIC ENZYMES IN LESIONS CAUSED BY *Creontiades dilutus* (Stål) (MIRIDAE)

8.1. ABSTRACT

The functional significance of the salivary pectolytic enzymes of *C. dilutus* is investigated. The maceration of bean sections by pectinase is examined histologically. Elution of amino nitrogenous compounds, and of plant allelochemicals and oxidation products from bean sections, is enhanced when incubated in pectinase. Such enhanced elution in conjunction with mechanical laceration is discussed with reference to the feeding strategy of mirids.

8.2. INTRODUCTION

Smith (1926) found that wherever the potato plant had been fed upon by the mirid, *Lygus pabulinus* Linn., the tissue for a considerable distance surrounding the puncture site was killed. He examined affected tissue histologically and demonstrated its progressive collapse. These symptoms, other than mechanical damage, were absent when plant tissue was punctured by the bug's ovipositor or with a sterile needle. Using slices of potato, Smith (1920) compared the effect of feeding of three species of bug, two of which, *Plesiocoris rugicollis* and *L. pabulinus,* he considered "harmful" to apple and potato respectively. The other, *Psallus ambiguus*, did not produce necrotic lesions on its host plant. From punctures made by the former two, a small drop of clear fluid exuded which increased in volume over several hours, eventually evaporating and leaving an area of blackened, dead cells. He reasoned that the appearance of the droplet was a direct result of the toxicity of the salivary secretions which, in its progressive destruction of cells, brought about their loss of turgidity and the exudation of their contents. No such exudation of fluid, nor any blackening occurred with the "harmless" species. He then removed the salivary glands from individuals of each species, placed them on potato discs and from the results obtained concluded that the toxic substances originated in the glands.

Strong and Kruitwagen (1968) isolated the activity of a polygalacturonase from the posterior lobe of the salivary glands of the mirid, *L. hesperus* and postulated that pectic enzymes were advantageous in that they liberated cell constituents thus

making them available as a food source. Strong (1970) observed that during feeding copious amounts of saliva were liberated, presumably containing pectinases from the posterior lobe; then the fluids released by laceration and enzymic action were ingested. Laceration, salivary secretion and ingestion is repeated until the tissue within the reach of the stylets is destroyed and depleted of fluids. He considered, however, that the action of stylet laceration in relation to the development of necrosis was only minimal as little obvious damage was caused by mechanical injury with fine needles. Miles (1972) coined the term "lacerate and flush feeding" for those Heteropteran families (Lygaeidae, Pyrrhocoridae, Miridae and some Pentatomidae) which use mechanical laceration and which do not secrete a complete stylet sheath.

Although some researchers have reported a role for bacterial and fungal pectinases in the degradation of plant tissue (Basham and Bateman 1975, Strand and Mussell 1976, Ishii 1978), similar studies of the role of the insect salivary pectolytic enzymes in the liberation of available cell constituents, thus making them available as a food source (Strong and Kruitwagen 1968, Miles 1987a), remain largely speculative i.e., the action of the enzyme during feeding itself has been implied rather than observed. So too is the action of the stylets in lacerating cell walls (Strong 1970, Miles 1972). The release of plant compounds during incubation in pectinase solutions and salivary preparations is investigated below, and compared with the effect of artificial (ultrasonic) maceration.

8.3. MATERIALS AND METHODS

8.3.1. Histological examination of plant tissue

Transverse sections containing one or two unbroken layers of cells of seedling broad bean stem were placed in 200 µl of either water or 0.1% pectinase (500 U/gm from *Rhizopus* sp.) and incubated at 25°C. At 24 hr then at 2, 3 or 6 days, sections were placed in Carnoy's fixative for 2 hr, rinsed in 100% ethanol, transferred to 95% ethanol, stained in 1% light green in 95% ethanol, washed in 100% ethanol with several changes, placed in 50% xylol in ethanol then 100% xylol and mounted in Canada balsam.

8.3.2. Incubation of bean sections in pectinase

In each of the experiments described in 8.3.3, coarse transverse sections 5 mm in length were cut from the stem of a broad bean seedling, incubated in 0.5 ml of water or 0.1% pectinase (500 U/gm from *Rhizopus* sp.) at 25°C and assessed as indicated.

8.3.3. Elution of amino nitrogen from bean sections by pectinase

8.3.3.1. Elution of amino nitrogen from bean sections by pectinase

After incubation as described in 8.3.2. for 20 hr, the tubes containing bean sections were shaken. Samples of 10 µl of water and 0.1% pectinase (controls), and of the water and pectinase solutions in which the sections were incubated (tests) were taken, 100 µl ninhydrin solution (3% ninhydrin in 2-methoxyethanol) and 100 µl acetate cyanide (0.001% NaCN in acetate buffer composed of 360 gm sodium acetate trihydrate and 66 ml glacial acetic acid per litre) was added and the mixtures were boiled on a water bath for 15 min, 150 µl was taken, made up to 1.2 ml with 50% propan-2-ol and read spectrophotometrically in 1.5 ml plastic cuvettes against water at 570 nm. In addition, one sample from each treatment (tests and controls) with an optical density value closest to the mean for the group was taken and scanned from 200-700 nm.

8.3.3.2. Elution of amino nitrogen from bean sections by pectinase (with maceration)

After incubation as described in 8.3.2 for 20 hr, the tubes were partially macerated for 1 min using an ultrasonic probe (Branson sonifier cell disruptor B15). Three degrees of maceration were used. The output control was set on "1, continuous" and the "% duty cycle" was set on 1, 2 and 4 respectively. The "% duty cycle" is the effective ultrasonic output of the probe and hence a measure of the degree of maceration. All tubes were then shaken, 10 µl of each was taken and treated as described in 8.3.3.1 for the detection of amino nitrogen.

[Note: This experiment was undertaken concurrently with 8.3.3.1 in which no ultrasonic treatment was used, and which thus provided "no maceration" controls for experiment 8.3.3.2. On the other hand, the following three experiments: 8.3.3.3, 8.3.3.4 and 8.3.4 were undertaken on separate occasions and did not permit similar comparison. Whilst every care was made to standardise experimental procedure and each experiment permits valid comparison within itself, high variances in the data indicated an intrinsic variability of the systems under investigation which precluded meaningful comparison *between* the successive experiments.]

8.3.3.3. Elution of amino nitrogen from bean sections by pectinase *vs* time

After incubation as described in 8.3.2, at 0.5, 1, 2, 4, 8 or 24 hr, 10 µl each of water and 0.1% pectinase (controls), and of the water and pectinase solutions in

which the sections were incubated (tests) were taken and treated as described in 8.3.3.1 for the detection of amino nitrogen.

8.3.3.4. Elution of amino nitrogen from bean sections by salivary gland extract from *C. dilutus* vs time

The salivary glands of 20 adult *C. dilutus* were dissected out under isotonic saline and placed in 1 ml water overnight, after which they were ground with a glass rod, centrifuged at 13,000rpm for 5 min, the supernatant removed and 0.5 ml placed in each of two 1.5 ml Eppendorf tubes. A single transverse section 5 mm in length was cut from the stem of a broad bean seedling and incubated in 0.5 ml of either water or one of the salivary gland extracts. At each of 1, 2, 4 and 8 hr, 10 µl of water and extract (controls), and of water and extract solutions in which the sections were incubated (tests) were taken, and treated with ninhydrin for the detection of amino nitrogen as described in 8.3.3 above.

8.3.4. Elution of other plant chemicals and oxidation products from bean sections by pectinase

Transverse sections 5 mm in length were cut from the stem of a broad bean seedling and incubated in 1 ml of either water or 0.1% pectinase (500 U/gm from *Rhizopus* sp.) at room temperature (about 18°C). At each of 1, 2, 5, 23 and 48 hr the tubes were shaken, the stem sections removed, and the solutions read spectrophotometrically in 1.5 ml plastic cuvettes against water at peak absorbance of 284.4 nm. One sample from each treatment (tests and controls) was taken and scanned from 200-700 nm for each time of incubation.

8.4. RESULTS

8.4.1. Histological examination of plant tissue

At 24 hr in pectinase the sections had decolourised, the epidermal cells had partially come away from the remainder of the sections (Fig. 8.1), the integrity of the cells had collapsed and the section, rather than maintaining its shape as in other treatments, folded around the forceps when manipulated. At 2 days the circle of epidermal cells had become almost completely disassociated from the remainder of the sections and the cell walls appeared somewhat thinner and fragile. At 6 days the ring of epidermal cells had come away completely from the remainder of the sections. In water the xylem vessels in the upper sections were darker (i.e. more oxidised) due

to their proximity to the air-solution interface, but they were otherwise visibly unaffected.

8.4.2. Incubation of bean sections in pectinase

Sections in water maintained a green colouration with a slight darkening of the xylem vessels (Table 8.1). In pectinase the cut surfaces became expanded and had blackened considerably (Fig. 8.2). This is thought to be due to the destruction of cell walls and the consequent release of cell wall bound phenols and the activity of plant derived oxidases. Beyond this blackening the cells had attained a green to pale brown translucent appearance and had become increasingly softened.

8.4.3. Elution of amino nitrogen, other plant chemicals and oxidation products from bean sections by pectinase and salivary gland extract from *C. dilutus*

8.4.3.1. Elution of amino nitrogen

An increased elution of amino nitrogen was detected from 5 mm long transverse bean stem sections incubated in 0.1% pectinase at 25°C for 20 hr (5.325 equivalents of leucine/ml ± 1.289 SD) compared to sections in water alone (1.563 ± 0.396 SD). These data are depicted spectrophotometrically (Fig. 8.3) with a spectral scan from 450-700 nm. After as little as 30 min incubation, more amino nitrogen was released from bean stem sections incubated in each of commercially available pectinase (data not shown) and salivary gland extract of *C. dilutus* (Fig. 8.4) than in their respective controls. Ultrasonic maceration (Fig. 8.5) increased the elution of amino nitrogen by about three-fold.

8.4.3.2. Elution of plant chemicals and oxidation products

Consistent with the elution of amino nitrogen, that of plant chemicals and oxidation products detected spectrophotometrically at 284.4 nm (Fig. 8.6) is linear up to about 8 hr. Beyond this time, the rate of elution decreased. Spectral data is shown only for 23 hr incubation, at which time a peak was observable at 285.6 nm (Fig. 8.7).

8.5. DISCUSSION

Plant cells are cemented together with intercellular materials in the middle lamella comprising cellulose, pectic substances and protein. Endopectolytic enzymes are able to digest pectin polymers causing tissue maceration (Bateman and Millar 1966, Strand and Mussell 1976, Ishii 1978), with release of cell wall bound

Fig. 8.1. Incubation of transverse stem sections of broad bean for three days in each of water and 0.1% pectinase (500 U/gm from *Rhizopus* sp.): top, in water; bottom, in pectinase.

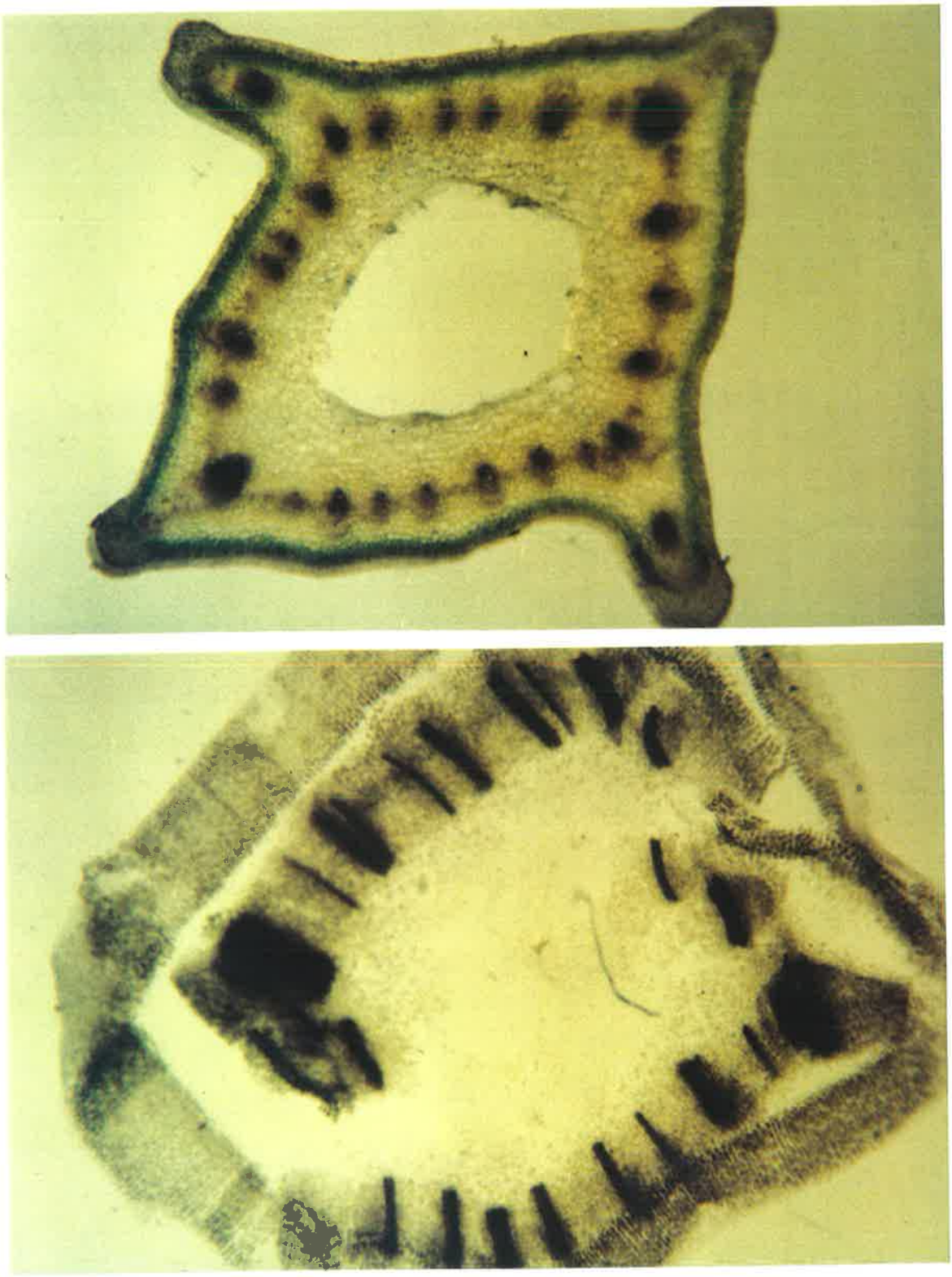


Fig. 8.2. Transverse stem sections of broad bean incubated in water (left) and 0.1% pectinase (500 U/gm from *Rhizopus* sp.)(right). Top, dorsal aspect, bottom, lateral aspect.

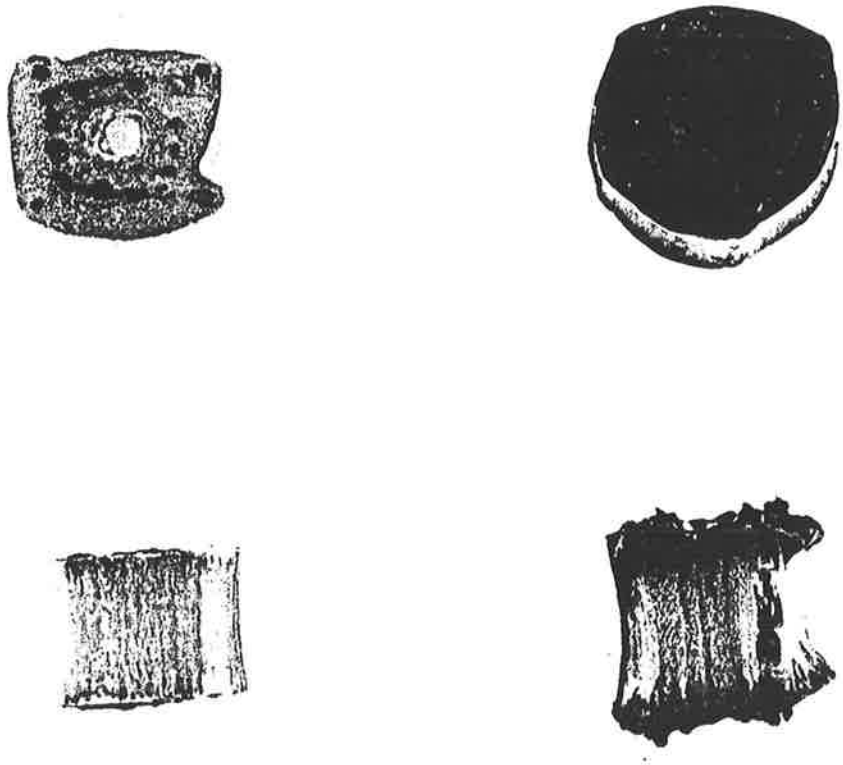


Table 8.1. Descriptions of bean sections incubated in water and 0.1% pectinase (500 U/gm from *Rhizopus* sp.).

Incubation time	Water	Pectinase
1 hr	Unchanged (sections green and turgid)	Cut epidermal edge slightly darkened
2 hr	Unchanged (sections green and turgid)	Cut epidermal edge slightly darkened
5 hr	Unchanged (sections green and turgid)	Darkening of the cut epidermal edge extends marginally within section; distinct decomposition of sub-epidermal cells; section with translucent or "water-soaked" and somewhat spongy appearance
24 hr	Unaltered appearance except that xylem bundles became light brown-grey in colour	Xylem bundles black, cut epidermis and cut surface dark grey to black; discolouration extends some distance within section; epidermal layers separated from remainder of section in vicinity of cut surfaces; section with translucent or "water-soaked" appearance and spongy texture

Fig. 8.3. Elution of amino nitrogen from 5mm bean stem sections incubated in 0.1% pectinase (500 U/gm from *Rhizopus* sp.) and water at 25°C for 20 hr. Spectrophotometric scan from 450-700 nm after reaction with ninhydrin: A, stem section in pectinase, B, stem section in water, C, pectinase control, D, water control.

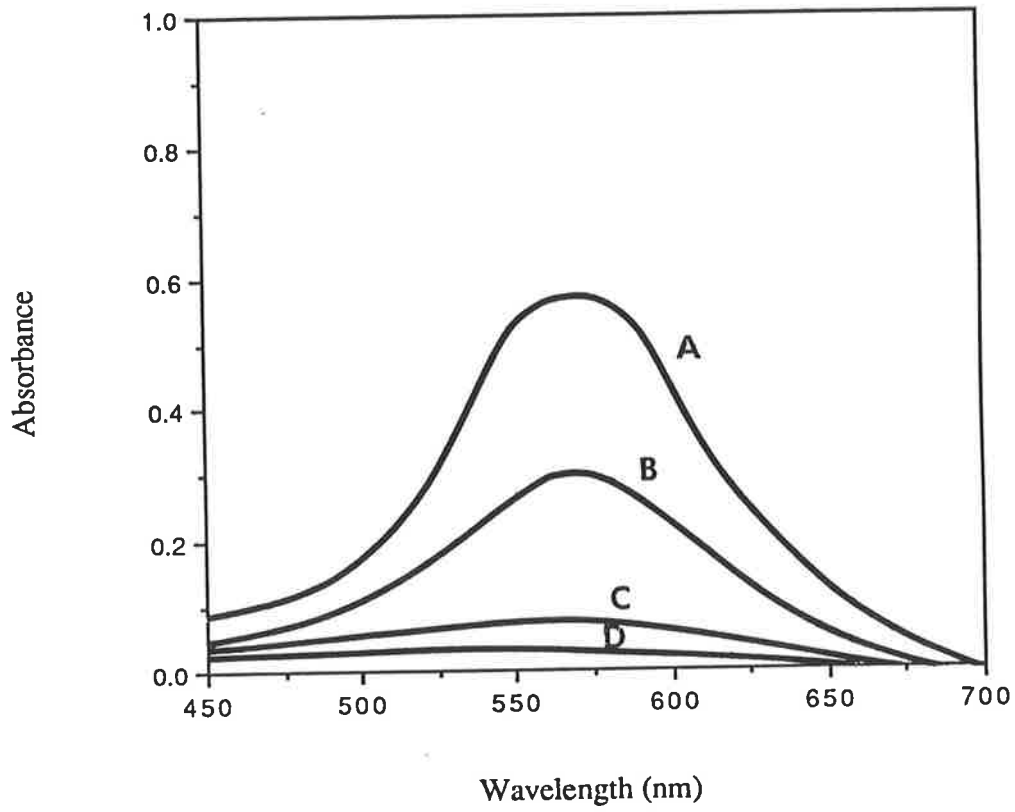


Fig. 8.4. Elution of amino nitrogen (as mM equivalents of leucine) from bean sections incubated in salivary gland extract of *C. dilutus* and water.

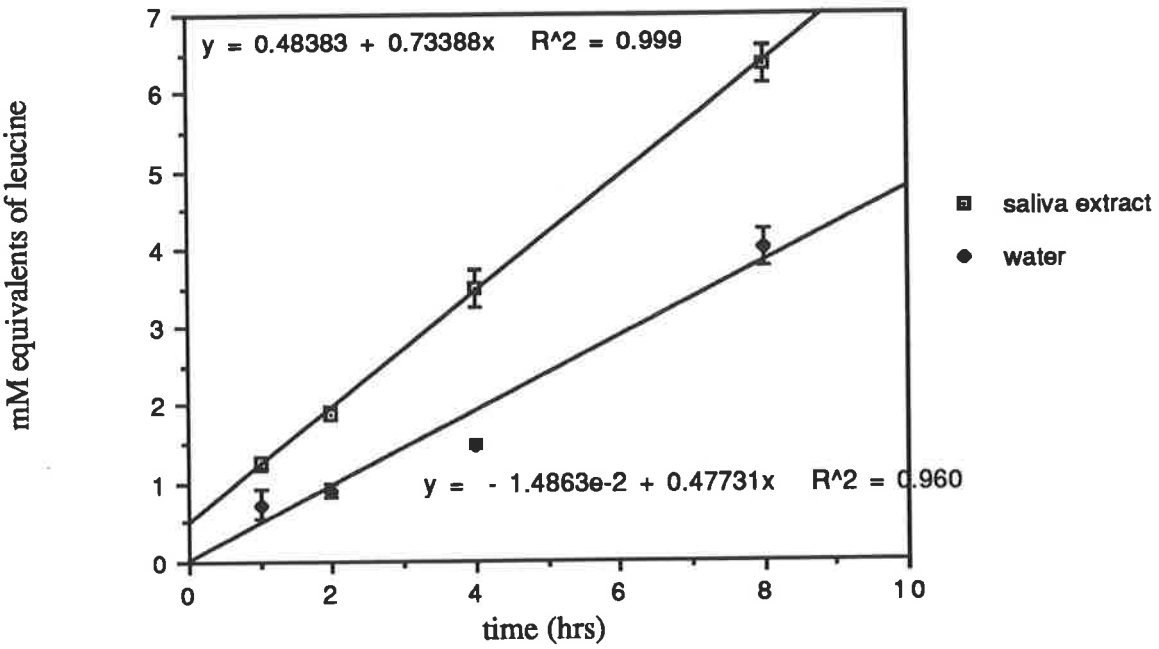


Fig. 8.5. Elution of amino nitrogen from 5 mm bean stem sections incubated in 0.1% pectinase (500 U/gm from *Rhizopus* sp.) and water at 25°C for 20 hr with following ultrasonic maceration.

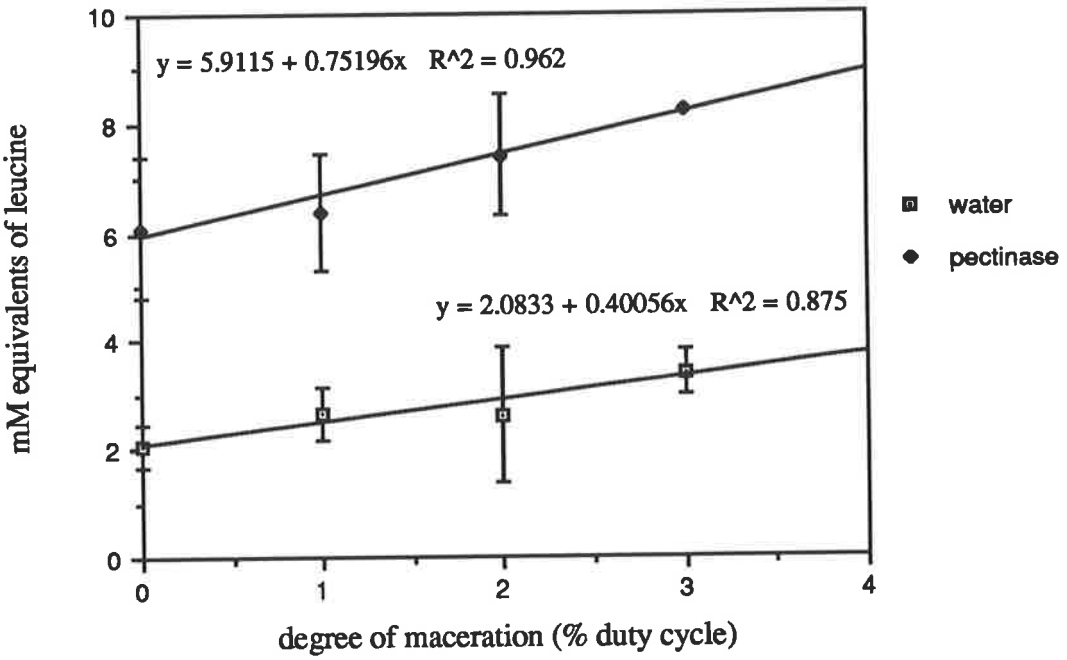


Fig. 8.6. Elution of phytochemicals and oxidation products from sections of broad bean seedling 5 mm in length, incubated at 25 °C in 0.1% pectinase (500 U/gm from *Rhizopus* sp.).

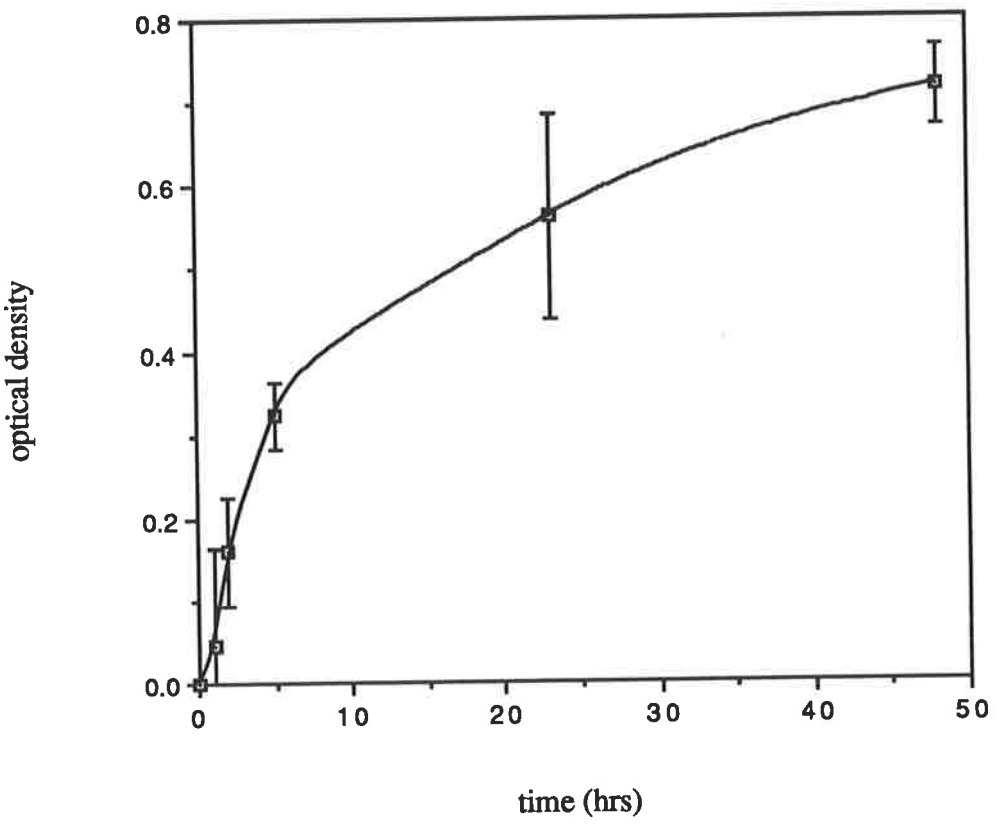
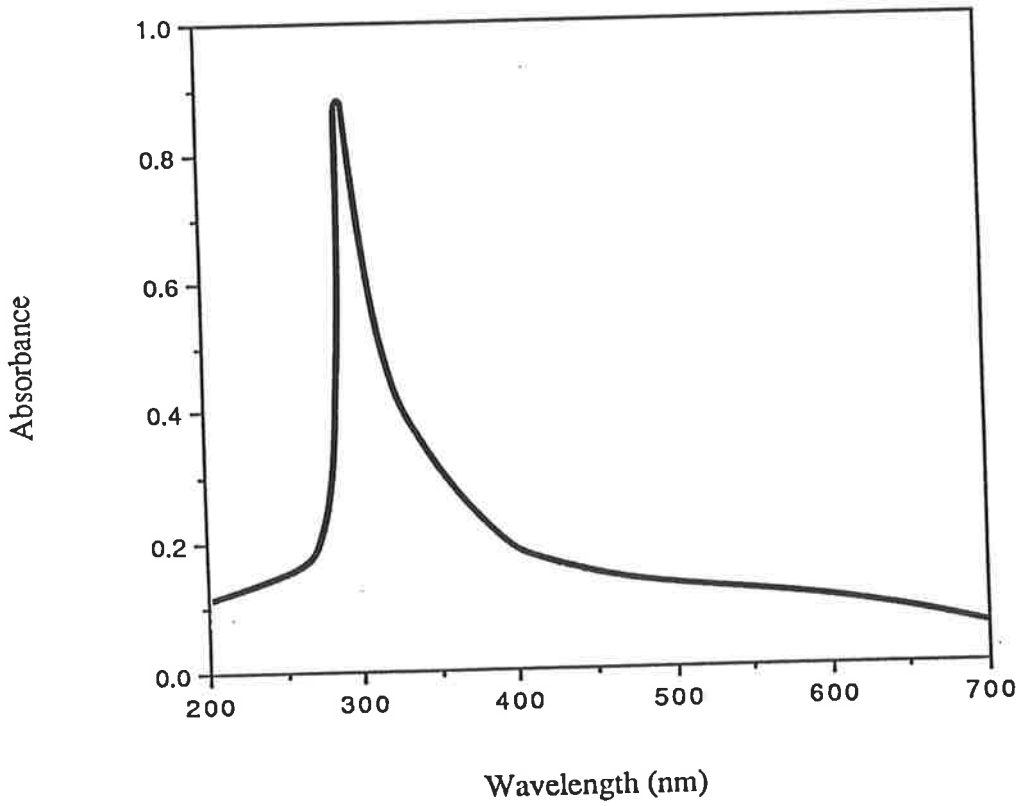


Fig. 8.7. Elution of phytochemicals from 5 mm bean stem sections incubated in 1 ml 0.1% pectinase (500 U/gm from *Rhizopus* sp.) and water at room temperature for 23 hr. Spectrophotometric scan from 200-700 nm: subtraction spectrum (stem section in pectinase *vs* stem section in water, with controls for pectinase only and water only subtracted).



proteins and neutral polysaccharides, which may be of nutritional benefit to the feeding bug. Pectic acids *per se* do not appear to be nutritive to mirids (Varis *et al.* 1983).

Consistent with the findings of Ishii (1978), who used endopectin lyase and endopolygalacturonase purified from *Aspergillus japonicus* on cell walls of potato tuber, the salivary gland extracts of *C. dilutus* increased the availability of soluble compounds extractable from bean sections.

Stem sections incubated in the salivary extracts underwent a number of changes consistent with the degradation of plant tissues that occurs in the presence of fungal and bacterial pectolytic enzymes. Thus, sections in saliva became progressively oxidised and rapidly assumed a blackened appearance. Furthermore, sections incubated in saliva and commercially available pectinases became progressively macerated and individual cells, particularly the stem epidermis, dissociated from formerly coherent structures.

Although pectolytic enzymes digest pectic polymers that comprise the cell wall, injury to plant cells is also characterised by loss of the ability of the plasmalemma to function as a semi-permeable membrane, such that damaged cells fail to accumulate and retain the vital stain, neutral red, and suffer a concurrent loss of water and electrolytes (Basham and Bateman 1975). Presumably the pectinase of the saliva of *C. dilutus*, which also has surfactant properties and is secreted unrestrained by the presence of a coherent stylet sheath, is also responsible for prominent water-soaked lesions produced.

The question of whether mechanical laceration by bugs' stylets serves to break down cell components and, in doing so, creates the lesions distinctive of some Heteropterans is problematic. Miles (1972) observed the vigorous activity of the stylets of the lygaeid, *O. fasciatus* which led to the fluidisation and removal of pockets of cells in milk-weed seeds, and reported on similar activities for *D. koenigii* in cotton seeds (Saxena 1963). This form of feeding was termed 'lacerate and flush' (Miles 1972). *H. clavifer*, however, was observed to use very little stylet movement once penetration had been achieved, yet this insect removed pockets of cells far beyond the possible reach of its stylets (Miles 1987a). Experiments reported here confirm that mechanical (ultrasonic) maceration in conjunction with enzymic maceration increases the elution of amino nitrogen compounds over the latter alone. In this context, however, these experiments do not elucidate to what extent stylet laceration may actually be used by mirids; such a mechanism would seem technically difficult to demonstrate.

It has previously been suggested that the salivary endopolygalacturonase of mirids is multifunctional. The primary function is undoubtedly, as shown above, to facilitate the elution of available solutes by the enzymic maceration of the midlamella of plant tissue. It has also been postulated, however, that its action may also aid in the initial penetration of the bugs' stylets into plant tissue during feeding (Strong 1970). Several observations preclude this as a viable mechanism. The penetration of plant tissue occurs both inter- and intracellularly and is particularly rapid, even by low instar nymphs. This is suggestive of a mechanical insertion too rapid for possible enzymic tissue degradation. Indeed, many other Hemiptera are able to penetrate plant tissues equally as fast without the aid of a salivary pectinase.

CHAPTER NINE

GENERAL DISCUSSION

9.1. The salivary glands and properties of insect saliva

The principal salivary glands of *C. dilutus* comply with the fundamental bilobed condition of Cimicomorpha, comprising an anterior and posterior lobe and an additional accessory duct terminating in a bulbous gland or salivary reservoir. Those of *M. profana* are apparently pentalobate. The anterior, anterolateral and medial lobes appear to be derived from a primitive anterior lobe and the lateroposterior and posterior lobes from an ancestral posterior lobe, analogous to the anterior and posterior lobes, respectively, of *C. dilutus*. Unlike that of *C. dilutus* the accessory duct in *M. profana* terminates without a prominent reservoir. This attribute is probably indicative of the fundamentally different feeding strategies of these insects, as discussed later.

The anterior, anterolateral and median lobes of *M. profana* form water insoluble gels consistent with their contributing to the formation of a stylet sheath. The posterior lobe contributes sucrase (sucrose α-D-glucohydrolase, EC 3.2.1.48) almost certainly as the only carbohydrase to the watery saliva. This enzyme was found in the salivary glands of all five coreids tested and as such appears to be a consistent familial character. There was no definitive evidence of additional sucrase being secreted from the digestive tract. The salivary sucrase of *M. profana* was found to have a pH optimum of 7.25, with MW ca 66,000 and exhibited weak maltase activity. During secretion, high sucrase activity coincided with high pH. The only other enzyme secreted in the watery saliva was a catechol oxidase derived from the accessory duct. The precise function of the lateroposterior lobe remains unknown but it appears to contribute at least two proteins to the saliva. Up to 58 µl watery saliva was secreted by an individual insect which contained free amino acids up to 1.8 µg/µl and possessed marked surfactant properties as indicated by persistent 'bubbles' in saliva taken into a pipette tip. Such wetting properties most likely cause the initial 'water soaking' of plant tissues during feeding and would undoubtedly aid in the penetration of salivary solutes in plant tissue.

Both a pectinase (endopolygalacturonase) and an amylase were detected in the salivary glands of *C. dilutus*. The MW of the pectinase was estimated at 105,000. In the salivary glands its presence was restricted to the posterior lobe. This is consistent with the source of the supposed principle salivary hydrolase of *M. profana*. Further characterisation of the salivary amylase of *C. dilutus* was not attempted. As with *M. profana*, too, *C. dilutus* secreted catechol oxidase into

artificial agar diets. Fundamentally distinct, however, is the propensity for *C. dilutus* to produce, at most, an incoherent stylet sheath, often limited to the occurrence of an occasional granule of stylet sheath-like material along the path taken by the insects stylets.

9.2. Physiological response of host plants to insect feeding

Feeding by *M. profana* causes terminal wilting acropetal to the feed site on young, small diameter shoots of their host plant. Surfactant properties of the saliva cause water soaked lesions accompanied by an initial increase in water content, all of which precedes progressive senescence of the shoot tip. Laterally branched stylet tracks target cells in the vicinity of the vascular bundles initiating mainly acropetal discolouration especially of xylem vessels, yet some adjacent bundles, too, were similarly affected. Feeding punctures, indicated externally by the presence of stylet sheath flanges, occur in rows at a distance of about 0.3 mm, invariably as a basipetal sequence. It is postulated that this successive basipetal adjustment of feed sites is necessary for the insect to counter the advancement of progressive tip senescence.

Similarly, *C. dilutus* causes water soaked lesions around the feed site. It feeds preferentially on the flower racemes and seed heads, a food source apparently obligatory for juvenile survival (Hori and Miles 1993). Abscission of the flower buds and seed pods occurs with remarkably few feeding punctures, and stem collapse, consistent with the intrinsic activity of salivary pectinase, was observed with somewhat higher insect densities.

9.3. A comparison between the feeding strategies of coreids and mirids

Inherent similarities within Heteropteran families of the qualitative complement of salivary enzymes (see Chapter One), such as sucrase in Coreidae and pectinases and amylase in Miridae, allow for the comparisons and conclusions drawn here to be extended to other species in these two economically important families of the Heteroptera.

In the context of the presumed predigestive role of salivary enzymes, the occurrence of amylase and pectinases in the saliva of mirids can readily be explained. Although pectic substances do not appear to subserve a specific dietary need, the purely biochemical maceration of the intercellular pectic matrix caused by salivary pectinases secreted into host parenchyma, with the demonstrable release of both cell wall bound proteins and neutral polysaccharides, must surely be of nutritional benefit to the feeding bug. Surfactant properties of the saliva and lack of a functional stylet sheath would undoubtedly assist in the rapid penetration of saliva into tissues and allow pockets of cells, beyond the reach of the stylets, to be made available to the feeding bug. This is consistent with the large volumes of watery saliva (derived from

the enlarged accessory gland) thought to be secreted then reingested during feeding. It is problematic, however, whether continuous mechanical laceration by the bugs' stylets increases the effectiveness of chemical maceration and hence contribute to ingestion of nutrients. Indeed it is experimentally demonstrable that some degree of mechanical laceration does increase the amounts of nutrients that are released by tissues bathed in pectinase. *In vivo*, however, laceration is necessarily limited to the reach of the stylets and falls short of the area observed to be affected by salivary pectinases, e.g. in Miles (1987a).

The detection of amylase, too, is not surprising. Many of the facultative seed feeders, inclusive of mirids secrete such a salivary enzyme (e.g. see Nuorteva and Laurema 1961). Its presence enables the extra-intestinal maceration of starch granules to a more readily ingested form. Unlike *M. profana*, *C. dilutus* was not tested for the presence of digestive enzymes in the digestive tract. It would be of interest to determine whether the presence of one particular predigestive enzyme in the salivary glands, be it amylase, sucrase (or proteinase for carnivorous Heteroptera), precludes its necessity elsewhere in the digestive tract, or regularly requires the presence of some other enzyme.

While a direct, dietary explanation could readily be found for the presence of a pectinase in the saliva of *C. dilutus*, the occurrence of sucrase in coreid saliva, given that sugars are not a limiting dietary resource, required a novel explanation. *M. profana* is able to inject possibly small volumes of watery saliva, localised by the formation of a stylet sheath. The accessory gland (salivary reservoir) is perhaps consequently relatively undifferentiated. In affected plants, cells in the vicinity of vascular bundles are selectively targeted and lateral branches of the stylet track effectively increase the region accessed at each feeding puncture. As with the mirid, infiltration of saliva into the apoplast is undoubtedly aided by the surfactant properties of the secretion. Salivary sucrase is able to hydrolyse endogenous sucrose, essentially doubling the osmotic effects of sucrose at the feed site, thus inducing a strong osmotic gradient along which a general flow of nutrients may occur. Such a gradient is especially possible since the insect, for reasons already adduced, would seem to use minimal quantities of saliva in achieving its effects. This effect on its own, especially if strictly localised, was not thought to be sufficient to explain the rapid response shown by plant tissues to the insect's feeding; rather, it was considered plausible that the insect was able to intervene in the physiology of the plant causing elevated concentrations of monosaccharides and consequent vascular unloading.

Previous work on phloem transport (as reviewed in Chapter One), while still to some extent controversial, has indicated the importance of sugars and plant invertases in the induction of phloem unloading at sink sites. The feed site of

M. profana is clearly a sink site, and concurrent phloem unloading of solutes has now been demonstrated. On suitable food plants the insect simultaneously destroys other, connecting meristematic sinks (its competitors), possibly by the sheer volume of liquids removed and certainly by causing acropetal necrosis (see also Neal 1993). This may well be promoted by the secretion of salivary oxidases which are apparently capable of inducing a general 'leakiness' in plant cell membranes. It seems likely, however, that sucking alone does not provide the insect with sufficient food. As indicated above, it achieves this by inducing a stimulus in the plant that enhances phloem unloading. This is done by the secretion of a salivary sucrase, effectively an enzymic mimic of the plant's own invertase.

In placing the present study within the context of current knowledge, it appears that much of what is known of the salivary physiology of Heteroptera is still characterised by a number of significant gaps and some inconsistencies. The present study has strengthened some past interpretations of the origin in the glands of precursors of the stylet sheath, but in a way that indicates the inadequacy of knowledge about the origins and physiological functions of the various divisions of the glands. The main contribution of the present work is to introduce an essentially new concept in bug feeding (that driven by an osmotic process) and to indicate that sucrase is a major constituent of the secreted saliva of some coreids; that perhaps it is a characteristic of all members of the family and therefore involved in a further characteristic of these insects, namely the production of terminal wilting in actively growing shoots. A possible role for the enzyme based on its intervention in the vascular function of the food plants is presented but it may be pointed out here that the concept implies induction by the insects of a short term, dynamic response on the part of the plant and not merely passive post feeding necrosis resulting from loss of cell contents and simple destruction of cell function.

APPENDIX ONE

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ADDENDUM

Similarity of 'pilocarpine induced' saliva to that normally secreted during feeding

It is by no means assumed that saliva secreted under the influence of pilocarpine solution (p. 44) is identical to that secreted during feeding. However, in the context of previous studies on the physiological role of salivary secretions, particularly as they relate to feeding strategies and plant damage, the techniques reported here represent orders of magnitude of greater relevance than techniques using whole insect, insect head or, indeed, salivary gland macerate. Once, however, the secretion in the saliva of a particular enzyme from this so-called 'pure' saliva has been demonstrated, extracts of entire salivary glands may provide a more convenient, and equally valid, enzyme source for subsequent analyses.

'Buffered' salivary gland extracts

The terms 'buffered extracts' (p. 45) refer to salivary gland extracts prepared as follows: 1 volume of 1 M sodium acetate was added to 5 volumes of salivary gland extract and the pH was further adjusted to particular values in the presence 1 volume 0.4% indicator solutions using either 0.1 M HCl or 0.1 M NaOH. Initial pH values of 'buffered' extracts approximated 7: about 7 µl HCl were required for adjustment to pH 5, 2 µl to pH 6 and 1 µl NaOH to pH 8. The combination of sodium acetate with acid, except for pH 8, formed the buffer system. However, except for experiments to determine pH optima of sucrase, pH of preparations were not considered critical, and except where stated, were not further adjusted.

The use of 'positive controls' for specific enzyme tests

Although not mentioned specifically in the text and in order to provide positive controls for the detection of salivary enzymes (see section 4.3.4), experiments also included known sources of particular enzymes. For the amylase test, a preliminary experiment using the peanut trash bug, *Elasmolomus sordidus* (F.) (Heteroptera: Lygaeidae) provided a positive reaction, which, not only provided evidence that this species possesses a salivary amylase, but that the method was capable of detecting its occurrence elsewhere. This test was confirmed using 5 µl human saliva. Similarly, and in order to validate the negative response for pectinase in the coreid, pectinase was detectable in the mirid, *C. dilutus*, and from 0.1% pectinase (500 U/gm from *Rhizopus* sp.). For a positive test for sucrase, 5 µl 0.1% yeast invertase from *Candida utilis* was used. For proteinase, 5 µl 0.1% aqueous papain failed to produce a positive control: there was, however, a positive reaction to proteinase in the third ventriculus of the midgut of *M. profana*. Similarly, a positive result for phosphatase in salivary gland extracts, but not in the saliva, confirmed its presence as an endogenous enzyme in *M. profana* and, once again, provided a suitably positive test. No commercially available enzyme source for lipase was used.

Possible microbial contamination during enzyme analyses

No attempts, other than what is considered normal procedure in a biochemically related laboratory, were made to counter non-specific contamination by bacteria, yeasts or fungi during incubations of enzymes with their respective substrates (see sections 4.3.4, 8.3.3-4). Mostly, incubation times were considered too short for any such contamination, but nevertheless, and regardless of the duration of experiments, the use of adequate controls were utilised to negate possible effects.

Discussion on phloem transport in plants (see section 6.5)

There are two routes by which plant metabolites and salts diffuse in plants. One is via the apoplast which comprises the intercellular spaces and cell walls. The other is via the symplast which comprises the cytoplasm of the plant, which is interconnected by small pores, termed plasmodesmata, in the cell walls. The two are not sealed off from one another and at the local level there is evidence that metabolites, plant hormones, enzyme elicitors, and phytoalexins and other defensive compounds, diffuse between the apoplast and the symplast. Long distance transport in plants (via the symplast) is facilitated by elongate phloem sieve tubes. These cells do not have nuclei but are controlled of companion cells with which they are connected by plasmodesmata. The mechanism of transport of plant metabolites, first proposed by Münch (1930), is thought to be driven by an osmotic process along a concentration gradient of sucrose whereby production of sucrose in source tissues (for example, and in the case of *M. profana* feeding on *A. iteaphylla*, roots and mature phyllodes) causes a high osmotic pressure in cells thus inducing the absorption of water. The consequential increase in turgor pressure forces the loading via the plasmodesmata of a solution of sucrose (and accompanying diffusible solutes) into the phloem sieve tubes. Conversely, at sink tissues (eg. meristematic shoot tips) sucrose is actively unloaded, inducing an osmotic flow and accompanying diffusion of metabolites out of the sieve tubes as well as a lower pressure within them.

The unloading process is thought to involve the hydrolytic enzyme, invertase, located on the membranes of the companion cells. When the enzyme is activated the disaccharide, sucrose surrounding the phloem, is hydrolysed to the monosaccharides, glucose and fructose, essentially doubling the osmotic pressure of the cytoplasm surrounding the sieve tubes relative to its contents, resulting in an osmotically driven flow of metabolites from the sieve tubes into the apoplast.

The 'osmotic pump' hypothesis for coreid feeding, propounded in this thesis, suggests that the salivary sucrase, functionally identical to plant invertase and secreted into plant tissue during feeding, induces a similar osmotic gradient and consequential flow of plant metabolites from the phloem sieve tubes into plant tissues surrounding the phloem. Indeed, the demonstrable release of metabolites into the apoplast (estimated as concentration of amino acids) following the introduction of the breakdown products of sucrose experimentally supports this essentially new concept of Heteropteran feeding.