PHYSIOLOGICAL AND STRUCTURAL ASPECTS
OF FLORAL NECTAR SECRETION

by

ARTHUR RALPH DAVIS

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Corrections to Ph.D. Thesis

"Physiological and structural aspects of floral nectar secretion"
A.R. Davis, Plant Cell Biology Group, Research School of Biological Sciences, The Australian National University, Canberra, A.C.T. 2601, Australia

Chapter 1
Page 1.6, line 2 - change "Sempervirum" to "Sempervivum"
1.7, lines 5 and 19 - change "dictysomes" to "dictyosomes"
1.16, line 5 - delete the first occurrence of "passively"

Chapter 2
Page 2.4, 2nd paragraph, line 10 - change "comparison" to "comparisons"
2.4, 2nd paragraph, line 13 - change to "and concentration, and were examined by light microscopy."
2.29, line 7 - change "indifferent" to "not different"
2.36, start of second paragraph, add the word "Although"
2.43, line 7 - change to "nectary starts to degrade several days after..."
2.50, line 9 - delete the second occurrence of "of"

Chapter 3
Page 3.7, line 8 - change to "a significant, 3-fold increase in the ...
3.8, section 3.3.5., line 2 - change to "of in situ flowers."
3.10, 2nd paragraph, line 7 from bottom - change to "along the tips of all projections ...
3.12, line 10, change "that" to "those"

Chapter 4
Title Page - remove the second "I" in "ARABIDIOPSIS", and insert it to the last word "ACTIVITY"
Page 4.6, section 4.2.7, line 7 from bottom - change to "The bud lengths..."
4.21, section 4.3.5, line 1 - change "3.115" to "4.115"
4.28, section 4.3.6.2, line 8 - change to "in a basipetal fashion ...
4.33, final paragraph, line 2 - change to "genotypes (Table 4.3, right)."

Chapter 5
Page 5.5, line 3 - indent, to make a paragraph
5.9, line 4 from bottom - change to "that"
5.15, line 5 - change to "5.73 ± 1.30"
5.15, second line above the table, change to "or 85% of the ...
5.28, final paragraph, line 3 - change to "The second event ...
5.28, final paragraph, line 4 - change to "the first practice ...
Figure Caption 5.7 - change to "five of the nine ..."
Figure 5.13 - in bottom left, change ratio to "Fructose/Glucose"
Chapter 6
Page 6.5, penultimate paragraph, line 3 - change to "That is, even ..."
   6.5, final paragraph, line 3 - change to "Hirschfeldia"
   6.6, final paragraph, line 2 - change to "removed from the load ..."
   6.10, final paragraph, line 2 - change to "in order"

Chapter 7
Page 7.9, final paragraph, line 6 from bottom - change to "nectar is too much richer in hexose ..."
   7.10, line 8, change to "Kronestedt-Robards"

BIBLIOGRAPHY
Change Ziv et al. (1987) to "(Dianthus caryophyllus)"
FRONTISPICE

**Top** - The author’s wife Diane and eldest son Stephen beside plants of *Echium plantagineum* L. utilized in preliminary studies of nectar secretion and pollination.

**Below** - The base of the style of a flower of *E. plantagineum* that had been exposed to multiple insect visits. After staining with aniline blue, eight pollen tubes are discernible in the transmitting tissue between the two vascular bundles.
STATEMENT

All the research reported in this thesis
is original and my own
and has not been submitted
for any other degree

Arthur Ralph Davis
ACKNOWLEDGEMENTS

Many people deserve special mention for their assistance to me during the course of this research. I thank the members of my supervisory committee, Dr. D.B. Bittisich and Prof. G.D. Farquhar, for their very helpful discussions and interest in the progress of the work, particularly during the initial stages when important foundations were being laid. Dennis also supplied the seed of *Vicia faba cv. Aquadulce*, made arrangements for the plot kept in the Department of Botany's propagation area, and helped to translate relevant parts of the Italian works of Villani (1905) and Bosia and Pescarmona (1972), for which I am grateful.

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ABSTRACT

This study concerned various physiological and structural aspects of floral nectar secretion in three species: Arabidopsis thaliana (L.) Heynhold, Echium plantagineum L. and Vicia faba L. Microscopic, biochemical and immunological techniques were employed to investigate the development and possible function of nectary stomata, the relationship between stomatal number and their distribution with respect to total nectar-sugar production, changes in content of specific sugars in nectar as flowers age and are pollinated, the contribution of nectary starch to nectar sugar, the development of nectary vasculature, and the relationship between nectar-sugar types and the quantity of endomembranous systems within the nectariferous cells. In the field, a promising technique was developed that allows the direct evaluation of the efficiency of flower visitors as pollinators. Furthermore, investigation of a rare instance of mixed loading of pollen from two different species during the same foraging trip of an individual honey bee provided unique insights into bee foraging behaviour.

On the floral nectaries of A. thaliana and V. faba, stoma-like structures develop at positions where initial nectar droplets are later visible. Passage of nectar through the pores of these structures was observed in fababean. Their development is asynchronous and often contiguous. Because the guard cells of these structures only rarely contract sufficiently to close their pores, they are considered as "modified stomata". The limitation on their guard-cell movement is attributed to incomplete substomatal spaces in which contact is maintained between guard and subepidermal cells, and mechanical features of the walls of the guard cells, including the occurrence of circumferentially-arranged microfibrils and a complete cuticle. Guard cells of mature stomata are not connected to adjacent epidermal cells by plasmodesmata. In V. faba, direct measurements of pores of modified stomata showed that they reach their
maximum aperture a few days before the onset of secretion, and average pore widths remain constant throughout the day and night, unlike those of leaves. Also, in both species, open and even immature modified stomata can occur on old post-secretory glands undergoing degradation.

The physiological evidence is also against a rôle for the nectary stomata in the regulation of nectar flow. Guard cells of nectaries were not observed to recover from plasmolysis, nor did the pores of these modified stomata normally close when the cells were plasmolysed. Nectary stomata of *V. faba* neither underwent plasmolysis nor closed when exposed to concentrations of abscisic acid or Ca\(^{2+}\) that induce closure in leaves. Localization of K\(^+\) ions within guard cells on nectaries was weak, contrary to those cells of leaves. Guard cells of nectaries of *A. thaliana* and *V. faba* contained large quantities of starch, even after the cessation of nectar secretion, unlike other cells of the same glands. In fababean, but not in *Arabidopsis*, evidence for a system of pore occlusion was detected. A comprehensive literature survey of the occurrence of nectary stomata is presented as Appendix 1.

An investigation of the floral nectary of three cultivars (Aquadulce, Blandine, Long Pod) of *V. faba* showed marked differences in the lengths of nectary projections and in the total number and distribution of modified stomata on the projections. A detailed examination of five selections of the first cultivar revealed significant, positive linear relationships between projection height and total number of stomata for both cultured (*r* = 0.751) and *in situ* (*r* = 0.769) flowers. However, the total number of modified stomata per nectary of cultured flowers was significantly and inversely related (*r* = -0.436) to nectar sugar production. The percentage of modified stomata borne on the top quarter of nectary projections, on the other hand, was significantly and positively correlated (*r* = 0.466) with nectar sugar production. It was concluded that because the connection between nectary stomata and the potential amount of nectar sugar produced by a nectary is not strong (i.e., *r* < 0.5), counts of nectary stomata and
the distribution patterns of stomata on the gland were not recommended as primary selection criteria in breeding programmes of *V. faba*.

The nectar-secreting tissue in flowers of *Arabidopsis thaliana* forms a complete gland encircling the androecium. Proliferation of the nectary tissue occurs at the lateral positions, abaxial to the filaments of the short stamens, and at up to four median positions, situated between the bases of the long stamens and petals. All of these protuberances are variable in size, shape and symmetry. The gland is supplied directly by vasculature consisting solely of phloem. Vascular supplies to the gland vary within racemes or individual flowers, and are not necessarily equal, symmetrical or present. A major apoplastic route for nectar movement and release is inferred because sieve elements, companion and phloem-parenchyma cells, as well as other subepidermal cells of the gland, commonly border intercellular spaces. A symplastic route, centred on plasmodesmatal connections between glandular cells, is also available for pre-nectar movement. As in the case of *V. faba*, the "modified stomata" of the nectary do not regulate the flow of nectar during the secretory phase.

In wild-type flowers of *A. thaliana*, starch accumulates in plastids of the nectary tissue during the bud stages, especially as anthesis approaches after the vascular supply to the gland has matured. This starch stains indicatively as amylopectin. Flowers bearing nectar, whether *in situ* or *in vitro*, were found to possess nectary starch no longer, suggesting that the breakdown products of the starch augmented the nectar sugar arriving from the phloem during secretion. However, because equal quantities of nectar sugar were produced by cultured flowers of a starchless mutant (TC7), starch deposition and degradation are not essential steps in the nectar secretion process of *A. thaliana*.

A survey of previous ultrastructural studies of nectaries is presented in Appendix 2, along with published analyses of their dominant nectar sugars. There was no connection between relative sucrose content of the nectar and the relative quantities of
endomembranous systems (reticulum, dictyosomes, etc.) in nectariferous cells of secreting glands. However, extracellular transformation of nectar sugar usually is not accounted for in these sugar analyses, and the possibility remains that the endomembranous systems do provide a protective route for sucrose from enzymes of the general cytosol.

A novel technique was developed which simply, directly and quantitatively evaluated flower visitors as pollinators of *E. plantagineum*, based on microscopic detection of the fluorescence of the callose content of stylar pollen tubes stained with aniline blue. The technique utilized previously-unvisited flowers to demonstrate that although autogamy was possible, it was not predominant: permanently-bagged, intact flowers had very low numbers of pollen tubes at their style bases (usually none), and emasculated flowers and those with all their pollen still present (intact flowers), when exposed to a lone honey bee visit, usually had the same numbers of tubes. Within treatments (intact, emasculated), honey bee visits for pollen, nectar or both, did not result in differences in pollen-tube number, nor was there any connection between degree of pollination and time spent by a single honey bee per virgin flower.

Although most honey bees attending virgin flowers carried pellets of *E. plantagineum* pollen on their hind legs, as a result of grooming actions to gather grains from their bodies, they were still as effective as pollinating agents as bees lacking corbicular pollen. The results of a scanning electron microscopic study of a case of mixed loading, involving distinguishable pollens of *E. plantagineum* and *Hirschfeldia incana* (L.) Lagrèze-Fossat that had been packed as segregated loads in the pollen baskets of a honey bee, concur with this finding: both pollen types were detected in all fields of pollen scanned, suggesting that honey bees are inefficient at or incapable of grooming all grains from the body. The nectar of *H. incana* was found to contain fructose and glucose only, in stark contrast to the nectar of *E. plantagineum*, suggesting that similarity in nectar-sugar composition was not the stimulus to visit both species.
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CHAPTER 1

CURRENT ISSUES IN THE BIOLOGY OF
NECTAR, NECTARIES AND NECTAR SECRETION

1.1 INTRODUCTION

Nectar secretion is a widespread process within the reproductive cycle of many flowering plants. It is the result of an interaction between the floral nectar (made up of carbohydrates and proteins) and the insects and bees after pollination and fertilization (Pankratz and Bollen 1963, Shabal 1967, 1972). The floral nectary is a specialized structure in the flower that produces nectar. The process of nectar production is influenced by various factors, including environmental conditions and plant hormones.

Nectar secretion is a crucial aspect of plant reproduction, as it attracts pollinators and provides them with food. The nectar production and its attraction to potential pollinators are essential for successful pollination. In many plant species, nectar production is not always coincident with the flowering stage. For instance, some plant species that undergo apomixis may still produce nectar (Webster et al. 1982) and floral nectar secretion is not always restricted to the flowering stage. Epaphus (1932) has reported many species for which post-floral nectar has been reported, and Vogel (1977) has described discoveries of carinatella in post-floral nectar of Lamium and Myosotis. In addition, Kessler (1983) reported that ants attracted by the post-flowering activity of the floral nectary of Mentha spicata depend heavily on this species.

Owing to their importance in plant reproductive biology and commerce (nectar being the precursor of honey), nectar and the glands that secrete it have been subjected to considerable research. Many of the earliest works, such as the first anatomical investigations of nectaries (Caspar 1848) and several treatments that followed (e.g., Heer 1891, Börner 1876, Böhm 1917, Kessler 1917, Pohl 1926, Zürcher 1926, and numerous works by Davidson), have an emphasis on morphological and anatomical descriptions of these secretory structures.
1.1. INTRODUCTION

Floral nectar secretion is a widespread process within the reproductive cycle of a
great many flowering plant species. It usually commences at anthesis and ceases after
pollination and fertilization (Pankiw and Bolten 1965, Shuel 1961, 1978). The floral
events of anthesis, nectar secretion, anther dehiscence and stigma receptivity, and
changes in corolla colour and abscission, appear to be coordinated and under hormonal
that "nectaries" - the universal term applied in 1735 by Linnaeus (Bonnier 1879) to
describe the sites of nectar secretion - often are associated with pollination, wherein the
nectar, sought as a food source by many animals, is secreted at floral positions
conducive to incidental transfer of pollen to the stigma, and to pollen dispersal, by
Generally, nectar secretion persists for a longer period in unpollinated flowers (Bonnier
1879, Shuel 1961, 1978). There are a few exceptions to this relationship between
nectar production and the attraction of potential pollinators. For instance, some plant
species that undergo apomixis may still produce nectar (Webster et al. 1982) and floral
nectar secretion is not always restricted to the flowering stage. Daumann (1932b) lists
many species for which post-floral secretion has been reported, and Vogel (1977)
summarised discoveries of ant visitation to post-floral nectar of Lamium and
Myrmecodia. In addition, Keeler (1981) reported that the ants attracted by the post-
flowering activity of the floral nectary of Mentzelia deterred herbivory of this species.

Owing to their importance in plant reproductive biology and in commerce
(nectar being the precursor of honey), nectar and the glands that secrete it have been
subjected to considerable research. Many of the earliest works, such as the first
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1926, Zimmermann 1932, Feldhofen 1933, and numerous works by Daumann), have
an emphasis on morphological and anatomical descriptions of these secretory
structures, but hold much physiological information as well. Significant research on physiological aspects of nectar secretion followed, principally in several laboratories in Europe (e.g., Agthe, Beutler, Frei, Frey-Wyssling, Lüttge, Matile, Schnepf, Ziegler and Zimmermann) and in Canada (Shuel). Concurrent analyses of nectar chemistry and how they relate to pollination ecology (e.g., Maurizio, Percival, Wykes) are still being continued and expanded today (e.g., Baker and Baker, Corbet, Freeman, Gottsberger).

With the advent of transmission electron microscopy, investigations of ultrastructural features of the cells comprising the glands have been numerous (e.g., Fahn, Figier, Schnepf). Since the first studies by Mercer and Rathgeber (1962), Wrischer (1962), Eymé (1963, 1966, 1967) and Schnepf (1964a,b), the fine structure of nectaries from approximately ninety species has now been investigated, to varying degrees, in less than three decades. In this respect, much effort has been directed to the elucidation of cellular mechanisms of pre-nectar formation, movement and eventual release of nectar. The nectaries of many more species have been observed with scanning electron microscopy, and this important technique has greatly aided investigations of external anatomical features as they relate to glandular function.

Currently there is a growing research interest worldwide in improving agricultural production to meet ever increasing demands. Nectar is the major attractant for many flower visitors and certainly plays a key role in crop pollination (Simpson and Neff 1983, Shuel 1989). For example, studies such as those of Pedersen (1953) and Murrell et al. (1982a,b) have demonstrated the promising results of increased nectar-sugar yields that are attainable by selection and breeding in legume crops, and the ensuing increases in seed production due to increased bee visitation (Pedersen 1953). There can be little doubt that continued research on nectaries and nectar and the mechanisms which govern its production, secretion and reabsorption will provide important data fostering fruitful applications to agriculture, horticulture and honey production.
1.2. NECTAR

Nectar is essentially a solution of sugars in water, though it may contain minor amounts of several other substances (Shuel 1975). The latter include amino acids, minerals, enzymes, organic acids, essential oils and ions (Beutler 1953, Shuel 1975, Lütge and Schnepf 1976, Baker 1977, Fahn 1979a). Lütge (1961, 1977) showed that the amount of these "associate compounds" accompanying nectar sugar is related to the structural complexity of the gland. Most nectars tested have been found to be acidic, nectar pH ranging from 2.75 to 9.0 (Beutler 1930, Vansell 1944b, Baker 1977, Baker et al. 1978a). Recently, Prys-Jones and Willmer (1992) found the pH of nectar of Lathraea to be as high as 11.5.


The immediate origin of nectar has not been identified and may not be the same for all species. Beutler (1953) proposed that starch, tannins, and protein stored in the glandular tissue are the substances from which the sugar in nectar is derived. However, Schnepf (1969) reported that in Gasteria, stored polysaccharides were too limited to provide the nectar sugar, and in Abutilon spp., Findlay et al. (1971) estimated that the total sugar secreted is at least seven times that present in the glandular tissue at the onset of secretion. Kenoyer (1917) demonstrated that darkening
of plants of *Canna, Fagopyrum esculentum* and *Impatiens sultani* reduced the quantity of floral nectar sugar and the amount of sugar produced by foliar glands of the latter species, whereas when only the flowers were shaded, they secreted fully as much sugar as uncovered ones. Girnik (1967) observed in *Tilia* and *Lespedeza* that flowering and nectar secretion were closely related to the amount of illumination received by the leaves. Other shading experiments involving *Echium vulgare* and *Symphoricarpus* (von Czarnowski 1952), *Eucalyptus melliodora* (Nuñez 1977) and *Asclepias syriaca* (Southwick 1984) have provided similar results. Maclachlan (1940) gave indirect evidence that in trees and shrubs, nectar sugar may be derived from stored carbohydrates. By bark-ringing and defoliating branches of horse chestnut, Wykes (1952b) showed that carbohydrate supply can limit nectar production. Ringing plants of *Antirrhinum majus* just below the flowering spike resulted in a 60% reduction in nectar sugar yield per flower, and indicated that nectar sugar is translocated from some distance below the inflorescence (Shuel, unpubl.).

1.3. **NECTARIES**

Caspary (1848) designated nectaries into the broad categories of 1) floral, consisting of nectaries occurring in flowers, and 2) extrafloral, comprising nectaries occurring on vegetative parts of plants. Brongniart (1854) referred to the ovarian nectaries of many monocotyledons as septal nectaries. Many detailed studies have been carried out on plant species possessing both floral and extrafloral nectaries (Butler *et al.* 1972, Elias *et al.* 1975, Elias and Gelband 1976, Baker *et al.* 1978b, Durkee *et al.* 1981, Durkee 1982, Davis *et al.* 1988a,b).

The position of the nectary in the flower varies with species (Percival 1965, Fahn 1979a). Fahn (1953) suggested an evolutionary shift in position of floral nectaries in an acrocentripetal direction i.e., from the perianth to the style. In recent years, great strides have been taken to delineate the taxonomic distribution of various character states of floral nectaries in the Magnoliophytina, largely based on nectary position and
1.5

structure (Smets 1986, 1988, Smets and Crescens 1988). Some species, such as *Limnanthes nymphoides* (Daumann 1931), can have nectaries at two distinct locations within their flowers e.g., at the base of the ovary and on the perianth.

Structures of floral nectaries are diverse, ranging from relatively simple secretory surfaces e.g., *Fragaria* (Esau 1977), *Lotus corniculatus* (Murrell et al. 1982b), *Paeonia* (Frey-Wyssling and Häusermann 1960, Elias 1983), to multicellular outgrowths e.g., *Brassica* spp. (Frei 1955, Leung et al. 1983, Davis et al. 1986), *Cucumis* spp. (Kartashova and Nemirovich-Danchenko 1968, Collison and Martin 1975), *Vicia* spp. (Bonnier 1879, Davis et al. 1988a), to arrangements of unicellular secretory trichomes e.g., *Caltha palustris* (Peterson et al. 1979), *Lonicera japonica* (Fahn and Rachmielevitz 1970) and multicellular trichomes e.g., *Abutilon* spp. (Findlay and Mercer 1971a,b, Gunning and Hughes 1976, Fahn 1979b, Robards 1984, Kronestedt et al. 1986, Robards and Stark 1988), *Hibiscus rosa-sinensis* (Sawidis et al. 1987a). Within a genus, there is a positive correlation between size of the nectary structures and nectar production e.g., *Citrus* (Fahn 1949), *Lamium* (Gulyás 1967).

As nectaries are situated on the surfaces of plants, nectar is an external plant secretion. Nectar can escape from nectaries in several ways, depending on modifications of the nectary epidermis and its usual cuticular covering (Fahn 1979a). In floral nectaries of some monocotyledons and especially dicotyledons, hundreds of examples exist where stoma-like structures occur in the epidermis (see Appendix 1), and it has been demonstrated that nectar exudes through pores of these modified stomata e.g., *Anethum, Mirabilis* and some Rosaceae (Bonnier 1879), *Citrus* (Fahn 1953), *Vinca* (Fahn 1979a). Schmid (1985) reported that on the septal nectaries of monocotyledons, trichomes and stomata are rare, and when stomata are present, nectar does not emerge through their pores. Radtke (1926) emphasised that the presence of stomata on the nectary surface is a familial trait, although it has become clear since then that nectaries of Cucurbitaceae and Scrophulariaceae (Vogel 1977, 1981) are families that contain species with nectary trichomes and species that bear stomata.
(Appendix 1), and Bonnier (1879) reported both the presence and the absence of nectary stomata within a genus (*Sempervirum*). Modified stomata are also known from gymnosperm (*Ephedra*) and fern (*Pteridium*) nectaries (Lloyd 1905, Bino *et al.* 1984, Power and Skog 1987). In addition, nectar can escape directly from epidermal cells or trichomes through: 1) a ruptured cuticle *e.g.*, *Clerodendrum* (Maheshwari and Chakrabarty 1966), *Passiflora* (Durkee 1982), *Ricinus communis* (Reed 1923, Baker *et al.* 1978a), *Turnera* (Elias *et al.* 1975); 2) pores in the cuticle *e.g.*, *Abutilon* (Findlay and Mercer 1971b); or 3) a permeable cuticle *e.g.*, *Lonicera japonica* (Fahn and Rachmilevitz 1970, Fahn 1979a,b). Furthermore, there exist cases where both stomata and trichomes occur at the same sites of nectar secretion *e.g.*, flowers of *Aristolochia* spp. (Daumann 1959), *Koenigia delicatula* (Ronse Decraene and Smets 1991a), *Melittis melissophyllum* (Stadler 1886), *Prunus domestica* (Bonnier 1879) and *Tropaeolum majus* (Rachmilevitz and Fahn 1975).

Rachmilevitz and Fahn (1975) proposed that the most primitive type of nectar secretion was from epidermal cells and that the development of an impermeable cuticle was then followed by the advent of mechanisms that enabled nectar to pass through the cuticle or by the development of internal secretory parenchyma, wherein all cells of the nectary are capable of secretion, coupled with exudation through modifications of the epidermis, primarily stomata (Rachmilevitz and Fahn 1973, 1975). Where stomata occur, there is no special differentiation of the nectary epidermis (Caspary 1848, Bonnier 1879). Specialised epidermal cells from which nectar is eliminated are called nectary "secretory cells" (Fahn 1979a,b). In the epithelium of the cyathial glands of *Euphorbia*, solitary "passage cells" ("Diffusionsporen"; Feldhofen 1933) characterised by a relatively thin cuticle are thought to be involved in nectar release (Schnepf and Deichgräber 1984).

Often, secretory cells possess wall protuberances along their outer walls. Wall ingrowths which are un lignified, secondary cell wall deposits protruding into the cell cytoplasm and enveloped by plasma membrane are characteristic of transfer cells (Pate
and Gunning 1972, Peterson 1982). Secretory transfer cells in floral nectaries may have ingrowths that are papillate/filiform e.g., *Musa* (Fahn and Benouaiche 1979), *Vicia faba* (Gunning and Steer 1975, Davis et al. 1988a), or labyrinthine e.g., *Epipactis atropurpurea* (Pais 1986), *Gasteria trigona* (Schnepf 1964a, 1969), *Lonicera japonica* (Fahn and Rachmilevitz 1970, Fahn 1979a,b). Dictysomes are particularly abundant in the pre-secretory stages of those nectaries in which wall ingrowths occur and probably contribute to the formation of these protuberances (Durkee 1983a). The existence of multiple foldings of the plasma membrane, without accompanying wall ingrowths, has been demonstrated in epidermal cells of floral nectaries of some species of the Asclepiadaceae (Schnepf and Christ 1980, Christ and Schnepf 1988).


Apart from the secretory cells, nectaries typically contain specialized parenchyma cells and have some type of vascular association. In addition, intercellular
spaces are common. Ultrastructural studies of numerous nectaries fixed during secretion (Eymé 1966, 1967, Fahn and Rachmilevitz 1970, Zandonella 1970c, Figier 1971, Findlay and Mercer 1971b, Rachmilevitz and Fahn 1973, 1975, Eriksson 1977) reveal that nectary parenchyma cells are generally small, isodiametric, and thin-walled. Numerous plasmodesmata are usually present between them. These subglandular parenchyma cells have relatively large nuclei and dense cytoplasm which is rich in ribosomes, endoplasmic reticulum and mitochondria. Generally, however, the hyaloplasm of these subglandular cells is less dense and neither the endoplasmic reticulum nor the dictyosomes show the usual degree of development and swelling found in the secretory cells (Durkee 1983a). The degree of vacuolation varies; in some cases vacuoles are well-developed for the duration of secretion (Figier 1971, Findlay and Mercer 1971b, Durkee et al. 1981), whereas in Ricinus and Silene vulgaris, many small vacuoles occur during secretion (Zandonella 1970c, Baker et al. 1978a).

Floral nectaries may have a vascular supply or may completely lack special vascularization. In the former case, the vascular association can be 1) direct, where vascular bundles enter the nectary tissue, or 2) indirect, lateral diffusion occurring from vascular traces destined for adjacent floral organs (Frei 1955, Kartashova 1965, Esau 1977). In families like the Fabaceae, Frei (1955) found a relationship between nectary size and the extent of the vascular supply. In vascularized nectaries, the vein endings usually terminate a few cell layers below the secretory cells (Frei 1955, Durkee 1983a), but can be very close to the epidermis e.g., Helianthus annuus (Sammataro et al. 1985).

Floral nectary vascularization has been studied extensively by Frei (1955) and Kartashova (1965). Of 366 species from many different families, 39.6% lacked vascularization, 47.8% were supplied with phloem only, and 12.6% had both xylem and phloem (see Fahn 1979a). Within the latter category of nectaries, Frei (1955) never found xylem to predominate. The type of nectary vascularization may vary within the family or even between species of the same genus e.g., Salvia (Dafni et al.
1988), Centaurea (Frei 1955). For instance, although the floral nectaries of C. cyanus, C. jacea and C. montana all bear stomata (Gulyás and Pestf 1966), in the former vascularization is lacking, in the second a direct supply of phloem alone exists, whereas in the final species both xylem and phloem are found (Frei 1955). Fahn (1979a) has tabulated the data of Frei and Kartashova on the occurrence of the three nectary innervation types within several families.

Agthe (1951) and Frey-Wyssling and Agthe (1950) found an association between type of vascular tissue supplying the nectary and nectar concentration. The concentration of nectar sugar from nectaries supplied exclusively by phloem was higher than that from those supplied by both phloem and xylem. This relationship between the vascular structure to the nectary and the composition of nectar points to a phloic origin of the sugar in nectar (Esau et al. 1957). In the monocotyledon Fritillaria imperialis, Van Die et al. (1970) used 14 C-labelling to locate the source of nectary sugars in photosynthesizing leaves and to follow pathways of translocation: the nectaries secreting most 14 C-labelled sugar were on the same orthostichy as the 14CO2-treated leaf.

Ultrastructural studies (Figier 1971, Findlay and Mercer 1971b, Wergin et al. 1975, Durkee 1982, 1983a,b) reveal that nectary phloem is often accompanied by companion cells. These nucleated cells usually stain more densely than those of the neighbouring subglandular tissue, and are rich in organelles including mitochondria, dictyosomes, ribosomes, plastids and endoplasmic reticulum. The protoplasts of companion cells are characteristic of metabolically-active cells; these cells, contiguous with phloem sieve elements, by way of compound plasmodesmata, are involved in sugar unloading (in the case of nectaries) from the phloem conduits (Esau 1977).

Nectary companion cells may possess wall ingrowths e.g., Vicia faba (Ziegler 1965, Figier 1968a, 1971, Gunning and Pate 1969, Browning and Gunning 1977, Hughes and Gunning 1980, Davis et al. 1988a), Impatiens holstii (Figier 1972b,c), and are therefore also transfer cells (Pate and Gunning 1972). In nectaries of Abutilon
1.10

(Findlay and Mercer 1971b), *Gossypium* (Wergin et al. 1975, Eleftheriou and Hall 1983a), *Passiflora* (Durkee 1982, 1983) and *Vigna* (Kuo and Pate 1985), companion cells lack wall ingrowths. Absence of ingrowths, however, does not mean that these cells are not functioning in solute transfer (Gunning et al. 1968, Esau 1972, 1977).

The characteristic densely-packed arrangement of the nectary parenchyma cells is usually interrupted by numerous, small, intercellular spaces e.g., *Trifolium* (Eriksson 1977), *Tropaeolum* (Rachmilevitz and Fahn 1975) and *Vicia* (Davis et al. 1988a). In the last two cases, wall ingrowths of companion cells and parenchyma cells, respectively, oppose the intercellular spaces. These spaces are relatively well developed in the subepidermal nectary tissue in glands exuding nectar via stomata (Fahn 1979a). In *Brassica*, *Vicia* and *Vinca*, the spaces are particularly well developed in the distal part of the gland, near the stomata, and the size of the spaces decreases gradually towards the base of the nectary. Furthermore, parts of the wall facing these spaces are covered by cuticle (Rachmilevitz and Fahn 1973, Gunning and Steer 1975, Davis et al. 1986, 1988a).

1.4. THE PROCESS OF NECTAR SECRETION

Much research has been directed towards understanding the mechanisms of nectar secretion. Most of the earlier investigations were physiological. With the advent of electron microscopy, the more recent efforts have concentrated on linking ultrastructural features to the nectar secretion process. Nectar secretion, and some hypotheses relating to the secretion mechanisms, have been reviewed recently by Lüttge and Schnepf (1976), Lüttge (1977), Schnepf (1977), Fahn (1979a, b), Zandonella et al. (1981), Durkee (1983a), Fahn (1988) and Findlay (1988).

Before the advent of ultrastructural studies of nectaries, there were two general theories on the basic mechanism of nectar secretion: 1) that sugar and water excretion occur as distinct phases, and 2) that secretion occurs as one phase (Shuel 1952). In the first case, Trelease (1920) considered water secretion as being analogous to guttation
1.11

and dependent on root pressure. Bonnier (1879) presented evidence for a positive relationship between secretion and root pressure. Both Wilson in 1881, cited by Helder (1958), and Trelease (1920) reported that washing away the secretion from the nectary caused the cessation of nectar secretion; they concluded that osmotically-active substances (e.g., sugars) are secreted on the nectary surface and water is subsequently drawn out of the nectary tissue. However, Radtke (1926) found that rinsing was effective in stopping secretion of old, exhausted nectaries only, and Pfeffer (1897) and Radtke (1926) showed that root pressure could not be regarded as the driving force because flowers floating on sucrose solutions still secreted nectar. Furthermore, the persistence of nectar secretion in turgid flowers of badly-wilted plants indicated that the process is not dependent on a hydrostatic pressure transmitted through the vascular system of the whole plant (Shuel and Shivas 1952).

The discovery that each nectary is provided with a phloem supply either by neighbouring vascular strands or directly by special glandular traces (Frey-Wyssling 1955) led to the view that nectar is secreted phloem sap (Agthe 1951, Frei 1955, Frey-Wyssling and Agthe 1950). Agthe (1951) postulated that the sieve tube contents are forced through the glandular tissue by an excess pressure caused by a sugar surplus. The observation that secretion of nectar coincided with cessation of growth in the organ with which the nectary is associated, led to the conclusion that secretion resulted from a surplus in the assimilate stream. Hence, nectaries were regarded as simply "sap valves" (Frey-Wyssling et al. 1954, Helder 1958). Shuel (1956), however, found that appreciable quantities of very dilute nectar were secreted by excised snapdragon flowers cultured on sugar-free or low-sugar media, and Ziegler and Lütte (1959) reported secretory activity of the extrafloral nectaries at the base of senescing, yellowing leaves of *Viburnum opulus*. Therefore, initiation of secretion apparently does not depend on a build-up of sugar. Also, culture of flowers on varied sugar concentrations indicated that the passage of all of the water present in nectar is not contingent on sugar transport (Shuel 1959). Furthermore, the discovery of a dual effect
of indole acetic acid on nectar secretion (Matile 1956, Shuel 1964) suggested that a partial independence of movement of the solute and solvent components of nectar occurs (Shuel 1964). Auxin was found to act on the secretory process of the nectary cells rather than on movement of sugar towards the gland (Shuel 1978).

The conclusion that nectaries are not just valve-like organs for the exudation of phloem sap, but are real glands with a characteristic active metabolism (Frey-Wyssling et al. 1954), has been supported by studies of enzymes. For example, by employing chromatographic and/or radioisotope techniques, sugar transformations have been noted in the nectar of several species. Zimmermann (1953) reported that cut shoots of Impatiens holstii secreted only sucrose when placed in glucose or sucrose solutions. He identified invertase and a transfructosidase enzyme in Impatiens nectar. Zimmermann (1954) determined an abundance of fructose, as well as sucrose, glucose, and oligosaccharides in nectar of Robinia pseudoacacia; however, the phloem sap contained only sucrose. A transglucosidase was identified. Frey-Wyssling et al. (1954) found that nectaries of Euphorbia pulcherrima secreted invertase and transformed $^{14}$C-glucose to radiolabelled sucrose and fructose in nectar. However, Ziegler (1965) found that when sucrose labelled in the glucose moiety was supplied to Abutilon nectaries, well over half of the radioactivity remained in the glucose component of sucrose in the nectar, demonstrating that sucrose inversion is not essential during secretion. Sugar transformations occurred in nectar of excised snapdragon flowers cultured on media of various sugars, and sucrose was usually found in nectar chromatograms (Shuel 1956). Shuel concluded that the sugar transformations and consistent differences in quantity of secretion on several sugar substrates, which were taken up and assimilated to a similar degree, strongly suggested a measure of localized control over secretion. Nichol and Hall (1988) found that sugar ratios (sucrose/fructose/glucose) were maintained constant in nectar secreted by cultured extrafloral nectaries of Ricinus supplied with various concentrations of sucrose alone, in the medium. On the other hand, Zaurolov (1969) found that three
enzymes of glycolysis (phosphoglucomutase, aldolase and enolase) were not directly related to the process of nectar secretion in flowers of pumpkin and *Asclepias cornuti*. Also, it is noteworthy that the unstructured nectaries of the *Paeonia calyx*, which could not be distinguished by the cytochemical Gomori test (commonly used for the determination of acid phosphatase activity; Glick 1949), secreted sucrose only; other Gomori-positive unstructured nectaries secreted glucose and fructose in addition to sucrose (Frey-Wyssling and Häusermann 1960).

In addition, supportive evidence that nectar secretion is an active process has been obtained from experiments using various enzyme inhibitors. For instance, at concentrations which caused no visible injury to excised flowers of *Antirrhinum majus*, enzyme inhibitors depressed both concentration and quantity of sugar in nectar (Shuel 1959). Brown (1959) reported similar findings with many of the same inhibitors. Shuel (1959) found a sulphydryl enzyme, phosphate transfer, and acetate metabolism to be involved; however, the level at which enzymatic reactions participated in the secretion process was not evident. Respiratory inhibitors and uncouplers of oxidative phosphorylation were found to decrease nectar sugar secretion in isolated nectary segments of *Abutilon* (Ziegler 1956, Findlay et al. 1971). Because secretion by nectaries floating on water could also be inhibited, Findlay *et al.* (1971) concluded that the metabolically-linked process is not solely to do with sugar uptake.

Under conditions of varied sugar concentration and an atmospheric humidity close to saturation, the sugar concentration of nectar from excised flowers of snapdragon was found to be almost identical with that of the culture medium (Shuel 1956). Similarly, Agthe (1951) and Brown (1959) found that isolated nectaries of poinsettia and snapdragon, respectively, did not concentrate the sucrose solutions upon which they floated. These results suggested that nectaries do not move sugar against a concentration gradient (Shuel 1956, 1959). However, Lütte (1961) noted that isolated nectary tissue was readily plasmolyzed when placed in the nectar it secreted, which suggested that a water potential gradient from the cells to the nectar may exist.
Plasmolysis, however, did not occur in situ where water balance of the gland tissue was maintained by water supply via the transpiration stream.

Most evidence, therefore, indicates that nectar secretion requires the expenditure of metabolic energy. It is not certain, however, where the metabolic step(s) occur (Vis 1958, Schnepf 1969, Lüttge 1971, Lüttge and Schnepf 1976). Several possibilities have been suggested. Because the sugars of nectar are provided mainly by the phloem, the transport of sucrose out of the conductive elements is one possible site of metabolic control of nectar secretion (Lüttge 1971, Lüttge and Schnepf 1976). The secretory pathway from the phloem to the actual site of elimination on the nectary surface may vary, and is related to the particular anatomy of the gland. For instance, Schnepf (1969) suggested that structureless nectaries, lacking secretory cells (e.g., Paeonia), might be reliant on a phloem "pump" for secretion. Nectary anatomy also determines to what extent the phloem translocate is subject to modification during the secretory process (Lüttge 1961). The companion cells of the phloem probably play an important role in the loading and unloading of sieve tubes from the surrounding parenchyma (Lüttge 1971). Figier (1968) has shown that acid phosphatase is associated with the plasma membrane lining the wall ingrowths of companion cells in extrafloral nectaries of Vicia faba. However, isolated nectaries of many different flowers have been observed to secrete nectar, and therefore the active step supplying sugar to the gland tissue may occur further within the nectary (Lüttge and Schnepf 1976).

An additional driving force may be associated with the modification of sugars by nectary cell metabolism. Although fructose and glucose are often present in similar amounts, when their ratio in fully-hydrolyzed nectar is different from unity, metabolic interactions of the migrating sugar are indicated (Lüttge 1971). In addition, transformation of sugars by the nectary could contribute to the maintenance of a concentration gradient (Lüttge 1971, Reed et al. 1971, Lüttge and Schnepf 1976). For instance, the diffusion of sucrose into the nectary tissue followed by its transformation to glucose and fructose could maintain concentration gradients favouring the further
diffusion of sucrose into the nectary and nectar, and simultaneously maintain a water potential gradient for the passive movement of water (Reed et al. 1971). The formation of trisaccharides (Zimmermann 1953, 1954) may also assist in maintaining gradients. The abundance of mitochondria in nectaries may be related to both requirements for increased carbohydrate metabolism and for increased energy transfer in the nectary cells (Lüttege and Schnepf 1976). Many of the enzymes required for sugar transformation are very active in nectaries, and these may be at the nectary surface or in nectar itself (Fekete et al. 1967, Lüttege and Schnepf 1976). However, Reed et al. (1971) noted that some active process must also be present which prevents diffusion into the medium of sugars formed in isolated nectaries of Abutilon.

Another proposal is that the driving force may involve active secretion at the outer nectary plasma membrane (Lüttege 1962, Schnepf 1969, Figier 1971, Heinrich 1975b). This view is largely supported by the abundance of acid phosphatase in the nectary secretory cells, especially near the plasma membrane (Lüttege 1971). Lüttege and Schnepf (1976) noted that cells strategically located where substantial short-distance sugar transport occurs often display conspicuous acid phosphatase activity. In fact, such activity has been considered by Ziegler (1956), Vis (1958) and Frey-Wyssling and Häusermann (1960) as a prerequisite for genuine secretory tissue in nectaries. Heinrich (1975b) has also identified acid phosphatase associated with endoplasmic reticulum, dictyosomes and mitochondria in nectaries of Aloe. With regard to the significance of Gomori-positive reactions in numerous nectaries, Lüttege and Schnepf (1976) cautioned that acid phosphatase is non-specific and concluded that it remains unresolved whether sugar or carrier phosphorylations are involved in the carbohydrate movement across nectary plasma membranes.

Nectary trichomes such as those of the well-studied Abutilon nectary are a special case. The stalk cell at the base of each trichome has anticlinal walls which are cutinized, and hence only very slightly permeable to sugar solution (Findlay and Mercer 1971b, Reed et al. 1971, Gunning and Hughes 1976). The average sugar
content in *Abutilon* nectaries is highest within these hairs (Reed *et al.* 1971). As the stalk cell is relatively impermeable to sugar diffusion, Reed *et al.* (1971) postulated that active sugar transport into the hair would be required at this location. An increase in sugar concentration within the hair would decrease the water potential, and hence water would passively accompany the sugar flow passively. The resultant increase in hydrostatic pressure within the hair would account for passage of the solution through the small cuticular pores. Findlay *et al.* (1971) have calculated that the ratio of phosphate-derived energy available from oxidative phosphorylation to the amount of sugar transported, without taking into account other phosphate-consuming processes in the cells, is low. They concluded that a direct correlation of sugar phosphorylation and sugar transport is not readily supported by the evidence. Fekete *et al.* (1967) also found low levels of sucrose-phosphate-synthetase in nectaries of two species.

Although physiological evidence has provided much support for some type of active transport in nectaries (i.e., eccrine secretion), understanding of the process of nectar secretion is still incomplete. Recent ultrastructural studies of nectaries have contributed much detail and have led to further postulates. Because the rates of transmembrane sugar fluxes in nectaries are very high, the large secretion rates might be achieved by structural adaptations as well as biochemical and physiological features (Lüttege and Schnepf 1976). For instance, plasmodesmata, the intercellular connections that permit symplastic transport (Gunning and Robards 1976), usually occur in nectaries between phloem sieve tubes, companion cells, parenchyma cells and secretory cells (Figier 1971, Wergin *et al.* 1975, Gunning and Hughes 1976). The number of plasmodesmata is often especially high between secretory cells and also between sieve tubes and companion cells (Lüttege and Schnepf 1976). Although Gunning and Hughes (1976) agreed that pre-nectar must pass through the stalk cell protoplast in *Abutilon*, they determined that the required sugar flux across the stalk cell plasma membrane would be 3-4 orders of magnitude greater than published values for other plant cell membranes. Instead, they suggested that the bulk of the sugar solution
travels through the low resistance pathway provided by the abundant plasmodesmata in the distal periclinal wall of the stalk cell. Also, plasmodesmata interconnect all the cells of the trichome (Findlay and Mercer 1971a,b, Gunning and Hughes 1976). Therefore, unlike Reed et al. (1971), Gunning and Hughes (1976) concluded that the stalk cell is probably not the location where the driving force for nectar secretion is generated, and inferred that the apical cells of the trichome are involved; mitochondria and endoplasmic reticulum cisternae were found to be most abundant there.

A granulocrine secretion theory, based exclusively on electron microscopic observations, has also been proposed. Support for this view was extensive and included work by Eymé (1966), Fahn and Rachmilevitz (1970, 1975), Figier (1971), Findlay and Mercer (1971b), Rachmilevitz and Fahn (1973, 1975), Heinrich (1975a) and Cecchi Fiordi and Palandri (1982). Briefly, the granulocrine secretion theory involved the transport of pre-nectar in vesicles, from cell to cell. When the vesicles reached the plasma membrane, it was postulated that membrane fusion occurred and the sugar solution was released into the cell wall area. Pinocytosis and exocytosis continued until the secretory cell was reached; here, the fluid was released to the exterior by a final membrane fusion. The vesicles involved were thought to be derived from the plentiful endoplasmic reticulum, and possibly from dictyosome, cisternae (Schnepf 1969, Fahn 1979a,b).

Although vesicles are often seen in close association with secretory cell plasma membranes, Lüttege and Schnepf (1976) regard the evidence for granulocrine secretion as circumstantial. Also, not all nectaries possess the structural make-up required to support this theory. In Passiflora, for instance, the lack of abundant endoplasmic reticulum and dictyosomes in the secretory cells and the existence of numerous mitochondria suggested that an eccrine secretion system is operating (Durkee et al. 1981). They postulated that "starchy nectaries", those accumulating starch prior to the onset of secretion, lack the granulocrine mode of secretion. Instead, in nectaries where endoplasmic reticulum is present, Durkee (1983a) suggested that it is the site for
manufacture of those enzymes necessary for the transport process. Figier (1969) viewed the endoplasmic reticulum of the petiolar gland of *Mercurialis annua* as having a role in protein transport. Pais (1986), however, has suggested that the endoplasmic reticulum in the floral nectary of *Epipactis atropurpurea* is involved in carrying sugar derived from the massive quantities of starch stored there, contradicting the postulate of Durkee *et al.* (1981) above.

Another ultrastructural feature common to many nectaries is the possession of cell wall ingrowths. Gunning and Pate (1969) suggested that wall protuberances develop in any anatomical situation where adverse surface area-volume relationships occur between donor and receptor compartments of the transport pathway. The enlarged plasma membrane surface may provide an increased area for fusion of vesicles derived from endoplasmic reticulum e.g., *Lonicera* (Fahn and Rachmilevitz 1970, Fahn 1979a,b), may provide more room for membrane-bound carrier mechanisms, or may facilitate passive elimination by diffusion (Lüttgte and Schnepf 1976). The mere presence of wall ingrowths does not allow any definite conclusion to be drawn concerning the secretion mechanism, though clearly the ingrowths would favour eccrine much more than granulocrine processes.

Lüttgte and Schnepf (1976) and Schnepf (1969) proposed that because of the diversity of both nectary types and nectar compositions that have been identified, both eccrine and granulocrine secretion mechanisms may be possible. Either mechanism would involve movement of pre-nectar in the symplast, the living part of the plant cells. However, it has been proposed that in some cases, exudation of nectar occurs strictly via apoplastic movement through the intercellular spaces and cell wall. For example, Frey-Wyssling and Häusermann (1960) could not detect typical nectary cells in the unstructured nectaries on the perianth of *Paeonia*, and concluded that these nectaries have a close affinity to hydathodes. Also, Vasiliev (1969, 1971) maintained that the apoplast is the main route of nectar flow from the phloem endings. Davis *et al.* (1988a) provided evidence in support of this in the floral and stipular nectaries of *Vicia*
faba, wherein relatively large numbers of wall ingrowths in the companion cells of nectar-bearing glands were opposed to intercellular spaces and cell-wall junctions of adjacent cells. According to Schnepf (1977), in the various nectaries studied by Vasilijev, plasmodesmata were particularly infrequent in glands actively secreting nectar. Vasilijev (1969, 1971) suggested that the sugar solution is released from the phloem and travels through the free space between the nectary cells to the exterior; activity of nectary cells is directed solely toward the manufacture of sugar-modifying enzymes. Findlay (1988) noted that inversion of phloem-supplied sucrose by the extracellular enzyme acid invertase accords with the view that at least part of the pre-nectar pathway is apoplastic. Schnepf and Deichgräber (1984) concluded that the apoplast was a major pathway for pre-nectar movement in several species of Euphorbia. However, at least in the case of secretory trichomes, an uninterrupted apoplastic pathway of pre-nectar from the phloem (Vasilijev 1969, 1971) is difficult to envisage. The cutinized walls of the stalk cells e.g., Abutilon (Findlay and Mercer 1971b, Gunning and Hughes 1976), Lonicera (Fahn and Rachmilevitz 1970), Vicia (Figier 1971), Vigna (Kuo and Pate 1985) are apoplastic barriers (but see Zellnig et al. 1991 for Abutilon). Hence, pre-nectar must at least pass through the symplast of the stalk cells, in order to reach the secretory cells above. Incrustations similar to the supposed apoplastic barriers of trichome stalk cells are also found within the extrafloral nectaries of species of Aphelandra, where the glands are multicellular outgrowths (Durkee 1983a, 1987).

1.5. AIMS OF THE THESIS

The literature on nectaries, and even more so the current research emphasis, is biased toward ultrastructural and cytochemical studies of the wonderfully-intricate nectary trichomes of a single plant family, the Malvaceae: Abutilon (Robards 1984, Robards and Oates 1985, Kronestedt et al. 1986, Robards and Stark 1988, Sawidis et al. 1989b, Zellnig et al. 1991); Gossypium (Eleftheriou and Hall 1983a,b); Hibiscus
(Sawidis et al. 1987a,b, 1989a, Sawidis 1991). Indeed, much of the impetus for these studies revolved around earlier work on Abutilon in Europe and in Australia (Mercer and Rathgeber 1962, Findlay and Mercer 1971a,b, Findlay et al. 1971, Reed et al. 1971, Gunning and Hughes 1976).

While the Malvaceae have proved useful for these investigations, nectaries composed of multicellular trichomes are much less common than simpler structures bearing what appear to be counterparts of the stomata found on the epidermis of leaves and known to regulate photosynthetic and transpirational gas exchange. There is a general belief in the literature that nectary stomata are incapable of regulating nectar flow (Helder 1958, Zandonella 1967c, 1972, Fahn 1974, 1979a). However, for no nectary has the development, anatomy and ultrastructure of these stoma-like structures been examined and combined with rigorous examination of their physiology, to confirm or deny the existence of a regulatory function. The second chapter of the thesis is devoted to filling this gap in knowledge. Selection of the floral nectary of Vicia faba L. stemmed from an earlier study (Davis et al. 1988a) which showed stomata, in statistically meaningful quantities, to be located at the tip of a multicellular outgrowth (i.e., not a depression) that would allow relatively convenient access for such an investigation. Another point in favour of V. faba is that its foliar stomata have been studied extensively, thus providing a basis for comparison with the counterparts in nectaries. Chapter 3 extends the work on V. faba and is devoted to a related study of interest from the standpoint of plant breeding. It involved addressing the question of whether there is a correlation between the number and distribution of modified stomata on the floral gland of V. faba with the total amount of nectar sugar produced.

While conducting my Ph.D. studies there has been a veritable explosion of information concerning the developmental and molecular genetics of floral organogenesis, particularly in Arabidopsis thaliana (L.) Heynhold and Antirrhinum majus L. Perhaps surprisingly, from the standpoint of their general biological significance, the floral nectaries of these two species have received little, if any,
attention in these recent investigations. Familiarity with floral nectaries of the Brassicaceae (Davis et al. 1986) and the strong emphasis on A. thaliana in modern plant research prompted a comprehensive study of the development, anatomy, vasculature and ultrastructure of the floral gland of this increasingly important species. Questions addressed during this part of the investigation concerned quantitative changes in fine structure of the gland's epidermal, subepidermal and vascular cells throughout development, and an opportunity to revisit the possible regulatory role of stoma-like structures during nectar secretion. The availability of a starchless mutant of this species provided material for preliminary investigations of the importance of nectary starch deposition prior to the commencement of secretory activity, and of any differences in nectar-sugar yield or composition between the wild-type and starchless mutant, the final topics covered in Chapter 4.

Nectaries of Echium plantagineum L., an introduced plant species highly favourable to Australian apiarists and their honey bees, but whose weedy nature and increasing distribution have raised the ire of many an Australian farmer and grazier, were the focus of the latter part of my studies. In Chapter 5, nectar-sugar composition and constancy with floral age, as well as the influence of pollination and pollen-tube growth on reabsorption of nectar sugar, were investigated. Echium then provided material for an enquiry that led into the broader aspects of nectary function, introduced at the start of the present literature survey. Nectar is a lure for pollination vectors. The development and potential of a promising, novel technique which directly and quantitatively discriminates and evaluates insect visitors as pollinators of "virgin" (previously-unattended) flowers, based on the detection of pollen tubes at the base of the style, are presented in the latter part of this chapter. The final research paper, Chapter 6, concerns a scanning-electron microscopic study of a relatively rare occurrence of mixed-loading of pollen, and nectar collection, from two plant species during the same foraging trip by an individual honey bee, and the implications of these results are discussed in terms of honey bee foraging behaviour. These studies reinforce
the need to pay due attention to the biology of nectar and nectaries in plant breeding programmes.

As well as being a summary of the main results of the thesis, Chapter 7 also includes an analysis of all previous ultrastructural studies of nectaries whose nectar-sugar composition is adequately known, to determine whether there is a link between the relative quantities of endomembranous content in nectariferous cells and the proportions of sucrose and hexoses (fructose, glucose) in nectar. This analysis was spurred by the results of the study of the floral nectary of *A. thaliana* and the postulate of Gunning and Hughes (1976) that endoplasmic reticulum of the secretory cells of the *Abutilon* nectary trichomes may provide protection to nectar sucrose from cytoplasmic enzymes.
CHAPTER 2

THE MODIFIED STOMATA OF THE FLORAL NECTARY

OF VICIA FABA L.

I. DEVELOPMENT, ANATOMY, ULTRASTRUCTURE

AND PHYSIOLOGY

Most of the developmental, anatomical and ultrastructural aspects covered in this chapter have been published in Protoplasma (1992) 166(3/4): 134-152.
2.1. INTRODUCTION

The secretion of nectar is important both in floral biology and in commerce. Nectar is released from the secretory cells of nectaries either by passage through a permeable, porous or ruptured cuticle, or through pores of structures referred to as "modified stomata" (Fahn 1979a). The latter structures are known to occur in many angiosperm families and are found commonly in the nectary epidermis of flowers of Fabaceae (see Appendix 1), wherein the dominant stomatal function of gas exchange is replaced by one of passing sugar solutions to the exterior (Ziegler 1987).

There are but brief studies of the development of nectary stomata (Picklum 1954, Zandonella 1972, Teuber et al. 1980), and only two published transmission electron micrographs of their guard cells (Eriksson 1977, Sammataro et al. 1985). Despite the fundamental difference in function between nectary and foliar stomata, there are no physiological comparisons between nectary and foliar stomata within the same species, with the exception of Medicago sativa L. (Teuber et al. 1980). The floral nectary of Vicia faba L. possesses these stoma-like structures (Gunning and Steer 1975, Davis et al. 1988a). Stomata on leaves of this species have been well described [for references see Allaway and Setterfield (1972) and Wille and Lucas (1984)], and there exists a wealth of data from studies of guard-cell physiology of leaves of this species. Accordingly, V. faba was chosen for a detailed investigation of the development, anatomy, ultrastructure and physiology of nectary stomata. The aims of the study were to ascertain any differences between the modified stomata of the floral nectary and the stomata of leaves, and to determine whether release of nectar is regulated by these structures on the gland's surface.
2.2. MATERIALS AND METHODS

2.2.1. Plant material

Plants of *Vicia faba* L. cv. "Aquadulce", or cv. "Blandine", a tannin-free variety, were grown throughout the year from seed in an outdoor plot directly exposed to sunlight. The multi-stemmed plants were watered as required. Unless specifically stated, all observations reported here are based on cv. Aquadulce.

Bud length was found to be a reliable index of flower age. For some of the physiological experiments, buds were classified as: *Stage A* - buds 16.0-18.0 mm long from corolla tip to calyx base, approximate gynoecial length (i.e., distance from calyx base to stigma tip) 8.5-10.9 mm, with petals light green and corolla tip exsert from calyx lobes, approx. 96-108 h before onset of nectar secretion; *Stage B* - buds 18.0-19.0 mm, gynoecium 11.8 - 12.5 mm, greenish-white corolla, about 72-96 h prior to secretion; *Stage C* - buds 20.5-22.5 mm, gynoecium 13.0-14.3 mm, corolla pale greenish-white and banner petal still tightly closed over other petals, approx. 48 h before secretion begins; *Stage D* - mature buds 25.5-28.0 mm, gynoecium 16.4-18.3 mm, corolla white with banner closely surrounding wing petals, usually approaching or just reaching anther dehiscence, ≤ 24 h pre-secretion.

2.2.2. Sequential scanning electron microscopy of live material

The development of individual modified stomata of the nectary was followed by making sequential high resolution impressions using a low viscosity vinyl polysiloxane material [Permagum\textsuperscript{R} (ESPE) obtained from Rudolf Gunz Co., 63 Ann Street, Surry Hills, N.S.W.] according to the general procedure of Williams and Green (1988). Pre-secretory flowers of various stages were dissected to remove basal portions of the calyx, androecium, and the keel petals (see Fig. 2.1). An impression of the exposed nectary projection was prepared by surrounding it with Permagum\textsuperscript{R}. After 5-10 min, the adhering, hardened impression was peeled from the nectary with tweezers, and the
bud placed, ventral side up, on a moistened filter paper in a petri dish and kept in the dark at 20-23°C. Up to three further impressions of each nectary projection were prepared, over 24 h, returning the buds to darkness after each impression was taken. After the final impression, the projection was dissected, mounted in fluorescein diacetate and viewed with a Zeiss fluorescence microscope. In all material presented here the cells remained capable of taking up and hydrolyzing fluorescein diacetate at the end of the sequence of observations.

Under the dissecting microscope, the impressions were inserted in LR White plastic resin (London Resin Company Limited, Basingstoke, Hampshire, England) in gelatin capsules, and all air bubbles expelled. After polymerization at 60°C for 24 h, the facsimiles of the nectary projections were carefully extracted, mounted vertically (to allow viewing access of all modified stomata on the projection) on aluminum stubs for scanning electron microscopy, coated with gold (approx. 60 nm) in a Polaron E5000 Sputter Coater, and photographed on a Cambridge Stereoscan 180 or 360 scanning electron microscope at 25 or 20 kV, respectively. Comparison of successive replicas allowed the development of individual modified stomata to be followed.

2.2.3. Stomatal development and aperture in flowers of various stages

To investigate the maturation of floral nectaries and the development and any change in aperture of their modified stomata, numerous flowers of various stages of growth were measured for gynoecial length, dissected to the nectary/receptacle and fixed separately, immediately after collection, in 3 ml of 2% glutaraldehyde / 2% paraformaldehyde in 25 mM K phosphate buffer, pH 6.8, at room temperature. After sitting overnight in fixative, tissues were washed twice in 25 mM K phosphate buffer, pH 7.0, before placement for 1 h in cold 1% OsO₄ in the same buffer. Following several rinses in buffer and then distilled water, tissues were immersed for 1 h in cold 2% OsO₄. Tissues were rinsed three times in distilled water, dehydrated in a graded
ethanol series and then critical-point dried in a Balzers CPD Model 010 using liquid CO₂. Nectaries were then mounted and viewed by scanning electron microscopy as outlined in 2.2.2. Further specimens were flash-frozen in liquid N₂ slush (-230°C), gold-coated and then viewed directly at -180°C using a Hexland CT1000A Cryo Trans cold stage attached to the Cambridge 360 scanning electron microscope.

For each flower, all modified stomata were photographed individually in face view, accomplished by appropriate orientation of the stub bearing the nectary projection, at a film magnification of 1440x. Aperture widths were calculated directly from these films as the maximum distance between sister guard cells, 90° to a line joining the two ends (i.e., the cell-wall junctions) of the pore (see Fig. 2.49). The accuracy of this method was checked by measuring pores of modified stomata on the distal quarter of the gynoecial face of nectary projections (see Fig. 2.1, 2.3) first in fresh material and subsequently re-measuring the same modified stomata after preparing the projections for scanning electron microscopy by the critical-point drying method. For these comparison, projections from pre-secretory flowers were mounted in distilled water and those from secretory flowers were mounted in sucrose solution previously prepared to mimic nectar in respect of the predominant sugar species (see Table 3.2, page 3.7) and concentration by light microscopy. After measuring pores on the fresh material, the projections were processed as described above. The length of the nectary projection along its abaxial, staminal face was also measured from flowers of the developmental study.

2.2.4. **Anatomical investigation of nectary stomata**

2.2.4.1. **Bright-field microscopy**

To examine the gross structure, orientation and insertion of floral parts near the nectary, fresh flowers of different stages were dissected to various degrees, and photographed under a Wild-Heerbrugg Photomakroskop M 400 dissecting microscope.
Hand-sectioned fresh material was mounted in iodine potassium iodide, ruthenium red, hydroxylamine-ferric chloride, zinc chlor-iodide, Sudan black B or Nile blue solutions (Jensen 1962) or in various stains (ferric chloride, aniline-KIO₃, vanillin-HCl, nitrous acid) for phenols (Reeve 1950, Mace 1963) and examined using a Zeiss microscope.

Exudation of nectar was observed directly by mounting flowers, dissected to the nectary, in immersion or paraffin oil on glass slides.

Sections, 1.5-2.0 µm thick, were cut on a Reichert Ultramicrotome OmU3 with glass knives from nectaries fixed and washed as in 2.2.3, but then dehydrated in a graded series of ethanol (without prior post-fixation) before embedding in LR White resin (2.2.2). The sections were heat-fixed to slides before staining with 1% toluidine blue O in benzoate buffer, pH 4.4 (O’Brien and McCully 1981).

2.2.4.2. Polarized light microscopy

Modified stomata of fresh material (nectary projections whole or thinly sectioned by hand) mounted in water were viewed on a Nikon Optiphot-Pol microscope fitted with rectified polarisation optics. Epidermal peels of leaves were also mounted in water, for comparative observations of birefringence in foliar stomata.

2.2.4.3. Fluorescence microscopy

To examine the walls of nectary guard cells for cutin or callose, resin-embedded sections were mounted in 0.01% auramine O in 50 mM K phosphate buffer (Marin et al. 1988) or aqueous aniline blue (Galway and McCully 1987) and photographed with exciter filter BP450-490 and barrier filter LP530, or 2XKP500 and LP530, respectively. Fresh material was mounted in 0.1% Calcofluor White M2R New (Gunning and Hughes 1976) and examined using filters UG1, LP530. Fresh and sectioned material were also checked for autofluorescence.
2.2.5. **Ultrastructural investigation of nectary stomata**

Buds and turgid, open flowers containing nectar were dissected to the nectary/receptacle, immediately immersed in fixative for 2 h, rinsed with three changes of phosphate buffer, post-fixed in 1% OsO₄ in buffer for 2 h, washed again in buffer, distilled water and then dehydrated in an ascending series of acetone solutions. Tissues were embedded in Spurr’s resin (Spurr 1969) and ultrathin, silver sections cut using a Dupont Instruments diamond knife on a Reichert-Jung Ultracut E microtome. Sections were stained for 20 min in 2% uranyl acetate and 10 min in 0.02% lead citrate (Venable and Coggeshall 1965) and viewed with a Hitachi 500 or 600 transmission electron microscope at 100 kV.

To quantify changes in cell area, vacuolation and organelle density throughout the development of modified stomata, electron micrographs of guard mother cells and guard cells sectioned at the midpoint, both longitudinally and transversely, were prepared at 15,000x magnification. The micrographs were covered by a grid of 1.5-cm squares, and the number of intersection points counted, such that the total area of the cell profile and that of the vacuome, cytoplasm and nucleus, could be estimated (Steer 1981). Numbers of plastids, mitochondria, microbodies and spherosomes per cell profile were counted and expressed per cytoplasmic area. Details of the plastids were compared with those provided by Allaway and Setterfield (1972) for foliar stomata.

Maximum widths of the dorsal, ventral, outer and inner walls (see Fig. 2.41 and Sack 1987) were measured (to the middle lamella, and including ridges but not cuticle, where applicable) from electron micrographs of ten, randomly-selected guard cells of open modified stomata (transverse section) and of both end walls from five guard cells (longitudinal section).

2.2.6. **Stomatal densities**

Five leaf segments were chosen at random and three fields of each photographed
normal to the leaf surface at 1500x using scanning electron microscopy. The number of stomata (all were mature) per mm$^2$ of leaf area was determined for each field, and the mean calculated. For the modified stomata of floral nectaries, all stages ranging from Immature-1 to closed structures (see Table 2.1, page 2.12) on the projections of five mature buds from the same plant were counted. The nectary projections were treated as tall, narrow pyramids for calculation of surface area, and the density of modified stomata determined both for the entire projection and the distal quarter of each projection.

### 2.2.7. Variation of stomatal aperture in field conditions throughout the day

At approx. 4-h intervals over 36 h, a leaflet and a pair of buds (one each of Stage B and D) were collected from one of eight flowering stems of the same plant. The two pre-secretory flowers were taken from the same raceme. The leaflet was the most proximal of six or seven of the mature compound leaf of the node bearing the sampled inflorescence, and a piece (approx. 4 x 3 mm) cut from the centre of the leaflet’s left side was immediately fixed, as were the two floral nectaries. Tissues from all collection intervals were prepared together for scanning electron microscopy (2.2.3). All nectary stomata (33-69), and 53-56 foliar stomata of the abaxial epidermis, were photographed and apertures measured as before (see 2.2.3).

### 2.2.8. Experimental plasmolysis of guard and epidermal cells

Spence (1987) has warned that caution must be exercised when interpreting the results of physiological experiments based on epidermal peels of leaves, because their guard cells are not normally exposed to solutions. However, the same cannot be said for nectary stomata, which can serve as exits for nectar and are usually bathed by accumulating exudate. Indeed, it seems proper to examine nectary tissue in solutions. Accordingly, pre-secretory flowers at stages A, C and D were collected from each of three randomly-chosen plants and nectary projections, excised approx. 0.5 mm below
their tips, were immediately placed in a droplet of 0.02% aqueous neutral red on a microscope slide under a cover slip. After 3-5 min, when the dye was visible within nectary cells, the isolated projections were perfused with a concentrated solution of mannitol, sucrose or CaCl$_2$, and the slides placed in the dark. Fresh solution was provided regularly and liberally during the time-course experiment (below). Operations were conducted at 20-22°C.

The percentages of plasmolysed guard cells of mature modified stomata and epidermal cells were determined from the projection tips at each of six times (10 and 30 min, 1, 2, 4 and 6 h) later. The projections were examined by microscopy in dim green-light, which does not influence stomatal aperture in leaves (Bittsnich et al. 1987). Slides were returned to darkness immediately after each examination. Three buds per stage per plant were examined for each freshly-prepared solution (1.0 M mannitol or sucrose, pH 5.8; 0-100 mM CaCl$_2$, pH 5.4).

Incipient plasmolysis, the condition wherein 50% of cells show any degree of plasmolysis, was calculated for nectary and foliar guard and epidermal cells 10 min or 24 h after exposure of projections or leaf pieces to graded (stepwise by 0.1 M) solutions of either mannitol or sucrose.

2.2.9. Influence of nectar-solute concentration on pore aperture of nectary stomata

To manipulate the solute concentration of nectar, bases of 5-6 mature buds (stage D) were inserted through holes in Nescofilm$^R$ covering 25 ml vials full of freshly-prepared sucrose solution, pH 5.6 (see Fig. 3.3). The vials were then sealed with three layers of film in 100-ml beakers containing either 5 ml distilled water or 25% H$_2$SO$_4$, to expose the secreted nectar to two different relative humidities within the beakers (Shuel 1952). The sealed beakers were wrapped in aluminum foil and placed in a growth chamber at 25°C. Three treatments were investigated: 1) 0.15 M sucrose, high
relative humidity, 2) 0.5 M sucrose, high relative humidity, 3) 0.5 M sucrose, low relative humidity. All buds in the experiment were excised from the same plant, and twenty buds were cultured per treatment.

Twenty-four hours after the bud-culture began, nectar was collected by destructive sampling using a combined capillary-wick method. Flowers were individually removed from the beaker (covered again) and the reflexed standard petal pulled back. Any nectar at the standard base and basal gaps of the androecium (see Fig. 2.1, 2.2) was collected using Drummond MicrocapsR (1 µl). The height of the nectar volume in the capillary was measured immediately, and its concentration determined using a Bellingham and Stanley (Tunbridge Wells, U.K.) pocket refractometer, with readings corrected to 20°C. Partial removal of the androecium allowed access to nectar that was pooled around the nectary projection (see Fig. 2.3). The residual exudate was carefully swabbed using wicks of Whatman No. 1 filter paper (McKenna and Thomson 1988). The wicks were labelled and stored for up to a month, in a desiccator, and then soaked in distilled water (usually 1.0 ml), shaken periodically, and finally centrifuged. The sensitive spectrophotometric phenol-H$_2$SO$_4$ technique of Roberts (1979) was employed to quantify residual sugar thus dissolved from the paper wicks. The total amount of nectar sugar per flower was therefore a summation of the capillary (product of volume times concentration) and wick collections.

Immediately after nectar harvest, five randomly-chosen flowers per treatment were dissected to the nectary/receptacle, fixed and prepared for scanning electron microscopy (2.2.3). The mean aperture of open modified stomata and the total number of open, immature and closed modified stomata were determined (2.2.3). A further five presecretory buds of culture age were similarly processed.

2.2.10. Localization of potassium ions in guard cells

The cobaltinitrite technique described by Humble and Hsiao (1970) and Fischer
(1971) was utilized to stain for $K^+$ in guard cells. Immediately after buds and nectar-secreting flowers were dissected to the nectary/receptacle, and pieces of leaf or epidermal peels were taken, the tissues were immersed or floated (strips) on a $0.5 \text{ M } \text{Na}_3\text{Co(NO}_2)_6$ solution in $0.1 \text{ M acetic acid}$ for 30 min. Thereafter, tissues were washed for 1 min in cold distilled water, placed in $1\% \text{ (v/v) aqueous ammonium sulphide}$ for two min, mounted on glass slides and then observed and photographed using light microscopy. Some tissues, prior to immersion in sodium cobaltinitrite solution, were placed in or on $100 \text{ mM KCl}$ solution for 30 min, and rinsed for 10 min in distilled water.

2.2.11. **Natural and experimental secretion of abscisic acid in floral nectar**

Capillaries (Drummond Microcaps$^R$) were used to draw nectar from fully-open flowers of various plants in the plot. The banner petal was carefully pulled back and off. Nectar residing on this petal was immediately collected, and then only from the edge of the nectar droplet distant from the petal base, to avoid any artefactual contamination caused by petal leakage or flower wounding. These precautions were taken because abscisic acid (ABA) has been detected in at least the gynoecia of buds and open flowers of *V. faba* (Diethelm *et al.* 1988). Exudate was either analysed directly for ABA content or the capillaries frozen at -20°C until later analysis.

Buds of stage D from various plants were cultured according to the *in vitro* technique (2.2.9) in darkness on $0.3 \text{ M } [10\% \text{ (w/v)}]$ sucrose solution, pH 5.6, containing 0 to 5 mM $(\pm)$-2-cis-4-trans-abscisic acid (Sigma; approx. 99% purity - Lot 127F-0079) for 24 or 36 h. Then, flowers were dissected and nectar collected and frozen as above. In several cases, the nectary projection was then immediately excised, mounted gynoecial-face-up under a coverslip on a microscope slide and perfused with weak sucrose solution of the appropriate concentration. Because the modified stomata
on the distal quarter were always covered by standing nectar, in situ, they were selected for examination of guard-cell plasmolysis. Thereafter, some of these projections were fixed and processed conventionally for scanning electron microscopy (2.2.3).

Frozen nectar was thawed, diluted as required, and directly analysed for exogenous ABA using an ELISA technique based on monoclonal antibodies (Sigma) for (+)-cis-trans-ABA (Weiler et al. 1986). The quantities of (+)-cis-trans-ABA in the nectar samples were determined from standard curves, and nectar concentrations of the plant-growth regulator were calculated in terms of nectar dry weight.

2.3. RESULTS

2.3.1. Nectary orientation, nectar escape and accumulation

The morphology of the floral nectary of *Vicia faba* is shown in Figs. 2.1-2.3. It consists of a disk (Fig. 2.3, 3.1a, 3.2a) situated on the receptacle between the androecium and gynoecium. A prominent outgrowth arises from this disk (Fig. 2.3, 3.1a, 3.2a), opposite the free stamen (Fig. 2.1). This projection is vascularised by phloem only, the column of sieve elements originating from five ventral vascular bundles supplying the staminal column (Fig. 2.1, 2.5).

The first nectar appears concurrent with or shortly after anther dehiscence, as the banner petal just begins to loosen before it ascends above the wing and keel petals. The initial droplet gathers around the tip of the projection, either between it and the gynoecium, or on the staminal column (Fig. 2.3). Although absent on the disk portion of the nectary, numerous modified stomata are located on the projection, particularly at the tip (Fig. 2.5-2.7). Nectar can be observed to escape through these stomatal pores (Fig. 2.4). As the production of nectar continues, the increased volume accumulates at
the base of the cavity formed by the androecium around the gynoecium. Nectar may also exude around the free stamen to collect at the base of the banner petal.

2.3.2. Ontogeny and features of guard mother cells

Features of guard-mother and guard cells described here and in the next sections are summarised in Table 2.1, which gives features of developmental stages of modified stomata categorized as "Immature-1", "Immature-2", "Immature-3", "Open" and "Closed".

<table>
<thead>
<tr>
<th>Stomatal stage</th>
<th>Pore</th>
<th>Substomatal space</th>
<th>Vacuole</th>
<th>Plastids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guard mother cell</td>
<td>None</td>
<td>None, or just beginning</td>
<td>Few, small vacuoles with contents clear or dense</td>
<td>Elongate. Peripheral reticulum abundant. Starch practically absent.</td>
</tr>
<tr>
<td>Immature-1</td>
<td>None. Very shallow depression, externally</td>
<td>Small, incomplete</td>
<td>Many, small vacuoles with clear contents</td>
<td>Spherical, or becoming so. Peripheral reticulum abundant or declining. Starch grains numerous.</td>
</tr>
<tr>
<td>Immature-2</td>
<td>None. External depression deepening, and expanding longitudinally</td>
<td>Enlarging, incomplete</td>
<td>Many, small vacuoles with clear contents</td>
<td>Spherical. Peripheral reticulum reduced. Starch-laden.</td>
</tr>
<tr>
<td>Immature-3</td>
<td>None exposed. Outer cuticle intact, or just tearing, otherwise complete</td>
<td>Enlarging, or often complete</td>
<td>Large, solitary vacuole. Cytoplasm peripheral</td>
<td>As above.</td>
</tr>
<tr>
<td>Open</td>
<td>Partially or completely exposed between guard cells</td>
<td>Complete</td>
<td>As above</td>
<td>As above.</td>
</tr>
<tr>
<td>Closed</td>
<td>Completely occluded. As above</td>
<td>As above</td>
<td>As above</td>
<td>As above.</td>
</tr>
</tbody>
</table>

Paradermal cell divisions of the nectary disk, and the subsequent elongation of these newly-created cells, generate the nectary projection (Fig. 2.10a, 2.11a, 2.12). The guard mother cells of the projection are typically pentagonal in longitudinal section, and often arise from oblique, asymmetrical divisions of certain narrow, rectangular epidermal cells (Fig. 2.10b, 2.13a middle, 2.15). These precursors may
divide acropetally or basipetally, to yield guard mother cells of various sizes (Fig. 2.15, 2.16, 2.18, 2.19a). When these parent epidermal cells are adjacent, guard mother cells often develop contiguous to one or more others. The occasional occurrence of three or four adjacent modified stomata in a longitudinal file (Fig. 3.6b, 3.7), and the finding of a modified stoma completely surrounded by others (Fig. 3.7) indicates that divisions of each precursor cell can yield more than one guard mother cell (also see Fig. 2.10b).

The first guard mother cells originate on the extreme tip of the differentiating projection (Fig. 2.10b) of very small buds. As the projection lengthens, these cells may develop at any location on the gynoecial face (Fig. 2.12, 3.4). However, they are only occasionally found on the basal 3/4 of the staminal face, even in mature flowers (but see Selection A, Fig. 3.5, 3.6a).

Apart from their characteristic diminutive size and five-sided shape, guard mother cells also differ from their larger, sister cells, and other epidermal cells, with regards to vacuolar contents. In the former, the vacuoles are usually small, electron-translucent and abundant (Fig. 2.16, 2.18) around the centrally-located, spherical nucleus (Fig. 2.16, 2.18, 2.19a). In other cell types, however, the vacuoles are larger and stain darker in transmission electron micrographs (Fig. 2.16), they give a positive reaction (red) for phenols using aniline-KIO₃, and their contents autofluoresce as yellow-green with UV light excited in the blue range. The cell plate separating each guard mother cell from its precursor cell, as well as the four other thin walls of the guard mother cell, may possess plasmodesmata (Fig. 2.16, 2.19b). Endoplasmic reticulum, curled dictyosomes and a small number of spherosomes occur in the cortical cytoplasm, and the elliptical plastids, located around the nucleus, possess thylakoids and plastoglobuli but usually lack starch.
2.3.3. **Ontogeny and features of guard cells**

2.3.3.1. *Division of guard mother cells*

Although no preprophase band of microtubules was observed in the relatively few sections of appropriately-aged guard mother cells which were obtained, the site of the future division of guard mother cells into sister guard cells is usually predictable from the patterns of wall insertion in nearby cells (Fig. 2.16). In the majority of guard mother cells the cell plate usually attaches to the wall recently formed to separate the guard mother cell from its sister, epidermal cell, and to the wall opposite, thereby yielding four- to six-sided sister guard cells (Fig. 2.16, 2.19a) having their long axes parallel to the epidermal cells (Fig. 2.15 centre, 2.16, 2.19a). However, the cell plate, which eventually differentiates into the ventral (pore) walls of the modified stomata, occasionally aligns perpendicular to the long axis of the epidermal cells, resulting in transversely-arranged guard cells (Fig. 2.15 top right). The cell plate fluoresces strongly with aniline blue (Fig. 2.19b). The division is not apparent on the external surface of the guard mother cell until the pore starts to form in the new wall. Neighbouring cells do not become morphologically differentiated as subsidiary cells (Fig. 2.13, 2.15).

2.3.3.2. **Microtubules and cell wall**

Microtubules are located in the cortical cytoplasm of the guard cells of modified stomata, immediately below the plasma membrane (Fig. 2.25, 2.31, 2.36). Usually the microtubules (Fig. 2.25) radiate from the pore site (ventral walls) toward the dorsal and end walls (Fig. 2.36). Microfibrils closest to the plasma membrane are congruent with the microtubules, e.g., microfibrils radiating from the pore (Fig. 2.36). Dictyosomes are found throughout the peripheral cytoplasm. Their numerous vesicles contain a flocculent material and, with coated vesicles, are often located near the plasma membrane (Fig. 2.25, 2.31, 2.36, 2.37). Strands of rough endoplasmic reticulum are also characteristic components of the guard-cell periphery (Fig. 2.21, 2.25, 2.31, 2.36).
Ridges of wall material are found at the cuticular faces of the outer and inner walls (Fig. 2.31 top left, 2.35, 2.39-2.41, 2.42a,b,d,e, 2.44, 2.49, 2.57, 2.58, 2.82b, 3.8a, 3.12d), founded on microfibrils which are often oriented perpendicular to the underlying ones, as can be seen in guard cells sectioned in both transverse (Fig. 2.50) and longitudinal (Fig. 2.51) planes. These ridges are hidden externally by an ethanol-soluble wax covering which is preserved by low-temperature scanning electron microscopy (compare Fig. 2.49 and 2.52).

Nectary and foliar guard cells were compared by examining the birefringence of their cell walls. The same pattern of birefringence, a Maltese-cross arrangement, was detected in foliar stomata and in modified stomata of nectaries (Fig. 2.63, 2.64), indicative of a net radial orientation of microfibrils centred on the pore (Fig. 2.8). However, in modified stomata, there was a marked reduction in intensity of birefringence, indicative of lesser optical path differences, and outer, circumferential ridges composed of wall microfibrils oriented at approx. 90° to the radial microfibrils below them were evident (arrows in Fig. 2.64b,c). Transverse sections of open modified stomata demonstrate the thinness of the guard-cell walls. The outer and inner walls are thicker at their centres than at the ends (Fig. 2.43). The inner walls averaged 1.16 ± 0.070 (S.E.) μm, whereas the mean of the outer walls of the same ten, randomly-selected guard cells, was 0.69 ± 0.033 μm. At the equatorial position, the ventral walls averaged 0.47 ± 0.044 μm, more than double the thickness of the dorsal ones, 0.20 ± 0.008 μm. The end walls were 0.35 ± 0.039 μm thick. Taken together, the thinner walls and reduced optical path differences indicate that guard-cell walls of modified stomata are quantitatively different from, but qualitatively similar to, the corresponding walls of leaf stomata.

All walls of guard cells fluoresce when exposed to Calcofluor White M2R New
(Fig. 2.7), and the presence of pectinaceous materials was verified by staining with hydroxylamine-FeCl$_3$ and ruthenium red. Although the walls of guard cells showed a faint yellow-green autofluorescence, they reacted negatively with all stains for phenols tested here.

2.3.3.3. **Plasmodesmata**

All cell types (sister and non-sister guard, epidermal and subepidermal) which border an immature guard cell may be connected to it by plasmodesmata (Fig. 2.21, 2.24). They are located in relatively thin-walled primary pit fields (Fig. 2.21) (showing callose fluorescence in Fig. 2.19b) at an average density of $29 \pm 9.1$ (S.D., $n = 6$ fields) per $\mu$m$^2$. In guard cells of open modified stomata, complete plasmodesmata were no longer observed. Although apparently intact on the epidermal- and subepidermal-cell side of the wall adjacent to a guard cell, the plasmodesmata apparently end blindly in overlying wall material deposited by the guard-cell protoplast (Fig. 2.23). Furthermore, with the eventual development of the substomatal space, the cell wall between guard and subepidermal cells, previously traversed by plasmodesmata, may become sheared and, if so, lined with cuticle (Fig. 2.23).

Similarly, some plasmodesmata between young sister guard cells may transect the wall even at sites (Fig. 2.16) where the pore will ultimately separate the cells. In the case of adjacent non-sister guard cells, plasmodesmata present in immature modified stomata (Fig. 2.16) later become covered by microfibrils from both sides (Fig. 2.22), probably rendering them non-functional.

2.3.3.4. **Development of the pore and substomatal space**

Initiation of the substomatal space either precedes or coincides with commencement of development of the pore, soon after division of the guard mother cell. The substomatal space first opens directly below the future pore, and below the dorsal and end walls, of the guard cells, and cuticular material is laid down over the
resulting exposed surface of the guard-cell walls (Fig. 2.21, 2.31, 2.32).

Externally, the first indication of a developing pore is a slight central depression on the surface of the immature modified stomata (Fig. 2.15 lower centre, 2.33a top). Splitting of the shared wall proceeds inwards and towards the ends of the cells. Stages of this process are shown in Fig. 2.10b, 2.11b upper centre, 2.15 bottom left, 2.31, 2.33a, and 2.34. Formation of the pore is further advanced at the inner walls (Fig. 2.39). The separation occurs medially along a pre-formed electron-translucent layer (Fig. 2.25, 2.31, 2.39). Cuticular material is deposited on the exposed pore-wall surfaces (Fig. 2.31, 2.35-2.37), which in fresh material then stains bright yellow with zinc chlor-iodide.

The cuticle covering the outer surface and the strands across the substomatal space usually remain intact during the initial stages of pore formation (Fig. 2.32, 2.39). The cuticle at the inner side of the future pore ruptures first (Fig. 2.40) and the remaining cuticle then tears, at variable positions, to open the pore to the outside (Fig. 2.11b,c, 2.13b,c, 2.14, 2.56, 3.8b, 3.12c). Externally, ledges on the outer walls contain microfibrils covered by the original cuticle (Fig. 2.31), and ventral walls and ledges at the inner region of the pore are covered by new cuticle.

The substomatal cavity enlarges while the pore develops. Further separation of walls between the guard and subepidermal cells connects substomatal spaces with other intercellular spaces (Fig. 2.40, 2.41) and can interconnect substomatal spaces of neighbouring modified stomata (Fig. 2.44). The entire continuum is lined by a cuticle (Fig. 2.41) which is auramine-O positive (Fig. 2.53) and may link guard and underlying nectary cells (Fig. 2.43, 2.44).

Tissue distortions during growth of the nectary projection may deform the modified stomata. As the pores of neighbouring modified stomata develop, on the tip or upper flanks of the nectary projection, arches may form when turgor forces of adjacent guard cells are in opposition (Fig. 2.6). Very rarely, twisting or gross
separation of guard cells allows subepidermal cells to expand into the pores (Fig. 2.53, 2.82a).

One instance was recorded of gross modifications of normal development resulting in two isolated guard cells lacking pores, but nevertheless each with the usual kidney shape (Fig. 2.9).

2.3.3.5. Organelles

Like guard mother cells (Fig. 2.16), newly-formed guard cells contain numerous, relatively small vacuoles, mostly with clear contents (Fig. 2.16, 2.17, 2.19a, 2.20, 2.33 top). These soon fuse into one large vacuole per cell, concurrent with the development of the stomatal pore (Fig. 2.33 centre and bottom, 2.35), confining the cytoplasm to the cell periphery (Fig. 2.35).

Observations were made of mitochondria, microbodies, spherosomes and plastids, organelles described qualitatively and quantitatively for foliar guard cells of *V. faba* (Allaway and Setterfield 1972, Pallas and Mollenhauer 1972a). Mitochondria were most numerous (Fig. 2.26), spherical or oblong in shape, had prominent cristae and contained dense globules in their matrices (Fig. 2.28-2.30, 2.36-2.38). Like mitochondria, the number of microbodies per µm² of sectioned cytoplasm remained constant as modified stomata enlarged from the guard mother cell to the open state (Fig. 2.26). Microbodies were relatively scarce (Fig. 2.26), spherical, ovoid or irregular in shape, contained a flocculent material (Fig. 2.21, 2.37, 2.38) and were usually located beside spherosomes (Fig. 2.37, 2.38). The latter were distinctly osmiophilic (Fig. 2.29, 2.32, 2.34-2.36, 2.41), sometimes densest in their centres (Fig. 2.25, 2.28, 2.30, 2.31, 2.37, 2.38), and lacked a membrane. Apart from their three-fold rise in number per cytoplasmic area during stomatal development (Fig. 2.26), their average diameter also increased from $0.30 \pm 0.092$ (S.D., n = 16) µm in guard mother cells to $0.52 \pm 0.13$ µm (n = 16) from guard cells of open modified stomata. By contrast, spherosomes were rarely observed in epidermal cells (Fig. 2.33a, 2.44-2.48).
Table 2.2. Ultrastructural features of plastids throughout development of the modified stomata of the floral nectary of *Vicia faba* L., in thin sections.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Stomatal Stage</th>
<th>No. plastids examined*</th>
<th>Plastid size (µm)</th>
<th>Plastoglobuli</th>
<th>Grana</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Length</td>
<td>Width</td>
<td>No. per plastid</td>
<td>Diameter (µm)</td>
</tr>
<tr>
<td>Nectary Guard</td>
<td>Guard mother cell</td>
<td>48</td>
<td>0.98 ± 0.77</td>
<td>0.42 ± 0.019</td>
<td>4.2 ± 1.19</td>
<td>0.055 ± 0.0030</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.32-2.27)</td>
<td>(0.18-0.69)</td>
<td>(0-13)</td>
<td>(0.022-0.107)</td>
</tr>
<tr>
<td>Immature-1</td>
<td>25</td>
<td></td>
<td>1.01 ± 0.092</td>
<td>0.43 ± 0.029</td>
<td>1.6 ± 0.56</td>
<td>0.056 ± 0.0139</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.36-2.02)</td>
<td>(0.24-0.71)</td>
<td>(0-6)</td>
<td>(0.026-0.118)</td>
</tr>
<tr>
<td>Immature-2</td>
<td>19</td>
<td></td>
<td>1.51 ± 0.219</td>
<td>0.76 ± 0.073</td>
<td>4.2 ± 2.15</td>
<td>0.048 ± 0.0032</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.46-4.99)</td>
<td>(0.20-1.42)</td>
<td>(0-23)</td>
<td>(0.015-0.101)</td>
</tr>
<tr>
<td>Immature-3</td>
<td>25</td>
<td></td>
<td>2.31 ± 0.113</td>
<td>1.74 ± 0.100</td>
<td>3.0 ± 0.39</td>
<td>0.047 ± 0.0045</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.72-3.16)</td>
<td>(0.39-2.59)</td>
<td>(1-5)</td>
<td>(0.016-0.131)</td>
</tr>
<tr>
<td>Open</td>
<td>18</td>
<td></td>
<td>2.34 ± 0.202</td>
<td>1.90 ± 0.166</td>
<td>5.7 ± 0.73</td>
<td>0.047 ± 0.0026</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.79-3.66)</td>
<td>(0.52-3.10)</td>
<td>(1-8)</td>
<td>(0.016-0.095)</td>
</tr>
<tr>
<td>Leaf*</td>
<td>Open</td>
<td>10</td>
<td>3.9 ± 0.7</td>
<td>2.8 ± 1.3</td>
<td>13</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3.2-5.0)</td>
<td>(2.2-4.1)</td>
<td>(3-21)</td>
<td>(19-40)</td>
</tr>
</tbody>
</table>

*Columnar mean values followed by the same letter are not significantly different using 2-tailed t-tests, α=0.05.

+All plastid profiles present in three guard mother cells or three guard cells per developmental stage.

§Data from Allaway and Setterfield (1972).
As nectary guard cells matured, there was a 2.5-fold decrease in plastid number per µm$^2$ of cytoplasm (Fig. 2.26). In guard mother cells and guard cells of the Immature-I stage (Table 2.1, page 2.12), elongated plastids (Fig. 2.27) surrounded the nucleus (Fig. 2.16). In later stages of guard-cell development, starch grains predominated as the plastids became more rounded (Fig. 2.17, 2.28-2.30, 2.35, 2.39, 2.41; Table 2.2). The starch stained pinkish-red with iodine potassium iodide, indicative of amylopectin. The peripheral reticulum evident in plastids (Fig. 2.27, 2.28) was much reduced when they were starch-laden, as in open modified stomata (Fig 2.30). The number of grana and thylakoids per granum (Fig. 2.27) became fewer as the guard cells matured (Fig. 2.28-2.30; Table 2.2) and a densely-osmiophilic material accumulated in the plastids (Fig. 2.35, 2.39, 2.41). Compared with epidermal cell plastids, they showed reduced chlorophyll fluorescence. The plastids of epidermal and subepidermal cells remained elongate, and were never as filled with starch (Fig. 2.17, 2.32, 2.35 bottom left, 2.44) as those in guard cells, although sometimes plastids in subepidermal cells immediately below a pore were particularly starch-abundant (Fig. 2.40).

The nuclei were cylindrical-elliptical in shape (Fig. 2.16, 2.19, 2.20), often lobed (Fig. 2.32, 2.40 left), and fluoresced yellow-green when unstained, accounting for the fluorescence in Fig. 2.19b. Although sometimes shifted in the mature guard cell (Fig. 2.42a, showing guard cells found from preceding sections to be non-plasmolysed), the nucleus typically remained approximately midway between the end walls throughout cell development (Fig. 2.16, 2.19), usually nearer the ventral wall (Fig. 2.20, 2.33, 2.35). In anticlinal sections, the nucleus may be located closer to the inner or outer walls (Fig. 2.39-2.41, 2.43). Nuclear size remained constant throughout development (Fig. 2.26), and nuclear pores averaged 38.5 ± 3.8 per µm$^2$ of nuclear surface.
For comparative purposes, organelle data from guard cells of open stomata of
nectary and leaf (Allaway and Setterfield 1972) are listed in Table 2.3.

Table 2.3. Comparison of numbers of certain organelles per cell profile in thin sections, between guard
cells of leaf and floral nectary in Vicia faba L.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Stomatal Stage</th>
<th>No. cells examined</th>
<th>Mitochondria (M)</th>
<th>Plastids (P)</th>
<th>M/P</th>
<th>Microbodies</th>
<th>Spherosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf*</td>
<td>Open</td>
<td>5</td>
<td>22.6 (7-49)</td>
<td>6.2 (3-11)</td>
<td>3.6 (1.9-4.5)</td>
<td>0</td>
<td>8.4 (0-31)</td>
</tr>
<tr>
<td>Nectary</td>
<td>Open</td>
<td>10*</td>
<td>25.0 ± 3.19</td>
<td>4.70 ± 0.91</td>
<td>6.46 ± 0.88</td>
<td>2.20 ± 0.63</td>
<td>11.3 ± 1.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12-45)</td>
<td>(1-10)</td>
<td>(4.25-13.0)</td>
<td>(0-6)</td>
<td>(5-20)</td>
<td></td>
</tr>
</tbody>
</table>

*Data from Allaway and Setterfield (1972)
+With the nucleus sectioned at its midpoint, five guard cells were randomly selected in each of transverse
and longitudinal section. The guard cells represent seven modified stomata from the same pre-secretory
nectary.

2.3.4. Occlusion of the pores of modified stomata

When the nectary guard cells are plasmolysed by nectar, or by experimental
treatments, pores that were open usually remain so (see section 2.3.8). However, pores
may be closed by occlusion (Fig. 2.11b,c, 2.54, 2.58). An osmiophilic material,
distinct from the cuticle and consisting of granules about 1/30th the size of the waxy
surface globules shown in Fig. 2.52, accumulates on the cuticle, perhaps via ramifying,
osmiophilic cuticular channels (Fig. 2.54 top left, 2.55). The occluding material
collects along the ventral and outer walls (Fig. 2.54, 2.57, 3.8a) and may even stretch
across the pore as an occluding film (Fig. 2.54, 2.58) at a level below the outermost
ledges. This film is not a derivative of the original cuticle; remnants of the outer
cuticle may also contribute to pore blockage, however (Fig. 2.54, 2.58).

The identity of the osmiophilic, occluding material remains unresolved.
However, it is known that it can be found across pores of the tannin-free cultivar
Blandine (Fig. 2.57, 2.58), and it stains slightly darker than the outer cuticle and
cuticular remnants of the ledges, using the lipophilic stains Sudan black B or Nile blue,
and with neutral red.

This occluding substance may cover some later-developing modified stomata, such as those of the lower gynoecial face of the projection, before their pores ever completely formed; the cuticular channels may be evident in young guard cells (Fig. 2.25). Although occluded pores are more common in post-secretory nectaries, modified stomata may be found closed even on pre-secretory nectaries (Fig. 2.11c, 2.54, 2.61).

### 2.3.5. Stomatal development and aperture in flowers of various stages

In the present experiments, the guard cells of fixed nectary tissue were never observed to move, nor to close their pores; Collison and Martin (1975) reported the contraction of stomatal pores when the electron beam of their scanning electron microscope was directed onto unfixed nectaries of cucumber.

<table>
<thead>
<tr>
<th>Floral stage</th>
<th>No. guard cells plasmolysed per modified stoma</th>
<th>No. modified stomata</th>
<th>Difference (%) Mean ± S.E. Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-secretory</td>
<td>None</td>
<td>6</td>
<td>10.2 ± 3.6</td>
</tr>
<tr>
<td>Secretory</td>
<td>None</td>
<td>6</td>
<td>16.6 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>One</td>
<td>4</td>
<td>26.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>1</td>
<td>31.9</td>
</tr>
</tbody>
</table>

*Mean values followed by the same letter are not significantly different, using 2-tailed t-tests, with α=0.05

It was possible to measure pore widths of the same open modified stoma using light, and then, scanning electron microscopy. When neither guard cell was plasmolysed, the difference between width of nectary stomatal pores was slight (averaging 10-17%, Table 2.4; and compare Fig. 2.81a and b). In nine of twelve modified stomata, the pore width measurements were greater using scanning electron
microscopy. It is noted that the stomata, when observed with bright-field optics, are not always in absolute face view, a condition which was assured consistently in the scanning electron microscope by mechanical orientation of the stub. These data indicate that measurements of pore widths taken from nectaries processed for scanning electron microscopy, were reasonable estimates of true widths. However, where one or both guard cells of a modified stoma were plasmolysed, conditions observed less frequently in these tests, larger differences in pore width measurements between light and scanning electron microscopy resulted (Table 2.4, previous page). However, in three of the five modified stomata, the pore measurements were larger using scanning electron microscopy, suggesting that better pore orientation still played a significant role in the percentage difference. Post-fixation and dehydration of guard cells which were non-plasmolysed when fixed (e.g., pre-secretory nectaries) did not cause plasmolysis of these cells. Therefore, it cannot be ruled out that a portion of the differences observed in pore measurements of non-plasmolysed modified stomata may be the result of at least some movement of ventral walls of plasmolysed guard cells during critical-point drying.

Figures 2.61 and 2.62 show relationships between bud/flower size, length of the nectary projection, stage of development of modified stomata (Table 2.1, page 2.12), and pore sizes. Maximum average pore width is reached in a young bud stage where the corolla is just emerging from the sepal tips, and maintained until nectar secretion commences (Fig. 2.61, 2.62). Note that mean pore widths can differ by up to 5 μm (Fig. 2.61, 2.62, 2.71); the same plant is represented in Fig. 2.62 and 2.71. Mean pore aperture of the secreting glands (N) decreases, an event partially attributable to pore occlusion (Fig. 2.61) (i.e., a completely occluded modified stoma was assigned a zero pore width). The frequency of occluded pores in post-secretory flowers was greater (up to 56%; Fig. 2.62).
As the fertilized legume lengthens to approximately 30 mm, its increased girth causes the nectary projection to fracture (Fig. 2.59), possibly along sieve elements, before eventually disintegrating (Fig. 2.60). Even at this late stage, an estimated eight weeks after nectar secretion had ceased, several modified stomata remained open, among numerous occluded ones.

2.3.6. Stomatal densities

The mean density of modified stomata on whole nectary projections was almost three times greater than that of stomata on the mature abaxial leaf surface (Table 2.5, and compare Figs. 2.68b and 2.69). In the top quarter of the nectary projections, the density of modified stomata averaged over twelve times that of foliar stomata. Whereas modified stomata of the floral nectary of *V. faba* are often contiguous (Fig. 2.10-2.13, 2.82a), only very occasionally (about 0.2% frequency) were adjacent stomata detected on leaves (Fig. 2.65, 2.66). Here, a relatively large percentage [80.4 ± 3.54 (S.E.), range: 73.8 - 94.1, n = 5] of modified stomata was located on the top portion of nectary projections [this percentage is highly variable, averaging 34.7 - 82.1 in another study (see Table 3.1D, opposite page 3.6)]. The density of foliar stomata is somewhat higher in other studies of *V. faba* (Fischer 1971, Yera *et al.* 1986, Jones 1987).

| Table 2.5. Stomatal densities of *Vicia faba* tissues. Data denote numbers of stomata or modified stomata per mm² of leaf or nectary projection surface. |
|---|---|---|---|
| Tissue | n | Mean ± S.E. | Range |
| Abaxial leaf surface | 15 | 39.8 ± 1.55 | 28.0 - 49.6 |
| Nectary projection - entire | 5 | 117.0 ± 15.5 | 81.4 - 168.0 |
| - distal quarter only | 480.7 ± 61.2 | 301.6 - 635.7 |

Table 2.5. Stomatal densities of *Vicia faba* tissues. Data denote numbers of stomata or modified stomata per mm² of leaf or nectary projection surface.
2.3.7. **Daily fluctuations in aperture of foliar versus nectary stomata**

Pore widths of modified stomata were monitored (and compared with foliar stomata) to see whether there were any changes that might be indicative of regulation of nectar flow. Whereas mean apertures of foliar stomata fluctuated considerably, average pore widths of the modified stomata of nectaries of buds of stages B and D remained constant (Fig. 2.71). Although larger apertures can be induced experimentally (Fig. 2.67), mean pore width of leaf stomata immediately fixed at the plot was always smaller than that of nectary stomata; in fact, **maximum** foliar apertures approximated the **average** apertures of those of nectaries (Fig. 2.71). At each interval, many more (up to 57%) leaf stomata were closed, whereas modified stomata on the glands of these predominantly pre-secretory flowers rarely appeared closed, and then, usually by occlusion (section 2.3.4).

2.3.8. **Experimental plasmolysis of guard and epidermal cells**

The osmotic relationship of foliar stomatal guard cells and neighbouring epidermal cells have been well studied. To see whether modified stomata of nectaries display the same osmotic phenomena, nectary projections were immersed in osmotica of graded strength and values of incipient plasmolysis determined. It emerged that there are pronounced differences between foliar and nectary stomata in respect of osmotic values and in the ability of the cells to equilibrate with externally-supplied osmotica. Nectaries from buds at different stages were examined, and the effects of mannitol were compared with the effects of sucrose, in the knowledge that sucrose is the major component of the nectar that bathes the modified stomata of *V. faba* nectaries under natural conditions (see section 3.3.2).

Some noteworthy differences occurred when mannitol or sucrose was used as the plasmolyticum. With 1.0 M mannitol, plasmolysis of guard cells of modified stomata of nectaries located on excised projections did not differ with bud age and stabilized under 20% (Fig. 2.72). However, 1.0 M sucrose caused slightly greater incidence of
plasmolysis of nectary guard cells of buds of stages C and D, with plasmolysis above 20% by 6 h. Nectary epidermal cells of Stage-A buds showed identical sensitivity regardless of osmoticum species, while epidermal cells of mid-sized buds plasmolysed sooner and those of mature buds showed much higher levels of plasmolysis, with mannitol rather than with sucrose (Fig. 2.72).

Regardless of cell type, incipient plasmolysis occurred at higher sucrose concentrations in dissected nectary projections of mature buds than in mature leaf pieces (Table 2.6). The osmotic potential of nectary guard cells diminished between 10 min and 24 h, whereas in nectary epidermal cells it increased with time in the sucrose solution such that incipient plasmolysis was only reached at considerably higher concentrations, then exceeding that of nectary guard cells.

Table 2.6. Incipient plasmolysis of guard (GC) and epidermal (EC) cells in the distal quarters of dissected nectary projections of mature buds and of abaxial surfaces of mature leaves of *Vicia faba* determined after different periods of exposure to a range of sucrose solutions.

<table>
<thead>
<tr>
<th>Exposure Period</th>
<th>Nectary</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GC</td>
<td>EC</td>
</tr>
<tr>
<td>10 min</td>
<td>1.75 M</td>
<td>1.15 M</td>
</tr>
<tr>
<td>24 h</td>
<td>1.15 M</td>
<td>1.7 M</td>
</tr>
</tbody>
</table>

During these experiments, it was sometimes possible to follow over time, individual modified stomata, aligned in face view, to observe plasmolysis (which usually occurred first at the end walls of the guard cells [Fig. 2.75b]) and to determine whether loss of turgor in guard cells could allow sister cells to move towards one another and reduce the pore aperture. Indeed, such movements can occur (Fig. 2.75a-f, 2.76a,b, 2.77a,b). However, only in the case of modified stomata with relatively small apertures at the time of first exposure to concentrated sucrose solution (Fig. 2.77a,b),
can pores actually shut. Usually, pores were maintained in an open state, despite plasmolysis (Fig. 2.78), even in an isolated modified stoma (Fig. 2.80).

Neither nectary guard cells nor epidermal cells of mature buds (stage D) suffered any significant plasmolysis when exposed in the dark to CaCl$_2$ at 100 mM for up to 6 h.

2.3.9. **Influence of nectar-solute concentration on pore aperture of modified stomata**

It is well established that nectar-solute concentration can be modified by supplying excised flowers with varying quantities of sucrose (Shuel 1967, 1978), and that solute concentration in nectar is related inversely to relative humidity (Shuel 1952, Corbet *et al.* 1979). Excised flowers of *V. faba* cultured at high relative humidity on 0.5 M sucrose secreted nectar with a higher concentration of solutes than those on 0.15 M sucrose at the same high humidity (Fig. 2.73b). When flowers were cultured on 0.5 M sucrose, differences in relative-humidity treatment affected the concentration but not the total quantity of sugar in the nectar (Fig. 2.73b), indicating that originally the same nectar volumes were secreted but evaporation from the standing nectar occurred to different degrees because of the variation in relative humidity. These disparities in nectar characteristics occurred without any differences in the number of modified stomata per nectary or in the relative proportions of immature and open modified stomata (Fig. 2.73a).

Mean pore widths of nectary modified stomata decreased from the pre-secretory to the secretory stage (Fig. 2.73c). Moreover, the higher the concentration of the nectar, the narrower were the pores. A significant, negative, linear regression was determined for this relationship (Fig. 2.74a). The greatest reduction in average pore aperture occurred between the mature pre-secretory buds and those buds cultured on 0.15 M sucrose at high relative humidity (Fig. 2.73c), although this difference was not
2.27 statistically significant (two-tailed t test, P = 0.055). The gradual, non-significant reductions in mean pore width of nectar-bearing (cultured) flowers (Fig. 2.73c) matched the incremental, significant increases in nectar-solute concentration (Fig. 2.73b).

In this study, differences in the percentage of fully-occluded pores of modified stomata between culture treatments and pre-secretory buds were insignificant (Fig. 2.73a). These pores were assigned a zero aperture because they are apparently no longer available to pass nectar and because the maximum distance between ventral walls of sister guard cells could not be measured precisely. The significant correlation of Fig. 2.74a, however, occurred independently of pore occlusion (Fig. 2.74b). Instead, pore occlusion occurred randomly, even in pre-secretory buds (see Fig. 2.61), and was not significantly related to nectar-solute concentration (P = 0.144), although all cultured flowers had some occluded pores (Fig. 2.74b).

Cultured flowers had more modified stomata than mature (pre-secretory) buds fixed at the time of culture initiation (Fig. 2.73a left), probably because their 24 h period in culture provided time for division of guard mother cells and additional differentiation of other modified stomata. The percentage of immature modified stomata remained unchanged whether buds were cultured or not (Fig. 2.73a), possibly reflecting tearing and clearance of overlying cuticles of immature modified stomata of stage 3 (Fig. 2.40) brought about by the exuding nectar itself.

2.3.10. Influx of potassium ions

Guard cells of foliar stomata of *V. faba* typically have a high concentration of potassium ions relative to adjacent epidermal cells and marked fluctuations accompany stomatal regulation (Raschke 1979). Large black crystals of cobaltous sulphide could be detected in the stomata and other cells of leaves after application of sodium cobaltinitrite (Fig. 2.69), indicative of K⁺. Pores were larger and the reaction stronger
in tissues that had been incubated in KCl solution (Fig. 2.67, 2.70). However, even with prior KCl treatment of nectaries and after thorough washing, usually any black precipitate seen in association with guard cells of open modified stomata was extracellular (Fig. 2.68a,b), occasionally within the pore. Only occasionally were small crystals located inside epidermal cells of nectary projections, and guard cells always scored less than Standard #1 (i.e., less than 5% of guard-cell area) (Fischer 1972). Whereas the cobaltinitrite experiments caused foliar stomata to close (compare Fig. 2.67 and 2.70, and see Humble and Hsiao 1970, Fischer 1971), modified stomata of the nectary remained open (Fig. 2.68a,b).

2.3.11. Natural and experimental secretion of abscisic acid in floral nectar

The plant-growth regulator abscisic acid (ABA) is known to have a strong promotory effect on stomatal closing in leaves of *V. faba* (see Raschke 1987), and because nectaries of excised flowers will secrete various non-sugar compounds added to culture media (Shuel 1967), the application of exogenous ABA was deemed an attractive means to explore effects of ABA, if any, on nectary modified stomata.

Nectar collected from seven flowers aged two-three days post-anthesis, picked from different plants, yielded sugar concentrations of 40.7 - 57.5% and always contained the physiologically-active (+) isomer of ABA at concentrations ranging from 1.79 - 7.28 μM (0.091 - 0.472 μg ABA/100 mg solute). This demonstrates that ABA is not excluded from floral nectar in vivo.

Mature buds of fababean were cultured on highly concentrated solutions of exogenous ABA to determine whether ABA had any effect on nectary guard cells at much higher concentrations than those which were encountered in vivo. When buds of stage D were cultured on 0.3 M [10% (w/v)] sucrose solution containing 0 - 5 mM (±) *cis-trans*-ABA, the (+) isomer was always detected in floral nectar (Table 2.7, next page). The conditions of high relative humidity during culture ensured minimal post-
Table 2.7. Range of (+) cis-trans-ABA concentrations in nectar and plasmolysis of guard cells of modified stomata of the nectary of *V. faba* flowers cultured as mature buds at high relative humidity on 0.3 M sucrose solutions with or without (±) cis-trans ABA

<table>
<thead>
<tr>
<th>Culture period (h)</th>
<th>No. buds cultured</th>
<th>(±)-ABA concentration in medium (µM)</th>
<th>Nectar solute concentration (%)</th>
<th>(+)-ABA concentration in nectar (µM)</th>
<th>(µg/100 mg solute)</th>
<th>No. modified stomata examined guard cells plasmolysed/ guard cells examined (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>11.0 - 19.1</td>
<td>0.13 - 0.46</td>
<td>0.031 - 0.084 71 (2) 0/140 (0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>0.264</td>
<td>14.9 - 23.8</td>
<td>0.25 - 0.26</td>
<td>0.028 - 0.046 64 (2) 0/115 (0)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>1.32</td>
<td>12.3 - 16.7</td>
<td>0.48 - 0.69</td>
<td>0.085 - 0.144 64 (2) 0/115 (0)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>2.64</td>
<td>12.9 - 23.0</td>
<td>0.34 - 0.86</td>
<td>0.046 - 0.162 135 (4) 1/244 (0.41)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>100</td>
<td>26.4</td>
<td>12.4 - 16.8</td>
<td>5.40 - 6.28</td>
<td>0.985 - 1.258 66 (3) 0/117 (0)</td>
</tr>
<tr>
<td>36</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>13.8 - 22.1</td>
<td>0.29 - 0.40</td>
<td>0.054 - 0.055 107 (4) 23/203 (11.3)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>100</td>
<td>26.4</td>
<td>13.7 - 20.6</td>
<td>2.52 - 4.75</td>
<td>0.414 - 0.787 242 (7) 37/467 (7.9)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1000</td>
<td>264</td>
<td>12.1 - 17.0</td>
<td>39.0 - 110</td>
<td>7.27 - 17.4 201 (7) 10/381 (2.6)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5000</td>
<td>1320</td>
<td>8.4 - 12.9</td>
<td>584 - 1590</td>
<td>161 - 471 63 (4) 13/106 (12.3)</td>
</tr>
</tbody>
</table>
secretion evaporation of water from the nectar, as indicated by the similarity between the nectar and the medium in respect of solute concentrations. As the sucrose concentration of the medium was held constant, there was a close relationship between ABA concentrations in medium and nectar, a phenomenon reported for other, "foreign" substances in floral nectar of *Vicia* (Davis *et al.* 1988b). Levels of plasmolysis in guard cells were low, and even with a concentration of ABA in the medium as high as 5 mM, only a low percentage (statistically indifferent from the control) of the open modified stomata bore guard cells exhibiting plasmolysis after 36 h (Table 2.7; Fig. 2.81a), and modified stomata of the nectary failed to close (Fig. 2.81b, 2.82a,b).

### 2.4. DISCUSSION

The present observations on the ontogeny of modified stomata of the floral nectary of *V. faba* extend previous studies by Picklum (1954) (*Trifolium pratense* L.), Zandonella (1972) (*Portulaca grandiflora* Hook) and Teuber *et al.* (1980) (*Medicago sativa* L.). Picklum (1954) found immature stomata with angular-shaped guard cells which later developed into larger stomata similar to those of fababean. Zandonella (1972) also used light microscopy to trace stomatal development and found that guard mother cells could not be distinguished easily from the polygonal epidermal cells of nectaries of young buds. Immature stomata had relatively small, angular-shaped guard cells with many tiny vacuoles. Before the pore formed, the numerous plastids had enlarged and the walls rounded up as the guard cells, containing a large vacuole, expanded. Nectary stomata were often contiguous. No mention was made of a cuticular rupture or a material deposition, to create or obstruct the pore, respectively. In some cases, large stomata each containing three or four thin-walled guard cells were
observed. In *V. faba*, all modified stomata had two guard cells, or in the case of suspected incomplete development, just one. In *P. grandiflora*, foliar stomata are paracytic (having two subsidiary cells), while the nectary types lack subsidiary cells. A further discrimination between nectary and leaf stomata was found in the Apiaceae (Gupta *et al.* 1965). Teuber *et al.* (1980) followed the ontogeny of nectary stomata using scanning electron microscopy. Pores were revealed by eventual cuticular rupture. Judging from the lack of external evidence of the division of guard mother cells into guard cells in fababean, and the presence of a central depression when the pore has begun, I suspect that the significant surface dimpling of structures in their Figs. 13 and 14 do not represent "stomatal mother cells", but rather immature stomata classified here as stage-2 or stage-3 (i.e., their so-called stomatal mother cells have already divided into guard cells). They detected various stadia of stomatal development at a bud stage, but seldom observed immature stomata in open florets. On the contrary, in large buds of *V. faba*, and at the onset of secretion, up to seventy-three per cent of modified stomata were immature (Selection A; Fig. 3.4, 3.5). In alfalfa, no reference was made to pore occlusion. Feldhofen (1933, Fig. 127) illustrated the tearing of the overlying cuticle of a stoma on a *Crataegus* nectary.

During the stages of wall separation in the immature modified stomata of the floral nectary of *V. faba*, the volume fraction of cytoplasm increases gradually but that of the vacuome does so dramatically, probably driving the final rupture of internal and external cuticles allowing pore formation. The guard cells of immature stage-3 and of open modified stomata are cytologically identical, indicating that cuticular rupture is the ultimate step in stomatal pore formation. Exudation of nectar may also aid rupture, and probably assists in clearance of remnants of cuticular coverings. That a chemical means (e.g., a cutinase) is responsible for such cuticular disintegration seems unlikely, because the new cuticle, which lines the pore and substomatal space completely, would
also be vulnerable to attack.

In several respects, the modified stomata of the floral nectary are very similar to the foliar stomata of *V. faba* and other Fabaceae studied. In both it is common to find various developmental stages of stomata within a very local area (Lurie 1977, Galatis *et al.* 1982); both lack subsidiary cells (Pallas and Mollenhauer 1972a, Galatis *et al.* 1982, Palevitz and Hodge 1984), have substomatal spaces, and pore development begins at the inner paradermal walls, while the external ones are still intact (Carr *et al.* 1980, Galatis and Mitrakos 1980), with wall splitting occurring along an electronlucent band (Singh and Srivastava 1973, Galatis and Mitrakos 1980) that culminates in a ruptured, overlying cuticle (Pallas and Mollenhauer 1972a). Moreover, the fate of plasmodesmata reported here for fababean nectaries appears identical in guard cells of legume leaves (Singh and Srivastava 1973, Willmer and Sexton 1979, Galatis and Mitrakos 1980, Galatis *et al.* 1982), including *V. faba* (Wille and Lucas 1984), although their frequency in pit fields (30/µm²) is higher than in fababean leaves (15-20/µm², Pallas and Mollenhauer 1972a). In addition, plasmodesmata in sunflower are evident in an immature stoma of the nectary (Sammataro *et al.* 1985), but Sanchez (1977) did not detect them in mature guard cells of leaves.

There are some noteworthy differences between nectary and leaf stomata. The latter are normally solitary and uniform in distribution. Only occasionally do they occur in pairs, and then they occupy various orientations to one another, like the modified stomata of the nectary. These stomata of the floral gland occur at high densities, especially at the projection tips, the major site of initial nectar escape.

Although similar in basic ultrastructural appearance to those of the leaf guard cells (Allaway and Setterfield 1972, Pallas and Mollenhauer 1972a), plastids of guard cells of open modified stomata of nectaries differ in several respects. They are smaller,
have only 1/15th the number of grana per plastid with an average of only 2.5 thylakoids per granum, a 60% reduction in number and 35% decrease in size of plastoglobuli, considerably larger quantities of starch and possess a conspicuous osmiophilic substance. Also, the ratio of mitochondria/plastids in nectary guard cells of open modified stomata is double that in leaves (Allaway and Setterfield 1972, Pearson and Milthorpe 1974).

In several physiological respects, stomata of leaves and nectaries on intact plants behave differently. Throughout the day, over half of foliar stomata could be found closed, whereas nectary modified stomata remained open and average pore width remained constant, regardless of changes in ambient temperature and photoperiod. Even pores on exposed nectary projections did not shut when kept in darkness (Fig. 2.13, and for *Medicago* see Teuber *et al.* 1980), the usual state experienced by these relatively-protected nectaries in situ, wherein they are closely covered by three floral whorls (see Fig. 2.1). On the other hand, foliar stomata are relatively exposed and adapted to "sensing" the environment, and their fluctuations and low mean apertures appear related to the cool (Stalfelt 1962), very windy, overcast and drizzling conditions which prevailed during the experiment, and are in accordance with data obtained from a similarly-affected intact potted plant of *V. faba* (Stalfelt 1963; Fig. 2). The lack of "classical" stomatal behaviour of foliar stomata (Fig. 2.71) may be, in part, attributable to the relatively old leaves (i.e., at nodes bearing the sampled racemes) examined.

The osmotic potential of guard cells and epidermal cells on floral nectary tips of *V. faba* differed with bud age and length of exposure to osmoticum. Because guard cells mature sooner than epidermal cells on the top quarter of projections and because pre-secretory nectary cells of *V. faba* normally accumulate sugar and starch during the course of development, it is not unexpected that variation in guard-cell plasmolysis
between the three different bud ages was relatively low compared to that of epidermal cells. There is a possibility that plasmolysis was reversible in epidermal cells of mature buds exposed to sucrose for relatively long periods, because the percent plasmolysis diminished, and osmotic potential increased, with time. Probably sucrose of the plasmolyticum was gradually accumulating in epidermal cells by cell-to-cell transport from below and/or was being directly absorbed. In guard cells, a small number of experiments failed to demonstrate recovery from plasmolysis when osmotic stress was eventually reduced (e.g., Fig. 2.76).

In *V. faba*, guard cells of nectaries have higher osmotic potentials than those of leaves. Although both types of cell contain similar numbers of plastids, which remain constant throughout development (Lurie 1977; present study), nectary plastids contain more starch. Zandonella (1967c) also reported this discrepancy in starch content as a major difference between nectary and foliar stomata of *Phytolacca esculenta* and *Silene vulgaris*. Furthermore, because the nectary guard-cell ratio of mitochondria/plastids is about twice that in leaves, it seems likely that precursors or products of starch synthesis or metabolism, respectively, may be responsible for the high osmotic potential of nectary guard cells. Indeed, the driving force for the increase in guard cell turgor associated with pore development may involve precursors of starch production; the quantity of plastid starch increases dramatically during ontogeny of modified stomata. Interestingly, this increase in guard-cell starch content of nectaries occurs while they are enveloped by floral parts (i.e., under low light intensities), as it does in foliar stomata of non-photosynthetic, etiolated plants of *V. faba* (Lurie 1977).

Mean pore width of nectary modified stomata decreased in two phases, that is, with both the onset of secretion and with exposure of modified stomata to increasing nectar-solute concentrations over time. At least up to nectar-solute concentrations of
50%, the greatest rate of decrease in mean pore aperture occurs at the start of secretion, upon wetting. Zandonella (1967c) proposed that guard-cell movements on the floral nectary of *Phytolacca* were the result of hydration of epidermal cells adjacent to the stomatal apparatus. An as yet unexplored possibility is that the pH of *V. faba* nectar (ranging from 5.4-6.2, using Merck pH-indicator strips) is sufficiently high to cause a decrease in the elastic modulus existing in nectary guard-cell walls before secretion; Jinno and Kuraishi (1982), Bittsnich *et al.* (1987) and Kondo and Maruta (1987) have recorded increases in foliar stomatal pores of epidermal peels of *V. faba* as pH of the floating medium was decreased, for example, from 6.5 to 3.

The second, less-rapid phase of the closing motions of modified stomata which occurs when nectary modified stomata of *V. faba* are subjected to a gradually-increasing osmoticum concentration (e.g., standing nectar exposed to decreasing relative humidity during the day), appears related to a decrease in guard-cell turgor. In the nectary, is it possible that guard-cell starch undergoes some degradation that increases turgor, to counteract closing movements? The starch in guard-cell plastids of both leaf and nectary stains pinkish-red with IKI, indicative of amylopectin, or "transitory" starch (Küster 1956). It is well established that the malate and sugars often produced from stored starch act as counter ions to potassium influx and as another source of osmoticum, respectively, during stomatal opening of fababean leaves (Allaway 1973, Tallman and Zeiger 1988, and references therein). In experiments causing guard-cell plasmolysis of the modified stomata of the nectary, the plastids in the retracted protoplasm often still contain starch, however, suggesting that starch degradation is not rapid enough during acute exposure to high osmoticum, to prevent plasmolysis.

Zandonella (1972, p. 186) found that nectary guard cells exhibited some, but limited, flexibility in wall movement, so were not "stomates rigides". The results with
V. faba confirm his findings (1967c, 1972) with Silene vulgaris, and the earlier reports of Daumann (1930a,c, 1932a, 1974), Feldhofen (1933, Fig. 14, which appears to show plasmolysis), and Frey-Wyssling and Häusermann (1960). Examination of individual modified stomata of the fababean nectary indicated that most pores do not close shut, even upon reduction of turgor pressure leading to plasmolysis. However, relatively narrow pores may be completely shut by guard-cell movements, without plasmolysis. Using scanning electron microscopy, only rarely were modified stomata of secreting glands found to be completely closed (other than by occlusion), and usually such modified stomata were crowded on the projection tip by other modified stomata, suggesting that only a relatively narrow pore had originally been attained. In Phytolacca spp., however, Zandonella (1967c) found that most stomatal pores of the floral nectary closed before plasmolysis was evident. Like this form of nectary modified stomata, guard cells of leaves only exhibited plasmolysis after the stomatal pore disappeared (Fig. 2.79); during these movements leading to pore closure, the guard cell walls intimately shadowed the plasma membrane, such that withdrawal of the latter could only be discerned when the walls could move no further. At this time, accumulations of wall material are noticeable at the end walls of leaf (but not nectary) stomata (arrows in Fig. 2.79). It is possible that these protrusions into epidermal cells were mistaken by Shah and Gopal (1969, Fig. 24) for newly-created cells, leading to their "budding theory" (Fig. 21-28) to explain ontogeny of adjacent stomata on leaves of Lathyrus sativa. In the few instances where multiple stomata have been detected on leaves of fababean, I have found no evidence to support their claim that division of a guard cell can give rise to an eventual, adjacent stoma.

In leaves, stomata of V. faba rely on transfer of potassium ions across the guard-cell plasma membrane, to cause turgor changes associated with initial opening movements of stomata (e.g., Fischer 1971, Outlaw 1983, Tallman and Zeiger 1988).
However, once the modified stomata of nectaries open ontogenetically, the pores rarely close entirely, by guard-cell movements, and hence the absence of strong $K^+$-localization within their guard-cells and epidermal cells perhaps is not surprising. Perhaps the $K^+$-pumps present on the plasma membrane of guard cells of leaves are inoperative, or lacking entirely, in modified stomata of the nectary. The fact that potassium ions occur at millimolar levels in nectar (Nicolson and Worswick 1990) exuding from other glands possessing apparently inactive surface stomata (e.g., *Echium, Eucalyptus*; personal observation) indicates that a ready supply of these ions would be available to allow guard-cell movements caused by turgor changes, and hence a shortage of $K^+$ cannot explain the inoperability or lack of recovery from guard-cell plasmolysis of these modified stomata.

$Ca^{+2}$ at 5 mM is known to have inhibitory effects on pore aperture in foliar stomata of *V. faba* (Fischer and Hsiao 1968, Pallaghy 1970, Fischer 1972, Schroeder and Hagiwara 1989), similar effects were not detected in nectary projection tips, where the guard-cell protoplasts of modified stomata failed to respond to exogenous $Ca^{+2}$. This result demonstrates that the abundant crystals of (presumably) calcium oxalate in the nectary disk of fababean (Davis *et al.* 1988a; Fig. 2.5), at the point where the phloem supply branches into the nectary projection, do not serve the function of preventing exposure to calcium ions of modified stomata on the projection. The pores remain open for nectar exit, irrespective of whether they are exposed to $Ca^{+2}$. It would be of interest to determine what the levels of $Ca^{+2}$ in the floral exudate are.

Stomata on detached leaves or epidermal peels of *V. faba* close when exposed to $(\pm)$ ABA at concentrations less than 10 $\mu$M, by uptake or when floated, respectively (e.g. Horton 1971, Raschke *et al.* 1975, Hornberg and Weiler 1984, Kondo and Maruta 1987). Despite the physiologically-active $(+)$cis-trans and relatively inactive $(−)$cis-trans isomers of ABA naturally occurring in *V. faba* (Everat-Bourbouloux 1987) and
both being present in the media prepared for bud-culture experiments, modified stomata bathed in nectar containing up to 1.5 mM (or 4.5 µg/mg solute) concentrations of the \((+\text{cis-trans-ABA})\) not only remained open, but their guard-cell cytoplasms did not withdraw from the relatively rigid cell walls. The nectar pH is within the range in which ABA induced closure of stomata in leaves of another species (Hartung 1983). This lowered sensitivity in guard cells of the modified stomata of nectaries to ABA suggests that the high-affinity receptor sites for ABA on the plasma membrane of leaf guard cells (Hornberg and Weiler 1984) are either absent or significantly fewer, or modified stomata are geared for a much higher ABA-threshold before a response occurs, or else that in modified stomata of nectaries there exists some disruption to the system that is active in foliar stomata. Detection of ABA in nectar eventually secreted by buds excised when mature and then cultured on ABA-lacking medium indicates that the sesquiterpene is present in flowers before nectar secretion commences (and see Diethelm et al. 1988), and perhaps that ABA can be synthesized in flowers. However, the higher ABA concentrations in nectar collected from flowers that had been secreting for at least two days in the field than from in vitro nectar suggests a supplemental import of ABA into flowers during the period of nectar secretion, possibly reaching the nectary predominantly by the vascular supply of phloem alone (Davis et al. 1988a). Support for the present experimental evidence with \(V.\ faba\) comes from the recent study of Lipp (1990), who traced the ABA in honey back to various sources, including nectar. He found that only the physiologically active \((+\text{cis-trans isomer of ABA})\) could be detected in the exudate from field-gathered flowers of \(Lamium\ album\) and \(Brassica\ napus\). It is noteworthy that these two species also possess stomata on their nectary surfaces (Gulyás 1967, Polowick and Sawhney 1986), those of the latter, at least, appearing inoperative (Davis et al. 1986). In addition, these results agree with the scanning electron microscopic study of Teuber et al. (1980), wherein an absence of
nectary stomatal pores with widths below 1 µm was determined from racemes of *Medicago* cultured in solutions of compounds (other than ABA) that closed the pores of foliar stomata. However, details of whether the flowers were of a secretory stage, whether these inhibitors of stomatal metabolism reached the nectar, and whether the guard-cell protoplasm responded by plasmolysis, were not disclosed. Furthermore, Jung and Lütge (1980) demonstrated that ABA had no effect on $K^+$ transport, and only slight effects on sugar secretion, in nectaries of three species (including the floral gland of *Acer platanoides*, which also bears stomata; Haragsim 1977).

Important differences in guard-cell movement between nectary and foliar stomata of fababean are directly related to structure of the guard-cell walls. Whereas both types contain walls composed of microfibrils radiating from the pore region, walls of modified stomata are relatively uniform in thickness. From two electron micrographs of essentially-mature leaf guard cells in approximately equatorial transverse section (Pallas and Mollenhauer 1972a, Fig. 1; Allaway and Milthorpe 1976, Fig. 1b), the thickest regions along the inner-ventral and outer-ventral walls (4.4 µm and 2.5 µm, on average) are about four to five times those in nectary guard cells, whereas dorsal walls were only 45% thicker. The birefringence pattern from the relatively thin-walled modified stomata, when mature, is approximated by immature leaf stomata. It is interesting to note that leaf guard cells incorporated less $^{14}$C-glucose into their walls when kept in the dark than in the light (Takeuchi and Kondo 1988). Only low light intensities are naturally experienced by modified stomata of the nectary, until floral parts absciss long after secretion has ceased.

In modified stomata of the nectary, the cuticle covering the outer wall of the guard cells is about half the thickness of that over adjacent epidermal cells (Fig. 2.44, 2.46, 2.47, 2.53), although the outer walls of the guard cells are over twice the width of the primary walls of epidermal cells.
One of the main issues addressed in the present investigation was the possibility that nectary stomata regulate the flow of nectar. The structural and physiological points arising from the preceding discussion will now be incorporated into a consideration of this aspect of their biology.

The first point is that pore sizes do not correlate with the onset and cessation of secretion. Maximum pore aperture is reached in a bud stage where the corolla is just emerging from the calyx, about four days before nectar secretion commences. This mean width is maintained throughout the day and night until then. At the onset of, and during nectar secretion, and even after cessation of secretion, it is common to find that many modified stomata are still immature. Despite the occlusion of up to half of the pores in nectaries which have ceased to secrete nectar, many remaining modified stomata survive in an at least partially open state. Verification of this point for members of the Phaseoleae has been provided by Waddle and Lersten (1973). Deposition of occluding material occurs unpredictably, sometimes even before the pores are fully open, and some pores may be completely blocked before nectar secretion even begins. There is therefore no evidence from development of modified stomata for regulation of secretory activity.

The physiological and structural evidence is also against a regulatory role. When their guard cells are plasmolysed, the modified stomata ordinarily remain open, indicating that guard cells cannot move sufficiently to close the pores. Possible reasons for this failure in movement include:

a) The modified stomata are often adjacent, sometimes practically surrounded by others, and develop with various pore orientations, such that their guard cells may become shifted or twisted as a result of opposing turgor forces during expansion of maturing, non-sister guard cells. Buckling may even occur, and then it seems even less apparent how the pores could be physically shut by guard-cell movements. Within a
modified stoma, unequal cell division of the guard mother cell may also cause guard-cell misalignment.

b) The cuticle surrounds all free surfaces of each guard cell, and lines the entire continuum of stomatal pore-substomatal space-intercellular spaces, including partial surfaces of certain epidermal and subepidermal cells. Substomatal spaces are often incomplete, such that subepidermal cells may remain attached to guard cells from below, or sometimes these cells even extend within the pore, often fused by cuticle, thereby restricting guard-cell movement. Even when modified stomata are not adjacent, the epidermal cells appear incapable of reflexion, to accommodate a closing guard-cell movement. Fahn (1967) earlier made reference to such a cuticle.

c) The variably-sized, external wall ridges of microfibrils and cuticle may have a significant mechanical role in maintaining the open state of the pore. These ridges become more obvious after cell expansion, and because they are on the external surface of the wall, it is difficult to determine how they originate. The microfibril component probably represents an original layer of the wall of the guard mother cell that separated into patches when the radially-oriented microfibrils of the expanding guard cells were being deposited. As in the ledges which become obvious on the outer paradermal walls when the external cuticle ruptures, the microfibrils in these variable ridges on the outer and inner walls are oriented circumferentially. What may be similar ridges, covered by cuticle, are evident on the guard cell walls of foliar stomata of *V. faba* (Pallas and Mollenhauer 1972a), giving a "wrinkled" appearance externally (Pallas and Mollenhauer 1972b). However, because of the consistently thinner walls of the guard cells in fababean nectaries, these external ridges and the ledge would seem likely to have a greater restrictive influence than in the thicker guard cell walls of leaves. Zandonella (1967c) could not account for the failure of guard-cell movements that would allow pore closure on the floral nectaries of *Silene vulgaris*, but suggested that
the guard-cell walls were instrumental in that failure. Later (1972, p. 186) he proposed that guard cells of nectaries could probably move as much as their foliar counterparts, but because of the larger apertures on the glands, the pores could not be fully closed. Whether circumferential ridges of external microfibrils occur on nectary and leaf stomata of this species, is unknown. Similar ridges are discernible on open stomata of the sunflower nectary (Sammataro et al. 1985, Fig. 4A) and on an immature one of leaves (Sanchez 1977, Fig. 10). They are also evident on nectary guard cells of Floerkea prosperpinacoides and Limnanthes douglasii (Link 1992, Fig. 1D, F), Glycine max (Erickson and Garment 1979, Fig. 4), Kigelia pinnata (Subramanian and Inamdar 1985, Fig. 2B, C), Medicago sativa (Teuber et al. 1980, Fig. 15), Phaseolus vulgaris (Webster et al. 1982, Fig. 7, 8), Tecomaria capensis (Subramanian and Inamdar 1989, Fig. 2E), and Tropaeolum majus (Rachmilevitz and Fahn 1975, Fig. 1C, D), but the present study is the first in which they have been examined by transmission electron microscopy and implicated in conferring some rigidity to the guard-cell walls.

d) An unidentified osmiophilic substance located among the radially-oriented microfibrils, often the innermost ones, in some guard cell walls, may also be significant. Localization of a phenolic substance was demonstrated in the guard cell walls of Ophioglossum vulgatum leaves (Peterson et al. 1975). Cross-linking of phenolic materials within cell walls can regulate flexibility (see Taiz 1984). This osmiophilic material has not been reported for foliar stomata of Vicia.

e) Certain ontogenetic arrangements and orientations of modified stomata lead to fortuitous development of non-stomatal gaps between outer walls in the projection tip in about three percent of nectaries, thereby providing additional exits for exudate. In hydathodes, such gaps were called "apical openings" (Stevens 1956). Because many of
these apical openings cannot be fully contracted, even if guard cells move freely, their presence is taken as additional evidence that nectar release and flow is not finely regulated by the modified stomata of fababean nectaries. Furthermore, Daumann (1932b) showed gaps between epidermal cells of the stoma-bearing floral nectary of Jasminum.

In summary, development of the modified stomata is not closely correlated with the onset of nectar secretion, and nor is there evidence for changes in pore width indicative of a regulatory role. The evidence strongly suggests a passive role for the modified stomata and a non-regulatory pathway for nectar escape from the gland. Zandonella (1972) also concluded that the role of the stomata in nectar secretion was "entièremennt passif". The unknown signal for the commencement of nectar secretion does not coincide with a synchronous, initial opening of the modified stomata and must originate elsewhere. Phloem unloading may be the key, with the possibilities existing for nectar movement both apoplastically, directly to the cell walls and intercellular spaces, and symplastically (Davis et al. 1988a). Plasmodesmata are common between nectary cells beyond the sieve elements, providing a continuous symplastic route until cells lining intercellular, or even substomatal, spaces, are reached. Wall ingrowths present in these cells suggest the transfer of pre-nectar constituents into the apoplastic continuum, from which pre-nectar could reach the exterior even if pore occlusion occurs in some modified stomata.

Rosen (1936) stated "it is not certain that, in addition to being secretory, (the pores of the floral nectary stomata of some Rosaceae) may not serve at times for the passage of air; and it is quite conceivable that following nectar flow and after the tissue has aged, they may serve as ordinary stomata." In fababean, I have not investigated this possibility directly. During nectar secretion, at least, movement of nectary guard cells of fababean is apparently limited mechanically by connections maintained with some subepidermal cells, the cuticle and circumferential ridges of their walls, etc. If
thicker walls (to overcome the limitations of the ridges and cuticle), an enlarged substomatal space, and some physiological mechanism allowing turgor changes of the protoplasm, are requisites for nectary guard cells to move freely, and are still attainable developmentally in the same guard cells after secretion has ended, then the sequence of nectar secretion before gaseous exchange seems to allow the possibility. However, several major points argue strongly against the existence of a dual role in *V. faba*. The first is that the nectary degrades several days after secretory activity has ceased, and although it is pale green (i.e., has plastids containing chlorophyll), the nectary projection bearing the modified stomata underlies the expanding gynoecium and largely remains covered above an encroaching abscission zone, even after abscission of most floral parts. The green, growing fruit itself has stomata (Davis, pers. obs.) and is probably photosynthetic (Crookston *et al.* 1974, Andrews and Svec 1975, Atkins *et al.* 1977, Flinn *et al.* 1977, Blanke 1990). The next point is that I have not found evidence that nectary guard cells in *Vicia* can recover from plasmolysis, which is a necessary condition for guard-cell movements by turgor. Whether pore occlusion would still allow gas exchange, remains unknown. Indirect evidence that the nectary pores remain fixed, and do not regulate gas exchange, is the discovery of entrance of fungal hyphae through them, in post-secretory nectaries. Others (Rosen 1936, Mansvelt and Hattingh 1987, Wilson *et al.* 1990) have demonstrated the entry of pathogens into nectary tissue through stomatal pores on the surface. This liability to pathogenicity may explain the natural degeneration of the nectary post-secretion. Certainly, there is no evidence for a concurrent passage of nectar and air in floral nectaries of *V. faba*, and Ziegler (1987) has emphasised the consequences that liquid water has on the process of photosynthesis.

There is accumulating evidence in the literature on leaf stomata which suggests
that stomatal density and ability to respond to changing microenvironmental conditions are dependent on the conditions which were ambient during stomatal initiation and development. Higher relative humidities led to higher stomatal densities (Bakker 1991). Stomata formed on leaves in high-humidity conditions, such as in tissue-culture vessels, usually have larger apertures which appear fixed (Johanssen et al. 1992; personal observations with Helianthus annuus), guard cells with relatively thin walls (Ziv et al. 1987, Marin et al. 1988), and a lack of responsiveness of the guard-cell protoplasm to ABA (Ziv et al. 1987). These features also pertain to root stomata (Tarkowska and Wacowska 1988), which likewise form under high relative humidities. There seems to be a concurrence between the occurrence of thin walls of guard cells and the inability of the guard-cell protoplasm to respond to ABA. Perhaps the plant's "perception" of high humidity conditions leads to development of large-apertured stomata where the lack of a regulatory function is not critical, and may be necessary, to maximize gas exchange. Asynchrony of stomatal development on individual leaves in situ, and the growth of new leaves, may be important adaptations for plants to cope successfully with changes in ambient conditions which may govern development of different types of stomata.

The structural and physiological similarities, as well as high densities, between the modified stomata of the nectary and those of leaves growing in vitro are striking. By reducing the high humidity to which they are exposed during development, could nectary stomata form which are able to regulate pore apertures by being ABA-responsive? Under such conditions, with ABA being expelled in nectar, would the pores be available for nectar exit? Humidity inside developing flower buds may be high enough to encourage the development of non-regulatory stomata on many floral parts, not just nectaries. The available data, however, indicate that high humidity is not alone in dictating the lack of development of a regulatory role in nectary stomata. If
one considers the case of the adaxial and abaxial surfaces of sepals, the former being exposed to the high-moisture conditions **inside** flower buds, while the latter are exposed to lower humidities **outside** the buds, it is interesting to note that the abaxial stomata of the calycine nectaries on closed buds of *Paeonia* have been found to be non-regulatory (Zimmermann 1932, Frey-Wyssling and Häusermann 1960). Whether non-nectary stomata on the same abaxial surface are also incapable of closing, by guard-cell movements, remains to be determined.

The thicker wall regions in modified stomata of nectaries have implications for guard-cell movement, as can be seen from comparisons of individual modified stomata during the course of turgor loss and plasmolysis of their guard cells. Although the width of modified stomata, from the dorsal wall of one guard cell to that of its sister, declines with volume reduction of guard cells, pore widths also decrease due to the flattening (reduction in distance between the outer and inner walls) of guard cells, thereby causing the ventral walls to approach each other. Foliar stomata of *V. faba* behave in a similar fashion (Raschke 1979). A major adaptation in foliar stomata allowing complete closure of pores in terrestrial environments where water retention is of paramount importance, involves the thickening of inner and outer walls near the ventral wall, which overcomes any inhibition caused by the ledges and any circumferential ridges, and surrounding cuticle. These thickened walls, however, require systems that permit large differences in turgor pressure to be attained in order that stomata close rapidly, i.e., ABA response, or to cause stomatal opening, i.e., K⁺ pumps. As detailed above, both these systems appear to be lacking or inoperative, in modified stomata of the floral nectary of *V. faba*.

In fababean, closure of some modified stomata of the floral nectary occurs by occlusion of pores. Although it is not absolutely certain that Permagum™ impressions provide an accurate account of occlusion, the occluding film has been observed by
light, scanning electron and transmission electron microscopy. Apparently this phenomenon has been recognized only once before in nectaries, using scanning electron microscopy of the glands on stipes of *Pteridium aquilinum* (Power and Skog 1987). An early description of the floral nectary stomata of *Parnassia palustris* (Behrens 1879, p. 312-313) may refer to an occluding material, and the sketch of an obstructed pore of a nectary stoma of a flower of *Buxus sempervirens* (Daumann 1974) appears to be confirmed by scanning electron microscopy (Smets 1988, Fig. 7 left centre). Similarly, occlusion of a pore may be illustrated in Fig. 2 (top right) of Murrell *et al.* (1982b)'s study of the floral nectary of *Lotus corniculatus*. The strongest resemblance to the occluding film observed with *Vicia* is seen in material in the bacterial-filled pore of the floral nectary of *Crataegus* (Wilson *et al.* 1990). The dark-black substance illustrated in many of the figures of Rosen (1936) may also represent occlusion of nectary pores of Rosaceae infected by *Erwinia*. Hydathodes intergrade structurally and physiologically with extrafloral nectaries (Radtke 1926, Frey-Wyssling and Häusermann 1960, Elias and Gelband 1977, Belin-Depoux 1989); Perrin (1970) also demonstrated the lack of pore closure of hydathodes when their guard cells were plasmolysed. Occlusion has also been implicated in hydathodes of Ranunculaceae (Mortlock 1952, Stevens 1956) and illustrated in those of wheat (Maeda and Maeda 1987), and the "exudate" in pores of hydathodes of rice stomata infected with *Xanthomonas* bacteria (Fig. 5B, 6B of Mew *et al.* 1984), suggested as a possible response to the infection, appears similar to the occluding material discovered in *V. faba*. Partially- or fully-occluded stomatal pores (or immature stomata) probably account for the "partly or entirely" or "permanently closed" forms of the lower gynoecial faces of the floral nectary of *Phaseolus*, observed using scanning electron
microscopy (Webster et al. 1982). The variability in numbers of modified stomata which are closed may reflect the differences in pore width and symmetry found on an individual nectary. Some pores fail to be blocked, and occasionally, fungal hyphae have been observed to enter such pores of post-secretory nectaries.

How the occluding film forms across the pore is puzzling. Perhaps the film is stretched across some pores as the ventral walls separate developmentally. Little is known about occlusion, such as why some pores are blocked but not others. There seems to be no relation to nectar-solute concentration. Although the phenomenon is more prevalent in post-secretory glands [also see Daumann’s (1974) description for Buxus], the fact that occluded pores can be found on pre-secretory glands indicates that the material for occlusion can be deposited by individual stomata, and need not be transported in nectar, from elsewhere.

Because of the similar staining intensity, it is a possibility that the occluding material of the pores of the floral nectary of fababean originates from the osmiophilic substance of the large, starch-bound plastids. Pore occlusion has apparently not been reported for the foliar stomata of V. faba, whose plastids lack this osmiophilic material (Allaway and Setterfield 1972, Pallas and Mollenhauer 1972a,b). A similar osmiophilic material is present among the plastids of the guard cell in a secreting nectary of Trifolium pratense (Eriksson 1977). In fababean nectaries, this substance is usually manufactured within plastids before the pore is completely formed, although some occluded pores have guard cells lacking starch. In nectaries, starch may be the main precursor for production of spherosomes, some of which could be extruded, carrying osmiophilic material to the cell surface. Epidermal cells rarely have spherosomes. However, these organelles are detectable in development well before the osmiophilic substance in plastids is apparent, raising the question as to whether different spherosomes (e.g., uniform, versus distinctly osmiophilic in their centres)
have different origins and fates within nectary guard cells.

The isolation of guard-cell protoplasts from their cell walls has allowed important investigations into foliar stomatal physiology in V. faba (e.g., Schnabl et al. 1978, Weiler et al. 1982, Shimazaki et al. 1983, Schroeder et al. 1984). Despite the cuticle covering the free surfaces of the thin-walled guard cells of nectary stomata in this species, the harvesting of guard-cell protoplasts using enzymes which digest cell-wall materials, may still be possible. If a cutinase is found necessary to free the protoplasts, it would be of interest to observe the pores of nectary stomata during digestion of the cell walls, to monitor the cuticle’s contribution to rigidity of these modified stomata. Because of the high densities of modified stomata on the distal portions of nectary projections in V. faba, the use of projection tips should ensure a high-frequency harvest of guard-cell protoplasts that could permit further, more detailed investigations of the modified stomata of the nectary.

There is little evidence to suggest that modified stomata are themselves important contributors to nectar production in V. faba. Supporting evidence comes from the facts that a) starch can still be found in plastids of guard cells, although it has disappeared from all other nectary cells, at the cessation of secretion; b) although epidermal and subepidermal cells of secreting nectary projections are transfer cells (Pate and Gunning 1972, Davis et al. 1988a), wall ingrowths directed toward adjacent guard-cell walls from these cells are rare, as well as being totally absent within guard cells; c) after prolonged exposure to sucrose solution mimicking nectar, guard cells have lower osmotic potentials than epidermal ones, and d) plasmodesmata have been occluded before the modified stomata mature, so that the symplastic pathway (if important in nectar assimilation) to nectary guard cells is apparently no longer functional. Instead, the major functions of modified stomata in V. faba seem to be
those of permitting nectar escape from the gland and perhaps enhancing reabsorption of uncollected nectar, and for these purposes they appear to be well suited to maintain open pores, even under conditions that lead to plasmolysis.

2.5. SUMMARY

The floral nectary of *Vicia faba* L. consists of a disk which surrounds the gynoecium. As the flower bud matures, a tapered projection arises by cell divisions and expansion at a position opposite the free stamen. Modified stomata develop from pentagonal guard mother cells on this projection, and are often contiguous, but because development of modified stomata is asynchronous, many stages can occur in a local area. The density of modified stomata on the nectary projection is especially high on the projection tips, where nectar initially escapes. Plasmodesmata, which connect guard mother cells and guard cells of developing modified stomata with adjacent cell types, become covered by cell wall material on the guard-cell side in mature modified stomata. The numerous small vacuoles of guard mother cells and young guard cells are replaced by a single large one as the guard cells expand. Pore development occurs concurrent with this expansion as the ventral walls separate at a designated location leading to rupture of the inner, and then outer, cuticles, to reveal the pores of mature modified stomata.

The stomata are considered to be "modified" because the guard cells of mature stomata only rarely can contract sufficiently to close their pores. Several structural features may contribute to this inability to close the pores by guard-cell movements: incomplete substomatal spaces, in which contact between guard and subepidermal cells is maintained; a cuticle which lines all free surfaces of guard cells; ridges of circumferentially-arranged microfibrils along the outer (and sometimes inner) walls; an unidentified osmiophilic wall material. Pores of modified stomata reach their maximum pore aperture dimensions a few days before the onset of secretion. Also,
open, and even immature, modified stomata occur on old post-secretory glands undergoing degradation. The lack of close correlation between development of modified stomata and secretion suggest that nectar flow is not finely regulated by these structures.

Apical openings, which develop fortuitously between modified stomata on about three percent of nectary projections, would appear to provide additional exits for nectar.

A comparison of physiological responses between the modified stomata of the floral nectary and the stomata of leaves of has revealed several significant differences. Average pore widths of the nectary stomata on large buds remained constant throughout the day and night, contrary to those of leaves. Mean pore width of the modified stomata of nectaries was directly related to nectar-solute concentration, but decrease in mean pore width was proportionately greatest with the onset of nectar secretion, upon hydration of guard-cell walls. Experiments with plasmolytica demonstrated that sister guard cells of the nectary only occasionally closed their pores by movements, even though they were plasmolysed, whereas guard cells of leaves were found to be plasmolysed only after their pores had shut. Recovery of guard cells of nectaries from plasmolysis was not observed. Nectary stomata neither underwent plasmolysis nor closed when exposed to concentrations of Ca\(^{2+}\) that induce stomatal closure in leaves. Abscisic acid (ABA) was always detected in nectar of flowers growing in vivo. Furthermore, the results of experiments involving K\(^{+}\)-localization and the secretion of large quantities of ABA in nectar suggested that guard cells of the modified stomata either lack K\(^{+}\)-pumps and ABA-receptor sites, or that these systems are inoperational, in nectaries. All physiological information gained in this study is consistent with the conclusion based on structural aspects that the modified stomata do not have a regulatory role in nectar secretion by flowers of V. faba.

Although there is no evidence for regulation of pore aperture, pores may become
occluded. In these cases, an osmiophilic material, released through channels in the cuticle of outer and ventral walls, is deposited across the pore as a film. Although occluded pores can occur on very young buds, they are more common on secretory and post-secretory nectaries.
Figs. 2.1-2.9. Flower and nectary morphology of *Vicia faba*

**Fig. 2.1.** Transverse section (T.S.), stained with toluidine blue O, showing location of nectary projection (Pj) between gynoecium (G) and staminal column (SC). Note two gaps (arrowheads) between latter and free stamen (FS). Wing (W), keel (K), banner (B) petals, calyx (Ca). The vascular bundles $X_1$, $X_2$, and $X_3$ are also shown in Fig. 2.5. Bar = 250 µm

**Fig. 2.2.** Base of flower dissected of corolla and upper part of calyx (Ca), to reveal androecium with gaps (arrowheads) between free stamen (FS) and staminal column (SC). Pedicel (Pd). Approx. x 15

**Fig. 2.3.** The nectary projection (Pj) arises from the disk (D). The first nectar (arrow) is situated on attached remainder of SC, opposite projection tip when SC is inserted to its original position. Asterisk corresponds to that of Fig. 2.1, 2.2. Approx. x 28

**Fig. 2.4.** Tip of projection (Pj) showing droplets of nectar (arrowheads) escaping into immersion oil. Bar = 100 µm

**Fig. 2.5.** Birefringence (white areas) in projection (Pj) (staminal view) and part of receptacle (R) in fresh material dissected from bud (gynoecial length 9.5 mm). Phloem sieve tubes (arrows) enter projection base and sometimes ramify while extending toward tip. The designations ($X_1$, $X_2$, $X_3$) of vascular traces present correspond to those of Fig. 2.1. Several modified stomata of projection tip show Maltese-cross birefringence (arrowheads), and crystals of calcium oxalate at base (double arrowheads) are also birefringent. Bar = 100 µm

**Fig. 2.6.** Portion of projection tip of fresh material stained with neutral red, showing modified stomata, often adjacent and in various orientations. Each modified stoma consists of two guard cells (GC) surrounding pore (P). Several modified stomata have formed an arch, thereby providing a view through nectary at aligned stomatal pores (*). Bar = 20 µm

**Fig. 2.7.** Cell walls (arrowheads) of modified stomata of different developmental stages on projection tip of fresh material, stained with Calcofluor White M2R New. Pore (P). Bar = 30 µm

**Fig. 2.8.** Facial view of developing modified stoma, demonstrating birefringent regions (arrowheads) in Maltese-cross arrangement. Bar = 10 µm

**Fig. 2.9.** Isolated guard cells near middle of gynoecial face of projection. Bar = 25 µm
Figs. 2.10-2.14. Light and sequential scanning electron microscopy of development of nectary projection and modified stomata of *Vicia faba*

**Fig. 2.10a.** Resin replica of base of young bud (gynoecial length 3.5 mm) representing the initiating nectary projection arising from disk (D) between gynoecium (G) and remainder of staminal column (SC). Note developing modified stomata, often adjacent, on projection surface. **Bar = 100 µm**

**b.** Region of projection tip outlined in **a,** showing modified stomata at various stages of development, including fully mature stomata with pore (P) and ledges (arrow), and four immature ones with central depressions (arrowheads). It is interpreted that epidermal cells *E*₁ and *E*₂ had divided obliquely to yield (*₁ + 1) and (*₂ + 2), respectively. Subsequent divisions separated *₁ from 1, and *₂ from 2. Pentagonal cells *₁ and *₂, like 1 and 2, could also be precursors of modified stomata. **Bar = 50 µm**

**Fig. 2.11a.** Replica (gynoecial length 4.6 mm) showing an elongating nectary projection with several modified stomata (arrowheads) at its tip. **Bar = 100 µm**

**b.** Boxed area of **a,** showing modified stomata of various stages. Note cuticle (white arrowhead) rupturing to reveal antechamber of maturing stomata, and pores (P) of mature ones. Ledges (arrows) and developing pore (black arrowhead) evident. **Bar = 50 µm**

**c.** Sequential replica prepared 140 min later, showing apparent occlusion of pores (clear arrows) and tearing of surface material (double arrowheads), and further opening of pore (P). **Bar = 50 µm**

**Fig. 2.12.** Scanning electron micrograph of gynoecial face of projection (Pj) of critical-point-dried nectary from secreting flower (gynoecial length 18.5 mm) showing many mature modified stomata at tip, and several immature ones further down (arrowheads). Modified stomata are absent on disk (D). **Bar = 250 µm**

**Fig. 2.13a.** Replica of tip of gynoecial face of nectary projection from advanced bud (gynoecial length 17.0 mm). **Bar = 100 µm**

**b-d.** Successive replicas of the modified stomata outlined in **a.** Replicas of **c** and **d** prepared 4.5 and 14.5 h after that of **b.** In **b,** arrowheads denote overlying cuticles which have torn, in **c,** to reveal newly-formed pores (P). The resultant ledges (black arrows) are evident. White arrows of **b** indicate artefactual damage to this replica, apparently caused by forceps when peeling away impression. **Bars = 15 µm**

**e.** Cytoplasmic fluorescence of guard (*) and epidermal cells of **d,** using fluorescein diacetate. Absence of fluorescence (star) corresponds to damaged area of **d.** **Bar = 15 µm**

**Fig. 2.14a.** Light micrograph of unstained, obliquely-oriented maturing modified stoma focused on external, tearing cuticle (arrowhead) and newly-formed ledges (arrows). **Bar = 20 µm**

**b.** Focus on mid-region of same stoma to show that the inner aperture of the pore is already open.
Figs. 2.15-2.17. Immature stages of modified stomata of floral nectary of *Vicia faba*

**Fig. 2.15.** Replica of staminal face immediately below tip of projection of advanced bud (gynoecial length 16.0 mm), showing several central, immature modified stomata surrounded by mature ones at tip and edges. Guard mother cells (*) and other modified stomata with tearing external cuticles (arrowheads) and ledges (arrows) are evident. Bar = 50 µm

**Fig. 2.16.** Longitudinal section (L.S.) of guard mother cell contiguous with two modified stomata (immature stage-1) amidst epidermal cells (E) located on extreme tip of developing projection similar to that of Fig. 2.10a. Location of predicted cell division of guard mother cell is shown (*). Vacuoles (V) are numerous, small and usually free of the dark contents of large ones of epidermal cells. Sites where plasmodesmata are visible at higher magnification are indicated by arrows, and elongate plastids (Pl) contain little starch. Nucleus (N). Bar = 5 µm

**Fig. 2.17.** Immature modified stomata near those of Fig. 2.16, with guard cells of very different shape. Starch grains in plastids (Pl). Bar = 5 µm
**Figs. 2.18-2.25. Development of modified stomata of floral nectary of *Vicia faba***

**Fig. 2.18.** L.S. stained with Toluidine Blue O (TBO), of pentagonal guard mother cell at prophase, located adjacent to obliquely-sectioned, mature modified stomata of two guard cells (GC) and pore (P) on extreme tip of projection of young bud. Bar = 20 µm

**Fig. 2.19a.** L.S. showing two contiguous, pentagonal guard mother cells (GMC) and immature modified stoma composed of two guard cells (GC), separated by new ventral wall (arrow). Bar = 10 µm

b. Section adjacent to a, stained with aniline blue. Note positive reaction in newly-formed ventral wall (arrow) and in presumed pit fields (arrowheads) along walls of both guard mother cells and guard cells.

**Fig. 2.20.** L.S. of two adjacent, immature modified stomata on extreme tip of projection of small bud, showing new ventral walls with pore thickenings (arrows). Bar = 10 µm

**Fig. 2.21.** T.S. of pit field between GC (left) of immature modified stomata similar to that of Fig. 2.19, and epidermal cell (right). Numerous plasmodesmata (Ps), in L.S. in thin wall (W) portions separated by middle lamella (ML). An osmiophilic deposition (*) and lining (arrows) are evident along intercellular space (IS). Vesicles, microbody (MB), vacuole (V). Bar = 1 µm

**Fig. 2.22.** T.S. of end walls between non-sister guard cells of mature modified stoma, illustrating a new wall layer with different microfibril orientation, apparently occluding a plasmodesma (arrow). Bar = 0.2 µm

**Fig. 2.23.** T.S. through wall junction of plasmolyzed guard cell (left) of mature modified stoma, and subepidermal cell (right), of secreting nectary. Wall material (W) occludes two plasmodesmata (Ps). This glancing section of the edge of the substomatal space reveals cuticular lamellae in osmiophilic material (arrows) lining space. Dictyosome (D), mitochondria (M) and membranous reticulum (mR) are present in subepidermal cell. Bar = 0.5 µm

**Fig. 2.24.** Glancing section of wall (W) between guard and subepidermal cell, revealing plasmodesmata (Ps) in T.S. in pit field. Bar = 0.1 µm

**Fig. 2.25.** Oblique section near end of immature modified stoma, showing upper half of thin ventral walls separating sister guard cells. Dictyosomes (D) with numerous vesicles (Dv), often in close proximity to plasma membranes (double arrowheads), and coated vesicles (Cv), prevalent in cortical cytoplasms. Rough endoplasmic reticulum (rER), spherosomes (L), portion of small vacuole (V), mitochondrion (M), electron-lucent material in the intervening wall (black arrows), microtubules (arrows) along outer walls, cuticle (*) with osmiophilic channels (white arrows). Bar = 0.5 µm
Fig. 2.26. Changes in area (µm$^2$) of nuclear (N), cytoplasmic (C) and vacuolar (V) components in cell-section profiles, and in number of organelles per µm$^2$ of anucleate cytoplasm, throughout development of modified stomata of pre-secretory floral nectaries of \textit{Vicia faba}. All cells have been sectioned longitudinally, and examined at the nuclear midpoint. Organelles represented are mitochondria (light circles), plastids (squares), spherosomes (dark circles) and microbodies (triangles), and are shown as means ± standard errors. Number of guard mother cells and guard cells analysed were: GMC (7), Immature-1 (6), Immature-2 (4), Immature-3 (3), Open (5)
Figs. 2.27-2.30. Organelles of guard cells of modified stomata of various developmental stages on the floral nectary of *Vicia faba*. Figs. 2.27, 2.28, and 2.30 are from pre-secretory glands.

**Fig. 2.27.** Elongate plastid of guard cell of modified stoma of immature stage-1, showing plastoglobuli (Pg), thylakoids (Ty) forming distinct grana (Gr), and a peripheral reticulum (arrows). Bar = 0.5 µm

**Fig. 2.28.** Mitochondria (M), smooth (sER) and rough (rER) endoplasmic reticulum, spherosomes (L), dictyosomes (D) and vacuoles (V) of guard cell of immature modified stoma. The plastids contain a starch grain (S), plastoglobuli (Pg), thylakoids (Ty) and peripheral reticulum (arrows). Bar = 1 µm

**Fig. 2.29.** Two circular plastids with plastoglobuli (Pg), thylakoids (Ty), several starch grains (S) and peripheral reticulum in guard cell of maturing modified stoma on secreting nectary. Nucleus (N). Bar = 1 µm

**Fig. 2.30.** Starch (S)-laden plastid of guard cell of open modified stoma. Note the reduction in peripheral reticulum (arrows) in the plastid. Cell wall (W). Bar = 1 µm
Figs. 2.31, 2.32. Features of developing pores of immature modified stomata from a pre-secretory floral nectary of *Vicia faba*

**Fig. 2.31.** Outer-wall (OW) region of modified stoma of immature stage-2, in transverse section, near the end of the developing pore (star), showing detached original, outer cuticle (arrowhead) composed of thin epicuticle (open arrows) covering thick endocuticle (thick black arrows), stretching between the outer ledges. Newly-formed cuticle (smaller black arrows) is laid down over the newly-exposed edges of outer/ventral walls as the pore widens and deepens. The cuticle-covered ledges and circumferential ridge (top left) contain wall microfibrils sectioned transversely (*) (i.e., running into the page), which are distinct from the orientation of the more obliquely-sectioned microfibrils (direction indicated by the double-arrowed black lines) below them. Below the plasma membrane are microtubules (small arrows) sectioned obliquely to transversely. Intervening the future ventral (pore) walls (VW) is an electron-lucent layer (arrow). Organelles present include dictyosomes (D) and associate vesicles (Dv) containing flocculent material, plastids (pl), mitochondria (M), spherosomes (L), rough endoplasmic reticulum (rER). Vacuoles (V). Bar = 0.5 µm

**Fig. 2.32.** Portion of sister guard cells (centre, and bottom left) of a transversely-sectioned modified stoma of immature stage-2, adjacent to epidermal (E) and subepidermal (SE) cells. Note the expanding substomal space (right star) below the dorsal wall (DW) of the central guard cell and the enlarging pore (left star) at the inner walls (IW) of the sister guard cells. A strand of cuticle (arrowhead), which may include pectinaceous material, stretches across the ventral pore region. Note the cytoplasmic furrow (area between arrowheads) in the lobed nucleus (N). Bar = 2 µm
Figs. 2.33-2.38. Pore development of modified stomata of floral nectary of *Vicia faba*

Figs. 2.33a,b. Immature modified stomata of various developmental stages near base of gynoecial face of nectary projection (unstained, fresh material), photographed at different levels of focus. Note difference in vacuolation. In *a*, external portion of pore of young modified stoma is just forming (arrowhead), and spherosomes of both modified stomata are evident (arrows) above vacuoles (*V*). Bars = 20 µm

Fig. 2.34. Glancing L.S. of developing pore of modified stomata of Fig. 2.33 (similar to that of Fig. 2.33a top), showing cuticular material (*) in depression separating ventral walls. Bar = 1 µm

Fig. 2.35. L.S. of maturing modified stoma on extreme tip of pre-secretory nectary, demonstrating peripheral cytoplasm surrounding large vacuoles (*V*). Plastids (*Pl*), spherosomes (*L*), nucleus (*N*). ER cisternae line the cell walls, particularly at developing pore. This modified stoma is surrounded by six other vacuolated guard cells (of four stomata), and epidermal cell (*E*). Arrows indicate the ridged outline of outer walls of two guard cells. Intercellular spaces (*IS*). Bar = 5 µm

Fig. 2.36. Outer-ventral wall region of guard cell of open modified stoma showing microfibrils radiating (white lines with arrows) from pore (*P*) lined by cuticle (arrows). Microtubules (arrows) underlie the plasma membrane. Mitochondria (*M*), spherosomes (*L*), endoplasmic reticulum (*rER*), coated (*Cv*) and dictyosomal (*Dv*) vesicles, plastid (*Pl*) (arrow). Ventral wall (*W*). Bar = 1 µm

Fig. 2.37. L.S. of pore (*P*) region of mature modified stoma showing osmiophilic lining (*,arrow*) at pore tip. Microbody (*MB*) situated near aggregation of spherosomes (*L*). Dictyosomes (*D*) with numerous vesicles (*Dv*), endoplasmic reticulum (*rER, sER*), mitochondrion (*M*) and plastid (*Pl*). Bar = 0.5 µm

Fig. 2.38. Ovoid microbody (*MB*) abutting spherosome (*L*). Mitochondria (*M*). Bar = 0.25 µm
Figs. 2.39-2.43. Light and transmission electron microscopy of modified stomata of floral nectary of *Vicia faba*, showing pore development and differential thickening of guard-cell walls.

Figs. 2.39-2.41. Transverse sections taken near the middle of the developing pore, through progressive stages of immature modified stoma (Fig. 2.39, 2.40) and an open modified stoma (Fig. 2.41), on the gynoecial face near the projection tip of a pre-secretory nectary. In Fig. 2.39, pore development [separation of the ventral walls (VW)] is more advanced at the inner (IW), than at the outer (OW), paradermal walls. The formation of the ledges, connected by cuticle (arrowheads), is evident at both faces of the developing pore. In Fig. 2.40, the thin, intact outer cuticle lies across the top of the otherwise, fully-formed pore. In Fig. 2.41, remnants of the ruptured cuticle (arrowheads) occur at the top of the newly-revealed pore. Concurrent with pore development, note the changes in vacuolation and displacement of the cytoplasm, and the degradation of wall material (*), resulting in intercellular spaces (IS) and leading to the manifestation of the substomatal space (SS). In Fig. 2.41, a cuticle (black arrows) entirely surrounds the guard-cell walls, and those of the subepidermal cells, such that the substomatal space is completely lined. Several ridges (clear arrows) on the outer walls of these and adjacent guard cells, and along the inner walls of Fig. 2.40 and 2.41, are evident. Bars = 5 µm

Fig. 2.42a-e. Serial, longitudinal sections (2.0 µm thick) through the tip of the gynoecial face of a nectary projection of a secretory flower, stained with TBO. The pore (P) between two guard cells (triangles) of a mature modified stoma in a and b eventually leads to the substomatal and intercellular spaces in d and e; the latter (*) occur between subepidermal cells (dark squares). Wall ingrowths (arrowheads) occur at the tips of these subepidermal cells (d) and in some epidermal cells (clear squares) (b,d,e). Longitudinal ridges (clear arrows) are evident on the outer walls of incoming guard cells; compare this wall’s thickness to the inner walls of the outgoing, central modified stoma in e. A longitudinally-arranged cuticular patterning (black arrows) occurs on the epidermal cells of this nectary. Bar = 40 µm

Fig. 2.43. Median longitudinal section in the anticlinal plane of a guard cell (triangle) of a mature modified stoma from the nectary projection of a bud, stained with TBO. Both walls are thicker in their middles (arrows) than at their ends. The inner is much thicker than the outer wall. The inner walls of adjacent epidermal cells (clear squares) and those of the subepidermal cells (solid squares) are relatively thin. A substomatal space (*) separates the guard cell from subepidermal cells, although a cuticular connection (arrow) remains. Bar = 10 µm
**Figs. 2.44-2.47.** Features of transfer cells from the same nectar-bearing gland of a flower of *Vicia faba*

**Fig. 2.44.** Extreme tip of a longitudinally-sectioned nectary projection showing three guard cells (GC), with plasmolysed cytoplasm, above two epidermal (E) and several subepidermal cells. Wall ingrowths (wl) are evident in several of the latter. A strand of cuticle is marked (arrowhead) between a guard and subepidermal cell. Note the large, confluent intercellular spaces (*), and the ridges (open arrows) along the outer walls of the rightmost guard cell. Vacuoles (V), nucleus (N). Bar = 10 µm

**Fig. 2.45.** Outer region of epidermal (transfer) cell, showing primary wall (W) with microfibrils relatively densely packed, and attached wall ingrowth (wl) of loosely-aggregated fibrils. A multi-layered, membranous myelin body in the vacuole (V) appears to be continuous with the plasma membrane and connected by trabecules (double arrowheads) to the wall ingrowth. Mitochondrion (M), membranous reticulum (mR). Bar = 0.5 µm

**Fig. 2.46, 2.47.** Outer junctions between epidermal (E) and plasmolysed guard cells (GC) showing wall ingrowths (wl) along the outer primary wall (W) of the epidermal cells, but their scarcity along outer (OW) and dorsal (DW) walls of the guard cells. Nucleus (N), vacuole (V), membranous reticulum (mR), cuticle (arrows). Bars = 1 µm in Fig. 2.46, 10 µm in Fig. 2.47.
Fig. 2.48. Epidermal cell of a secreting floral nectary of *Vicia faba*, showing higher magnification of boxed area of Fig. 2.47, depicting masses of membranous reticulum (mR) which may originate from dictyosomal (D) activity, by vesicle coalescence. Mitochondria (M), with weakly-developed cristae, are found near the tonoplast of the vacuole (V). What may be initial stages of the multi-membranous myelin figure of Fig. 2.45, are arrowed in the small profile of the vacuole (V). The primary wall (W) underlies secondary material resembling wall ingrowths (wl). Bar = 1 µm
Figs. 2.49-2.60. Guard-cell walls, wax, cuticle, pore occlusion and fate of post-secretory gland of *Vicia faba*

**Fig. 2.49.** Scanning electron micrograph of critical-point dried nectary projection demonstrating circumferentially-oriented ridges (clear arrows) on walls of guard cells (triangles) among epidermal cells (squares). Ledges (black arrows). Horizontal line with arrows marks measured pore width. Bar = 10 µm

**Fig. 2.50.** Wall layers of immature modified stoma. The outermost microfibrils (*) are covered by cuticle (black arrows) and constitute the ridges (clear arrows) as seen in Fig. 2.49. The ridges lie external to wall layers with microfibrils radiating from the pore region. Bar = 1 µm

**Fig. 2.51.** Section showing microfibrils (*) at right angles to those of Fig. 2.50, in this case running along a cuticle-covered ridge with underlying wall layers. Bar = 0.5 µm

**Fig. 2.52.** Low-temperature scanning electron micrograph of open modified stoma on flank of projection. Note waxy globules on surface of guard and epidermal cells. Symbols as in Fig. 2.49. Pores (P). Bar = 10 µm

**Fig. 2.53.** Fluorescence microscopy with auramine-O demonstrating cuticle covering epidermal and guard cells, substomatal and intercellular spaces (arrowheads). Subepidermal cell immediately below pore (P). Bar = 10 µm

**Fig. 2.54.** Oblique section of mature modified stoma from pre-secretory nectary, showing a sheet of osmiophilic material (double arrowheads) deposited across the pore (P) and along outer and ventral walls of sister guard cells. Note remnant of cuticle (arrowhead) at the ledge incorporated into pore blockage by loose covering of deposition. Aggregations of deposited granules are also evident at occlusion site. Cuticle (black arrows) lines guard cells and substomatal space above subepidermal cells. White arrow (top left) denotes channels in cuticle covering outer wall (W). Nucleus (N), mitochondria (M), vacuole (V), plastids (Pl). Bar = 2 µm

**Fig. 2.55.** Cuticle lining outer wall (W) of guard cell and bearing osmiophilic channels (white arrows) extending to external surface where spherical osmiophilic granules (double arrowheads) are evident. Bar = 0.2 µm

**Fig. 2.56.** Scanning electron micrograph of maturing modified stoma undergoing rupture of overlying external cuticle (arrowheads), to reveal pore. Potential ledges (arrows). Bar = 10 µm

**Fig. 2.57.** Scanning electron micrograph of mature modified stoma of tannin-free cultivar Blandine, partially occluded by material (*, double arrowheads) over pore. Ledges (solid arrows), circumferential ridges (clear arrows). In this micrograph, as in Fig. 2.58, high magnifications revealed a definite blockage traversing the pore as seen in section view in Fig. 2.54. Bar = 10 µm

**Fig. 2.58.** Scanning electron micrograph of completely-closed modified stoma of tannin-free cultivar Blandine, with occluding material on outer and ventral walls (double arrowheads) and across pore (*). White arrowhead indicates what may be a large remnant of original outer cuticle incorporated into pore blockage. Black solid arrows at bottom show portions of ledges still visible, not covered by occluding material. Ridges (clear arrows). Bar = 10 µm

**Fig. 2.59.** Scanning electron micrograph of staminal face of projection (Pj) at base of fruit (G) (gynoecial length 30.5 mm) with longitudinal fracture (arrow). Arrowheads indicate open modified stomata, circles denote areas of crushed/collapsed cells. Disk (D). Bar = 250 µm

**Fig. 2.60.** More advanced stage than Fig. 2.59, illustrating the separation of halves of projection by radial expansion of fruit (gynoecial length 44.5 mm). Bar = 250 µm
Fig. 2.61. Developmental study of flowers of pre-secretory (n = 26) and secretory (N; n = 1) stages, sampled from several inflorescences of the same plant (May 18, 1988). The percentages of open (clear squares), immature (triangles), and closed (black squares) modified stomata (see Table 2.1, page 2.12, for developmental stages) per nectary projection are shown. Mean pore widths are plotted with standard errors. The secretory flower had a small amount of nectar at the projection tip (see Fig. 2.3)
Fig. 2.62. Developmental study of flowers of pre-secretory (n = 1), secretory (N; n = 3) and post-secretory (n = 12) stages, sampled from several inflorescences of the same plant of *Vicia faba* (September 19, 1988). Check Fig. 2.61 for symbol explanations. The nectary projections of the four largest beans exhibited folding on the gynoecial side, and that of the longest fruit was splitting longitudinally (see Fig. 2.59), so that some modified stomata may have been hidden or destroyed.
Figs. 2.63-2.70. Preparations from fresh tissue comparing stomata of floral nectary and leaf of *Vicia faba*

Figs. 2.63, 2.64. Patterns of birefringence revealed by polarized light from stomata of similar dimensions. Bar = 20 µm

**Fig. 2.63a,b,c.** A mature stoma of abaxial leaf surface, rotated through 45° and 90°

**Fig. 2.64a,b,c.** A mature modified stoma on nectary projection face, rotated through 45° and 90°. Note the dark lines in the birefringent areas (open arrows), thought to represent the surface ridges of circumferentially-oriented microfibrils

Figs. 2.65, 2.66. Adjacent foliar stomata. Bars = 20 µm

**Fig. 2.65.** Nomarski-interference micrograph of immature stomata on abaxial peel

**Fig. 2.66.** Mature, adjacent stomata on leaf piece stained with neutral red

Figs. 2.67-2.70. Fresh tissue exposed to, and/or stained for, potassium ions. Bars = 20 µm

**Fig. 2.67.** Two mature stomata on leaf, after floating abaxial epidermis on 100 mM KCl for 5 h in dark

**Fig. 2.68a,b.** Modified stomata of nectary subjected to cobaltinitrite-(NH₄)₂S staining for potassium. Note scarce black precipitate (arrowhead) within guard and other epidermal cells in a, but crystals obvious externally (arrowheads) above same cells, in b

**Fig. 2.69.** Mature stomata on leaf peel stained with cobaltinitrite-(NH₄)₂S for potassium

**Fig. 2.70.** Mature stoma on leaf peel floated on 100 mM KCl for 25 min in light, before staining for potassium. Note large black crystals in guard cells
Fig. 2.71. Diel changes in mean (white bars) and maximum (slashed bars) pore aperture and percentage of pores completely closed (dots) in leaf stomata and modified stomata of the floral nectaries of a plant of *Vicia faba* growing outdoors (August 5-6, 1988). Standard error bars are given above the means. Air temperature (T) 0.5 m above ground, and prevailing photoperiod, are indicated. The third flower of stage D sampled had a small amount of nectar (N).
Fig. 2.72. Influence of time, bud age (young - stage A, circles; medium - stage C, triangles; mature - stage D, squares) and osmoticum (1.0 M sucrose or mannitol) on the percentage plasmolysis of guard cells of mature modified stomata (white symbols) and epidermal cells (black symbols) located on the top quarter of dissected nectary projections of *Vicia faba*. Each datum represents the mean ± S.E. for six buds. Error bars for guard cells have been omitted, for clarity.
Fig. 2.73. Characteristics of nectar and modified stomata of floral nectaries of *Vicia faba* cultured 24 h earlier as mature buds at different relative humidities (high, low) and on solutions of variable sucrose concentration (0.15, 0.5 M). Within groups of bars, bars identified by a different letter are significantly different at $\alpha = 0.05$, two-tailed t test.

a. Total modified stomata, and percentages of immature, open and closed stomata, per treatment. For comparison, data are also given for mature (pre-secretory) buds. Data are presented as means ± S.E. for five buds each.

b. Nectar volume (V) and solute concentration (C). Data are presented as means ± S.E. for twenty buds per treatment.

c. Pore widths (mean ± S.E.) for the various treatments; five buds per treatment.
Figure A: Bar graph showing Modified Stomata (n) for different conditions. 

- Non-cultured (presecretory) buds
- Low sucrose
- High humidity
- High sucrose
- High humidity
- High sucrose
- Low humidity

Figure B: Bar graph showing Volume (µL) for V and C conditions.

Figure C: Bar graph showing Concentration (%) and Pore Width (µm) for V and C conditions.
Fig. 2.74a. Relationship of pore width of modified stomata of the floral nectary with nectar-solute concentration in *Vicia faba*, for mature (pre-secretory) buds or flowers cultured 24 h earlier as mature buds at 0.15 M sucrose and high R.H.; 0.5 M sucrose and high R.H.; or 0.5 M sucrose and low R.H. Data points indicate the mean pore width ± S.E. for each nectary.

b. Plot of fully-occluded pores (as percent of total modified stomata) against nectar-solute concentration
A 0 Non-cultured (pre-secretory) buds

Y = 5.310 - 0.0322X

r = -0.587

P = 0.006

High sucrose, high humidity

High sucrose, low humidity

B

Occlusion (%) vs. Nectar Solute Concentration (%)

○ Non-cultured (pre-secretory) buds

○ Low sucrose, high humidity

○ High sucrose, high humidity

○ High sucrose, low humidity
Figs. 2.75-2.77. Experimental plasmolysis of guard and epidermal cells of floral nectary of *Vicia faba* with sucrose solutions. Focus is on maximum pore width. Bars = 20 µm

**Fig. 2.75a-f.** Series of light micrographs of an open modified stoma, part of another (top right), and epidermal cells near the tip of a nectary projection dissected from a mid-sized bud (stage C), originally stained with neutral red in distilled water and then perfused by 1.5 M sucrose for **a** 1.5 min, **b** 4 min, **c** 14.5 min, **d** 122 min, **e** 352 min, **f** 1026 min. Vacuoles (V). Withdrawal of the plasma membrane from guard-cell (arrowheads) and epidermal-cell (arrows) walls is indicated. Right guard cell is fully plasmolysed in **f**; compare pore width with **a**

**Fig. 2.76a,b.** Open modified stoma at tip of nectary projection dissected from mid-sized bud (stage C) and placed in **a** distilled water containing neutral red, immediately before perfusion with 1.6 M sucrose, and **b** 1440 min after the projection, exposed to 1.6 M sucrose for 1300 min, was perfused with distilled water. Note continual plasmolysis of guard cells (arrowheads), smaller pore width, and increased width of guard cells (compare w1 with w1', w2 with w2') due to flattening

**Fig. 2.77a,b.** Open modified stomata just below tip of dissected nectary projection of small bud (stage A) exposed to 1.0 M sucrose solution for **a** 17 min and **b** 251 min. Note reduction in pore widths and loss of guard-cell volume, despite absence of guard-cell plasmolysis except where indicated (arrowhead). Arrows denote plasmolysis of epidermal cells
Figs. 2.78-2.82. Results of plasmolysis and ABA experiments using floral nectary and leaf (Fig. 2.79) tissues of *Vicia faba*. Bars = 20 µm

Figs. 2.78-2.80. Plasmolysis experiments

Fig. 2.78. Plasmolysis of guard and epidermal cells (arrowheads and arrows, respectively) at tip of nectary projection from small bud (stage A) exposed overnight to 0.6 M sucrose solution containing neutral red. Note open pores of modified stomata, despite complete plasmolysis of some guard cells. The stars indicate non-stomatal, apical openings associated with the modified stomata.

Fig. 2.79. Completely-closed stoma on abaxial leaf piece incubated in 1.2 M sucrose solution, showing plasmolysis (arrowheads) of the guard cells. Focus on guard-cell protoplasm. Accumulated wall material at ends of guard cells (arrows).

Fig. 2.80. Modified stoma isolated from post-secretory nectary, demonstrating maintenance of a pore despite plasmolysed guard cells (arrowheads). Focus on guard-cell protoplasm. Note ridges (arrow) in an adjacent guard-cell wall.

Fig. 2.81, 2.82. Secretion of ABA in floral nectar by flowers cultured at high R.H. on 0.3 M sucrose solutions containing (±)cis-trans-ABA at 100 µM for 36 h (Fig. 2.81) and at 10 µM for 24 h (Fig. 2.82). Bars = 20 µm.

Fig. 2.81a. Note open pores and lack of plasmolysis in guard and epidermal cells after collection of nectar containing 430 ng (+)cis-trans-ABA/100 mg solute.

b. Scanning electron micrograph of a, showing pore of similar dimensions. Note cleft in outer walls (arrowheads) common to large-pored modified stomata.

Fig. 2.82a. Scanning electron micrograph of nectary projection tip found bathed in nectar of 71 ng (+)cis-trans-ABA/100 mg solute, showing large pores and subepidermal cells within pores (asterisks).

b. Higher magnification of top left area in a, showing apical openings (stars) between open modified stomata, ridges (open arrows) on outer walls of guard cells, and subepidermal cell (asterisk) below pore.
CHAPTER 3

THE MODIFIED STOMATA OF THE FLORAL NECTARY
OF VICTA FABA L.

II. STOMATAL NUMBER AND DISTRIBUTION
AS SELECTION CRITERIA FOR BREEDING
FOR HIGH NECTAR SUGAR PRODUCTION

3.1 INTRODUCTION

Nectar is a major attractant to bees and other flower visitors, and has a recognized role in crop pollination (McGregor 1976, Kevan and Baker 1983). Furthermore, because it is the precursor of honey, nectar yields are economically important to commercial honey production. Therefore, it seems a reasonable expectation that increasing nectar production should attract more foraging visits, thereby resulting in greater seed and honey yields (Shuel 1989).

Improving the genetic potential of nectar plants through selection and breeding can increase nectar harvests dramatically: genetic differences of several hundred percent have been reported among cultivars or selections of numerous legume species (Shuel 1989). It is necessary to determine anatomical and physiological characters important as selection criteria. However, because our understanding of the secretory process is not complete, selection procedures to date have been largely empirical, and include assessments of number of flowers per plant, number of flowering periods per year, flower aroma and colour, flower size, receptacle diameter, volume of functional phloem in the pedicel, and number of nectary stomata (Shuel 1989). The latter are found in many legume species (see Fabaceae in Appendix 1).

Results of investigations of a relationship between nectar volume and number of stomata per floral nectary of Lotus corniculatus and Medicago sativa have been inconclusive (Teuber et al. 1980, Murrell et al. 1982b). Research on developmental, anatomical, ultrastructural and physiological aspects of the nectary stomata of Vicia faba has demonstrated significant differences to those of leaves, most notably a loss of guard-cell movement, suggesting a passive, non-regulatory function for these modified stomata during nectar secretion (Chapter 2 of this thesis). Nevertheless, it was of interest to investigate whether the number of nectary stomata, and their distribution on
the nectary, bear any relationship to nectar production, and hence whether these could be useful selection criteria for use with fababean.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

Flowers of *Vicia faba* cv. Aquadulce and cv. Blandine were gathered from the outdoor plot during June-August 1990. During this period, daily maximum and minimum temperatures typically were 8-13°C and -3-6°C, respectively. Plants of cv. Long Pod were grown from seed in a growth chamber under constant light (60 µmol m⁻²s⁻¹) and temperature (22°C).

3.2.2. Culture of excised buds

An extensive study involving cv. Aquadulce was conducted, because initial investigations showed these plants to bear relatively large numbers of modified stomata on their floral nectaries. Five plants (selections) of 3-4 stems each, whose flowers exhibited different numbers of modified stomata per nectary, were chosen. Mature buds were cultured as described in 2.2.9, in random arrangement (Fig. 3.3), using freshly-prepared 10% (w/v) sucrose, 10 mM K phosphate solution, pH 6.8. After the vials with buds were placed individually in 100 ml beakers containing 5 ml distilled water, the beakers were sealed and wrapped as before and held at 25°C.

It was decided to culture the flowers to be analysed for nectar-sugar production and number of nectary stomata, because the five selected plants were among numerous ones sown in two rows near a wall which, at this time of year, did not permit equal sunlight to all selections. Accordingly, because the amount of illumination received by plants is a critical factor in nectar-sugar production (see section 1.2), this standardised, *in vitro* method was chosen. Culturing mature buds also removed from them any competition by other sinks (e.g., lengthening fruits, growing flower buds, etc.) within
3.3

the raceme and within the plant during the period in which they would have produced nectar, so that by this *in vitro* technique, it was possible to treat buds from the different selections as equally as possible. Culturing also overcame interference by insect visitation (possibly leading to removal of nectar or pollination of flowers), as well as avoiding vagaries in the weather from day to day.

Because it is crucial for nectar-production comparisons that nectar be sampled from flowers of the same age (Shuel 1989), the age of the buds was assessed by observing the plants in the mid- to late afternoon of the day preceding bud harvest. Very large buds, immediately distal on racemes to those flowers demonstrating partial or full reflexion of the standard petal, but themselves still fully closed (with the tips of the standard petal tightly overlapping the wing and keel petals), were labelled with coloured tape looped over the calyx base. At 9:00 a.m. next day, when *all* flowers appeared closed, the labelled buds from the five selections were collected and cultured as outlined above. Dissection of some buds showed them to be usually pre-secretory, occasionally with only a small bead of nectar at the projection tip. Thirteen to sixteen buds were cultured per selection.

3.2.3. Nectar collection and analysis

Thirty-six h after the bud-culture was initiated, nectar was collected as described in 2.2.9. Here, however, wicks were analysed for component sugars (sucrose, fructose, glucose) using the enzymatic method described by Kronestedt-Robards *et al.* (1989). As Wykes (1952a) could not detect maltose, melibiose and raffinose from paper chromatography of floral nectar of *V. faba*, no analyses were conducted for these minor sugars. Freshly-prepared standard solutions of fructose, glucose and sucrose (analytical grade) were similarly analysed, and the method could detect less than 50 ng of hexose. The enzymes used were hexokinase (HK) EC 2.7.1.1, 140 U/mg, #127-175;
glucose-6-phosphate dehydrogenase (G6P-DH) EC 1.1.1.49, 350 U/mg, #127-035; phosphoglucone isomerase (PGI) EC 5.3.1.9, 350 U/mg, #127-396 (all of analytical grade, from Boehringer-Mannheim); and invertase (β-fructosidase) EC 3.2.1.26, 122 U/mg, Grade VI, melibiase-free, from Sigma. The enzymes were diluted and stabilized in buffer solution (pH 7.1) of 0.05 M Hepes-K containing 0.1% BSA (Sigma), to yield 0.14 U (HK), 0.175 U (G6P-DH), 0.35 U (PGI) and 5U (invertase) per 5-µl aliquot added into final assay volumes of 800-900 µl. Buffers utilized were 10 mM Na acetate, pH 4.5, and 100 mM Tris-HCl, 5 mM MgCl₂, pH 8.1. The reagents were 30 mM ATP and 5 mM NADP (Sigma).

This enzymatic method is pH-dependent and based on catalysis of the following reactions:

Invertase, pH 4.5

Sucrose + H₂O → Fructose + Glucose

HK, pH 7.6

Fructose + ATP → Fructose-6-phosphate + ADP

PGI, pH 7.6

Fructose-6-phosphate → Glucose-6-phosphate

HK, pH 7.6

Glucose + ATP → Glucose-6-phosphate + ADP

G6P-DH, pH 7.6

Glucose-6-phosphate + NADP⁺ → Gluconate-6-phosphate + NADPH + H⁺

The oxidation of glucose-6-phosphate by NADP⁺ to gluconate-6-phosphate is the eventual reaction common during analysis of all three sugars, and the concurrent reduction of NADP⁺ to NADPH is measured spectrofluorometrically at 340 nm.
Nectar was also collected directly from flowers of identical age taken from the plants of two selections (A, K), for comparative sugar analysis. At this time, the length of the gynoecium was also determined (section 2.2.1). After nectar collection, flower receptacles bearing the nectary were fixed and processed for scanning electron microscopy as outlined in 2.2.3. For each selection, four or usually five nectary projections, representing identically-aged flowers randomly selected from the field plot (in situ) and from culture (in vitro), were photographed for counts of total nectary stomata. Only modified stomata (Immature stage-1 and older; see Table 2.1, page 2.12), not guard mother cells, were considered. The percentage of nectary stomata located on the top 1/4 of the projection’s length was also determined for in vitro flowers. Heights were measured along the staminal face of the nectary projections.

3.3. RESULTS

3.3.1. Number and distribution of nectary stomata and nectary-projection height in various cultivars

The data for cultivars Blandine and Long Pod are given in Table 3.1A (on reverse). All flowers per cultivar came from the same plant, indicating that there is considerable variation between mature flowers of individual plants. Although they did not differ in average number of modified stomata per nectary, the nectary projections of cv. Blandine were over twice the height of those of cv. Long Pod (Table 3.1A, and compare Fig. 3.1a,b with Fig. 3.2a,c). Projections of both cultivars bore the majority of their modified stomata near the tips, with very few being located on the mid-regions of the gynoecial (Fig. 3.1a,c, 3.2a,b) and staminal (Fig. 3.1b) faces. In flowers of cv. Long Pod, most modified stomata were found in a band traversing the extreme tip of the staminal face of the nectary projection (Fig. 3.2c).

Comparative data for the five selections of cv. Aquadulce are presented in Tables
Table 3.1A. Heights of nectary projections and numbers of modified stomata [Mean ± S.E. (Range)] in two cultivars of *Vicia faba*, from mature flowers growing in situ

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>No. flowers</th>
<th>No. modified stomata</th>
<th>Nectary projection (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blandine</td>
<td>5</td>
<td>29.4 ± 1.96 (23-35)</td>
<td>1.32 ± 0.099 (1.08-1.64)</td>
</tr>
<tr>
<td>Long Pod</td>
<td>5</td>
<td>25.6 ± 1.72 (19-29)</td>
<td>0.589 ± 0.042 (0.487-0.726)</td>
</tr>
</tbody>
</table>

Table 3.1B. Numbers of modified stomata per nectary [Mean ± S.E. (Range)] in normal and cultured flowers of five selections of *Vicia faba* cv. Aquadulce

<table>
<thead>
<tr>
<th>Selection</th>
<th>n In situ</th>
<th>n In vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>A**</td>
<td>5</td>
<td>208.4±9.61 (184-242)</td>
</tr>
<tr>
<td>Bns</td>
<td>4</td>
<td>92.25±6.54 (76-108)</td>
</tr>
<tr>
<td>Dns</td>
<td>5</td>
<td>102.8±6.12 (79-113)</td>
</tr>
<tr>
<td>Ens</td>
<td>4</td>
<td>128.0±13.64 (104-162)</td>
</tr>
<tr>
<td>Kns</td>
<td>5</td>
<td>62.2±5.06 (49-73)</td>
</tr>
</tbody>
</table>

Differences between values per selection are statistically significant (**, P<0.01) or not (ns, P>0.05)
Columnar values followed by a different letter are significantly different (P<0.05), using 2-tailed t-tests.

Table 3.1C. Heights (mm) of the nectary projections [Mean ± S.E. (Range)] in normal and cultured flowers of five selections of *Vicia faba* cv. Aquadulce

<table>
<thead>
<tr>
<th>Selection</th>
<th>n In situ</th>
<th>n In vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>A**</td>
<td>5</td>
<td>1.38±0.090 (1.05-1.57)</td>
</tr>
<tr>
<td>B**</td>
<td>4</td>
<td>0.893±0.033 (0.799-0.949)</td>
</tr>
<tr>
<td>Dns</td>
<td>5</td>
<td>0.656±0.040 (0.523-0.753)</td>
</tr>
<tr>
<td>Ens</td>
<td>4</td>
<td>0.923±0.018 (0.873-0.951)</td>
</tr>
<tr>
<td>Kns</td>
<td>5</td>
<td>0.894±0.036 (0.763-0.963)</td>
</tr>
</tbody>
</table>

Differences between values per selection are statistically significant (**, P<0.01) or not (ns, P>0.05)
Columnar values followed by a different letter are significantly different (P<0.05), using 2-tailed t-tests.

Table 3.1D. Total nectar sugar (µg) secreted [Mean ± S.E. (Range)] by cultured flowers and the percentages [Mean ± S.E. (Range)] of modified stomata located in the distal quarter of their nectary projections from the five selections of *Vicia faba* cv. Aquadulce

<table>
<thead>
<tr>
<th>Selection</th>
<th>n Percentage</th>
<th>Nectar sugar per flower</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>34.73±2.59 (24.54-38.60)</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>53.60±2.16 (50.56-62.16)</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>46.70±1.59 (40.71-49.43)</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>59.92±6.32 (46.77-77.14)</td>
</tr>
<tr>
<td>K</td>
<td>5</td>
<td>82.08±2.62 (75.38-90.00)</td>
</tr>
</tbody>
</table>

Columnar values followed by a different letter are significantly different (P<0.05), using 2-tailed t-tests.
3.6

3.1B and 3.1C. Flowers of selection A had significantly fewer nectary stomata in cultured buds (P<0.01) than *in situ* (Table 3.1B). In flowers of selection B, the height of the nectary projection was significantly greater *in vitro* (Table 3.1C). Flowers of selection A had approximately three times the number of nectary stomata as selection K (Table 3.1B, and compare Fig. 3.4, 3.5 with Fig. 3.12e, 3.13), and bore nectary projections over twice the length as in selection D (Table 3.1C, and compare Fig. 3.5 with Fig. 3.9, 3.10).

Regression of projection height on number of modified stomata per nectary was highly significant for both treatments (Fig. 3.15). Therefore, longer nectary projections possessed more modified stomata, in this cultivar.

The comparison of the lengths of five nectary projections of selection A measured fresh and then after conventional tissue preparation (dehydration, critical-point drying) for electron microscopy showed the latter measurements to be 83.7% those of fresh projections; indicating the importance of comparing only those nectaries which have undergone similar treatments.

In the left half of Table 3.1D, the proportions of modified stomata distributed in the top 1/4 of the nectary projections of the five selections, are given. Flowers of selection K bore over 80% of their stomata in these distal parts of the projections (Fig. 3.12a,b,e, 3.13, 3.14), whereas those of selection A possessed 65% of their stomata on the proximal 3/4 of projections (Fig. 3.4-3.6a), on average. The corresponding values for selections B, D (Fig. 3.9, 3.10) and E (Fig. 3.11a-c) were between these extremes, the differences in percentages for B and E not reaching statistical significance (Table 3.1D).

### 3.3.2. Nectar sugar production *in vitro*

The reaction curves charted during a typical enzymatic analysis of nectar sugar of *Vicia faba* cv. Aquadulce are shown in Fig. 3.16.
The average quantities of nectar sugar secreted 36 h after mature buds of the five selections were cultured on sucrose solution are shown in the right half of Table 3.1D (facing previous page). Flowers of selection K secreted about 4.5 times, and flowers of selection B about 3 times, as much sugar as those of selection A.

Floral nectars collected from flowers in the field were sucrose dominant, the ratios of sucrose/fructose/glucose (percent, by weight) averaging 79.9/11.1/9.0 and 80.0/10.8/9.2 for selections A and K, respectively. A comparison of nectar samples collected from flowers in situ and in vitro showed a significant, 33% increase in the mean sucrose/hexose (= fructose + glucose) ratio in cultured flowers of selection A (Table 3.2). However, in selection K, the corresponding increase was not significant. Both selections had proportionately more fructose in their nectars when cultured than in in situ flowers, the increases in the fructose/glucose ratios being highly significant (Table 3.2).

Table 3.2. Nectar-sugar ratios (Mean ± S.E.) of in situ and in vitro flowers of the five selections of Vicia faba cv. Aquadulce. F=fructose, G=glucose, H=hexose (= F+G), S=sucrose

<table>
<thead>
<tr>
<th>Selection</th>
<th>n</th>
<th>S/H</th>
<th>F/G</th>
<th>n</th>
<th>S/H</th>
<th>F/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>4.08a±0.35</td>
<td>1.26a±0.08</td>
<td>4</td>
<td>13.69a±2.12</td>
<td>3.04a±0.38</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>7.40b±1.47</td>
<td>5.03b±1.46</td>
<td>5</td>
<td>10.70ab±0.94</td>
<td>3.74a±1.27</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>10.70ab±0.94</td>
<td>3.74a±1.27</td>
<td>5</td>
<td>12.06ab±2.24</td>
<td>2.93a±0.24</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>4.52a±0.95</td>
<td>1.08a±0.049</td>
<td>5</td>
<td>7.26b±1.63</td>
<td>5.06a±1.09</td>
</tr>
<tr>
<td>K</td>
<td>5</td>
<td>4.52a±0.95</td>
<td>1.08a±0.049</td>
<td>5</td>
<td>7.26b±1.63</td>
<td>5.06a±1.09</td>
</tr>
</tbody>
</table>

Columnar values followed by a different letter are significantly different (P<0.05), using 2-tailed t-tests.

Regarding the sugar ratios of floral nectar collected from the culture experiments, there were no significant differences in fructose/glucose levels between selections (Table 3.2). However, the sucrose/hexose ratios of selections B and K were significantly lower than that of selection A (0.05 > P > 0.02).
3.3.3. **Relationship between nectar sugar production and total number of nectary stomata**

The linear regression of nectar sugar production (µg) per cultured flower (after transformation to the natural-logarithm scale) on the total number of modified stomata per nectary of the five selections was negative and statistically significant (Fig. 3.17a).

3.3.4. **Relationship between nectar sugar production and the distribution of modified stomata on the nectary projection**

The linear regression of nectar sugar production (µg) per cultured flower (after transformation) on the fraction of the total modified stomata located on the distal 1/4 of nectary projections of the five selections was positive and significant (Fig. 3.17b).

### Table 3.3. Gynoecial length (mm) [Mean ± S.E. (Range)] of normal and cultured flowers of the five selections of *Vicia faba* cv. Aquadulce

<table>
<thead>
<tr>
<th>Selection</th>
<th>n</th>
<th>In situ</th>
<th>n</th>
<th>In vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sup&gt;as&lt;/sup&gt;</td>
<td>11</td>
<td>18.96&lt;sup&gt;ab&lt;/sup&gt; ± 0.12 (18.3-19.4)</td>
<td>15</td>
<td>18.79&lt;sup&gt;ab&lt;/sup&gt; ± 0.09 (18.25-19.3)</td>
</tr>
<tr>
<td>B&lt;sup&gt;as&lt;/sup&gt;</td>
<td>4</td>
<td>18.55&lt;sup&gt;b&lt;/sup&gt; ± 0.16 (18.2-18.9)</td>
<td>14</td>
<td>18.49&lt;sup&gt;b&lt;/sup&gt; ± 0.15 (17.4-19.4)</td>
</tr>
<tr>
<td>D&lt;sup&gt;as&lt;/sup&gt;</td>
<td>3</td>
<td>19.18&lt;sup&gt;a&lt;/sup&gt; ± 0.14 (18.9-19.35)</td>
<td>15</td>
<td>18.59&lt;sup&gt;ab&lt;/sup&gt; ± 0.14 (17.3-19.4)</td>
</tr>
<tr>
<td>E&lt;sup&gt;as&lt;/sup&gt;</td>
<td>4</td>
<td>19.13&lt;sup&gt;a&lt;/sup&gt; ± 0.11 (18.8-19.3)</td>
<td>13</td>
<td>18.72&lt;sup&gt;ab&lt;/sup&gt; ± 0.14 (17.95-19.8)</td>
</tr>
<tr>
<td>K&lt;sup&gt;as&lt;/sup&gt;</td>
<td>6</td>
<td>19.12&lt;sup&gt;ab&lt;/sup&gt; ± 0.22 (18.4-19.8)</td>
<td>15</td>
<td>18.95&lt;sup&gt;a&lt;/sup&gt; ± 0.12 (18.2-19.6)</td>
</tr>
</tbody>
</table>

Differences between values per selection are statistically significant (**, P<0.01) or not (ns, P>0.05) Columnar values followed by a different letter are significantly different (P<0.05), using 2-tailed t-tests.

3.3.5. **Gynoecial length**

In Table 3.3 are given the mean lengths of the gynoecium for the five selections of cv. Aquadulce. Although, within selections, the gynoecia of *in situ* and flowers were always longer than those *in vitro*, none of these differences were statistically significant. The gynoecial length of flowers of selection B averaged significantly less than those of selections D and E *in situ*, and, *in vitro*, selection K (Table 3.3). However, cultured flowers of selection B produced quantities of nectar sugar which were not significantly dissimilar from those of selection K (Table 3.1D). Flowers of
selection B had the shortest gynoecia, but the second-longest nectary projections (Table 3.1C).

3.4. DISCUSSION

In previous attempts to relate numbers of nectary stomata to nectar production, Teuber et al. (1980) and Murrell et al. (1982b) counted stomata and measured volumes of nectar. However, because standing nectar can lose appreciable quantities of water by evaporation, it is better to use total nectar sugar as a measure of nectary output, because this parameter (the product of nectar volume times concentration) remains essentially constant during evaporation (see section 2.3.9 and Shuel 1961, 1989). The use of high humidities in the culture experiments also ensured low evaporative losses, and the thin exudates were relatively easier to gather by both capillary and wick, thereby permitting total nectar sugar collected to closely approach that amount actually present.

The regression of nectar sugar production per flower on the total number of modified stomata per nectary projection was significant yet negative, indicating that a true inverse relationship exists in this cultivar of *V. faba*. In agreement with this finding are the results of the studies of alfalfa and birdsfoot trefoil, wherein the varietal selections which possessed the greatest number of nectary stomata (Vernal 290, Teuber *et al.* 1980; Carroll 1, Murrell *et al.* 1982b) actually produced the least nectar per flower. The passive role of these modified stomata during the secretory process (Chapter 2 of this thesis) accords with the lack of a positive connection with total nectar sugar produced by the nectary. Concerning selection for high nectar-sugar production, it is recommended to select against large total numbers of modified stomata per nectary.
Although the total number of stomata per nectary was negatively related to nectar production, there is a significant positive correlation between nectar sugar production per flower and the percentage of modified stomata on the upper quarter of the projection. This relationship \((r=0.466)\) may therefore be useful as a secondary selection criterion for breeding purposes.

Exactly what governs stomatal development and position on the nectary projection of *Vicia faba* is unknown. This study has shown there to be considerable variation in total numbers of modified stomata, and their position on nectary projections, both between and within selections. Therefore, both genetic and environmental determinants are inferred. Although it has not yet been investigated, it seems reasonable to expect that the high-sugar producing selections, having relatively low total numbers of modified stomata that were situated predominantly on the top quarter of nectary projections, had larger supplies of phloem entering the projections. This seems probable, judging from the results of Murrell *et al.* (1982b) which demonstrated a strong correlation between phloem cross-sectional area of the flower stalk, and nectar volume. Could nectary phloem, or more specifically, a factor in its translocate, be responsible for suppressing stomatal initiation? It is envisaged that such an inhibitor would be in short supply in poorly-secreting glands (supposedly those with less phloem), which consequently produce further modified stomata. In this model, the modified stomata along the tips of the projections would be present, regardless of the amount of phloem entering projections of various genotypes, because these stomata begin to differentiate when the projections have hardly formed (Fig. 2.10a, 2.61) and are yet without vasculature. Inhibition of initiation of modified stomata on the faces of the elongating nectary projection would therefore, by default, result in large fractions of the total modified stomata being situated at the tip. Perhaps the quantity of sugar itself entering the gland during the period before secretion begins, or some other factor
stimulated by sugar influx, may be decisive in guard-mother-cell initiation. In this regard, the immediate, ad libitum availability of sucrose supplied in the culture medium to bases of buds nearing anthesis might account for the common reduction (four of the five selections, including the significant decrease for selection A) in total nectary stomata in cultured flowers as compared to the ones remaining attached to plants. From the standpoint of stomatal distribution, if there is a direct positive relationship between quantity of nectary phloem and nectar-sugar production, then this model, based on inhibition of stomatal initiation, is appealing, because it would account for the significant positive correlation between percentage of modified stomata located in the top quarter of projection tips and total sugar secreted.

Unfortunately, discrimination of high nectar-sugar producing selections was not possible by simply measuring the length of the gynoecium, an approximate estimate of flower size.

The proportion of fructose to glucose in nectar of in situ flowers of two selections (A, K) averaged more than 1.0. In addition, the quantity of sucrose always exceeded that of glucose and fructose; sucrose/hexose ratios were 4.08-4.52. These types of sugar ratios are not unlike those of several other legume species, such as Lotus corniculatus (F/G = 1.24, S/H = 1.66 - Maurizio 1959), Medicago sativa (2.0, 1.31 - Furgala et al. 1958), Robinia pseudoacacia (3.46, 1.31 - Maurizio 1959) and Trifolium pratense (3.44, 1.69 - Wykes 1953; 2.68, 1.69 - Maurizio 1959). In passing, it should be emphasised that paper chromatographic analyses are helpful but not preferred, because they are semi-quantitative at best. Such analyses of floral nectar of V. faba were categorized as S/F/G = ++/+++ (Wykes 1952a) and sucrose only (Davis et al. 1988a). For five other Vicia spp., Percival (1961) only occasionally rated floral samples as having slightly more fructose than glucose. Furthermore, Wykes (1952a) rated the floral nectars of M. sativa, R. pseudoacacia and T. pratense as ++/++++++, +++/+++ and +++/++, although for the latter species Percival (1961) did rate fructose as exceeding glucose in half the samples analysed.
The high fructose/glucose ratio in floral nectar of *R. pseudoacacia* was attributed to a nectar sucrase with transglucosidic properties, such that the enzymatic hydrolysis of sucrose [the only sugar found in *Robinia*’s phloem sap (see Zimmermann 1954), which has a pH of 7.0-7.4 (see Ziegler 1975)] yielded proportionately more fructose than glucose. It seems likely that a transglucosidase is also operating in *V. faba*. That the fructose/glucose and sucrose/hexose ratios were even higher, usually significantly so, in nectar of cultured flowers, indicates that the higher temperature, possibly different pH, continual darkness, etc., that these flowers were subjected to in vitro than in situ, must be responsible. Unlike *Robinia*, the pH of phloem sap of *V. faba* is apparently unknown, although the culture-medium pH utilized here (6.8) was intermediate to that employed in various other experiments involving fababean tissues (5.3, Delrot and Bonnemain 1978; 5.8-6.2, Delrot and Bonnemain 1979; 7.2, Peterson and Currier 1969; 7.2-8.0, Eschrich 1966, 1967). Matile (1956) found that pH 7 was optimal for nectar-sugar production by isolated nectaries of *Abutilon striatum* and *Euphorbia pulcherrima* floated on glucose solutions. Others have shown that alteration of pH can affect the biochemical pathways of nectar-sugar production (Fekete et al. 1967, Bosia and Pescarmona 1972). Temperature has also been shown to have a direct influence on nectar-sucrose levels (Nichol and Hall 1988, Villarreal and Freeman 1990).

Statistically-significant differences in sucrose/hexose ratios occurred between nectar of cultured flowers of selection A and selections B and K, but no such differences appeared in situ. One likelihood for the much higher levels of sucrose in floral nectar of selection A involves a differential activity of invertase, suggesting that plants within the same cultivar possess different enzyme kinetics. A similar conclusion might be drawn from field studies of nectar secretion in the legume *Glycine max* (Severson and Erickson 1984, Severson et al. 1987). This result is of interest to crop selection and breeding. For instance, selection for low glucose and high sucrose in nectar of *Brassica* spp. might delay the nuisance granulation that results in honey ela-
borated from this nectar (see Shuel 1989). There is no apparent reason for it, but it may be more than just coincidental that the two selections (B, K) which had strikingly similar ratios of floral-nectar sugar in the culture experiments also produced the most nectar sugar.

Two potential applications arising from the results of this chapter, which appear worthy of investigation in the context of bee visitation to plants of fababean in agricultural settings, involve height of the projection of floral nectaries from different selections capable of producing variable quantities of nectar, and manipulation of nectar-sugar ratios by culturing mature buds before exposing the open flowers to visitation. It is known that short-tongued bumblebees, such as *Bombus terrestris*, often obtain nectar of the fababean flower after chewing a hole in its calyx (Beutler and Schöntag 1940, Tasei 1976, Newton and Hill 1983). Such visits do not result in pollination, and other bees, like *Apis mellifera*, may become opportunistic and habituated to these holes instead of visiting flowers legitimately ("positively"; Tasei 1976), from the front (see Stoddard 1987). In my field plots, where bees capable of biting holes in flower bases were absent, a few honey bees were observed to try valiantly to reach copious accumulations of nectar by squeezing through the petals of blossoms from the front, but although they could gather pollen, and trip flowers, because of insufficient tongue length, their nectar-seeking activities went unrewarded. Owing to the position of the tip of the nectary projection (from whence nectar is released initially, at least) being below the gynoecium and oriented 180° from the gaps of the androecium from which accumulated nectar must pass to reach the corolla, where legitimate nectar-gatherers seek it (see Fig. 11b of Beutler and Schöntag 1940), it would be interesting to determine whether glands bearing relatively-short projections, but capable of secreting very large quantities of nectar, would allow honey bees to reach the exudate successfully, and hence maintain a stronger interest by *Apis mellifera* in this crop species. Similarly, it might be worthwhile to determine whether
combinations of projection length and nectar-secretory capability of the gland can alter the frequency of flowers chewed and subsequently robbed from the base, to preserve the nectar photosynthate for "positive" foragers.

The second application concerns the studies of Wykes (1952c), wherein honey bees were shown to have preferences, while foraging at dishes of sugar syrup for solutions of sucrose/fructose/glucose at 1/1/1, above any other sugar alone. To my knowledge, this preference has never been rigorously tested within a single plant species under approximate field conditions. If the results of the culture experiments with *Vicia faba* can be extrapolated across other legume species bearing similar nectar-sugar ratios *in situ*, then manipulation of sugar proportions by culturing large numbers of relatively-small flowers (e.g., alfalfa, birdsfoot trefoil, red clover) as mature buds under conditions of different pH, temperature, etc., and then making these flowers (with others of similar age, from the parent plants, so that only nectar-sugar characteristics are variable) available for honey bee visitation, might provide valuable answers to the question of the preference of *Apis mellifera* for certain nectar-sugar ratios while foraging on crops.

### 3.5. SUMMARY

This investigation of the floral nectary of three cultivars of *Vicia faba* L. showed marked differences in the lengths of the nectary projections and in the total number and distribution of modified stomata on the projections.

A detailed examination of five selections of one of the cultivars (*Aquadulce*) revealed the existence of up to a two-fold difference in mean projection height, and a three-fold difference in average total number of modified stomata per nectary. Significant, positive linear relationships between projection height and total number of
stomata were determined for both cultured and in situ flowers.

When flowers of the selections were cultured on sucrose solution, there was a 4.5-fold difference in average amount of nectar sugar produced. Significant differences also occurred in nectar-sugar ratios (fructose/glucose, sucrose/hexose) in cultured versus in situ flowers of the same selections. The total number of modified stomata per nectary was inversely related ($r=-0.436$) to nectar sugar production. The percentage of the total modified stomata which were located on the top quarter of projections ranged from 35-82% between selections, on average. This fraction of the modified stomata borne on the tips of projections was positively correlated ($r=0.466$) with nectar sugar production. These last two correlations may prove helpful in agricultural breeding programs aimed at increasing nectar production and cross pollination of Vicia faba.
Figs. 3.1, 3.2. Scanning electron micrographs of representative floral nectaries of *Vicia faba* cv. Blandine and cv. Long Pod

**Fig. 3.1a,b,c.** Flower 1 from a plant of cv. Blandine.
- **a.** View from above. Note projection (Pj) and disk (D) of nectary which surrounds the gynoecium (G). Modified stomata (arrows) at projection tip. Insertion points of free stamen (FS) and staminal column (SC) are evident. Bar = 500 µm
- **b.** Staminal face of nectary projection (Pj) showing cylindrically-shaped epidermal cells oriented longitudinally, several modified stomata (arrows) at tip and one farther down (arrow). Bar = 250 µm
- **c.** Higher magnification of mid-region of nectary projection of **a,** showing small, cuboidal epidermal cells and two modified stomata (arrows). Bar = 100 µm

**Fig. 3.2a,b,c.** Flower A2 from a plant of cv. Long Pod.
- **a.** View opposite the gynoecial face of the nectary projection (Pj). Bar = 500 µm
- **b.** Higher magnification of **a,** demonstrating absence of modified stomata on the gynoecial face except for a few (arrows) at the extreme tip. Bar = 100 µm
- **c.** Staminal face of nectary with transverse band of modified stomata just below the projection tip. Bar = 200 µm
Fig. 3.3. Mature buds of *Vicia faba* cv. Aquadulce in vials at initiation of culture. A small amount of paraffin wax (arrowhead) added at the calyx base (after Shuel) held the buds vertically in place.
Fig. 3.4-3.8. Scanning electron micrographs of floral nectaries of *Vicia faba* cv. Aquadulce, Selection A

**Fig. 3.4.** Basal- and mid-regions of the gynoecial face of the nectary projection of flower A3 taken directly from field plot, with many immature modified stomata. Bar = 200 µm

**Fig. 3.5.** Staminal face of nectary projection from flower A21 of culture experiment. Note distribution of modified stomata extending from the tip to the mid-region. Bar = 500 µm

**Fig. 3.6a,b.** Flower A13 from culture experiment
  a. Mid-region of staminal face of nectary projection bearing numerous immature modified stomata. Bar = 50 µm
  b. Boxed area of a showing longitudinal file of three modified stomata, all of immature stage-2, which may have arisen sequentially from a single epidermal cell (E). Bar = 20 µm

**Fig. 3.7.** One side of mid-region of gynoecial face of projection of flower A1 sampled directly from the field plot, illustrating a chain of seventeen adjacent, predominantly-immature modified stomata among epidermal cells. One stoma is completely surrounded by guard cells (*), and the open arrows delimit a longitudinal file of four modified stomata. Bar = 50 µm

**Fig. 3.8a,b.** Flower A3
  a. Region near tip of the gynoecial face of Fig. 3.4, showing the intact outer cuticle (arrowheads) of two modified stomata of immature stage-3, and an open modified stoma with the pore (P) partially occluded by material (asterisk and double arrowheads). Note the ledges (arrows) and circumferential ridges (open arrows) along the outer walls of the modified stomata. Bar = 10 µm
  b. Maturing modified stoma near those of a, showing outer cuticle that has torn near the two end positions of the pore (P) (much of which is now evident), leaving a central strand (arrowhead) intact. Bar = 20 µm
Figs. 3.9-3.11. Scanning electron micrographs of floral nectaries of Selections D and E of *Vicia faba* cv. Aquadulce

**Fig. 3.9, 3.10.** Staminal faces of relatively short nectary projections of flowers of Selection D

**Fig. 3.9.** Flower 11 taken directly from plant D in the field. Few modified stomata (arrows) are below the top quarter of the projection, which has a broad tip. Bar = 200 µm

**Fig. 3.10.** Flower D6 from culture experiment, showing skewed projection tip. Staminal face bears a few modified stomata in the mid-region. Bar = 200 µm

**Fig. 3.11a,b,c.** Cultured flower E5

  a. Staminal face, illustrating stomatal density highest at the projection tip, with very few modified stomata on the lower three-quarters of the face. Bar = 200 µm

  b. Gynoecial face. Below the tip region, note modified stomata congregated along projection flanks, but their virtual absence on the face proper. Bar = 200 µm

  c. Tip of gynoecial face, showing numerous adjacent, mostly-mature modified stomata. Bar = 50 µm
Figs. 3.12-3.14. Scanning electron micrographs of floral nectaries of *Vicia faba* cv. Aquadulce, Selection K.

**Fig. 3.12a-e.** Flower K1, sampled directly from the field

a. Overview of nectary. Bar = 500 µm

b. Tip of gynoecial face of projection shown in a, showing many mature modified stomata with pores of wide aperture, especially along the perimeter of the face. Bar = 100 µm

c. Maturing modified stoma from box (left) of b, showing tearing of outer cuticle (arrowhead) from the cell-wall junction of the sister guard cells. An apical opening (star) is apparent here, at the junction with epidermal cells of the end walls of the guard cells of another modified stoma. Bar = 20 µm

d. Mature, transversely-oriented modified stoma from box (right) of b. Note subepidermal cell(s) beneath the pore (P) and obvious circumferential ridges (arrows) along outer walls of guard cells. Bar = 20 µm

e. Staminal face, showing but two (immature) modified stomata (arrows) below top quarter of projection. Bar = 200 µm

**Fig. 3.13.** Basal- and mid-regions of gynoecial face of nectary projection of field-flower K2, showing but ten immature modified stomata (arrows). Bar = 100 µm

**Fig. 3.14.** High magnification of slightly bi-lobed tip of nectary projection from cultured flower K12, demonstrating lack of modified stomata below the tip and edges of this staminal face. Bar = 50 µm
Fig. 3.15. Plots of the height of nectary projections of the five selections of *Vicia faba* cv. Aquadulce against the total number of modified stomata they bear, for *in situ* (A) and *in vitro* (B) flowers. Each point represents a floral nectary. The data were analysed, and the regression lines fitted, using Minitab (Ryan et al. 1985)
**Graph A**

- Equation: \( Y = 0.499 + 0.00380X \)
- \( N = 23 \)
- \( r = 0.769 \)
- \( P < 0.01 \)

**Graph B**

- Equation: \( Y = 0.437 + 0.00508X \)
- \( N = 24 \)
- \( r = 0.751 \)
- \( P < 0.01 \)
Fig. 3.16. Sample chart-recorded assay of (A) sucrose, fructose and glucose, and (B) glucose, then fructose, from filter-paper wick used to collect residual nectar from gland of field flower 1 of selection B of *Vicia faba* cv. Aquadulce. The wick was soaked in 1.0 ml distilled H₂O in an Eppendorf centrifuge tube, on ice, and shaken at regular intervals over 25-30 min before the tube was centrifuged for 3 min and the rinsate analysed.

In A, 10.0 µl of the rinsate was transferred by an ACCU-FILL 90 capillary pipette to the cuvette containing 200 µl Na acetate buffer and 5 µl invertase. During the 15 min incubation, the cuvette was agitated periodically, and 5 µl PGI, 5 µl G6PDH, 10 µl ATP and 20 µl NADP were added by Eppendorf pipettes. To shift the pH to alkalinity (7.6-7.8), 350 µl Tris-HCl buffer was introduced after the incubation period, along with 200 µl distilled H₂O. The latter was added because the cuvette had a minimum-volume requirement (730 µl) to obtain readings. The contents were well mixed and the cuvette inserted into the spectrofluorimeter. After stabilization, 5 µl HK was introduced by a plummer, to initiate the reactions. Total volume was 810 µl. Chart speed was 1 cm/min.

In B, 50 µl of the rinsate (five times the volume analysed in A) was mixed with 400 µl distilled H₂O, 350 µl Tris-HCl, 20 µl NADP, 10 µl ATP and 5 µl G6PDH, before insertion of the cuvette into the spectrofluorimeter. Addition of 5 µl HK initiated the quantitative determination of glucose. Total volume, 840 µl. Then, 5 µl PGI was introduced, for fructose-6-phosphate conversion to gluconate-6-phosphate. Total volume, 845 µl.

After correction for differences in quantity of rinsate analysed and in final volume, the quantity of sucrose was calculated by subtraction from A (214.2 µg, calculated from standards) of the fructose (14.40 µg) and glucose (12.38 µg) determined from the curves of B, to yield 187.42 µg of the disaccharide. For this sample, fructose/glucose = 1.16, and sucrose/hexose = 7.00.
Fig. 3.17A. Regression of total nectar sugar per flower, transformed to a natural-logarithmic scale, on the total number of modified stomata per nectary projection of cultured flowers of the five selections of *Vicia faba* cv. Aquadulce. Each point represents a floral nectary.

Fig. 3.17B. Regression of total nectar sugar per flower, transformed to a natural-logarithmic scale, on the fraction of the total modified stomata which are situated in the distal quarter of each nectary projection of cultured flowers of the five selections of *Vicia faba* cv. Aquadulce. Each point represents a floral nectary.

Data analysis was performed using Minitab (Ryan et al. 1985)
\[ Y = 6.087 - 0.0108X \]
\[ N = 24 \]
\[ r = -0.436 \]
\[ P = 0.035 \]

\[ Y = 3.666 + 2.431X \]
\[ N = 24 \]
\[ r = +0.466 \]
\[ P = 0.030 \]
CHAPTER 4

THE FLORAL NECTARY

OF ARABIDOPSIS THALIANA (L.) HEYNHOLD:

DEVELOPMENT, ANATOMY, VASCULATURE,

ULTRASTRUCTURE AND SECRETORY ACTIVITY

There are numerous references to the nectar tissue of the plant now known as Arabidopsis thaliana (L.) Heynhold. More recently, there are occasions where the tissue is illustrated, but not identified. Until 1935, all of these descriptions were textual and very general, although some had been supplemented with sketches. There were numerous descriptions in the nineteenth century. Rus (1832) did not distinct any nectary tissue ("kneippe Oelzern") in Arabidopsis thaliana. Ceballos (1872) found only lateral nectaries (opposite the stamens) in flowers of Dinophyllum Thallidum Cebal. Having overlooked the glandular tissue at the median position, opposite the long stamens, according to Bayre (1903). Under this same species name, Veltenovsky (1883) appears to have provided the first drawing (Table III, Fig. 41) of the lateral and median views of the nectary tissue in the flower base, showing that the stamens at both positions were connected, and that the greatest proliferation occurred at the lateral position at a fructifer lower to the sepal scale than at the median position. Glandular tissue was not found to link the nectary proliferations occupying median positions on the same side of the flower, however. Interestingly, Hallbrand (1879) stressed the absence of nectary tissue in Arabidopsis thaliana (pp. 11, 32, 33; Table 1 - Fig. 1.14).
4.1. **INTRODUCTION**

As is the case in many plant families, nectar is a major attractant for flower visitors (potential pollinators) to members of the Brassicaceae. Velenovsky (1883) identified nectary tissue in flowers of all of the 170 species of the family he examined. Within the family, nectaries are situated at the base of the androecium where their variation in size, shape, number and arrangement (Velenovsky 1883, Hildebrand 1879, Bayer 1905, Villani 1905, von Hayek 1911, Arber 1931a, Norris 1941) has been important for taxonomic purposes. The glands are directly supplied with vasculature consisting of phloem alone (Frei 1955, Kartashova 1965, Davis *et al.* 1986).

There are numerous references to the nectary tissue of the plant now known as *Arabidopsis thaliana* (L.) Heynhold. More recently, there are occasions where the tissue is illustrated, but not identified. Until 1988, all of these descriptions were textual and very general, although some had been supplemented with sketches. There were numerous descriptions in the nineteenth century. Kurr (1833) did not detect any nectary tissue ("keine Drüsen") in "Arabis thaliana". Celakovský, in 1872, found only lateral nectaries (opposite the short stamens) in flowers of "Stenophragma Thalianum". Celak., having overlooked the glandular tissue at the median positions, opposite the long stamens, according to Bayer (1905). Under this same species name, Velenovsky (1883) appears to have provided the first drawing (Table III, Fig. 41) of the lateral and median views of the nectary tissue at the flower base, showing that the tissues at both positions were connected, and that the greatest proliferation occurred at the lateral position at a location lower on the receptacle than at the median positions. Glandular tissue was not found to link the nectary protuberances occupying median positions on the same side of the flower, however. Interestingly, Hildebrand (1879) stressed the absence of nectary tissue in "Arabis Thaliana" (pp. 11, 32, 35; Table 1 - Fig. 1,1a).
Jordan (1886) described the process of nectar collection from the lateral parts of flowers of "Arabis Thaliana" by insects, and referred to median glands as "ohne Verrichtung (steril)" (literally, without function or performance).

Early in the present century, Bayer (1905) described connected nectary tissue between the median and lateral positions of the flower of "Arabis Thaliana L." and emphasised the variation in gland anatomy within the species. Initial flowers on an inflorescence, or late-forming florets on a multi-flowered raceme, often completely lacked secretory tissue, whereas those flowers which lacked a short stamen had additional glandular tissue in its place. In flowers of "Sisymbrium Thalianum L.", Villani (1905) described a continuous gland with proliferation at the lateral positions. Knuth (1908) reported that flowers of "Stenophragma Thalianum Celak." had six nectaries located at the insertions of the six stamens, but that "only the two at the bases of the short ones are functional, the other four being vestigial." Von Hayek (1911) sketched the floral nectary of "Stenophragma" ("Arabidopsis") as it would appear in telescoping view from the stigma (Table 9, Fig. 4b), and described slender connections between nectary tissue at the lateral and median positions that created a continuous glandular structure borne on the receptacle. Jaretzky (1928) found both median and lateral nectaries in "Stenophragma Thalianum Celak." and noted the morphological similarities in the glands to those of Arabis. Schulz (1936) reported that in "Arabidopsis Thaliana (L.) Heynhold" the lateral nectaries were partially or fully ring-shaped around the short stamens and were also connected with the median glands. Norris (1941) classified the glandular tissue of "Arabidopsis thaliana L." as a single annular nectary composed of a continuous zone around the receptacle. Similarly, Hegi (1958) found the nectary tissue to be united throughout the flower by narrow constrictions. Müller (1961) described the shape and location of lateral and median
nectaries in flowers of "Arabidopsis thaliana (L.) Heynh.", and found them to be more or less connected; the median nectaries appeared incapable of secretion. However, nectary tissue is conspicuously absent in his drawing of the flower of A. thaliana in Fig. 15b. Drescher and Kranz (1987) made reference to two "honey glands" on the receptacle. In a resin-embedded section of a flower of the Columbia ecotype, Davis (1988) appears to have published the first anatomical micrograph of nectary tissue in this species, showing dense, relatively-small parenchyma cells of the lateral portions of the gland, and stomatal pores in the epidermis. Bowman et al. (1989) reported the initial appearance of lateral nectaries at a floral stage when the stigmatic papillae first appear on the gynoecium, and reported that further nectaries develop later at the base of the other stamens. Hill and Lord (1989) published light and the first scanning-electron micrographs of nectary tissue in A. thaliana, but usually the glandular tissue was not identified as such. Smyth et al. (1990) reported that both median and lateral nectaries initiate later than the other floral organs, suggesting that their growth may be limited by available space and nutrients, and provided scanning electron microscopic evidence of the stomata on the nectary surfaces. Bowman and Meyerowitz (1991) stated that nectary tissue at the base of the short stamens matures approximately at the stage that papillae appear on the stigma. Bowman et al. (1991a) demonstrated the expression of the AG gene ("agamous") in fully differentiated floral organs, including nectary tissue at the base of a (probable median) staminal filament, and postulated that this gene may be involved in specifying cell fate late in flower development. Bowman et al. (1991b) referred to nectaries of sporadic presence at the base of stamens in wild-type and "pistillata" mutant flowers, and confirmed from ap2-2 homozygotes and the creation of the double mutant pi-1/pi-2 that nectaries can occur regardless of the presence of stamens, or any other third-whorl structures. Although it was not identified as such, Schultz et al. (1991) have provided unmistakable scanning-electron microscopic evidence of nectary tissue (Figs. 4A,B) in the homeotic floral mutant Flo10-1.
Currently there is considerable research interest in floral mutants of *A. thaliana*, especially in discovering the genetic and molecular basis of floral-organ development (Koomneef *et al.* 1983, Meyerowitz 1987, Komaki *et al.* 1988, Haughn and Somerville 1988, Bowman *et al.* 1989, Hill and Lord 1989, Kunst *et al.* 1989, Meyerowitz *et al.* 1989, Okada *et al.* 1989, Irish and Sussex 1990, Smyth *et al.* 1990, Smyth 1990, Bowman *et al.* 1991a, 1991b, Coen and Meyerowitz 1991, Drews *et al.* 1991, Goto *et al.* 1991, Meyerowitz *et al.* 1991, Schultz *et al.* 1991). Perhaps owing to its relatively small size and concealed position at the base of the flower, coupled with its manifestation relatively late in floral development, the floral nectary of *A. thaliana* has received only fleeting (if any) attention in these studies. In terms of plant reproductive biology, the importance of floral nectar secretion warrants a full investigation of the nectary tissue of this species. Accordingly, the present study was undertaken to clarify the status of the nectary and to investigate its development, anatomy, vasculature, ultrastructure and secretion, and to serve as a basis for further studies of the glandular tissue and nectar secretion in mutants of *A. thaliana*. In that light, some preliminary investigations of nectar secretion in the starchless mutant TC7 (Caspar *et al.* 1985) are reported here.

### 4.2. MATERIALS AND METHODS

#### 4.2.1. Plant material

Plants of *Arabidopsis thaliana* (L.) Heynh. var. Columbia were grown from seed in potting soil while exposed to continuous illumination (approx. 150 µmol/m²s at plant tops) from fluorescent lights, at 20°C. Flowers of different developmental stages were collected from many plants, for studies of the nectary. Because individual
4.5

races bear florets of various stages of development, it was often convenient to study units within an inflorescence, to minimize genetic variation.

4.2.2. **Bright-field microscopy**

Nectary tissue was dissected from freshly-harvested flowers and examined unstained or after exposure to solutions of iodine potassium iodide, Sudan black B or zinc chlor-iodide (Jensen 1962).

Resin-embedded sections of flowers were prepared from the primary inflorescences of plants. Prior to fixation, a petal was sometimes removed (e.g., Fig. 3.97a) to aid interpretation of flower orientation during serial sectioning. Because of their diminutive size, whole flowers were fixed overnight in separate vials and processed as described in section 2.2.4.1. Sections were cut at 1.5 - 2.0 µm thickness and stained with toluidine blue O (see 2.2.4.1).

4.2.3. **Scanning electron microscopy**

The technique of sequential scanning electron microscopy of live material, and the scanning electron microscopic examination of flash-frozen tissue, described in detail in sections 2.2.2 and 2.2.3, were also performed.

4.2.4. **Three-dimensional reconstruction of nectary vasculature**

The vascular supply to a lateral portion of one fully-developed nectary was reconstructed using a photographic enlarger and computer software (PC3D®, version 5.0, Jandel Scientific, Corte Madera, California, U.S.A.) which assembled the traces made of serial, 1.5-µm sections into a three-dimensional image. The image was drawn using a Roland DG (DXY-980A) plotter.

4.2.5. **Starch content of nectary stomata**

The relative quantities of starch in guard cells were estimated from light micrographs (3500x) of individual stomata observed in face view from dissected,
lateral portions of fixed nectaries stained with iodine potassium iodide. Each stoma was analysed three times using a transparent grid of 8-mm squares, and the mean number of intersection points covering starch and the guard cells (excluding walls and pore) determined.

4.2.6. **Fluorescence microscopy**

Resin-embedded sections were stained for polysaccharides with periodic acid-Schiff's reagent using acriflavine (pseudo-Schiff's reagent) (O'Brien and McCully 1981). Starch grains and nuclei stain green, whereas cellulose walls and nucleoli stain yellow-orange.

4.2.7. **Ultrastructural investigation**

Flowers of six developmental stages [0.9 mm bud, 1.5 mm bud, 1.9 mm bud, early-opening (still pre-secretory), open and nectar-bearing, and post-secretory] from many plants were fixed, embedded, sectioned and stained according to section 2.2.5 before nectary tissue was examined using Hitachi 500 or 600 transmission electron microscopes at 100 kV. The six floral stages correspond to stage 11, early stage 12, late stage 12, stage B2/B3, stage B4 and stage B5, respectively, according to the classifications of Müller (1961) and Smyth *et al.* (1990). The early-opening (B2/B3) stage is still pre-secretory, whereas the post-secretory (B5) stage no longer has nectar (because of its reabsorption), despite the lack of abscission of the lateral sepals or any other floral part. The buds lengths represent the distance from the deepest part of the pouch of the lateral sepals to the tip of the outermost median sepal.

Changes in cell area, vacuolation and organelle density throughout nectary development were determined by stereology (Steer 1981) from cells magnified 30,000 to 37,500 times. Micrographs of five epidermal- and ten subepidermal-cell profiles (chosen randomly) of lateral portions of nectary tissue were prepared from each of four flowers per developmental stage, and cell sections covered by a grid of 1.0-cm squares
such that the total cell area and that of the vacuome and cytoplasm could be estimated from counts of intersection points. Numbers of dictyosomes, microbodies, mitochondria and plastids per cell profile were counted and expressed per cytoplasmic area, for all developmental stages.

For the floral stadia of 1.9-mm bud to the post-secretory stage, plasmodesmatal frequencies were determined according to Gunning in Robards (1976) for anticlinal walls, sectioned transversely at 80 nm thickness, of epidermal cells, walls shared by epidermal and subepidermal cells, and walls shared by adjacent subepidermal cells. The average outer diameter of plasmodesmata was determined according to Robards (1976) for transversely-sectioned plasmodesmata in these thin sections.

4.2.8. **Nectar sugar production by cultured flowers**

Comparatively little nectar is produced by the small flowers of *A. thaliana*, and owing to the relatively open nature of the flower, the water of this nectar readily evaporates, leaving a very small volume of viscous exudate. Therefore, because of the technical difficulties of nectar collection from *Arabidopsis in situ*, it was decided to employ the high-humidity culture technique as outlined in section 2.2.9. Both the wild-type and a starchless mutant (TC7) derived from it (Caspar *et al.* 1985) were investigated for nectar sugar production.

Only buds at or near maturity, of 1.80-1.95 mm in length, were chosen for culture, as follows. Plants with a potentially-suitable bud were uprooted and the top of the primary raceme examined under a dissecting microscope. After measurement confirmed a bud’s qualification for the experiments, the rest of the inflorescence above the selected bud was removed by razor blade immediately above the junction made by that bud with the rachis. The raceme below the chosen bud was similarly removed by slicing the rachis just above the attachment point of the flower immediately below the
selected bud. Using tweezers, this final wound was placed at once through a small hole in the Nescofilm covering a freshly-prepared sucrose solution. All buds were taken from various positions on the primary inflorescence, from plants which were sown 32-39 days earlier. Ordinarily, five or six buds were cultured per vial. Vials were sealed in water-containing beakers and held in darkness at 25°C, exactly as described in 2.2.9.

Tissue remnants of the plants from which buds were taken for the experiments were placed in iodine potassium iodide solution and examined for starch. This precaution always was taken to confirm each plant’s starch status (as a safeguard against seed heterogeneity), because except for its starchless character, the TC7 mutant is phenotypically indistinguishable from the wild-type when grown in continuous light (and see Caspar et al. 1985).

Bud pedicels were inserted into either 0, 2 or 10% (w/v) sucrose solution, pH 5.6. Because of the relatively high humidity maintained within the beakers, nectar collected from the cultured flowers was representative of freshly-secreted nectar. Because of the dilute nature of the nectar, there was little doubt that close to all of the available exudate was collected when dabbed with filter paper wicks (McKenna and Thomson 1988), 24 h after these in vitro experiments were initiated. After initial attempts with anthrone (McKenna and Thomson 1988) and phenol-H2SO4 (Roberts 1979), nectar sugar and its composition were successfully determined by the enzymatic procedures outlined in section 3.2.3.

4.3. RESULTS

4.3.1. General anatomy of the nectary

In the mature flower, the floral nectary of A. thaliana is a continuous zone of glandular tissue which surrounds the base of the six staminal filaments (Fig. 4.1, 4.2). The nectary bears several lobes, the greatest proliferation of glandular tissue occurring
usually as bilobed outgrowths situated between the short stamens and the lateral sepals (Fig. 4.1, 4.3, 4.5). The nectariferous tissue of these lateral outgrowths narrows as it extends through the gaps between the petals and the short stamens (Fig. 4.3), the tissue connecting behind each short stamen (Fig. 4.2, inset of Fig. 4.3). The glandular tissue arches around the inner surfaces of the petal claws (Fig. 4.2-4.4) and then along the outer edges of the long stamens. Therefore, in *A. thaliana*, the glandular tissue continues uninterrupted around the entire androecium (Fig. 4.1, 4.2), so that technically there is only one nectary per flower. Multicellular lobes, up to four per flower, may arise at positions between the petal bases on the median sides of the flower, opposite the long stamens and facing the median sepals (Fig. 4.2, 4.4).

Stoma-like structures commonly are found on the extremities of these nectary outgrowths (Fig. 4.3-4.6) and allow nectar to pass to the exterior. Nectary stomata of the lateral outgrowths face toward pouches formed by the lateral sepals (Fig. 4.5, 4.6), where most of the nectar accumulates.

### 4.3.2. Nectary development

Classification of the floral stages examined for nectary development follows that system recently proposed by Smyth *et al.* (1990). Here, however, bud length was measured from fresh material (i.e., not after preparation for conventional scanning electron microscopy), using a dissecting microscope, as the distance from the bud tip to the depression of the lateral sepal pouches.

#### 4.3.2.1. Lateral outgrowths of the nectary

Development of nectary tissue at the lateral positions occurs relatively late in floral development, likely from initials present in buds of stage 7 to early stage 9. Before stage 7 there is virtually no space between the elongating short stamen and lateral sepal. Thereafter, as the developing anther of the short stamen becomes stalked,
a spatial separation appears between this lateral stamen and sepal (Fig. 4.7a,b, 4.8b). Small numbers of epidermal cells, which may be nectary initials, occur there.

Unmistakable glandular tissue, now developing as small swellings, is evident from buds late in stage 9 and in stage 10 (Fig. 4.9a, 4.10). A significant proportion of buds lacks one of the short stamens (Bayer 1905, Müller 1961, Smyth et al. 1990); in that case, the differentiating nectary is particularly noticeable at that lateral position, as can be seen by comparing Fig. 4.9b and 4.11b with Fig. 4.9c and 4.11c, respectively. As well as the developing outgrowths external to the short stamens, buds late in stage 9 to early in stage 11 already exhibit a few layers of cells extending around the base of the short stamen, between the growing petals (Fig. 4.11c). By late in stage 11 and early in stage 12, divisions of the nectary cells have resulted in the typical bilobed appearance of the lateral portions of the nectary (Fig. 4.12, 4.13, 4.14a, 4.16). Extensions of nectary tissue between the short stamen and the petals are now well established (Fig. 4.14b, 4.15, 4.16). Variation exists in the total amount of nectary tissue between buds of the same length (Fig. 4.14a, 4.16).

Concurrent with nectary expansion, the lateral sepals begin to differentiate pouches early in stage 11 (Fig. 4.11a) through to stage 12 (Fig. 4.5, 4.19a). Before these developmental stages, bases of the four calyx members are indistinguishable (Fig. 4.8a, 4.9a).

By the time buds reach 1.5 mm in length - about the middle of stage 12 - cell divisions have occurred to encroach into the region between the two nectary lobes (compare Fig. 4.16 with Fig. 4.17, 4.18). The lobes themselves continue to grow (compare Fig. 4.15 with 4.19b) by expansion and divisions. Epidermal cells now bear distinct ridges on their external walls (Fig. 4.17).

All regions of the lateral portion of the gland continue to expand for the duration of stage 12 (compare Fig. 4.26 with Fig. 4.19b; Fig. 4.20b,c with Fig. 4.18), as the
petals extend above the long stamens (Fig. 4.19a).

Late in stage 12, as the bud reaches maturity, increases in size of the lateral parts of the nectary are less dramatic, but continue while the flower opens (compare Fig. 4.20b with 4.21b). Once nectar secretion commences, nectary growth ceases (Fig. 4.21b-4.24b).

During the few days after which nectar secretion has ended, the androecial and perianth components begin to wither and finally abscind (Fig. 4.28). On the other hand, the lateral portions of the nectary persist at the base of the expanding silique (Fig. 4.24a, 4.25a, 4.28-4.31). Two to three days after abscission begins, these remaining lateral portions of the nectary collapse (Fig. 4.29-4.31) due to cell deterioration internally (Fig. 4.25b, 4.82, 4.83, 4.182). Concurrently, epidermal cells of the abscission zones expand into bulbous, rounded forms that surround and lie between the two lobes of the lateral parts of the nectary (Fig. 4.25a, 4.28-4.31). The epidermal cells of the nectary, like those in the abscission zones, continue to deposit a waxy product over their cuticles that gradually accumulates over the entire cell surface (Fig. 4.29-4.31). Initially the greatest accumulation occurs on the relatively exposed nectary parts (Fig. 4.32a,b). The wax appears to be deposited directly over the initial ridges of external cuticle seen in Fig. 4.27 and 4.28 (Fig. 4.32b).

4.3.2.2. Median outgrowths of the nectary

Like the bilobed portions of the nectary which surround the short stamens, nectary tissue at the median flower positions is found only after the sepal, petal, androecial and gynoecial primordia have differentiated. Nectary initials are obvious late in stage 9 (Fig. 4.36a-c), but could not be detected with certainty before then, where spatial restrictions between the petals and tetradynamous stamens are prohibitive (Fig. 4.33-4.35). Early in stage 11 (Fig. 4.37a), nectary cells are continuous between the filaments of the long stamens on each median side of the flower and begin to
extend between each long stamen and petal (Fig. 4.37b,c). However, not until late in stage 11, as the petal height approaches that of the tetradynamous stamens (Fig. 4.38a) does the nectary tissue extend as small outgrowths through the gaps between the petal and long-stamen bases (Fig. 4.38b-d), to connect with nectary tissue from the lateral sides of the flower, thus forming a continuous gland.

In glandular tissue of buds entering stage 12 (Fig. 4.39a), both periclinal and anticlinal divisions occur as the median portions of the nectary increase in size (Fig. 4.39b). These divisions may continue, particularly at discrete positions opposite the long stamens but adjacent to the petal claws, such that prominent multicellular outgrowths (Fig. 4.40, 4.41) may number four per flower (Fig. 4.2). These median projections are connected by up to four layers of glandular cells (Fig. 4.4).

Like the lateral portions of the nectary, stoma-like structures may occur on these median outgrowths (Fig. 4.42 right, 4.44, 4.45, 4.84). Other median projections bear enlarged or flattened epidermal cells of various shapes (Fig. 4.41 left, 4.43).

On post-secretory flowers, nectary outgrowths at the median positions may differ in shape, length (Fig. 4.46-4.48) and presence, even on the same flower (Fig. 4.2, 4.3, 4.48a,b, 4.49b, 4.51, 4.115). They have a similar fate to their lateral counterparts, the median portions eventually collapsing because of internal deterioration (Fig. 4.45, 4.84) and becoming surrounded by bulbous cells in the abscission zones (Fig. 4.42, 4.49a, 4.50) with their epidermal ridges (Fig. 4.47, 4.48a,b) becoming covered with wax (Fig. 4.49a, 4.50).

4.3.3. **The modified stomata**

4.3.3.1. **Ontogeny and division of guard mother cells**

The ontogeny of modified stomata in Arabidopsis thaliana is not synchronous with development of the floral nectary. Therefore, at any one time various developmental stages of modified stomata (see Table 2.1 for description of stages of V.
faba, page 2.12) can be detected close to one another on a single nectary (Fig. 4.17, 4.52a, 4.53a), and immature modified stomata may occur on post-secretory (Fig. 4.28, 4.60, 4.61, 4.68), even deteriorating (Fig. 4.67), nectaries. Modified stomata may develop from guard mother cells adjacent to one another (Fig. 4.57), such that modified stomata often exist side-by-side (Fig. 4.17, 4.60, 4.169, 4.172, 4.173, 4.180d, 4.181, 4.183). Adjacent epidermal cells do not become morphologically distinct as subsidiary cells (Fig. 4.54a, 4.55a).

The guard mother cells originate as diminutive, four- or five-sided cells that, after rounding up, appear elliptically-shaped amidst larger epidermal cells (Fig. 4.52b, 4.56). Internally, these cells are largely cytoplasmic, the vacuome being composed of numerous small vacuoles (Fig. 4.57, 4.65). Externally, the central region of the guard mother cell is concave (Fig. 4.52b, 4.56) and, on nectaries advanced in development, only the periphery of the guard mother cell displays the prominent ridges of ordinary epidermal cells (Fig. 4.52b).

Division of each guard mother cell yields two guard cells approximately equal in size (Fig. 4.65) - a modified stoma of immature stage-1 (see Table 2.1, page 2.12). The division is not evident externally at this time (Fig. 4.65), but becomes visible when the pore starts to form as a shallow depression in the new wall (compare Fig. 4.52b with 4.53b; Fig. 4.54b).

4.3.3.2. Features of guard cells

4.3.3.2.1. Microtubules and cell wall

Microtubules are found in the cytoplasm of guard cells directly below the plasma membrane (Fig. 4.76, 4.80). The microtubules radiate from the pore site (ventral walls) (Fig. 4.76) toward the dorsal and end walls, the microfibrils nearest the plasma membrane showing general congruence with the microtubules (Fig. 4.76).
In transverse sections of guard cells of modified stomata in which the pore has developed, the various walls vary in thickness (Fig. 4.71-4.73, 4.81). The inner walls (for terminology, see Fig. 2.41) are thickest, particularly in the regions nearest the ventral walls, with the corresponding position in the outer walls being next thickest. The dorsal walls are thinnest. In longitudinal section, the inner and outer walls of the guard cells are thickest at their midpoints (Fig. 4.77-4.79).

Ridges of wall material covered by cuticle are located on the outer walls of guard cells, but only on the peripheral portions and near the end walls (Fig. 4.53b, 4.69, 4.71-4.73, 4.79). These ridges can remain from the guard mother cell stage (Fig. 4.52b) and contain narrow files of wall microfibrils (Fig. 4.71) encased by cuticle, which in fresh material stains black with Sudan Black B. Less pronounced undulations in the external surfaces of guard cells can occur along the ventral walls lining the pore (Fig. 4.71-4.73, 4.78, 4.80) and along the inner walls lining the substomatal space (Fig. 4.71, 4.77, 4.79). The cytoplasmic face of the inner walls may also be very uneven (Fig. 4.80).

Despite an active search for them, no plasmodesmata nor their remnants were detected between guard cells, or between guard and adjacent cells. Admittedly, there were relatively few immature modified stomata available for investigation in A. thaliana, in contrast to the floral nectaries of V. faba, where modified stomata are more abundant (see Chapter 2). Still, the lack of plasmodesmata in mature modified stomata of the nectary agrees with previous studies (Brassica napus - Davis 1985, Davis et al. 1986; Vicia faba - Chapter 2).

4.3.3.2.2. Development of the pore and substomatal space

The substomatal space begins to form soon after guard mother cell division, first opening directly below the future pore, and the dorsal and end walls of the guard cells.
Initiation of this space precedes or coincides with pore development, the latter first becoming visible externally as a slight central depression on the surface of modified stomata of stage immature-1 (Table 2.1, page 2.12) seen in Fig. 4.53b and 4.54b. Separation of the shared wall proceeds inwards and towards the ends of the guard cells of modified stomata of stage immature-2 (Fig. 4.55b, 4.58, 4.59) along a pre-formed electron-translucent layer like the one commencing in Fig. 4.68. Formation of the pore is most advanced at the inner/ventral walls (Fig. 4.69, 4.71), which have thickened (Fig. 4.66, 4.69). Expansion of the guard cells (compare Fig. 4.54b with 4.55b) continues as a single large vacuole forms per cell (Fig. 4.69, 4.180c,d). During this stage an osmiophilic material is laid down over the resulting exposed surfaces of the guard-cell walls (Fig. 4.70, 4.71), as the original cuticle separates from the microfibrils (Fig. 4.70, 4.71).

Eventually the ventral walls separate, leaving an intact pore covered only by the original, external cuticle (Fig. 4.72) stretching between the outer ledges: immature stage-3 (Table 2.1). Variation is common, the outer cuticle being found taut across the pore (Fig. 4.60 right), undulating (Fig. 4.72), concave (Fig. 4.61, 4.71) or depressed within the pore (Fig. 4.52b left, 4.60 left, 4.73). These differences may be due to local, external influences on the cuticle caused by expanding, adjacent guard or epidermal cells, or by nectar accumulation from below. There are also marked differences in the quantity of cuticle deposited at the outer ledges (Fig. 4.63, 4.77). Evidence of previous cuticular rupture at the inner ledges of the guard cells is found in Fig. 4.71. In fresh material placed in zinc chlor-iodide, the cuticle stains bright yellow. Expansion of the substomatal space continues (Fig. 4.72 right).

Maturation of the modified stomata (Fig. 4.64) is achieved with rupture of the external cuticle (Fig. 4.53b left, 4.62, 4.63, 4.73). A complete cuticle-covered (Fig. 4.73-4.80) passage is now manifested from the exterior to the substomatal space. The space below the open pore may be narrow, like that shown in Fig. 4.89b.
4.3.3.3. *The occurrence of open stomata and specialized substomatal cells on post-secretory nectaries*

Nectar is no longer found at the lateral sepal pouches when the stigma extends a few millimetres above the petal tips [e.g., Müller's (1961) stage B5]. Examination of sections of deteriorating post-secretory nectary tissue revealed that the pores of modified stomata are open at a range of apertures, or are closed (Fig. 4.25b, 4.81-4.84). The same conclusions were drawn when post-secretory nectaries were observed with low-temperature scanning electron microscopy. Despite the technique’s preservation of the dense covering of waxy globules over guard cells and other nectary epidermal cells (Fig. 4.85-4.87), and cells of the abscission zone, pores of relatively large aperture were detectable because of the lack of wax accumulation there (Fig. 4.87). Results of this investigation did not reveal any counterpart of the occluding film frequently encountered in post-secretory nectaries of *Vicia faba* (Chapter 2). Rather, the only evidence for occlusion of the modified stomata of the floral nectary of *A. thaliana* would be by accumulation of the waxy globules over shut or narrow pores.

Densely-staining subepidermal cells immediately below most modified stomata of both median and lateral portions of the nectary (Fig. 4.45, 4.72, 4.81, 4.82, 4.84, 4.88a, 4.89a) are found late during the secretory, and then during the post-secretory, stages. Paradermal sections through modified stomata of the nectary reveal that these specialized subepidermal cells line the substomatal cavities (Fig. 4.89b). Apart from their staining intensity and location, the cells are distinctive by their unique abundance of vesicles (possibly of dictyosomal origin) and the presence of large amorphous crystals, throughout the cytoplasm (Fig. 4.88, 4.91). Most vesicles appear clear, while larger ones are opaque (Fig. 4.88b, 4.89c). Instances suggestive of vesicle fusion with, or detachment from, the plasma membrane at positions adjacent to other cells and at the substomatal or intercellular spaces, are common (Fig. 4.89c, 4.91). The
cytoplasmic crystals are densest at their cores (Fig. 4.88b,c, 4.91) and their pattern is
dissimilar to the orderly arrangement of smaller, vacuolar crystalline bodies (Fig. 4.90)
in the same cells. A further disparity of these specialized cells is the presence of some
plastid starch (Fig. 4.88b), a rare occurrence in subepidermal cells of post-secretory
nectaries.

4.3.4. **Vasculature**

The floral nectary of *A. thaliana* is supplied directly by vasculature consisting
only of phloem. The various regions of the gland are vascularised to different degrees,
the lateral portions being innervated most extensively.

4.3.4.1. **Lateral outgrowths of the nectary**

In the lateral parts of the gland of buds less than 1.8 mm long, mature sieve
elements were not detected in the gland interior (Fig. 4.92, 4.93, 4.120). In buds 1.9
mm or longer, and especially in flowers open and of various post-secretory stages,
mature sieve elements penetrated the interior of the lateral sections of the nectary (Fig.
4.94 - 4.96, 4.98i,j, 4.99a-h, 4.100, 4.101, 4.115).

The vascular supply to the nectaries of six wild-type flowers of the same raceme
was studied from serial sections (Fig. 4.115). At the time of fixation, all flowers
sampled were currently secreting or had secreted nectar, conditions which would
suggest that the nectary vascular supply had reached maturity. A computer-assisted
reconstruction (Fig. 4.100) of the vascular supply to a bilobed lateral outgrowth of a
transversely-cut wild-type flower (Fig. 4.98, 4.99; top of flower E in Fig. 4.115)
demonstrated that the vascular connections underwent branching within the gland. In
this instance, up to five separate traces of various length ended blindly in the glandular
tissue per lobe, the terminal sieve element of each trace often being dilated (Fig.
4.100).
The origin of the phloem supply to the lateral portions of the nectary can vary, even within the same gland. The phloem, which only enters the lateral portions of the nectary at the bases, originates from up to three receptacular vascular bundles. These are designated X₁, X₂A and X₂B in Fig. 4.98d and 4.98j. All nectaries studied were supplied by phloem alone branching from X₁ (Fig. 4.95, 4.98f,h, 4.115), the bundle which becomes the major (central) vein of the pouch-like, lateral sepals. The X₁ bundle, in which the xylem overlies the phloem, arches up and then down into the lateral sepal (Fig. 4.93 left). Therefore, in order that phloem reaches the lateral regions of the nectary, sieve elements with adjacent companion cells depart from the mainstream phloem and xylem (which both continue as X₁ into the lateral sepal; Fig. 4.98a,c) to both sides at a point below the summit of the vascular arch, on the receptacular side of the arch (Fig. 4.98h). One or two departing strands of phloem enter each lobe of a bilobed, lateral portion of the nectary, although the extent of vascularisation is neither necessarily symmetrical nor equal (Fig. 4.115, flowers D, E, F). The most extreme case detected is shown in the three-dimensional reconstruction of a lateral nectary (Fig. 4.100, which is the top portion of flower E in Fig. 4.115), where part of the X₁ bundle to the right lobe in Fig. 4.100 crossed the base of the lobe to join phloem from the X₂A bundle. The left lobe of this lateral outgrowth of the nectary, however, received a reduced vascular supply from the X₁ bundle. There also does not appear to be any consistent pattern in the vascular systems derived from the X₁ bundles between lateral outgrowths of the same nectary (Fig. 4.115).

Variation in vascular supply to the lateral part of a nectary also occurs with the amount of phloem leaving the vascular bundles X₂A and X₂B (e.g., top of flower D in Fig. 4.115). These two large bundles donate xylem and phloem for the marginal, major veins of both the lateral and median sepals, before reaching their destiny - the petals. Either before or after both xylem and phloem depart from X₂A and X₂B for the marginal major veins of the lateral sepals, one or two files of sieve elements with
companion cells depart from this phloem for the nectary (Fig. 4.100, 4.115). These files supply the nearest lobe of a lateral portion of the nectary, entering at its margin. Inside the gland, these traces may undergo branching and can head toward the central part of the nectary where they may come into close association with phloem traces originating from bundle X₁ (Fig. 4.100, rightmost lobe). Sometimes the phloem traces to the gland from bundles X₂A or X₂B are weak (e.g., top right of flower D, Fig. 4.115) or do not branch (e.g., bottom left of flower F, Fig. 4.115), or are lacking altogether (e.g., top of flower F, Fig. 4.115).

The lateral parts of the nectary are only innervated by phloem from below (Fig. 4.95), not from above. The vascular bundle X₃, which supplies the short stamen (Fig. 4.95, 4.99d,j, 4.100), and X₄, which supplies the gynoecium (Fig. 4.95, 4.99j), originate from a source shared with X₁ (Fig. 4.93 left, 4.95). No vascular connection to the nectary was ever detected from X₃ (or X₄). Adaxially, therefore, the lateral outgrowths of a nectary do not receive vasculature. Nonetheless, the phloem traces entering the nectary on the abaxial side do extend upward (Fig. 4.96, 4.99c-4.99h, 4.100).

4.3.4.2. Median outgrowths of the nectary

Compared to the lateral outgrowths, the median protrusions of the floral nectary of A. thaliana always receive a reduced supply of vasculature. For instance, the most deeply-penetrating supply of phloem that was detected in a median outgrowth is shown in Fig. 4.97b, wherein a single sieve element extended only into a few basal layers of the outgrowth. Almost exclusively, this phloem originates from the central, main vein at a point before this major vascular bundle, consisting of both xylem and phloem, enters the median sepal. These sieve elements and companion cells directed toward the median nectary protuberances branch from this central major bundle laterally, at the point where the sepal connects with the receptacle (Fig. 4.97c,d). In only one case was a direct supply of phloem evolving from a marginal vein of a median sepal noticed (top
left of flower D, Fig. 4.115).

As well as differing from the lateral outgrowths in their extent of vascularization, the median outgrowths also show greater variation in the existence of a direct supply of phloem. For example, even on the same nectary, phloem may only supply one side of the gland’s median processes (Fig. 4.115, flowers B-F), often to different degrees (Fig. 4.115, flower F). Furthermore, in flower D (Fig. 4.115), only one of the two vascular bundles supplying the large marginal veins of the median sepal was involved in providing a direct supply of phloem to a median process.

4.3.4.3. **Ultrastructure**

The phloem supply to the nectary consists of sieve elements, companion cells and adjacent parenchyma. Sieve elements thought to be maturing contain a cytoplasm that stains less densely than surrounding companion and parenchyma cells (Fig. 4.102 top). Mature sieve elements have a conspicuous lumen with parietal cytoplasm, are anucleate and contain mitochondria, plastids and stacks of endoplasmic reticulum (Fig. 4.101, 4.102 centre, 4.103, 4.104, 4.113, 4.114).

Adjacent sieve elements are connected by sieve plates. Sieve pores are constricted by electron-lucent regions (Fig. 4.101, 4.111, 4.112). Fluorescent regions within the walls between contiguous sieve elements stained with aniline blue suggest the presence of callose. The pores bear material resembling p-protein (Fig. 4.111, 4.112), which is also commonly found in the cell lumen (Fig. 4.102 centre, 4.103, 4.104, 4.107, 4.109).

Compound plasmodesmata occur between sieve elements and neighbouring cells of similar dimensions, the companion cells (Fig. 4.102 top, 4.110). The ribosome-rich companion cells stain densely (Fig. 4.101, 4.102, 4.104, 4.107-4.110). Dictyosomes, endoplasmic reticulum, mitochondria and plastids are also common (Fig. 4.102, 4.107-4.110); vacuoles are small. Wall ingrowths characteristic of transfer cells (Pate and Gunning 1972) were not encountered. Plasmodesmata also connect adjacent
Table 4.1. Number of wall junctions per cell and the percentage of those junctions which were intercellular spaces bordering epidermal, subepidermal, sieve-element or companion cells in thin sections of lateral outgrowths of the nectary from six developmental stages [designated according to Müller (1961) and Smyth et al. (1990)] of wild-type flowers of Arabidopsis thaliana. For the components of the nectary supply of phloem, a distinction was made between cells (Basal) which bordered large intercellular spaces as shown in Fig. 4.98j and 4.105-4.108, and those cells (Basal) otherwise within two cell layers of the nectary base.

<table>
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<tr>
<th>FLORAL STAGE</th>
<th>Bud 0.9 mm (stage 11)</th>
<th>Bud 1.5 mm</th>
<th>Early-Opening 1.9 mm</th>
<th>Nectar-Bearing (early stage 12)</th>
<th>Post-Secretory (late stage 12)</th>
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<td>Interior</td>
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<td>No. intercellular spaces ( % )</td>
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</table>
companion cells (Fig. 4.109).

Phloem parenchyma cells, which are larger than companion cells and sieve elements, may be connected to them by plasmodesmata (Fig. 4.104, 4.109, 4.114, 4.161). These parenchyma cells are less densely staining than companion cells and they bear larger vacuoles (Fig. 4.102, 4.110).

A noteworthy feature of the phloem traces that extend to the lateral portions of the nectary from vascular bundles $X_{2A}$ and $X_{2B}$ (Fig. 4.98j) is that the sieve elements, companion and parenchyma cells are in direct contact with large intercellular spaces below the nectary proper (Fig. 4.98j, 4.105, 4.106; Table 4.1 - Basal IS). Examination of resin-embedded sections stained with Sudan Black B has shown a very narrow, faint-positive layer between the intercellular space and the exposed cell walls of phloem. With transmission electron microscopy, a very thin (20 nm) layer resembling a cuticle is resolved at this location (Fig. 4.107, 4.108). These large intercellular spaces commonly contain products such as starch grains and membrane-bound material (Fig. 4.107, 4.108), suggestive of cellular disintegration.

Intercellular spaces, though much smaller, can be found associated with nectary vasculature in the gland interior, adjacent to both sieve elements (Fig. 4.103, Table 4.1) and companion cells (Fig. 4.101, Table 4.1).

4.3.5. **The relationship between the extent of vascular supply and the occurrence of modified stomata**

Figure 3.115 shows sketches of the nectary of six flowers, in telescoping view, from the same raceme. The drawings include the position of relative proliferation of glandular tissue and the corresponding number of modified stomata and vascular supply. From this information, the following three generalizations were drawn:

1) All lateral outgrowths of the nectary bore modified stomata and received
phloem (although I have seen one, during other work, without any modified stomata); 2) Within a flower, the number of modified stomata on a nectary process is a poor indicator of the vascular supply to the process. For instance, the bottom-right region of the nectary in flower F shows a lobe of a lateral outgrowth, and a nearby median outgrowth, each to bear the same number of modified stomata (four). However, there is a tremendous discrepancy in phloem innervation to each of these proliferations of glandular tissue, the median one receiving essentially none; and 3) At the same position of nectary tissue (i.e., lateral or median) of the same flower, there appears to be a positive relationship between the number of modified stomata borne by an outgrowth and the extent of the vascularization it receives. This relationship is most obvious for median protuberances. The presence of modified stomata on them always was indicative that they received at least some rudimentary vascular supply. However, the median outgrowths lacking modified stomata could still be supplied in this same, limited fashion (e.g., flowers B, D, F). The lateral outgrowth of the nectary (top of flower F) that lacked vascular connections from the petal bundles had fewer total modified stomata than its fully-supplied counterpart opposite.

4.3.6. **Cellular changes during nectary development, including changes in fine structure and in starch content**

4.3.6.1. **Lateral portions of the nectary**

4.3.6.1.1. Changes in fine structure of cellular contents

Epidermal and subepidermal cells of the highly-secretory, lateral portions of the floral nectary of *Arabidopsis thaliana* were examined stereologically, at random, at six stages of flower development (see section 4.2.7; Fig. 4.116). The epidermal cells selected were neither plasmolysed nor guard cells, and the subepidermal cells were not plasmolysed or of the specialised type shown in Fig. 4.88, and were non-vascular. At
the youngest bud stage (0.9 mm) investigated, cell volume of the epidermal and subepidermal cells is identical, despite the epidermal cells being dorso-ventrally flattened compared to the isodiametric glandular cells below them. Intercellular spaces are already evident between these cell types, at this young bud stage (Fig. 4.117, 4.119). In 0.9-mm buds, both cell types are similar cytologically (Fig. 4.116) except for the absence of starch in plastids of epidermal cells (Fig. 4.117, 4.119, 4.133). No starch grains are evident in nectaries of small buds of the starchless mutant TC7 (not shown).

However, when buds reach 1.5 mm in length, there are many noteworthy differences between epidermal and subepidermal cells. Expansion of the former, concurrent with more frequent divisions of the latter, probably account for the larger volume of epidermal cells at this stage (Fig. 4.116, 4.120). In epidermal cells, a single large vacuole (Fig. 4.120) now occupies three-quarters of the cell volume (Fig. 4.116), whereas the subepidermal cells usually remain multivacuolate, their vacuome still averaging less than half the cell volume (Fig. 4.116, 4.120). The nucleus of epidermal cells now resides near the inner periclinal wall (Fig. 4.120). Epidermal cells show no changes in number of mitochondria or plastids, whereas subepidermal cells contain 1.25 times more of each organelle in 1.5-mm than in 0.9-mm buds. Therefore, subepidermal cells now contain 1.8 times more mitochondria and 1.5 times more plastids than those of the epidermis. Dictyosomes are still apparent in both cell types and are 1.6 times more common in subepidermal cells, but remain relatively infrequent (Fig. 4.116). Plastids of epidermal cells now contain their largest quantity of starch throughout nectary development (Fig. 4.133). More starch grains are found in subepidermal cells (Fig. 4.120-122), especially close to the epidermis. Starch is absent in only half of all plastids in section (Fig. 4.133), but is completely absent in buds of the mutant TC7 (Fig. 4.123, 4.126). The original, smooth external surface of epidermal cells (Fig. 4.14b, 4.16, 4.117) is now studded with wall ridges (e.g., Fig.
4.24

4.146), covered by cuticle (Sudan black-B positive), that particularly decorate the distal-most cells of the nectary (Fig. 4.27, 4.28, 4.120).

In maturing buds (1.9 mm), as compared to 1.5-mm buds, subepidermal cells of the lateral portions of the nectary have expanded by over 60% in average sectional area, such that these cells now exceed the average sectional area of the cylindrical epidermal cells (Fig. 4.116). The large, solitary vacuole of each epidermal cell now occupies almost 80% of the cell's volume, double that of the multivacuolate (Fig. 4.124, 4.125) cells below (Fig. 4.116). Mitochondrial and plastid numbers remain constant in epidermal cells, with a low percentage of plastids harbouring starch (Fig. 4.133). The quantity of mitochondria in subepidermal cells does not increase after buds reach 1.5 mm long, so these cells still contain almost twice as many mitochondria as epidermal cells. Plastid numbers, on the other hand, increase rapidly (1.65 times) in subepidermal cells during this period; there are now 2.05 times as many plastids per cell than in young (0.9 mm) buds. Plastids are now 2.5 times as frequent in subepidermal than in epidermal cells. As a result of these changes, the ratio of mitochondria/plastids in subepidermal cells has dropped below two (to 1.7) for the first time (Fig. 4.116). During this explosion in plastid numbers, a high proportion of these organelles still bears one or more starch grains (Fig. 4.124, 4.125, 4.127, 4.133). These amyloplasts contain thylakoid membranes but grana are rare (Fig. 4.124). The pale-green colour of the glandular tissue may be attributable to low chlorophyll levels. No starch is detectable in glandular tissue of the TC7 mutant (Fig. 4.128). Dictyosomes are very infrequent in mature buds (Fig. 4.116).

When the growing petals first separate the sepal tips, the flowers do not yet bear nectar. There are few changes that have occurred since the last stage (1.9-mm buds) in the cell types investigated here. Both epidermal and subepidermal cells have continued to expand, now at similar rates. The vacuome, and the numbers of dictyosomes,
mitochondria and plastids per cell, have remained static (Fig. 4.116). The higher percentage of plastids that lack starch grains (Fig. 4.133), despite no further net change in plastid numbers, suggests that dissolution of starch in the subepidermal cells may be occurring. In fact, there is evidence of internal fractionation/fragmentation ("endocorrosion") of starch grains, for the first time. A high concentration of starch at the nectary base (e.g., Fig. 4.162) suggests a phloic origin of the precursors of these deposits; mature sieve elements are found there at this floral stage (see section 4.3.4.1). By this early stage of anthesis, the intercellular spaces have enlarged (Fig. 4.129).

Nectar is readily detectable in open flowers with petals beginning to reflex. During nectar secretion, subepidermal cells, and especially epidermal ones, decrease in cell volume. This decline in size may be due to cellular water loss from the vacuoles to the nectar; both cell types have reduced vacuomes during this period (Fig. 4.116). Subepidermal cells remain multivacuolate (Fig. 4.132). Occasionally, nectaries lose water excessively such that their outer cell layers show plasmolysis. For the first time in epidermal cells, increases occur in both the number of mitochondria (1.35 times) and plastids (1.65 times) per cell. Like epidermal cells, the subepidermal ones also display a reduction (to less than 1.3) in the ratio of mitochondria/plastids during nectar secretion. Here, however, the numbers of mitochondria have remained constant while plastid numbers have continued to rise (by 1.35 times) since the earliest stage of anthesis. Misrepresentation of true plastid numbers, because the irregular shape of some plastids (Fig. 4.132 bottom left) sectioned fortuitously could cause profiles from individual organelles to be counted more than once, may be offset by the general reduction in plastid size resulting from starch degradation within them (Fig. 4.132). Over 90% of plastid profiles of subepidermal cells now lack starch (Fig. 4.133). Within the lateral parts of the nectary, the disappearance of starch begins distally and
proceeds basipetally, so that generally the last starch to be metabolized is located at the nectary base (Fig. 4.130, 4.135, 4.136). The relative rapidity of starch dissipation is clearly evident in two consecutive flowers on a raceme; the flower in Fig. 4.163 was still at an early stage of anthesis, whereas the older flower, in Fig. 4.164, bore nectar. Again, no starch is detectable within glandular tissue of the mutant TC7 (Fig. 4.131). Dictyosomes continue to be extremely rare in both cell types (Fig. 4.116). In nectar-bearing flowers, profiles of stacked endoplasmic reticulum (Fig. 4.74, 4.118) are evident in 15% of epidermal cells. This minority of cells is thought to represent the transition from ordinary epidermal cells to those densely-staining, specialised cells in the region of the modified stomata, which become prevalent post-secretion.

The nectary cells of flowers in which nectar was last detectable one to one-and-one-half days earlier have large vacuomes (Fig. 4.116) that are always (epidermis) or often (subepidermal cells) composed of single vacuoles (Fig. 4.146, 4.151, but see 4.142). For the first time, the average vacuome of subepidermal cells exceeds half the cell volume (Fig. 4.116). Vacuolar inclusions that take many forms are found (Fig. 4.142, 4.148-4.152). Numerous plastoglobuli (Fig. 4.143, 4.145, 4.146, 4.152), patches of a densely-osmiophilic material (Fig. 4.142, 4.143, 4.145, 4.146), and a peripheral reticulum (Fig. 4.142, 4.144, 4.145) are evident in the plastid stroma. However, very little starch remains in plastids of either epidermal or subepidermal cells (Fig. 4.133, 4.142-4.146). In the epidermis there is no difference in numbers of plastids or mitochondria per cell at this stage, when compared with the youngest bud stage (0.9 mm) investigated. In subepidermal cells, the numbers of mitochondria, and particularly plastids, are significantly reduced from the nectar-bearing stage, suggesting that these organelles have undergone deterioration (or fusion?). That lytic processes are underway is supported by the initial appearance (Fig. 4.116) of numerous microbodies (Fig. 4.142, 4.143, 4.145, 4.152). The marked reappearance of dictyosomes (Fig. 4.143, 4.144, 4.146) at quantities 2.5 - 3 times more abundant per
cytoplasmic area than in 0.9-mm buds (Fig. 4.116), and occasional occurrences of well-developed networks of a membranous reticulum (Fig. 4.142, 4.145), may also be linked to cell-degradative processes. The stacked profiles of smooth endoplasmic reticulum in epidermal cells (Fig. 4.74, 4.118) remain or are even more evident at this post-secretory stage, and are particularly noticeable around plastids (Fig. 4.74, 4.89b,c, 4.90, 4.91). Cytoplasmic crystals identical in appearance to those of the specialised subepidermal cells (Fig. 4.88) may be found in the non-guard-cell epidermis (Fig. 4.74 left, 4.147). Intercellular spaces now often contain a flocculent material (Fig. 4.145). Since the young bud stages, the wall thickness of both epidermal (compare Fig. 4.117 with Fig. 4.143) and subepidermal (compare Fig. 4.119 with Fig. 4.142) cells has increased noticeably.

4.3.6.1.2. Plasmodesmata

The average outer diameter of plasmodesmata between subepidermal cells was found to be 42.4 nm. The frequency distribution of measured diameters was normal and centred around the mean. There was no significant difference in mean diameter between cells of early-opening, nectar-bearing and post-secretory flowers.

Tangential sections of walls between subepidermal cells of pre-secretory nectaries showed the plasmodesmata to be solitary or in groups of up to five, in a rather random distribution. In post-secretory nectaries, these small groups of plasmodesmata were sometimes found to be more concentrated, at up to 48/µm² (for fifteen plasmodesmata), though pit fields with very high concentrations like those shown for *Gossypium* (Eleftheriou and Hall 1983a, Plate 5F) and *Hibiscus* (Sawidis et al. 1987b, Fig. 6) nectary trichomes were never found.

Plasmodesmatal frequencies for epidermal and subepidermal cells of lateral outgrowths of the nectary of *A. thaliana* are listed throughout flower development in Table 4.2 (see reverse). In general, these frequencies increased progressively with flower age, doubling and trebling for epidermal-subepidermal and subepidermal-
Table 4.2. Characteristics of plasmodesmatal frequency as determined from transmission electron micrographs of transversely-sectioned cell walls in thin (80 nm) sections of lateral outgrowths of the floral nectary of wild-type flowers of *Arabidopsis thaliana* of four stages of development. These data represent measurements from a total of 16-20 epidermal cells, and 20 subepidermal cells, per floral stage.

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<th>Cell junction</th>
<th>Floral stages</th>
<th>Total wall length (µm)</th>
<th>Number of plasmodesmata counted</th>
<th>Plasmodesmata per µm wall length</th>
<th>Plasmodesmata per µm²</th>
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<td>Anticlinal interfaces of epidermal cells*</td>
<td>1.9 mm bud</td>
<td>110.7</td>
<td>45</td>
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<td>Early-opening</td>
<td>89.6</td>
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<td>Nectar-bearing</td>
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<td>0.694</td>
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<td></td>
<td>Post-secretory</td>
<td>87.2</td>
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<td>0.608</td>
<td>5.44</td>
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<td>Periclinal interfaces of epidermal* and subepidermal cells</td>
<td>1.9 mm bud</td>
<td>120.5</td>
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<td>Early-opening</td>
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<td>41</td>
<td>0.335</td>
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<td>Nectar-bearing</td>
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<td>47</td>
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<td>Post-secretory</td>
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<td>Interfaces of subepidermal cells, including intercellular spaces</td>
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<td>0.156</td>
<td>1.39</td>
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<td>Early-opening</td>
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<td>Post-secretory</td>
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<tr>
<td>Interfaces of subepidermal cells, excluding intercellular spaces</td>
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<td>361.2</td>
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<td>Early-opening</td>
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<td>Nectar-bearing</td>
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<td>Post-secretory</td>
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*Not including guard cells
subepidermal interfaces, respectively, between the stages of mature bud to post-secretion, so that they were highest after secretion had ceased. The plasmodesmatal frequency for the anticlinal walls of epidermal cells reached its peak in the nectar-bearing phase; these epidermal frequencies were the highest encountered in the nectary (Table 4.2).

For calculations of plasmodesmatal frequencies between subepidermal cells, the measured wall length opposing intercellular spaces was both included and excluded (Table 4.2). From the total wall lengths measured, the percentage of cell wall lining intercellular spaces ranged from 10.6-15.6 percent, indicating essentially no difference with developmental stage, such that intercellular spaces between subepidermal cells have approached maximum size at least by the stage of bud maturation (i.e., 1.9 mm), before secretion begins.

4.3.6.2. Median portions of the nectary

Ultrastructural features of the epidermal and subepidermal cells of the median portions of the nectary closely resemble those of the lateral parts. In opening and nectar-bearing flowers, epidermal cells are characterized by large solitary vacuoles that account for the majority of the cell volume, whereas subepidermal cells contain more cytoplasm and are multivacuolate (Fig. 4.138, 4.139). Starch can be found in plastids of mature buds (Fig. 4.134), in buds entering anthesis (Fig. 4.138), and in nectar-secreting flowers (Fig. 4.135, 4.136, 4.139). Like the lateral portions of the wild-type nectary, the starch content of the median parts dissipates in a basipetal fashion (stage B2/B3 - Fig. 4.138; stage B3 - Fig. 4.135; stage B4 - Fig. 4.136, 4.139) until little starch remains (Fig. 4.140). An instance where pseudo-Schiff's-positive material was detected in several cells of a deteriorating median portion, including a cell resembling the specialised, substomatal forms of Fig. 4.88, is shown in Fig. 4.141.
4.3.6.3. **Sieve-element plastids**

Plastids of sieve elements penetrating the lateral parts of the nectary in pre-secretory (Fig. 4.153, 4.155) and secretory (Fig. 4.154, 4.156-4.158) flowers are similar. They are commonly spherical to ovoid in shape, and are characterised by small numbers of regularly-arranged thylakoid membranes (Fig. 4.153-4.155, 4.157), patches of dense osmiophilic granules (Fig. 4.153, 4.154, 4.157, 4.158), and starch grains amidst a dense matrix (Fig. 4.153-4.158).

One instance of apparent plastid division (Fig. 4.154) was recorded, leading to production of two equal-sized progeny. Tubule-like structures which may represent the plastid type described by Oross and Possingham (1991) are detectable at the thin region of cleavage, suggesting their possible involvement in the division.

In post-secretory nectaries, the outline and shape of the plastids are less regular (Fig. 4.104, 4.110, 4.159-4.161). Large plastoglobuli are evident, and the matrix is less dense. Starch grains (Fig. 4.159, 4.160) and thylakoids are still detectable, although the latter are more uncommon and irregularly aligned than before. The dense osmiophilic granules present before and during nectar secretion were not found at this stage (Fig. 4.159-4.161).

4.3.6.4. **Changes in starch content of the modified stomata during nectary development**

To determine any changes in starch content of the modified stomata throughout nectary development, twenty-five consecutive flowers (the youngest being a 1.6-mm bud) from the same raceme of a plant originating from a seed sown twenty-nine days earlier, were collected. Representative lateral portions of the nectaries of these flowers, after fixation and staining, are shown in Fig. 4.162-4.168 and 4.170-4.173. Other examples are given in Fig. 4.169 and 4.174.

Despite their identical genetic background, being located on the same raceme, there are noteworthy differences in nectary morphology. The lateral outgrowths range from single-lobed, pad-like structures (Fig. 4.162, 4.165, 4.171b) to bilobed glands
with unequal (Fig. 4.163, 4.164) or equal (Fig. 4.166, 4.171a, 4.172, 4.173) lobe sizes. 

As was the case with the flower of Fig. 4.20, the two distinct morphologies of the lateral outgrowths of nectary shown in Fig. 4.171a and 4.171b, and in Fig. 4.180a and 4.180b, come from the same flowers, respectively.

The stark identification of the modified stomata by their reaction to iodine potassium iodide is obvious, particularly after the onset of nectar secretion (Fig. 4.164-4.174; compare with TC7 mutant in Fig. 4.180a-d). The nectary starch, including that in the guard cells, stains reddish-brown with iodine potassium iodide, whereas other floral starch, such as that remaining below the nectar-secreting gland (Fig. 4.166, top), stains dark blue to black. The sum of modified stomata on the two lateral portions within a nectary, from mature flowers of the same raceme, ranged from eight to twenty-two (Fig. 4.179), and the distribution of modified stomata on the gland surface can be highly asymmetrical (Fig. 4.166-4.168).

A stereological technique (see section 4.2.5) was employed to quantify the starch content of the modified stomata of the lateral outgrowths (only) of the nectaries of this raceme. All ontogenetic stages of modified stomata were considered equal, because of the difficulty in differentiating between them in fresh material. Furthermore, starch scores of 0 to 100 were assigned only to those modified stomata which were aligned in facial view. Otherwise, stomata were not examined for starch quantification. Because of this restriction, overall, only 63% of the total modified stomata present could be examined (Fig. 4.179). Representatives are shown in Fig. 4.175a-i.

The analysis revealed that the highest starch scores occurred in modified stomata at the stage of nectar secretion (Fig. 4.179, flowers 16-20), or immediately after secretion had ceased (Fig. 4.179, flowers 13-15). The lower scores of pre-secretory flowers (Fig. 4.179, flowers 21-25) suggest that starch was still accumulating in their
plastids. Support for this statement comes from the ultrastructural investigation of *Arabidopsis* nectaries, wherein immature modified stomata had smaller plastids with less starch (Fig. 4.176) compared to those with open pores (Fig. 4.177, 4.178).

Starch is still present in nectary stomata from relatively-old, post-secretory flowers (Fig. 4.182, 4.183) but scores averaged less (Fig. 4.179, flowers 2-12; see Fig. 4.168, 4.170-4.173) than those of nectar-bearing flowers (Fig. 4.179, flowers 16-20; see Fig. 4.164-4.167). These data indicate a net reversion from starch production to starch metabolism, after nectar secretion has ceased. Also evident in post-secretory nectaries is a greater variation in starch scores of modified stomata upon a single lateral portion of the nectary (Fig. 4.179, e.g., flowers 3, 8, 11, 14), and between average scores of the two lateral portions of the same nectary (Fig. 4.179, e.g., flowers 5, 11-13), compared to glands of nectar-bearing flowers (Fig. 4.179, flowers 16-20).

In the starchless mutant TC7, neither whole nectary tissue (including the modified stomata) stained positive for starch when placed in iodine potassium iodide (Fig. 4.180a-d), nor did resin-embedded sections of tissue stained with pseudo-Schiff’s reagent (Fig. 4.181) display a positive reaction indicative of starch, in the modified stomata, or elsewhere.

### 4.3.7. Nectar sugar production and composition in the wild-type and starchless mutant

Mature buds cultured on 0 or 2% sucrose solutions for 24 h had barely reached stage B2/B3 (Müller 1961), where petals were just visible above the sepal tips. None of these flowers, whether of the wild-type or starchless mutant, had secreted any nectar, despite the presence of a nectary. In the case of wild-type flowers, nectary tissue placed in iodine potassium iodide stained positive for starch.

After 24 h on 10% sucrose solution, mature buds had reached stage B3 or very-early stage B4 (Müller 1961). Although flowers of *A. thaliana* are normally
autogamous, these *in vitro* flowers remained indehiscent, probably because the high humidity conditions had prevented anther dessication. Therefore, although their stigmatic papillae were usually long, indicating apparent receptivity, these flowers remained unpollinated. All of these flowers bore nectar, sometimes with the droplets reaching the tip of the lateral sepal, at both lateral positions per flower. On the other hand, only one quarter (25.5%) of all flowers were found to have nectar droplets at any of the median processes, with the percentage being higher (31.6% of 19 flowers) in TC7 flowers than in the wild-type (21.4% of 28 flowers). Of these twelve cases where median outgrowths were found to be active in nectar secretion, only four (33%) flowers had nectar at at least two outgrowths. No flowers secreted nectar from all four median protuberances per flower. Where more than one median outgrowth was active, 75% of such flowers produced nectar at both median sides of the flower. In one such case, I suspect that nectar droplets produced individually by both median protuberances on the same side of a flower actually combined to eventually yield the large droplet collected opposite that median sepal: analysis revealed that that large droplet contained 0.850 µg sugar, while a smaller droplet produced by a single median outgrowth opposite the other median sepal of this same flower yielded about half as much sugar (0.322 µg).

Despite the occurrence of nectar production at some median outgrowths, the bulk of nectar sugar can be found in the pouches of the lateral sepals opposite the lateral outgrowths of the nectary. For instance, in wild-type flowers bearing nectar at a median position, only 25.0 ± 3.2% (n=4) of the flower’s total nectar sugar escaped at the median positions, whereas for the same situation in the starchless mutant, 17.8 ± 0.30% (n=3) of total sugar was found in nectar opposite the median sepal bases. On the other hand, wild-type flowers with secretory activity at at least one of the median outgrowths of the nectary tended to secrete more nectar sugar (2.449 ± 0.467 µg, n=6)
Table 4.3. Total nectar sugar and nectar-sugar composition (by weight) of wild-type and starchless-mutant flowers of *Arabidopsis thaliana* cultured as mature buds for 24 h on 10% (w/v) sucrose solution. Column A contains data from the first replicate, involving analyses of fructose and glucose only, whereas column B contains data from further flowers whose nectar was analysed for sucrose, fructose and glucose.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TOTAL NECTAR SUGAR (µg)</th>
<th>NECTAR-SUGAR COMPOSITION (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.E. (Range)</td>
<td>n Sucrose</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 1.744±0.186 (0.999 - 2.962)</td>
<td>15 2.244±0.243 (1.406 - 4.477)</td>
</tr>
<tr>
<td>Starchless TC7</td>
<td>11 1.806±0.164 (0.898 - 2.732)</td>
<td>6 1.450±0.196 (0.781 - 2.160)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Columnar means followed by the same letter are not significantly different using 2-tailed t-tests, \( \alpha = 0.05 \).

Table 4.4. Ratio of fructose to glucose in nectar of flowers of the wild-type and starchless mutant of *Arabidopsis thaliana* cultured as mature buds for 24 h on 10% (w/v) sucrose solution. In the case of nectar secretion by median outgrowths, calculation of the overall ratio per flower was weighted according to total nectar sugar secreted per flower.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>FRUCTOSE/GLUCOSE RATIO</th>
<th>Mean ± S.E. (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n OVERALL</td>
<td>n Lateral outgrowths only</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>25 0.938±0.028 (0.787 - 1.454)</td>
<td>25 0.922±0.027 (0.787 - 1.454)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starchless TC7</td>
<td>17 0.859±0.024 (0.712 - 1.163)</td>
<td>17 0.851±0.024 (0.684 - 1.163)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Columnar means followed by the same letter are not significantly different using 2-tailed t-tests, \( \alpha = 0.05 \).
than those flowers producing nectar at the lateral outgrowths only ($1.916 \pm 0.164 \mu g$, n=19), but this difference was not statistically significant ($t_{23} = 1.377, 0.2>P>0.1$). A similar result occurred with flowers of the starchless mutant, wherein the increase in yield of nectar sugar by flowers showing some secretory activity by the median outgrowths ($1.979 \pm 0.137, n=5$) over all other flowers ($1.555 \pm 0.165, n=12$) still fell short of statistical significance ($t_{15} = 1.547, 0.2>P>0.1$).

The relatively large quantities of nectar sugar secreted by each of the lateral outgrowths of a floral nectary were found usually to be similar. In a small number of cases, separate analyses were conducted and the amounts of sugar ($\mu g$) produced at each lateral position within single wild-type flowers were: (1.700, 1.605), (1.205, 1.380), (0.854, 0.665), (1.010, 0.991), and (0.907, 1.545). A similar situation occurred in a TC7 flower (0.822, 0.691).

A comparison of total nectar-sugar production between cultured flowers of the starchless mutant and the wild-type from two replicates (columns A,B of Table 4.3) showed the differences in means to be statistically insignificant. In the second replicate (column B), the greater difference in mean nectar sugar production between the wild-type and mutant seems, at least in part, to be attributable to the smaller average size of mature TC7 buds ($1.83 \pm 0.025 \text{ mm}$) available for that experiment (and fewer buds of the acceptable size were available), compared to the wild-type ($1.91 \pm 0.016 \text{ mm}$). On the contrary, the mean sugar values of column A represent buds which were much more evenly matched (TC7: $1.89 \pm 0.022 \text{ mm}$; wild-type: $1.87 \pm 0.023 \text{ mm}$).

The floral exudates were found to be hexose dominant, with sucrose contents being low for both plant genotypes (Table 4.3, left). A three-fold increase in sucrose content of the TC7 mutant was detected. Both the wild-type and starchless mutant had proportionately more glucose than fructose, the difference being greater in the mutant. An overall comparison showed that this difference in fructose/glucose ratio between
the starch-bearing and starchless genotypes was on the verge of statistical significance (Table 4.4, left). Separate analyses of nectar sugar from the lateral outgrowths and from the few median outgrowths that produced it demonstrated highly significant differences in fructose/glucose ratios between glandular positions within flowers of the same genotype (Table 4.4, right). However, when weighted according to the relative quantities of nectar sugar secreted at the two positions within a flower, the higher fructose/glucose ratio of the nectar produced by median outgrowths had only minor influence on the overall ratio per flower (Table 4.4). In wild-type flowers, only five (20.0%) flowers produced nectar with an overall fructose/glucose ratio exceeding unity; this percentage was even lower (5.9%) in the starchless mutant. For a small number of flowers, the fructose/glucose ratio for each of the nectar droplets at the lateral positions was analysed separately. There was general agreement between droplets for wild-type flowers: (0.866, 0.797), (0.792, 0.783), (1.212, 1.083), (0.933, 0.853), but the lone pair of values for a TC7-mutant flower showed a greater disparity (1.084, 0.830). For the first data pair presented for wild-type flowers, i.e., (0.866, 0.797), median nectar from two different sides of this same flower yielded the following fructose/glucose ratios (1.017, 1.117).

Staining of both lateral outgrowths of the nectary dissected from ten wild-type flowers cultured on 10% sucrose solution, immediately after their nectar was collected for analysis, always showed the complete absence of starch in the subepidermal cells of the gland interior. However, the modified stomata of the nectary surface stained reddish heavily, and starch in cells below the gland proper stained bluish-black (e.g., see Fig. 4.167), indicating that if it were still present within the nectary tissue, the starch should have been visible by staining.
4.4. DISCUSSION

4.4.1. General comments

The data obtained in this study of sectioned flowers of *A. thaliana* has consistently shown that the receptacular nectary tissue at the base of the six stamens is actually connected throughout the flower of this species. One of the weakest of these connections occurs between median protuberances of the same side of a flower, where no more than four cell layers of nectary cells have been observed in transverse sections. The slenderness of this alliance may account for the lack of a connection drawn between the median outgrowths in Velenovsky's (1883) first sketch of an otherwise united gland in *A. thaliana*. Therefore, because of its unity, it seems technically correct in this species to refer to the *entire* glandular tissue as a single nectary or whorl, and rather than utilize the common terms "lateral nectaries" and "median nectaries" to refer to unconnected proliferations of glandular tissue at these floral positions in Brassicacean flowers (such as in *Brassica napus* L. - Davis *et al.* 1986), to use instead descriptions like "lateral outgrowths of the nectary" and "median outgrowths of the nectary", respectively. This treatment of the glandular tissue of *A. thaliana* disagrees with that approach of Arber's (1931a), who, after finding (Fig. 1-6, and p. 30) that the glandular tissue in *Sisymbrium allaria* Scop. forms a continuous zone around the receptacle, such that the boundaries between "individual glands could not be sharply defined", still referred (p. 14) to various regions of tissue proliferation as separate entities (i.e., "glands"). On p. 175, she (1931b) treats the continuous gland (Fig. I-A6) in *Capsella bursa-pastoris* Medic. in the same fashion. This finding of continuous nectary tissue in *A. thaliana* supports the earlier observations of Bayer (1905), Villani (1905), von Hayek (1911), Schulz (1936), Norris (1941) and Hegi (1958). Furthermore, the telescoping views of Brassicacean nectaries provided by von Hayek
(1911) show some species to have a thin connection across the inner (adaxial) side of the filaments of the short stamens. However, this very thin strand of nectary tissue was not included in his sketch (Table 9, Fig. 4b) of *A. thaliana*, although it was referred to by Schulz (1936) and was indeed present by the secretory phase, at least, in the present study.

For taxonomic purposes, Davis and Heywood (1963) noted the lack of information on the morphological variability of the nectary tissue within individual species of the Brassicaceae. Subsequently, Dvorák (1965, 1967, 1968, 1970), Dvorák and Uhlírová (1967), Clemente and Hernandez (1978) and Avetisyan (1979) have undertaken detailed studies that demonstrate a high degree of variability in morphology of the glandular tissue within a single species. For instance, Avetisyan (1979) provided sketches of no less than 37 different forms of the lateral nectaries of *Arabis caucasica* Willd. In accordance with these studies, there is considerable variation in morphology and size of the outgrowths of the nectary of *A. thaliana*. First of all, that flowers can occur with only four long stamens and without a nectary (seen once on the first flower of a primary inflorescence) confirms the finding of Hildebrand (1879). However, a nectary is usually present, and then the lateral portions of the gland can be hemispherical, loaf-shaped, bilobed, or even trilobed. An individual flower may possess two of these shapes. It should be noted here that disparity in the size of the lobes of nectary tissue at the lateral position is not uncommon in large, pre-secretory and post-secretory flowers, so the suggestion that this difference has arisen as a result of differential completion of secretory activity of the lobes, causing "one nectary" to appear "shrunken, probably because it has already released its nectar" (Fig. 5J and its caption, Smyth *et al.* 1990), instead of occurring as a developmental variation, is likely incorrect. In the same way, great variation can be found at the median positions within a single flower, concerning the presence, shape and size of glandular protuberances.
Because this variation within flowers cannot be caused by genetic differences, physiological and/or environmental factors must be responsible. Available space on the receptacle and nutrients may govern the amount of nectary tissue that develops at each position (Velenovsky 1883, Bayer 1905, Schulz 1936, Smyth et al. 1990).

Nectary development, in comparison to the ontogeny of all other whorls of the wild-type *Arabidopsis* flower, initiates relatively late. The nectary initials appear early in stage 9 of Smyth et al. (1990) at the lateral positions, and somewhat later in that same stage at the median positions. By the end of this floral stage, unmistakable nectary tissue is detectable external to the short stamens, in agreement with the finding of Smyth et al. (1990). However, expansion of this glandular tissue continues right up until nectar secretion; it does not mature during stage 11 (Bowman et al. 1991). Not until stage 11 can glandular tissue be discerned easily at the median positions, refining the statement of Smyth et al. (1990) that nectary tissue at the median positions appears after the lateral tissue. Bayer (1905), Müller (1961) and Smyth et al. (1990) found that up to 26% of the flowers that they had analysed lacked a short stamen, and reported additional nectary tissue in its stead. This phenomenon was also observed on occasion in the present study (e.g., Fig. 3.174); in most cases, however, a loaf-shaped outgrowth occupied the normal nectarial position, without any glandular tissue in the stead of the missing lateral stamen. In young buds, development of nectary tissue was advanced at the lateral sides where a short stamen was absent. Hegi (1958) found that in the case where short stamens were absent, nectar droplets at the flower base no longer remained as separate entities, but now became confluent.

There has been some disagreement regarding the origin of nectary tissue in the cruciferous flower. Bernhardi (1838, p.137) referred to "rudimentis staminum duorum abortivorum ... saepe in glandulis conservatis", and Brown (1866, p.267) also regarded the nectary tissue as aborted androecial tissue. Eichler (1878, p.204), on the other
hand, referred to the "glandulae hypogynae" as "Emersionen des Torus". Hildebrand (1879, p.22), emphasising the case of *Dentaria bulbifera* where the lateral nectaries were particularly large when the anthers of the short stamens failed to develop, concluded that nectary tissue was not independently formed but rather was linked to the stamens. Velenovsky (1883), in support of Eichler’s view, emphasised that the presence of enlarged nectary tissue in the complete absence of a stamen (e.g., "Stenophragma") indicated that the glandular tissue was therefore not any part of the androecium. He argued, rather, that the close proximity of nectary tissue to the filament bases evolved not from abortive staminal tissue, but simply for reasons of enhancing plant reproduction; this position of the glandular tissue was paramount to allowing pollen contact and dispersal by nectar-seeking insects. Arber (1931a), on the grounds that the vascular supply to the nectary tissue consists solely of phloem and because this vasculature has a very heterogeneous origin within flowers of different Brassicacean species, concluded that the nectary tissue should only be considered as an outgrowth of the receptacle. Results of the present study of *A. thaliana* agree with her point, the floral nectary being innervated by phloem alone from various sources, including some which have no association with the vascular bundle which enters each filament.

One of the few structural features in common between the androecium and nectary of *A. thaliana* is the presence of stoma-like structures on their surfaces; however, the only floral whorl lacking stomata in this species is the corolla (Davis, pers. obs.). In addition, the developmental evidence is against the reduced-stamen model; the nectary initiates relatively late, well after the gynoecium, at about the time that the six recognized stamens are about to produce pollen grains (and see Smyth *et al.* 1990). This developmental pattern would seem abberant, if the nectary tissue was staminoidal. Recent genetic evidence obtained in this species using floral homeotic
mutants has also demonstrated a lack of an obligatory connection in development of the nectary tissue with the normal androecium (Bowman et al. 1991b). The final point is that the nectary and androecium suffer different fates during flower senescence; the nectary persists at the base of the expanding gynoecium and becomes covered with wax and engulfed by the bulbous cells of the abscission zone, whereas the recognized stamens abscind. It should be noted therefore, that the description of floral stage 17 by Smyth et al. (1990) - "all organs fall from green siliques" - is slightly incorrect, because both the gynoecium and nectary remain intact after abscission of the perianth and stamens. Furthermore, this phenomenon of persistence of nectary tissue is evident in B. napus (Davis, pers. obs.), Hirschfeldia incana (L.) Lagrèze-Foss. (bottom of Fig. 6.1), S. alliaria (Arber 1931b), and Daumann (1932b) lists a few species of the Brassicaceae that secrete nectar post-flowering.

The present study has demonstrated that the median outgrowths of the floral nectary of A. thaliana should not be considered as "steril" (Jordan 1886), "vestigial" (Knuth 1908) or incapable of secretion (Müller 1961). Although not usually active in secretion, in one wild-type flower, 33.7% of total nectar sugar was produced by the median protuberances of the gland. Previous investigations with A. thaliana had not guarded against post-secretory loss by evaporation of water from standing nectar, to demonstrate the secretory capacity of these glandular outgrowths. Utilization of culture experiments involving high-humidity conditions had earlier shown the ability of median nectaries of Brassica napus, also poorly supplied by phloem, to secrete nectar occasionally (Davis et al. 1986).

The lack of difference between total production of nectar sugar by flowers with or without active median nectaries may be related to the small sample size, a result of the low frequency of flowers secreting nectar at the median position(s). In most cases median outgrowths were supplied by phloem from only the main vein of the median
sepals, because only one of twelve median processes of five flowers that did receive a supply of phloem additionally did so from a vascular bundle eventually supplying a petal. Therefore, competition for phloic sugar between lateral and median proliferations of tissue of a single nectary appears to be low, and so any nectar sugar derived from the major bundle supplying median sepals would seem to be supplementary to the nectar sugar expelled at the lateral-outgrowth positions.

4.4.2. Nectary vasculature

The floral vasculature of Arabidopsis thaliana generally was found to be similar to that of other cruciferous flowers reported by Arber (1931a) and Norris (1941). The base of each petal is supplied by a single bundle and each sepal base receives three main veins, one central and two lateral (referred to as "marginal", below). An interesting feature is that the twelve (total) vascular bundles which enter the petals and the margins of the lateral and median sepals, are all derived from four receptacular bundles. Therefore, the central, major veins of the four sepals are the only perianth bundles which arise from the receptacle separately.

In agreement with the twenty-four species of Brassicaceae which had been studied to date (Arber 1931a,b, Frei 1955, Kartashova 1965, Davis et al. 1986), the floral nectary of A. thaliana has a direct vascular supply consisting of phloem alone. Norris (1941) also reported the lack of xylem vessel elements leading to the nectary tissue of Brassicaceae, although it is unclear from his brief treatment on p. 112 if or which of the sixteen species (including A. thaliana) he examined had nectary tissue that received phloem. There is good agreement between the extent of glandular vascularisation and the quantity of nectar secreted; in the culture experiments, each lateral outgrowth of the flower's nectary was found to secrete a relatively large volume of nectar compared to the smaller volume, or common lack, of nectar, found at each
median position of the gland. This discovery supports the case for *Brassica napus* (Davis *et al.* 1986 and references therein). It is yet unknown whether a direct innervation of phloem is a mandatory requirement for a median protuberance of the nectary of *A. thaliana* to produce collectable nectar. Investigation of the vascular supply to individual median outgrowths of flowers previously checked for the presence of nectar after mature buds have been cultured at high relative humidity, should shed light on this question.

Maturation of the sieve elements that penetrate the lateral outgrowths of the nectary of *A. thaliana* was never found to occur in buds less than 1.8 mm long, in accordance with the relatively late development of the nectary within the flower. This pattern of late maturation of the vascular supply to floral nectaries has been reported for *Passiflora* (Durkee *et al.* 1981) and several species of the Lamiaceae (Sharma and Singh 1982).

Variation in origin of the vascular supply to nectary tissue has been noted before in flowers of several species of the Brassicaceae and Ranunculaceae. Arber (1931a) reported that the glands of the former family are "supplied by extremely delicate vascular bundles (that) ... have no xylem, but consist exclusively of elements with the characters of protophloem. The slight development of the strands and their lack of lignification make it difficult to trace them to their source." In the species in which she succeeded, there is no indication whether more than one flower was investigated. It was concluded that the phloem which enters the nectary tissue may be donated by the bundles of the lateral sepals, of the petals, of the short stamens, and of the margins of the gaps left in the vascular cylinder by the short-stamen bundles (Arber 1931a). Norris (1941) added that nectary vascularisation in the Brassicaceae was never found to arise from the stele or the carpel bundles. Arber's (1931a) description of nectary
vasculature for *Arabis albida* Stev. matched that found here for the lateral outgrowths of the nectary of *Arabidopsis*, although a possible involvement of petal bundles was not disclosed for *Arabis*. The origin of vascularisation to the poorly-secreting median nectary tissue has received less attention. For *Brassica arvensis* L., Frei (1955) found that the weak phloem supply to the median glands originated from median-sepal and petal vascular bundles, similar to the case determined here for *A. thaliana*. Arber (1936) also investigated the vasculature of the epipetalous nectary in two or three petals of eight species of *Ranunculus*, but it is not clear whether the petals came from different plants, the same plant, or possibly even the same flower. In general, the nectary of *Ranunculus* always received xylem and phloem from the central petal bundle, with additional supplies occasionally derived from one or both marginal bundles. This heterogeneity in supply within a species is supported by the present work with *Arabidopsis*.

Until now, it appears that no study has addressed the question of whether significant variation in degree of nectary vascular supply occurs within nearby flowers of the same plant. This problem was tackled using a single raceme of *A. thaliana* (Fig. 4.115). It is clear that each lateral outgrowth of a floral nectary investigated showed more similarities in vasculature than the median protuberances. All lateral outgrowths received a phloem supply from the vascular bundle entering a lateral sepal as the main vein, although both lobes were not necessarily innervated equally. In all but one flower, lateral outgrowths were supplied by phloem branching from the vascular bundles eventually destined for the petals. In that flower, one outgrowth did not receive phloem from either of these petal bundles. Median protuberances, when they received any supply of phloem, always did so from the central vein of the median sepal opposite. The presence of nectary tissue did not guarantee a vascular supply. Median
outgrowths on one side of a flower generally received vascular connections to a more similar degree than within a flower. Only one such outgrowth received a phloem trace from the vascular bundle destined for a main marginal vein of a median sepal.

Therefore, not all floral nectaries of an individual inflorescence of *A. thaliana* receive the same quantity of phloem, so this variation is due to environmental/physiological factors rather than genetic ones, in this case. It seems reasonable that late-forming flowers - those positioned nearer the inflorescence tip - have more competition for photosynthate because of the growing sinks of the expanding siliques below them (e.g., see increasing diameter of the gynoecial vascular supply in Fig. 4.21a-4.25a), even though siliques can manufacture their own photosynthate. A decline in nectar production as a result of reduced vascular connections to the nectary, as well as a lower total quantity and (or) rate of sugar supplied to the gland, would therefore not be unexpected within the growing raceme. Indeed, Bayer (1905) has noted that glands are smaller in new flowers forming on large, many-flowered inflorescences of *A. thaliana*, and that nectar secretion in such flowers may not occur. Andreev (1928), cited by Beutler (1953), showed that in *Phacelia* the size of the nectary and nectar yield were lower in the upper parts of the plant, and in the tops (i.e., late-forming flowers) of inflorescences. Although not indicative of a definitive trend, it is worthy of mention that the one of six *Arabidopsis* flowers found to be lacking a phloem supply from the vascular bundles which eventually supplied the petals was the youngest studied (nearest the top) on the raceme. Perhaps the amount of phloem that develops in the gland interior is a function of the relative amount of sugar moving into the flower or gland itself.

Arber's (1931a) "suggestive tissue", as it was referred to by Norris (1941) in reference to the phloem-like strands that she found to penetrate the floral nectaries of various Brassicaceae, was also correctly recognized by Frei (1955) and Frey-Wyssling (1955) to represent a true supply of phloem. Since then, it has been considered as such
(Kartashova 1965, Fahn 1979a). Although reference was made to ultrastructural features of the phloem supplying the floral nectaries of *Brassica napus* (Davis 1985, Davis *et al.* 1986), the transmission electron micrographs presented here provide the first comprehensive and illustrated evidence that the "suggestive tissue" is indeed, truly, phloem.

Most ultrastructural studies of nectaries, like those of the floral gland of the crucifer *Diplotaxis erucoides* (Eymé 1966, 1967), have concentrated on subcellular features of nectary cells. Those investigations which include the fine structure of the vascular supply are in the minority (Durkee 1983a). The characteristics of immature sieve elements detected in the floral gland of *A. thaliana* - endoplasmic reticulum, mitochondria, p-protein, plasmodesmata, plastids with starch, vacuoles, and disintegrating cytoplasm - closely resemble those of other nectaries (Durkee *et al.* 1981, Durkee 1983a,b, Sammataro *et al.* 1985). In mature sieve tubes of the *Arabidopsis* nectary, these same features are found, as well as a parietal cytoplasm and sieve plates with pores between neighbouring elements, in accordance with previous studies (Figier 1968, 1971, 1972c, Vasilijev 1972, Wergin *et al.* 1975, Baker *et al.* 1978a, Durkee 1982, Durkee 1983a,b, Eleftheriou and Hall 1983b, Kuo and Pate 1985, Davis *et al.* 1986, 1988a, Sawidis 1991). However, the "refractive spherules" and conspicuously thickened walls of the mature sieve elements supplying the extra-reproductive nectary of the fern *Pteridium aquilinum* (Power and Skog 1987) are foreign to *A. thaliana, B. napus, V. faba* and apparently all other species referred to above.

With regard to the sieve plate and its pores, there are close similarities between the sieve elements of the *Arabidopsis* stem (Plates 5.3, 5.3a of Ledbetter and Porter 1970) and nectary. Both have conspicuous accumulations of callose-like material underlying the plasma membrane at the sieve pores, and multiple strands of p-protein within the pores. On the other hand, the numerous, scattered, electron-dense spherical
bodies along the length of mature sieve elements of the stem (Plate 5.3) were never encountered in the nectary counterparts.

Until now, no study had included an ultrastructural examination of sieve elements following the cessation of nectar secretion. Changes in plastids - their shape, dissolution of thylakoid membranes, acquisition of large plastoglobuli, but persistence of some starch - were the most noteworthy. Otherwise, the sieve tubes remain intact at the nectary base, even by the time that lateral outgrowths are undergoing collapse. Shuel (1961) suggested that reabsorption of sucrose by the nectary tissue of senescent flowers which had ceased to secrete nectar, might indicate that secretion is terminated by a failure in the supply of sugars to the nectary, rather than by a failure of the secretory mechanism. The present discovery indicates that the cessation of secretory activity of the nectary of *A. thaliana* was not caused by any destructive change in the phloem. Therefore, a shortage of sugar to the nectary appears likely, because the supply route of sugar to the nectary remained intact. There is also the possibility that the pathway of phloem might be an important route during reabsorption of constituents of standing nectar or pre-nectar within the gland and possibly for certain reclaimable products of lytic metabolism within the post-secretory gland, for their transport out of the gland, after nectar secretion has ended. That starch should be stored at all in plastids of sieve elements, let alone persist after secretion has ceased, seems an anomaly in the floral nectary of *A. thaliana*. Plastid starch in sieve elements has been reported earlier (Figier 1971,1972c, Durkee 1983b, Sammataro *et al.* 1985), but not found when sought in the cyathial nectary of *Euphorbia pulcherrima* (Annigeri and Rudramuniyappa 1983).

There are now several examples demonstrating that sieve elements penetrating nectary tissue may be bordered at some point by intercellular spaces. For instance, such spaces were detected adjacent to mature sieve elements of *A. thaliana* in the gland...
interior and are evident there in *B. napus* (Fig. 6, Davis *et al.* 1986). In fact, obvious intercellular spaces can be found abutting **immature** sieve elements, such as in the floral nectary of *Passiflora warmingii* (Fig. 11, Durkee 1983b) and a terminal element in the floral gland of sunflower (Fig. 5, Sammataro *et al.* 1985). Like *A. thaliana*, all these nectaries bear stomata. Apparently the largest intercellular spaces adjacent to floral-nectary phloem so far reported are these at the base of the lateral outgrowths of the *Arabidopsis* nectary, where much of the length of each sieve element and companion cell (in traces branching from the vascular bundles eventually supplying the petals and the major, marginal veins of the sepals) can be directly bordered by a voluminous space. In the nectary literature, the only other illustrations or references to intercellular spaces abutting companion cells are those of other floral nectaries bearing modified stomata - *P. warmingii* (Fig. 12 bottom, Durkee 1983b), *B. napus* (Fig. 6, Davis *et al.* 1986) and *V. faba* (Fig. 14-16, Davis *et al.* 1988a).

In passing, it is important to note that the cellular debris that was localized within the large intercellular spaces in thin sections of the base of the lateral outgrowths of nectary does not necessarily indicate that the spaces contain fluid *in situ*. The debris may have entered them only as a result of cell damage during the preparative steps of infiltration by fixative, acetone and then resin. Debris in the lumen of immature and nectar-producing septal nectaries of *Strelitzia reginae* Ait. has been illustrated in transmission electron micrographs before (Kronestedt-Robards *et al.* 1989). On the other hand, lysis of nectary tissue has been shown to be a normal part of the secretory process in some plants [e.g., extrafloral nectaries of *Sambucus nigra* (Fahn 1987) and certain ones of *Vigna unguiculata* (Kuo and Pate 1985, Pate *et al.* 1985)].

Companion cells of the *Arabidopsis* nectary stain densely like those in the stem (Ledbetter and Porter 1970) and in nectary tissue of *B. napus* (Davis *et al.* 1986). They
are similar in dimensions to the sieve elements they accompany. Their characteristic contents - dictyosomes, endoplasmic reticulum, mitochondria, nucleus, plastids, ribosomes, and small vacuoles - are in common with those described or illustrated elsewhere (Ziegler 1965, Figier 1968, 1971, 1972b,c, Wergin et al. 1975, Browning and Gunning 1977, Hughes and Gunning 1980, Durkee 1982, 1983a,b, Eleftheriou and Hall 1983b, Kuo and Pate 1985, Davis et al. 1986, 1988a). Unlike the companion cells of phloem supplying the floral and stipular nectaries of V. faba and the petiolar gland of Impatiens holstii (see page 1.9), wall ingrowths are lacking in the companion cells of the floral nectaries of B. napus (Davis et al. 1986) and A. thaliana. Companion cells of the floral nectary of A. thaliana can bear plasmodesmata in walls shared by sieve elements (like those in the stem; Ledbetter and Porter 1970), phloem parenchyma or other companion cells, similar to the situation in the floral nectary projection of fababean (Davis et al. 1988a).

4.4.3. The role of nectary starch in nectar sugar production

It has been recognized for over a century that, generally, starch accumulates in nectary tissue before the commencement of nectar secretion and declines thereafter, likely to form nectar sugar (e.g., Behrens 1879, Stadler 1886). Exceptions to this pattern include the nectaries of Corydalus cava Schweigg u. Koert. (Fumariaceae) (Radtke 1926) and several species of Asclepias [Stadler 1886, Ewert 1932 (according to Beutler 1953), Zaurolov and Pavlinova 1975], which do not store starch prior to nectar production, and Vinca major and V. rosea (Rachmilevitz and Fahn 1973, Plate 7A and p. 4), Gasteria trigona (Schnepf and Pross 1976), Trifolium pratense L. (Eriksson 1977, Fig. 12, 13) and extrafloral trichomes of cotton (Eleftheriou and Hall 1983a, p.116), which may bear starch in the nectary tissue after nectar secretion has ceased.
In the crucifer *Crambe grandiflora*, Böhmker (1917) reported that nectary starch is absent in open flowers, but that small quantities are found in a bud stage. The nectary of wild-type flowers of *A. thaliana* conforms to this pattern. Starch deposition in the subepidermal cells occurred before formation and maturation of the direct phloem supply, but was found to occur at a rapid rate especially in very large buds after the vascular supply had matured. Despite a doubling of plastid numbers per subepidermal cell during this nectary growth, numbers of starch grains per plastid remained constant or slightly higher. This result indicates that the predominant method of nectary starch accumulation in *A. thaliana* is by increasing the number of starch-storing organelles, rather than by significantly increasing the number of starch grains per plastid. This storage pattern seems to differ from that of the subepidermal cells of the floral nectary of large buds of *Passiflora warmingii*, where plastids of apparently constant number are shown to contain 4-8 starch grains each, in thin section (Fig. 10 and 14 of Durkee *et al.* 1981).

In future investigations, it would be interesting to determine if there are any effects in nectary tissue on plastid numbers per cell and quantity of starch stored per plastid, when conditions of photoperiod and temperature are held differently than the protocol of continuous illumination at 20°C employed in this study. For leaf tissues of *A. thaliana*, McCourt *et al.* (1987) found 1.15 times more plastids per cell when wild-type plants grew under constant light at 27°C than at 19°C, and Caspar *et al.* (1985) detected only 60% as much starch in wild-type plants grown under continuous illumination than those given a 12-h photoperiod.

That study of *P. warmingii* (Durkee *et al.* 1981), and one of the floral nectary of *Bignonia illicium* L. (Subramanian and Inamdar 1986) are two of the few that have closely followed the accumulation and decline in starch content before and after nectar secretion begins. There are some noteworthy differences from the nectary of
Arabidopsis. For example, in the other two species, starch storage occurs initially in the lower layers of nectary tissue and progresses toward the epidermis, although in *Passiflora* just before anthesis, the greatest deposition of starch occurs nearest the nectary epidermis. Then, even epidermal cells of the floral nectary of passionfruit contain a "massive accumulation" of starch. The norm, to which *Arabidopsis* accords, is for the epidermis to lack starch (Böhmker 1917). As in *Passiflora*, starch degradation occurs in the floral nectary of *A. thaliana* just prior to, but is not complete by, anthesis. When nectar is visible, the starch content of the nectary tissue of both species has disappeared, or largely so. However, in the passionfruit nectary starch dissipation was found to begin at the nectary base and to proceed toward the epidermis, the opposite of the pattern in *B. napus* (Fig. 6, Davis *et al.* 1986) and *A. thaliana*. Therefore, although occurring in different areas within the nectary, in both *P. warmingii* and *A. thaliana* the region of initial starch deposition is the same as for initial starch disappearance.

In contrast to the blue/black colour of the starch in the subepidermal cells of the lateral sepals and in the receptacle, the starch of the subepidermal cells of the nectary of *A. thaliana* (including that of the modified stomata) stains reddish-pink to -brown with iodine potassium iodide. Such a staining pattern is indicative of "transitorische Stärke" (Behrens 1879), or amylopectin (Küster 1956) in the nectary, suggestive that this form of starch is relatively easily metabolized to sugar. The rapidity of starch disappearance within the nectary as nectar secretion commences is another testimony to the occurrence of this type of starch. In all checks of nectary tissue of open wild-type flowers cultured on 10% sucrose solution (immediately after nectar removal), starch was always absent in the nectary interior. This result suggests that the dissipated starch contributed to the nectar sugar. However, at 0 and 2% solutions, flower opening and nectar production were not supported within 24 h, so the endogenous starch is not
on its own a sufficient resource. A difference between species may explain why Radtke (1926) found nearly as much starch at the cessation of secretion as was present just prior to secretory activity, in the nectaries of *Fritillaria imperialis* after flowers were cultured on 7-15 % sucrose solution.

Although there are reports that starch does not accumulate before commencement of secretion (see above) in nectary tissue capable of producing an exudate, the present study is the first to address the process of nectar secretion in a starchless mutant (TC7), the wild-type of which possesses nectary starch before secretion begins but loses it at the beginning of the secretory phase. Interestingly, not only did the flowers of the starchless mutant still produce nectar, they had the same levels of nectar sugar as those of the wild-type, in similar ratios. These results indicate that the enzyme phosphoglucomutase, which is involved in the catalytic transition of glucose-1-phosphate to starch, and is lacking in leaf chloroplasts of the TC7 mutant (Caspar et al. 1985), is not essential to the nectar secretion process in *A. thaliana*.

One possible explanation for the lack of disparity in total nectar-sugar production between wild-type and starchless flowers of *A. thaliana* is that the contribution of starch to total nectar sugar in the former, compared to that arriving as a direct influx of (non-starch) sugar from the nectary phloem, is relatively small. In that case, a difference due to the starch content may be insignificant. Another possibility is that at their introduction to culture, the buds of both genotypes contained the same quantities of nectary sugar, although in the mutant this carbohydrate was strictly in soluble form.

The present findings indicate that nectary starch deposition and degradation are not obligatory steps in the process of nectar secretion in *A. thaliana*. However, they fall short of estimating the relative contributions of various sources, which when combined, account for total nectar sugar production. In *A. thaliana*, it is presumed that
sugar may arrive in nectar 1) by the remobilization of nectary starch reserves, some of which form before the nectary vascular supply of phloem has matured, 2) from sugar held within the nectary at the start of secretion, but not present in the form of starch (this may be the initial sugar collectable as nectar), and 3) from sugar delivered directly to the nectary by its penetrating phloem after secretion has begun, very likely without ever being diverted into the form of starch. Future delimitation of each source of nectar sugar may be possible using radiolabelled sugars, culture experiments and analysis of nectary tissue itself. Zaurolov and Pankritova (1975) concluded that the bulk of pumpkin nectar sugar is formed from starch accumulated in the nectary, whereas in Asclepias cornuti Decsn. it is formed from products of photosynthesis flowing into the nectaries. In the floral nectary of Passiflora warmingii, Durkee et al. (1981) regarded the contribution to nectar of phloic sugar (not derived from nectary starch) as minor. Apparently the only datum that partially specifies the contribution of various nectary sources to nectar sugar production is that of Findlay et al. (1971), who found the amount of sugar present in the nectary at any time was at most only one-seventh that present in nectar secreted by the glandular trichomes of Abutilon.

The results of these experiments raise the question of why starch forms at all in nectary tissue of A. thaliana flowers, if the same amounts and ratios of nectar sugar occur in the nectar secreted by cultured flowers of the starchless mutant TC7. An emphasis on competition may account for such an accumulation of nectary starch by the plant, prior to nectar secretion. Because other sinks, including other flowers of the same plant and other parts (e.g., the gynoecium) of the same flower, are competing with a nectary for photosynthate, a storage form for sugar within the glandular tissue over the relatively long-term may provide the flower with a method that makes available at least some quantity of nectar sugar to attract potential pollinators following anthesis. Such a "precautionary measure" might be especially important in cases where unfavourable weather for photosynthesis, causing a reduction in photosynthate normal-
ly available for nectar production, coincides with flower opening. At the cellular level, it seems likely that storage of nectar sugar in the form of starch may be a favourable means of conserving the water necessary for other purposes, such as osmotic regulation, within nectary cells, thereby allowing more sugar to be "captured" and held within the gland prior to commencement of secretion. This scenario would suggest that the lack of formation of starch in the nectary of the TC7 mutant might limit the amount of sugar that could be stored before secretion begins. If this were the case, such a shortfall might not be recognizable from the measurements of total nectar sugar produced by cultured buds, where both sucrose and water were available ad libitum. The aforementioned difficulty in measuring nectar sugar produced by flowers in situ did not allow a direct comparison of the mutant and wild-type except by culturing.

The strong predominance of glucose and fructose in approximately equal quantities in the exudate of the floral nectary of the wild-type and starchless mutant of *A. thaliana* agrees with the findings of many previous assays of Brassicacean nectar (Wykes 1952a, Percival 1961, Davis et al. 1988b, and for *Hirschfeldia incana* (L.) Lagrèze-Foss. see Table 6.2, page 6.7). The present results indicate that even when flowers of *Arabidopsis* are cultured on pure sucrose solutions, the disaccharide is present only in minor quantities in floral nectar, and that the presence of nectary amylopectin - composed of subunits of α-D-glucose in α-1,4 and α-1,6 linkages - does not sway the sugar content of the nectar in favour of glucose. In fact, for unknown reasons, the wild-type (starch-bearing) gland produced nectar with a higher fructose/glucose ratio than the starchless mutant, although this difference fell just short of statistical significance. Interestingly, Durkee et al. (1981) were unable to detect glucose (but did find fructose) in the floral nectar of *P. warmingii* and *P. biflora*, despite the glands being starch-laden before secretion started and starch-empty after it
ceased. A likely explanation for the similar quantities of glucose and fructose in *A. thaliana* nectar is by the inversion of sucrose, but how sucrose is initially synthesized from the starch-derived glucose is unknown in nectary tissue of *Arabidopsis*. The finding that acid invertase activity in the mutant’s leaves was more than double that in the wild-type’s (Caspar *et al.* 1985) is consistent with the present findings of fructose and glucose, in approximately equal quantities, in nectar of both genotypes, but is inconsistent with a higher sucrose content in floral nectar of the mutant. Investigation of nectary invertase levels seems warranted. Also, a significant quantity of the hexose sugars was detected in stem and leaf tissues of the mutant, but how this differed from the wild-type was not quantified (Caspar *et al.* 1985).

The discovery that various regions of tissue on the same nectary regularly produce nectar with significantly different sugar ratios is also unique to nectar research. However, the reasons why the fructose/glucose ratios in nectar expelled from the median outgrowths exceed those of exudate from the lateral portions of the same gland, are completely unknown. Perhaps differential enzyme presence or activity, or differences caused by the quantity of phloem or pre-nectar, or the rate of secretion, are involved.

It would seem worthwhile to investigate the ultrastructure of plastids of the nectary parenchyma of the starchless mutant throughout development, for comparison to the results obtained here for wild-type flowers, to determine whether there are ultrastructural features indicative of starch deposition and disintegration. Also, do nectary cells of the TC7 mutant have the same number of plastids per cell, as the wild-type?

Irregularity in plastid shape and the prominence of plastoglobuli in the *Passiflora* nectary at anthesis (Durkee *et al.* 1981) were features also found in the present study of the *Arabidopsis* wild-type nectary, although plastoglobuli were not
nearly so common until after nectar secretion had ceased. During and after nectar production, dense aggregations of small osmiophilic granules in the plastids of *A. thaliana* were also detected, similar in appearance to the "ferritin granules" (phytoferritin) of the extrafloral nectaries of *Acacia* spp. (Marginson *et al.* 1985a, b). The significance of these changes in plastids of post-secretory nectaries of *Arabidopsis* are unknown.

The occurrence of starch in plastids of guard cells of the modified stomata and its persistence there after nectar secretion has halted is dealt with separately, below (see section 4.4.6).

**4.4.4. Ultrastructural evidence for the process of nectar secretion**

In stark contrast to several earlier studies of the fine structure of floral nectaries during the phase of nectar secretion, (see sections 1.3, 1.4), the endoplasmic reticulum is only a very inconspicuous feature of the cytoplasm of both epidermal and subepidermal cells of the nectary of *A. thaliana*. The same can be said for pre-secretory glands, wherein rough cisternae only occur singly, near the cell walls or plastids. In fact, a membranous reticulum of greater intracytoplasm usually appears **only after nectar secretion has ceased**, and then particularly in the specialised cells associated with modified stomata, and in certain other subepidermal cells. Therefore, significant involvement of endoplasmic reticulum in any type of granulocrine process during nectar secretion in *A. thaliana* is very unlikely. The same conclusion was drawn in a study of nectar-secreting flowers of *Brassica napus* L. (Davis *et al.* 1986).

Dictyosomes are relatively common in both epidermal and subepidermal cells of the floral nectary of *A. thaliana* before the commencement, and especially after cessation, of secretory activity of the gland. Only rarely were dictyosomes detected during nectar secretion, a finding in agreement with other ultrastructural studies of
floral nectaries of the Brassicaceae (*Diplotaxis erucoides* D.C. - Eymé 1966; *B. napus* - Davis *et al.* 1986) and of many other species (see section 1.3). In accordance with the characteristics of the endoplasmic reticulum discussed above, this temporal scarcity of dictyosomal vesicles is also taken as strong evidence against a possible granulocrine mechanism of nectar secretion in *A. thaliana*.

Cytoplasmic components emphasised by Eymé, and proposed to have a role in the secretory process of the floral nectaries of *Diplotaxis*, are the "vésicules membranaires" which contain "trabécules pelotonnés" (Eymé 1966 - Fig. 2 centre, 5; Eymé 1967 - Fig. 2, 3, 5 bottom, 7, 9) and occur during (1966) or preceding (1967) nectar secretion. These large vesicles, up to 800 nm in diameter, yield membranous invaginations when they fuse with the plasma membrane, especially at sites where plasmodesmata are numerous. The vesicles are also said to be confluent with the endoplasmic reticulum, and eventually to fuse with the tonoplast (Eymé 1966, 1967). He (1966) postulated that these vesicles may be involved in the transport of pre-nectar constituents from cell to cell, similar to pinocytosis, a granulocrine type of process. Interestingly, I have found no evidence for these structures throughout the various developmental phases of the *Arabidopsis* nectary.

Other, more common, structures in nectary cells, "les figures myéliniques", were first detected by Eymé (1966, 1967) in the floral nectaries of *Diplotaxis* and several Ranunculaceae. These structures are of unknown origin and function, and were considered to be progressively disengaged into the vacuole as cells aged (Eymé 1966 - Fig. 2 left, 6; Eymé 1967 - Fig. 5, 9-11). These multimembranous myelin bodies have since been detected in the cytoplasm (Zandonella 1970c, Figier 1971, Dumas 1975, Eriksson 1977, Schnepf and Deichgräber 1984, Davis *et al.* 1986, Mohan and Inamdar 1986, Sawadis 1991) or vacuome (Figier 1968b, 1969, 1972b, c, Zandonella 1970c, 1972, Tacina 1972, 1973, Rachmilevitz and Fahn 1973, Eriksson 1977, Sawidis 1991;
B. napus - Davis, pers. obs.; present study of A. thaliana) of cells of pre-secretory and secretory nectaries. Therefore, these multilamellar structures are neither necessarily associated with post-secretion nor indicative of a granulocrine process of nectar secretion. Figier (1968) suggested that these membranous bodies may be artefactual.

The eccrine model of nectar secretion concerns a metabolically-active, energy-dependent process of intercellular transfer of pre-nectar constituents (see section 1.4). Throughout the phases of gland development and nectar secretion in A. thaliana, mitochondria consistently remain the most numerous organelle in both epidermal and subepidermal cells. Therefore, there is ultrastructural evidence in favour of an eccrine mechanism of secretion by the nectary of Arabidopsis, but little support for the involvement of a granulocrine system.

The average outer diameter of plasmodesmata between subepidermal cells was found to be 42.4 nm, in very close agreement to those values reported for the septal nectaries of Billbergia nutans Wendl. (45 nm; Schnepf and Benner 1978) and nectary trichomes of Abutilon (43.6 nm; Gunning and Hughes 1976), Gossypium (40.9-42.4 nm; Eleftheriou and Hall 1983a) and Hibiscus (38 nm; Sawidis et al. 1987b).

With the exception of two developmental stages of the stalk cell of the floral-nectary trichomes of Abutilon striatum (Kronestedt et al. 1986), the data on plasmodesmatal frequencies determined here for A. thaliana are the first collected for any nectary in different stages of floral development. In the Abutilon study, the frequency of plasmodesmata in younger trichomes was not significantly different from that of fully-mature hairs. In the lateral outgrowths of the floral nectary of A. thaliana, there was a consistent trend for increased frequencies of plasmodesmata in post-secretory glands compared to pre-secretory ones. In the case of walls of subepidermal cells, these increases involved a doubling or trebling of plasmodesmatal frequency. Secondary formation of plasmodesmata by the subepidermal cells is indicated. For the epidermal-epidermal cell interfaces, the increase in frequency was much lower. It is
noteworthy that these differences in frequency of plasmodesmata have occurred when the glandular tissue (i.e., both the nectary and its average cell size) is very near or at maturity. Similarly, Vasilijev [according to (Schnepf 1977)] previously found that plasmodesmata in nectaries of several species, most of which bear stomata, were relatively rare during the active phase of nectar secretion.

Despite the increases in plasmodesmatal frequency which occur during glandular development leading to eventual nectar secretion in *A. thaliana*, those frequencies (3-6/µm²) are lower than the vast majority of values obtained for cell walls associated with active nectar secretion in extrafloral glands of *Ricinus communis* L. (14/µm²; Nichol and Hall 1988) and for various walls of nectary trichomes of *Abutilon* [11.6-12.6/µm² (Gunning and Hughes 1976; Gunning, in Robards 1976) and 4.0-12.0/µm² (Robards and Stark 1988)], *Gossypium* (1.6-15.4/µm²; Eleftheriou and Hall 1983a), *Hibiscus* (3.8-20.9/µm²; Sawidis et al. 1989) and *Vigna* (25 ± 3.5/µm²; Kuo and Pate 1985). The lower frequencies of plasmodesmata in *A. thaliana* may reflect less demanding symplastic fluxes. The function of the increase in plasmodesmatal frequency up to the post-secretory stage of nectaries of *A. thaliana*, remains unresolved. Perhaps events which occur in older glands, such as lytic and reabsorptive processes, or the deposition of waxy globules onto the outer walls of the epidermis, require symplastic transport and compartmentation.

The usually high frequencies of plasmodesmata in the anticlinal versus the periclinal walls of epidermal cells of the lateral outgrowths of the floral nectary of *Arabidopsis* suggest that the transfer of material between epidermal cells may be of some importance in maintaining turgor across the epidermis. Besides their probable function in release of some nectar constituents, their large solitary vacuoles suggest that cells of the epidermis play an important role as a "buffer zone" between accumulated nectar and the nectary cells below. The relatively low numbers of
plasmodesmata traversing the subepidermal-epidermal cell walls also demonstrate that this symplastic route may be of little significance in the transfer of pre-nectar constituents to the epidermis. That different walls of the same nectary cell can exhibit differences in plasmodesmatal frequencies was shown earlier for certain cells of the floral nectaries of *Hibiscus rosa-sinensis* (Sawidis *et al.* 1987b).

The frequency of microbodies and the ratio of mitochondria/plastids also change during nectary development in *A. thaliana*. The premier appearance of microbodies in both epidermal and subepidermal cells in the post-secretory stage examined suggests that there is a profound change in metabolic processes at that time, as reported earlier for other floral and extrafloral nectaries (Vasilijev 1969, Belin-Depoux and Clair-Maczulajtys 1975). In *Arabidopsis* the ratio of mitochondria/plastids gradually **decreases** during gland development, to reach 1.3-1.6 for both epidermal and subepidermal cells at the secretory phase. However, in the only other studies of nectaries that have quantified organelle changes during development, this ratio was found to **increase** gradually during development, to reach "about 5" (by number) in cells of the floral nectary of *Passiflora* (Durkee *et al.* 1981) and to 2-2.5 (by volume) in various cells of the floral trichomes of *Hibiscus* (Sawidis *et al.* 1989), during the active period of secretion.

In general, the relatively few and subtle changes during floral nectary development of *A. thaliana* agree with those minor changes reported for *Acer platanoides* (Vasilijev 1969) and *Aptenia cordifolia* (Meyberg and Kristen 1981), two other nectaries which bear stomata.
4.4.5. **Apoplastic and symplastic routes for pre-nectar movement from the nectary**

There is considerable structural evidence that the apoplastic route is very significant for the movement of pre-nectar in the floral nectary of *A. thaliana*. Uninterrupted pathways can exist from the pores of the modified stomata on the gland surface to the relatively narrow substomatal spaces and finer intercellular spaces below them. The latter spaces extend well into the interior of the various proliferations of nectary tissue. In thin sections of these subepidermal layers, it was found that during the nectar secretion phase, on average **three-quarters** of a sectioned cell’s wall junctions abutted intercellular spaces. These routes may extend to the innervating phloem tissue itself, because over a quarter of the wall junctions of thin-sectioned, individual companion cells were found to abut spaces, and indeed, a few instances (about 2% of such wall junctions) were noted where (especially) terminal sieve elements themselves bordered intercellular spaces within the gland interior. Therefore, in *A. thaliana*, the possibility exists for precursors of pre-nectar to be directly unloaded to apoplastic spaces from the phloic tissue.

It should be emphasised that movement of solutes can still occur apoplastically, external to the cytoplasm of nectary cells, without the development of intercellular spaces. However, it seems certain that the bulk flow of pre-nectar will be impeded less where the apoplast is expanded. The large apoplastic volume within the floral nectary of *A. thaliana* suggests it is important in nectar flow.

Regarding the rôle of sieve elements and companion cells which may branch into the base of lateral outgrowths of nectary tissue from the vascular bundles that supply the two major veins near the margins of the lateral sepals, it is unknown whether they provide sugar to pre-nectar. Large intercellular spaces, also reported in the ground parenchyma below the nectary tissue of *Crambe* (Böhmer 1917) may border the entire
length of certain of these cells, and it is possible that leakage of sugar to the apoplast occurs there. The very thin cuticle observed in the present work to border these intercellular spaces may serve in sugar retention within the phloic tissues. Thicker cuticles may line entire intercellular spaces between subepidermal cells nearer the nectary surface, where a relative impedance to pre-nectar flow from the cytoplasm through the wall may occur. It has also been suggested that these cuticular linings of the apoplast may assist in pre-nectar flow within the gland to the exterior (Lüttge 1971, Davis et al. 1986).

No apoplastic barriers like those described in *Aphelandra* spp. (Durkee 1987) or in the stalk cells of trichomes of many nectaries (Gunning and Hughes 1976, Eleftheriou and Hall 1983a, Kronestedt et al. 1986, Sawidis et al. 1989a, Sawidis 1991) were detected, so there is no known limitation to apoplastic flow in the *Arabidopsis* nectary.

Although the frequencies of plasmodesmata between subepidermal cells and between epidermal cells and those below them are small in comparison to those published for various trichomes - a matter that might also favour apoplastic movement of pre-nectar - the mere presence of plasmodesmata therefore makes possible symplastic, cell-to-cell movement of nectar precursors. Symplastic transport may be relatively more common toward the nectary base, where intercellular spaces bordering sieve elements and companion cells were much fewer, and those between parenchyma cells were smaller. Plasmodesmata were observed between all cell types, from the sieve element-companion cell-phloem parenchyma complex to the subepidermal-epidermal cell interface, so that a continual symplastic route for pre-nectar that culminates in nectar escape across the external epidermal wall (i.e., the apoplast), therefore exists. The use of apoplastic and symplastic dyes to trace the routes of pre-nectar movement may shed more light on these suppositions, in the future.
4.4.6. The modified stomata and the evidence against their regulation of nectar flow

In the floral nectary of *A. thaliana*, modified stomata were commonly detected on the abaxial surface of the lateral outgrowths and near the tips of the median ones. The highest density of modified stomata, where the lateral outgrowths face into the pouches of the lateral sepals, indicates the involvement of these structures in nectar escape from the gland, similar to the many cases studied by Zandonella (1972) and in *Vicia faba* (see Fig. 2.3, 2.4), where the initial site of nectar accumulation coincides with predominant aggregations of stomata. Up to fifteen and seven modified stomata were detected on individual outgrowths of the lateral and median positions, respectively, but in the relatively few flowers where all modified stomata were counted (not including these extremes), up to twenty-six (Fig. 4.115, flowers A and B) have been observed. The data in Fig. 4.115 and 4.179 show that there can be considerable variation in numbers of modified stomata per outgrowth within the same flower, and that the average number per flower generally declines with increasing flower position on the inflorescence. In fact, the latter situation represents the only cases where lateral outgrowths from large buds were found to lack any modified stomata.

Are the pores of modified stomata necessary to allow nectar to escape from the gland of *A. thaliana*? On one cultured flower of the TC7 mutant, a lateral outgrowth that did not produce any nectar also lacked stomata, whereas the other, secreting lateral outgrowth did bear stomata. Conversely, median outgrowths which bear nectar occasionally in flowers cultured as described in section 4.2.8 quite commonly lack modified stomata. My conclusion is that the modified stomata assist, but are not essential for, nectar exudation. This judgement is based on occasional observations of a detached surface cuticle with apparent nectar accumulation below it, on stomatal-bearing lateral outgrowths dissected from nectar-bearing flowers of this species. There
is support in the literature for the existence of nectaries without stoma-like structures which nevertheless secrete nectar. Furthermore, Daumann (1928) reported that in those nectaries of *Nicotiana* spp. which bear relatively few stomata, nectar may escape by rupturing the external cuticle. Feldhofen (1933) reported that, despite the presence of nectary stomata, the bulk or all of the nectar secreted by flowers of *Thunbergia erecta* and several species of the Gesneriaceae passes through other epidermal cells, and on p. 612 he lists six families found to secrete nectar both through stomata and by diffusion. Daumann (1932a,b, 1974) added further examples wherein the cuticle covering floral nectary tissue is lifted off or torn to release nectar, despite the presence of surface stomata.

Many developmental, morphological and ultrastructural features of the modified stomata on the floral nectary of *A. thaliana* - such as their ontogeny from pentagonal guard mother cells, their lack of subsidiary cells and adjacency to other modified stomata, the increase in starch and size of the vacuome in their guard cells during stomatal maturation, the regions of wall thickening and microfibril orientation in their guard cells, the progressive formation of the substomatal space and pore, the cuticle covering all exposed surfaces of their guard cells, and the wax globules secreted onto the outer guard-cell walls - are in accord with the more detailed study of these structures in *Vicia faba* (see Chapter 2).

Previous investigators have noted the usefulness of iodine stains to identify nectary guard cells by their abundance of starch (Behrens 1879, Bonnier 1879, Schönichen 1924), and that the guard-cell starch does not disappear as in the remainder of the nectary tissue (Daumann 1932a). Similar results were obtained in wild-type flowers of *A. thaliana* (e.g., Fig. 3.166). In this study the quantities of starch in the guard cells of nectaries were estimated and found to reach high proportions during the phase of nectar secretion. At first sight it would seem likely that precursors/by-
products of starch are important contributors to the creation of a high osmoticum within these cells, thereby maintaining relatively large apertures when challenged by the nectar passing through their pores and accumulating outside them. It is well established that stomata of leaves utilize degradation products of starch, such as malate and other organic acids (Allaway 1973, Tallman and Zeiger 1988) to help maintain high osmotic potentials in their guard cells. However, it is significant that starch is neither essential for development nor for maintenance of open pores of modified stomata on the nectary of flowers of the starchless mutant TC7. These modified stomata of the mutant flowers behave similarly to the wild-type, often being found with open pores on pre-secretory (Fig. 3.128) and post-secretory (Fig. 3.180d, 3.181 centre right) glands, despite never accumulating starch. Instead, precursors of starch production are probably involved in osmotic relations of the guard cells of nectaries of these mutant flowers. Further research on this point, as well as an investigation of the characteristics of osmotic potentials in foliar stomata (which regulate their pore apertures) of the TC7 starchless mutant, would seem to hold promise.

The starch content of modified stomata diminishes as the post-secretory flowers age, possibly a result of reclamation of sugars and other reserves from the nectary. That starch is still present in guard cells of old and deteriorating glands suggests that these cells do not secrete nectar themselves. Starch reserves in subepidermal cells of pre-secretory glands, on the other hand, disappear rapidly when nectar secretion commences.

The evidence obtained in this study of A. thaliana, like that with V. faba, is again contrary to the hypothesis that the pore apertures are finely regulated during nectar flow. In Arabidopsis, the strongest evidence against possible regulation is that open pores can be detected on the nectary of buds well before the onset of secretion, and on post-secretory flowers. Furthermore, the absence of a close synchrony between
development of these modified stomata and the commencement of nectar secretion, also favours a lack of regulation by these structures.

Although detailed physiological experiments were not conducted with this species, there is anatomical evidence that the pores probably remain open because of limitations on guard-cell movements that would allow their closure. The first point, which concerns nectaries of *A. thaliana* to a greater degree than those of fababean, is that the substomatal spaces are smaller than their counterparts in leaves because more contact is maintained between guard cells and the nectary cells immediately below them: these subepidermal cells probably limit guard-cell movements. Furthermore, in *A. thaliana*, as in *V. faba*, the cuticle surrounding the exposed guard-cell walls may impose a hindrance on flexibility of these cells, and hence, on regulation of pore apertures. Also, in both species, wall ridges composed of microfibrils (oriented perpendicular to the underlying microfibrils radiating from the pore) are located on the external surface of the outer guard-cell walls and are themselves encased by cuticle. Whereas in fababean individual ridges are longer and oriented circumferentially, and are positioned anywhere from the outer/ventral wall thickening to the dorsal wall, in *Arabidopsis* they are shorter and confined to regions near the dorsal walls. Coincident with this specific localization of ridges on the guard-cell perimeter, the outer/ventral and especially the inner/ventral wall regions of the guard cells of *A. thaliana* are considerably thicker than in *V. faba*, suggesting that the ridges may have much less restrictive influence in *Arabidopsis*. The apical openings between modified stomata, occasionally observed on floral nectaries of fababean, were never detected in *A. thaliana*. It is possible that the maintenance of open pores, as well as the greater surface area of epidermal cells due to the numerous ridges they bear externally, may enhance the reabsorption of nectar sugar.

Although similar in respect of their lack of a regulatory function during nectar
secretion, there exist major differences in the modified stomata of *A. thaliana* to those of *V. faba*. In *Arabidopsis* there is no evidence for pore occlusion by a film. Such a manner of pore obstruction, the function of which is unknown, was very commonly encountered in nectaries of fababean (see section 2.3.4). Pore obstruction may still occur in *A. thaliana* by accumulation of wax globules over narrow pores. On the other hand, the specialised cells surrounding the nectary guard cells of nectar-bearing or post-secretory flowers of the crucifer, were not found in nectar-bearing flowers of the legume. It should be noted that post-secretory flowers of *Vicia* were not examined to see if these cells exist.

Without knowing the identity of the crystalline materials they contain, only speculation can be forwarded as to the function of the developmental progression into specialised cells late in the nectar-secretory phase. There are some similarities between these specialised cells and those referred to in the Brassicaceae as "myrosin cells", whose contents have insecticidal properties (Lichtenstein *et al.* 1962, 1964). For instance, toluidine blue O gave a uniform light-blue hue to these specialised cells as well as to the large, idioblastic cells associated with vasculature in the flower receptacle (Fig. 3.5 bottom left and right). Schweidler (1910) had also detected myrosin cells in sepals, petals and siliques of this family. Also, during nectar secretion and net reabsorption, it is conceivable that plentiful supplies of glucose would be available from nectar to form substrates of the myrosinase enzyme, namely the thioglucosides (glucosinolates) (Iversen 1973). However, few ultrastructural similarities exist between these specialised cells and those myrosin cells of *Sinapis alba* L. (Werker and Vaughn 1974, 1976) and several other Brassicaceae (Bones and Iversen 1985). None of those studies showed the dense staining of the ground substance, the periplastidal endoplasmic reticulum, or the presence of amorphous cytoplasmic crystals like those found here in the *Arabidopsis* nectary. And unlike
many myrosin cells, these specialised cells in the nectary are not idioblasts. However, in *Brassica* spp., Sharma (1971) found many locations were myrosin cells differed from surrounding cells mainly in the nature of the cellular contents, not in cell size, and related that these types of myrosin cells were converted from otherwise normal parenchyma cells. These specialised cells of the floral nectary are located at surface regions that may be vulnerable to attack by pathogens and herbivores. Similarly, Schweidler (1910) detected myrosin cells in leaf epidermis of "*Stenophragma Thalianum* Celak." It is tempting to suggest that these cells have evolved to become a precautionary defensive mechanism against microbial pathogens which could attack the plant at an important site, just below the growing gynoecium, via penetration of any remaining pores of the persistent, post-secretory nectary. Fungal entry through these pores has been noted before (caption of Fig. 4.179).

4.5. SUMMARY

The nectar-secreting tissue in flowers of *Arabidopsis thaliana* forms a complete gland encircling the androecium. Proliferation of the nectary occurs at the lateral positions, abaxial to the filaments of the short stamens, and at up to four median positions, situated between the bases of the long stamens and petals. All of these protuberances are variable in size, shape and symmetry.

Nectary development occurs relatively late in floral ontogeny, glandular tissue first appearing as outgrowths at the lateral positions. Despite abscission of the perianth and androecium, the nectary of fruiting flowers persists and eventually deteriorates, becoming covered by wax and encroached by bulbous cells of the abscission zone.

The gland is supplied directly by vasculature consisting solely of phloem. The greatest innervation of phloem occurs at the lateral outgrowths, which soon after
anthesis exude most of a flower's nectar into the pouch-like lateral sepals opposite. Receptacular strands branch from the central major bundle of the lateral sepals and usually additionally from vascular bundles that jointly supply the petals and the main, marginal veins of the sepals. The median protuberances, which were only occasionally found to produce nectar, are relatively poorly supplied by vasculature that ordinarily originates only from the vascular bundle that becomes the main vein of the median sepal. Vascular supplies to the gland vary within racemes or individual flowers, and are not necessarily equal, symmetrical or present.

In wild-type flowers, starch accumulates in plastids of the nectary tissue during the bud stages, especially as anthesis approaches after the vascular supply to the gland has matured. This starch, contrary to that in cells elsewhere in floral tissues, stains indicatively as "transitory starch". Flowers bearing nectar, whether in situ or in vitro, were found to possess nectary starch no longer, suggesting that the breakdown products of the starch supplemented nectar sugar, but because equal quantities of nectar sugar were produced by cultured flowers of a starchless mutant (TC7), starch deposition and degradation are not essential steps in the nectar secretion process of A. thaliana.

The hexoses fructose and glucose predominated the nectar sugar from cultured flowers of both wild-type and starchless mutant. Nectar produced by median outgrowths of the glands contained proportionately more fructose than that expelled at the lateral outgrowths.

Studies of nectary ultrastructure of wild-type flowers throughout development showed a decline in cellular content of dictyosomes at the secretory stage, and their reinstatement post-secretion. Endoplasmic reticulum was always sparse, with the greatest quantities of a membranous reticulum occurring after nectar production had ceased. Similarly, plasmodesmatal frequencies were generally highest post-secretion, and even then were considerably lower than most literature reports for other nectaries. Microbodies (peroxisomes) were only detected in post-secretory glands. In
subepidermal cells the mean number of plastids doubled during nectary development, but never was found to exceed the average number of mitochondria, the most numerous organelle measured.

The proponderance of intercellular spaces, from the gland base to the substomatal spaces below the modified stomata on the nectary surface, and adjacent to all cell types within the gland (including sieve elements, companion cells and phloem parenchyma) strongly suggest the importance of an apoplastic route in nectar secretion by *A. thaliana*. There was no evidence that nectar flow was regulated by the starch-laden guard cells of the stomata, their pores being found open before secretion began and after it ceased. Also, the presence of immature stomata on post-secretory glands, indicating that stomatal development did not exactly correspond with the period of nectar secretion, is taken as further evidence that the stomata are not regulatory for nectar flow. As well as through the pores, nectar escape was also possible from elsewhere (epidermal cells and/or their junctions). However, the occurrence of plasmodesmata from sieve elements to epidermal cells allows the possibility for symplastic pre-nectar movement within the nectary tissue.
Figs. 4.1-4.6. General anatomy of the floral nectary of wild-type flowers of *Arabidopsis thaliana* at or near maturity.

**Fig. 4.1.** Sketch of the floral nectary (dark) in telescoping view, showing positional relationship with median (MSL) and lateral (LSL) sepals, petals (PL), long (LS) and short (SS) stamens, and gynoecium (G). The most prominent portions of the nectary (LN) occur at the lateral sides of the flower. Other nectary outgrowths (MN) occur opposite the median sepals.

**Fig. 4.2.** Transverse section at the level of the median portions of the nectary, showing continuous nectary tissue (arrowheads) around the receptacle.

**Fig. 4.3.** Low-temperature scanning electron micrograph of a lateral side of a flower that recently ceased nectar secretion, showing nectary tissue that skirts the adaxial surface of the petal claw, to connect the lateral (LN) and median (MN) portions. Modified stomata (MS) occur on the nectary surface. Insertion points of the lateral (LSL) and median (MSL) sepals and petals (PL) are indicated. **Inset.** Same view. Note the thin ridge of continuous nectary tissue (arrowhead) that runs along the adaxial side of the short stamen, which inserts at *.

**Fig. 4.4.** Continuous nectary tissue (arrowhead) around the receptacle. Eventually the two outermost vascular bundles (VB) of the receptacle each supply a long stamen. These bundles are derived from the central bundle (left) which will supply the septum of the gynoecium.

**Fig. 4.5.** Longitudinal section showing lateral portions of the nectary (LN) which extend into the pouch-like bases of the lateral sepals (LSL).

**Fig. 4.6.** Transverse section showing modified stomata (MS) of the lateral portions of the nectary facing into the pouches of the lateral sepals (LSL).

*Other abbreviations:* VS - valve of the silique (gynoecium)

*Scale bars*: Fig. 4.2 - 100 µm; Figs. 4.3 and inset, 4.5, 4.6 - 50 µm; Fig. 4.4 - 20 µm
Figs. 4.7-4.13. Pre-secretory development of the lateral portions of the floral nectary of *Arabidopsis thaliana*. Fig. 4.10 represents the starchless mutant TC7. All other micrographs represent wild-type flowers. Tissues in Figs. 4.7-4.9 and 4.11 come from the same wild-type inflorescence. Floral stages given are those of Smyth *et al.* (1990) and Bowman *et al.* (1991a).

Fig. 4.7a. Longitudinal section of bud at stage 7.

4.7b. Absence of nectary tissue external to the short stamens (SS).

Fig. 4.8a. Longitudinal section of bud which has only just begun stage 9.

4.8b. Possible nectary initials (arrowhead) at the lateral position of the flower.

Fig. 4.9a. Longitudinal section of bud late in stage 9 (x 100).

4.9b. Obvious nectary tissue (LN) at a position where this flower lacks a short stamen.

4.9c. At the other lateral position of this flower, the nectary outgrowth is less prominent.

Fig. 4.10. Longitudinal section of bud late in stage 9/early stage 10.

Fig. 4.11a. Longitudinal section of bud in stage 10/early stage 11 (0.7 mm long).

4.11b,c. Same situation as in Figs. 4.9b and c, respectively.

Fig. 4.12. Transverse section of bud of stage 11 and of length 0.9 mm. The labelled vascular bundle (VB) will supply the short stamen.

Fig. 4.13. Low-temperature scanning electron micrograph of lateral side of bud in stage 11 (length 1.0 mm) showing obvious nectary tissue at lateral position (LN). Insertion points of a petal (PL) and lateral (LSL) and median (MSL) sepals are indicated.

*Other abbreviations:* G - gynoecium; LS - long stamen; VS - valve of the silique

*Scale bars:* Figs. 4.7a, 4.8a, 4.9a, 4.11a, 4.13 - 50 µm; Figs. 4.7b, 4.8b, 4.9b,c, 4.10, 4.11b,c, 4.12 - 20 µm
Figs. 4.14-4.20. Pre-secretory development of the lateral portions of the nectary in wild-type flowers of *Arabidopsis thaliana*. Floral stages are given according to Smyth *et al.* (1990) and Bowman *et al.* (1991a).

**Fig. 4.14a.** Higher magnification of Fig. 4.13, showing bilobed outgrowth (LN) of nectary at lateral position.

**4.14b.** Further micrograph showing extension (arrows) of same nectary tissue between short stamen (SS) and petal (PL).

**Fig. 4.15.** Bud of late stage 11 sectioned longitudinally, showing several cell layers of nectary tissue at the lateral position (LN) and upward extension (arrow) of nectary cell layers.

**Fig. 4.16.** Low-temperature scanning electron micrograph of different bud of stage 11 (length 1.0 mm), showing conspicuous lobes of nectary tissue (LN).

**Fig. 4.17.** Low-temperature scanning electron micrograph of a lateral outgrowth of nectary (LN) from bud at early stage 12 (length 1.5 mm). Several modified stomata, and ridges (arrows) on most non-guard, epidermal cells, are evident.

**Fig. 4.18.** Transverse section of bud at early stage 12 (length 1.5 mm) showing many cell layers of a lateral portion of nectary (LN).

**Fig. 4.19a.** Longitudinal section of bud at stage 12 (length 1.7 mm), showing petals (PL) exceeding tips of long stamens (LS).

**4.19b.** Close-up of the multicelled nectary (LN) of Fig. 4.19a.

**Fig. 4.20a.** Transverse section of bud of late stage 12 (length 1.9 mm).

**4.20b.** Close-up of lateral portion at bottom of Fig. 4.20a, showing cleft between lobes of this outgrowth.

**4.20c.** Close-up of lateral portion at top of Fig. 4.20a, showing absence of cleft.

*Other abbreviations:* LSL - lateral sepal; MSL - median sepal

*Scale bars:* Figs. 4.14a, 4.19a, 4.20a - 50 µm; Figs. 4.18, 4.20b,c - 20 µm; Figs. 4.14b, 4.15-4.17, 4.19b - 10 µm
Figs. 4.21-4.25. Transverse sections near the midpoints of the lateral outgrowths of the nectary in flowers of nectar-bearing or older stages from the same raceme of Arabidopsis thaliana (wild-type). Higher magnification of the right-hand nectary tissue of a, is shown in b.

Figs. 4.21a,b. Flower [Müller (1961) stage B3, photograph p.372] at the onset of nectar secretion.

Figs. 4.22a,b. Nectar-bearing flower [Müller (1961) stage B4, photograph p.373] located two positions down from that of Fig. 4.21.

Figs. 4.23a,b. Nectar-bearing flower [Smyth et al. (1990) stage 15; Müller (1961) late stage B4, photograph p.373] located two positions down from that of Fig. 4.22. Note the reduction in cytoplasmic density of the nectary cells compared with those cells of Fig. 4.22b.

Figs. 4.24a,b. Post-secretory flower [Müller (1961) stage B6, photograph p.373; gynoecial length 8.6 mm, with only one long stamen remaining] located two positions down from that of Fig. 4.23. Note the increase in vacuolation of the subepidermal cells of the nectary compared to those of Figs. 4.21b-4.23b.

Figs. 4.25a,b. Post-secretory flower [Smyth et al. (1990) stage 17; Müller (1961) early stage B7, photograph p.374; gynoecial length 15.3 mm] located two positions down from that of Fig. 4.24. In Fig. 4.25b, collapse/deterioration (arrows) of cells within the outgrowth, is evident.

Note the absence of sepals (due to abscission) in Figs. 4.24 and 4.25, and the relative increase in diameter of the central receptacular vasculature to the expanding gynoecium, as flowers age.

*Scale bars: a - 100 µm; b - 20 µm*
Figs. 4.26-4.32. Lateral portions of the nectary of *Arabidopsis thaliana* (wild-type) from a mature bud and from post-secretory flowers of different ages. Figs. 4.27-4.32 are low-temperature scanning electron micrographs.

**Fig. 4.26.** Bud at late stage 12 of Smyth *et al.* (1990) (length 1.9 mm). Higher magnification of Fig. 3.5. Note the modified stomata with open pores (bottom right) on this lateral part of the nectary (LN).

**Fig. 4.27.** Nectary (LN) from an old flower [Müller (1961) very late stage B5/early stage B6] shortly after nectar has been reabsorbed. Note ridges (arrows) on epidermal cells, and open pore (right).

**Fig. 4.28.** Note open pores of the modified stomata on lateral portion of the nectary (LN) in this post-secretory flower [Müller (1961) stage B6] where petals and a long stamen have already abscinded and other stamens (SS, LS) are wilted. Note bulbous cells of the abscission zone (AZ).

**Fig. 4.29-4.31.** Areas of cellular collapse (arrows) in lateral portions of nectary (LN) of post-secretory flowers of Müller’s (1961) stage B7. Note the wax covering the persistent nectary parts (LN) and the enlarged bulbous cells of the abscission zone (AZ).

**Fig. 4.32a.** Side view of nectary (LN) extending from the abscission zone (AZ) showing waxy accumulation over epidermal cells in this post-secretory flower of stage B7 (Müller 1961).

**4.32b.** Higher magnification of area outlined in 4.32a, showing basal cells of nectary with ridges (small arrows) becoming covered with waxy globules/flakes (large arrows).

*Other abbreviations:* G - gynoecium; VS - valve of the gynoecium

*Scale bars:* Fig. 4.28 - 50 µm; Figs. 4.26, 4.27, 4.29-4.32a - 20 µm; Figs. 4.32b - 5 µm
Figs. 4.33-4.38. Development of the median portions of the floral nectary of *Arabidopsis thaliana* (wild-type). Bud stages have been assigned according to Smyth *et al.* (1990) and Bowman *et al.* (1991a).

Figs. 4.33a,b. Different longitudinal sections of the base of the same bud of stage 7, showing absence of nectary tissue between petals (PL) and long stamens (LS).

Fig. 4.34a. Longitudinal section of bud at very early stage 9.

4.34b. Higher magnification of Fig. 4.34a, showing longitudinal midpoint of petal (PL) and long stamen (LS) and the lack of nectary cells between them.

Fig. 4.35a. Longitudinal section of bud of early stage 9.

4.35b. Region of Fig. 4.35a magnified to illustrate the absence of glandular tissue between the long stamen (LS) and elongating petal (PL).

Fig. 4.36a. Longitudinal section of the base of a bud late in stage 9.

4.36b. Base of a long stamen (LS) and petal (PL) from a section preceding that of Fig. 4.36a, showing what appear to be initials of the median portion of the nectary (MN).

4.36c. Higher magnification of Fig. 4.36a, near the longitudinal midpoints of the long stamen (LS) and petal (PL), illustrating the absence of nectary cells.

Fig. 4.37a. Longitudinal section of bud of stage 10/early stage 11 (0.7 mm long).

4.37b. Base of long stamen (LS) in a section preceding that of Fig. 4.37a, depicting nectary tissue at the median position (MS). Note incoming petal (PL).

4.37c. Higher magnification of Fig. 4.37a, showing median portion of nectary (MN) between androecium and corolla.

4.37d. Section following Fig. 4.37a (= 4.37c), near the longitudinal midpoints of the long stamen (LS) and petal (PL) where nectary (MN) is restricted to only a few cells.

Fig. 4.38a. Longitudinal section of bud late in stage 11 (length 1.1 mm) with tips of the tetradynamous stamens (LS) still exceeding the maximum height of the lengthening petals (PL).

4.38b. Section preceding that of Fig. 4.38a, showing nectary cells at median position (MN). Note incoming long stamen (LS) and petal (PL).

4.38c. Section following Fig. 4.38b, but still preceding Fig. 4.38a (= 4.38d), showing nectary (MN) between androecium and corolla.

4.38d. Magnification of Fig. 4.38a, illustrating the long stamen (LS) and petal (PL) sectioned longitudinally near their midpoints, and the mound of nectary cells (MN) between them.

*Other abbreviations:* G - gynoecium; LN - lateral portion of the nectary; LSL - lateral sepal; MSL - median sepal; SS - short stamen

*Scale bars:* Figs. 4.34a, 4.35a, 4.36a, 4.37a, 4.38a - 100 µm; Figs. 4.33a,b, 4.34b, 4.35b, 4.36b,c, 4.37b,c,d, 4.38b,c,d - 20 µm
Figs. 4.39-4.45. Median parts of the nectary of *Arabidopsis thaliana* in wild-type flowers of various developmental stages [after Müller (1961), Smyth *et al.* (1990) and Bowman *et al.* (1991a)].

**Fig. 4.39a.** Longitudinal section of base of bud at early stage 12 where petal (PL) length now exceeds the anther tips of the long stamens (LS).

4.39b. Higher magnification of Fig. 4.39a, demonstrating recent anticlinal and periclinal cell divisions of the nectary (MN) located between the long stamen (LS) and incoming petal (PL).

**Fig. 4.40.** Oblique section through median side of bud of stage 12 (length 1.7 mm), illustrating two dome-shaped outgrowths of nectary tissue (MN) between filaments of the long stamens (LS) and the petal claws outside them.

**Fig. 4.41.** The nectary tissue of this transversely-sectioned, nectar-secretory flower of stage B3 is continuous (arrowhead) between two prominent outgrowths (MN) opposite the median sepal (MSL).

**Fig. 4.42.** Transverse section of a post-secretory flower of early stage B7 (gynoecium 15.3 mm long) showing large protuberances of nectary tissue (MN) with encroaching, bulbous cells of the abscission zone (AZ).

**Fig. 4.43.** Transverse section through secretory flower of stage B4 showing peculiar epidermal cell (arrow) capping the median outgrowth (MN).

**Fig. 4.44.** Median portion (MN) of nectary of Fig. 4.42 showing open pore and commencement of internal collapse of protuberance.

**Fig. 4.45.** Transversely-sectioned, post-secretory flower of Müller stage B7 (gynoecial length 17.6 mm) showing collapse (arrows) of nectary outgrowth. This flower was positioned on the inflorescence four below that of Fig. 4.44.

*Other abbreviations: G - gynoecium*

*Scale bars: Fig. 4.39a - 50 µm; Figs. 4.39b-4.45 - 20 µm*
Figs. 4.46-4.51. Low-temperature scanning electron micrographs of median protuberances of the floral nectary of *Arabidopsis thaliana* (wild-type).

Figs. 4.46-4.48. Range of shapes and sizes of median outgrowths (MN) showing ridges (arrows) on outermost epidermal cells (Figs. 4.47, 4.48) which are absent on young buds (Fig. 4.46). The different median protuberances of Figs. 4.48a and 4.48b are located on the same post-secretory flower (see Fig. 4.3).

Figs. 4.49a, 4.50. Older post-secretory flowers [Müller’s (1961) stage B7] illustrating the deposition of waxy globules (arrows) over the epidermal cells of nectary (MN) and abscission zone (AZ).

Fig. 4.49b. Median view of same flower of Fig. 4.49a, showing lack of nectary outgrowth at *.

Fig. 4.51. Lateral view of post-secretory flower [Müller’s (1961) stage B7] depicting two prominent nectary protuberances (MS) on one median side, but their absence on the opposite side (*).

*Other abbreviations:* G - gynoecium; LN - lateral portion of the nectary; PL - insertion point of petal; VS - valve of the siliqua

*Scale bars:* Figs. 4.49b, 4.51 - 50 µm; Figs. 4.46-4.49a, 4.50 - 10 µm
Figs. 4.52-4.55. Sequential scanning electron micrographs of resin replicas cast from Permagum® impressions of living tissue of Arabidopsis thaliana (wild-type) that demonstrate the external view of the developmental pathway of the modified stomata of the nectary. The modified stomata in b are enlarged from the lateral outgrowths of nectary (LN) shown in a.

Figs. 4.52, 4.53. Sequential micrographs of replicas prepared from impressions taken 315 min apart from a bud initially 1.2 mm long, at very early stage 12 (Bowman et al. (1991a)). The centre of the guard mother cell (GMC) of Fig. 4.52b becomes depressed (Fig. 4.53b, arrow), whereas the deep depression (large arrow) of the outer cuticle of the maturing stoma of Fig. 4.52b (left) ruptures (arrowheads in Fig. 4.53b) to reveal the pore (P).

Figs. 4.54, 4.55. Sequential micrographs representing the lateral portions of the nectary (LN) of a bud originally 1.0 mm in length, at stage 11 (Bowman et al. (1991a)) prepared from casts poured 23-1/4 h apart. The large arrow of Fig. 4.54b shows the inward depression (Fig. 4.55b) indicative of pore development in the immature stoma. The asterisks in Figs. 4.54a and 4.55a mark the progression of division of epidermal cells of the nectary and petal (PL) claw.

The small arrows of Figs. 4.52b and 4.54b define epidermal regions that become more deeply ridged in Fig. 4.53b and 4.55b, respectively. The large arrows of Figs. 4.52a and 4.55a show small pieces of Permagum® torn from the moulds during extraction of the resin.

Other abbreviations: LS - long stamen; LSL - remnant of the lateral sepal; SS - short stamen, or its insertion point

Scale bars: a - 20 µm; b - 5 µm
Figs. 4.56-4.64. Representatives of the various stages of development of modified stomata on the nectary of Arabidopsis thaliana (wild-type flowers). Figs. 4.56 and 4.58-4.64 are low temperature scanning electron micrographs.

Fig. 4.56. Elliptical guard mother cell (GMC) adjacent to five large isodiametric epidermal cells on lateral portion of pre-secretory nectary (see Figs. 4.13-4.14b).

Fig. 4.57. Two adjacent, highly-cytoplasmic epidermal cells thought to be guard mother cells (GMC) on a developing lateral part of nectary (LN) of young bud at early stage 12 (length 1.5 mm). Note slightly thickened regions of outer wall.

Fig. 4.58. Initial depression at future pore site at outer walls of guard cells of stoma of immature-stage 1. Pre-secretory nectary.

Fig. 4.59. A later stage of stomatal development, immature-stage 2, wherein the initial depression has proceeded both inwardly and towards the end walls. Pre-secretory nectary (see Fig. 4.17).

Fig. 4.60. Adjacent, larger immature stages, probably of stage 3, on a post-secretory gland. Both modified stomata appear to have the outer cuticle taut across the pore (small arrows), with an additional, depressed region (large arrow) on the left structure.

Fig. 4.61. Large modified stoma of immature stage-3, showing convex reflexion of the intact outer cuticle above the future outer ledges (arrows). Post-secretory nectary.

Fig. 4.62. Maturation of modified stoma as outer cuticle ruptures at two locations to reveal outer ledges (arrows). Pre-secretory nectary (see Fig. 4.17).

Fig. 4.63. Tearing of outer cuticle at final stage of development of modified stoma, showing large accumulation of cuticle (arrows) at ledges. Pre-secretory nectary (see Fig. 4.17).

Fig. 4.64. Open pore (P) of modified stoma on resin replica of pre-secretory nectary. Ledges (arrows).

Other abbreviations: LSL - lateral sepal; SS - short stamen

Scale bars: Fig. 4.57 - 20 µm; Figs. 4.56, 4.58-4.64 - 5 µm
Figs. 4.65-4.69. Sectioned lateral outgrowths (LN) of the nectary of wild-type flowers of Arabidopsis thaliana, showing early developmental stages of modified stomata.

Fig. 4.65. Apparent guard mother cell (arrowhead) and recently-divided sister guard cells (arrows) of modified stoma of immature-stage 1 on nectary surface of 1.5 mm bud of early stage 12 (Smyth et al. 1990).

Fig. 4.66. Different developmental stages of immature modified stomata showing guard cells of stage 2 (small arrows) and stage 3 (large arrows) in 1.7 mm bud of stage 12 (Smyth et al. 1990).

Fig. 4.67. Immature stoma of late stage 2 on surface of post-secretory nectary undergoing internal degradation (arrow). Müller (1961) stage B7.

Fig. 4.68. Transmission electron micrograph of transversely-sectioned immature modified stoma in transition from stage 1 to stage 2 from a post-secretory flower of stage B5 [Müller (1961)]. The thickening ventral walls of the sister guard cells are separated by a thin, relatively osmiophilic layer (arrows). Mitochondria (M), dictyosomes (D) with associated vesicles (Dv), spherosomes (L), endoplasmic reticulum (ER), plastid (Pl) with plastoglobuli (arrows), and part of a vacuole (V), are evident.

Fig. 4.69. Transmission electron micrograph of modified stoma of immature-stage 2 sectioned transversely at position "a - - - a" indicated on Fig. 4.67. Note the central depression (arrowhead) and the ridges (clear arrows) near the dorsal walls, on the outer stomatal surface, and the developing substomatal space (SbS) at the inner/ventral walls. Each guard cell now contains a large, single vacuole (V). In this (pre-secretory) nectary of a 1.9-mm bud of late stage 12 (Smyth et al. 1990), the plastids (Pl) of the subepidermal cells contain similar quantities of starch as those in this immature stoma. Nucleus (N), nucleolus (Nl).

Other abbreviations: LSL - lateral sepal; SS - short stamen

Scale bars: Figs. 4.65-4.67 - 10 μm; Fig. 4.69 - 1.0 μm; Fig. 4.68 - 0.5 μm
Figs. 4.70-4.73. Transmission electron micrographs of transverse sections of developmental stages of modified stomata on lateral outgrowths of the floral nectary of *Arabidopsis thaliana* (wild-type).

**Fig. 4.70.** Cuticle (wide arrow) along outer walls of guard cells of immature modified stoma of Fig. 4.68, showing slight depression (arrowhead) where original cuticle has become separated from walls at future pore site. A new osmiophilic layer containing cuticular lamellae (long arrow) is deposited over the exposed outer/ventral walls. Note the granular material (*) between the original and new cuticles.

**Fig. 4.71.** Modified stoma of immature-stage 2 (see Fig. 4.69) now sectioned at its midpoint (as indicated in Fig. 4.67, "b - - - b") showing separating outer cuticle (arrowhead), and inner and future outer ledges (small arrows). The substomatal space (SbS) - which is larger here than at the level sectioned in Fig. 4.69 - and the outer walls are covered by a cuticle (wide black arrows). Note the external ridges (wide clear arrows) near the dorsal walls of the guard cells, and on adjacent epidermal cells (E).

**Fig. 4.72.** Undulating outer cuticle (arrowhead) spanning the future pore (P), otherwise complete, in this immature modified stoma of stage 3 on the gland of a nectar-bearing flower. The cell walls of the guard cells are particularly thickened at the inner/ventral and outer/ventral interfaces, and bear starch-laden plastids (Pl). Note the wall degradation (*) at the expanding substomatal space below the dorsal guard-cell walls, and cuticle (wide black arrows) lining the space, pore and outer walls.

**Fig. 4.73.** Open modified stoma with remnants (arrowheads) of ruptured outer cuticle. Intercellular spaces (IS), part of the substomatal continuum, are well developed below the dorsal guard-cell walls. Even though the stoma of this nectar-bearing flower is now mature, note the continual contact of guard cells with subepidermal cells (SE).

*Other abbreviations:* N - nucleus; V - vacuole

*Scale bars:* Figs. 4.70-4.73 - 1.0 µm
Fig. 4.74. Transmission electron micrograph of a mature modified stoma in longitudinal section on a nectar-bearing flower of *Arabidopsis thaliana* of Müller (1961)'s stage B4. Mitochondria (M), spherosome (L), plastids (Pl) with large starch grains, nucleus (N), and numerous profiles of the single vacuole (V) per guard cell, are evident. Pore (P). Part of the substomatal space (*) below the dorsal wall of the upper guard cell, is apparent. In comparison, the six epidermal cells which surround the modified stoma are densely cytoplasmic and vesiculate (Ve), with crystals (Cr), and stacks of endoplasmic reticulum (ER) sometimes dilated (top right centre) or not (top left centre) that surround starch-lacking plastids (Pl). Note plasmodesmata (Ps; bottom right) between epidermal cells, and cuticle (arrow) on outer wall of epidermis (bottom left).

*Scale bar: 1.0 µm*
Figs. 4.75-4.79. Transmission electron micrographs of guard cells of open modified stomata on lateral outgrowths of the nectary of nectar-bearing, wild-type flowers of Arabidopsis thaliana.

**Fig. 4.75.** Pore (P) between ventral walls (W) lined by cuticle (arrows) and containing granular material (*) with associated dense globules (arrows) at the ends of the pore. Note the similar staining qualities of this material, these globules and the cuticle with the two pore-associated spherosomes (L).

**Fig. 4.76.** Glancing longitudinal section of inner/ventral wall (W) of modified stoma of Fig. 4.74 showing orientation of microfibrils (white double arrows) approximately parallel to the cytoplasmic cortical microtubules (arrows). Note pore (P) lined by cuticle (wide arrow).

**Fig. 4.77.** Anticlinal section nearing longitudinal midpoint of guard cell (GC). Note major accumulation of cuticle at outer ledge (black arrow) and surrounding the free surfaces of the cell and its substomatal space (*). Outer and inner walls are thickest at their midpoints (arrowheads). Ridges (clear arrows) occur on external surface of outer wall near end walls, and on epidermal cells (E).

**Fig. 4.78.** Longitudinal section through inner walls (W) of modified stoma of Fig. 4.74, showing pockets of cytoplasm near the end walls, and portions (*) of the substomatal space there. This is the approximate level where the pore (P) continues into the substomatal space. Note the dense cytoplasm of these particular epidermal (E) and subepidermal (SE) cells, and their abundance of vesicles.

**Fig. 4.79.** Anticlinal section near longitudinal midpoint of guard cell (GC) illustrating thickened middle portions (arrowheads) of the inner and outer wall, ridges (clear arrows) near end walls, and cuticle (black arrow) lining all free surfaces of the cell and its substomatal space (SbS).

*Other abbreviations:* M - mitochondria; N - nucleus; Pl - plastid; S - starch

*Scale bars:* Figs. 4.77-4.79 - 1.0 μm; Figs. 4.75, 4.76 - 0.5 μm
Figs. 4.80-4.87. Modified stomata on nectary surface of post-secretory, wild-type flowers of *Arabidopsis thaliana*.

Fig. 4.80. Transmission electron micrograph of the thickened, middle region of the inner/ventral wall (W) of a guard cell (the unlabelled one at the very bottom left of Fig. 4.88a) sectioned longitudinally, but obliquely in the anticlinal plane. The most recently deposited microfibrils (*), which radiate from the pore (see Fig. 4.76), and the microtubules (arrows) within the cytoplasmic invaginations of the wall, are both sectioned transversely. Older regions of this wall bear microfibrils oriented obliquely and even longitudinally, whereas the oldest parts of the wall - in the protuberances (clear arrows) that jut into the pore (P) very near to its continuation by the substomatal space - contain less-densely packed microfibrils that are again in transverse section. Note the cuticle (wide black arrows) lining the exposed wall surface. Mitochondria (M) and a spherosome (L) with nearby microbodies (MB) are evident.

Figs. 4.81-4.84. Examples of lateral (LN; Figs. 4.81-4.83) and median (MN; Fig. 4.84) portions of the nectary that demonstrate the presence of open pores (P) between sister guard cells. In Figs. 4.81, 4.82 and 4.84, certain epidermal and subepidermal cells (arrowheads) immediately beside guard cells or below their pores, respectively, remain densely cytoplasmic. Other nectary cells contain large vacuoles. Note the internal collapse (arrows) of the nectary tissue, and large cells of the nearby abscission zone (AZ).

Figs. 4.85-4.87. Low temperature scanning electron micrographs of the surface of lateral outgrowths of the nectary of flowers of stage B7 (Müller 1961), showing pores (P) ranging from shut or narrowly open, to those of wide aperture. Amorphous waxy globules (arrows) cover the outer walls and epidermal cells, and, to some extent, reduce the length and width of pores which remain open.

*Scale bars:* Figs. 4.81-4.84 - 10 µm; Figs. 4.85-4.87 - 5 µm; Fig. 4.80 - 1.0 µm
**Fig. 4.88.** Transmission electron micrographs of specialised epidermal and subepidermal cells of a lateral outgrowth of a post-secretory nectary of *Arabidopsis thaliana* of stage B5 [Müller (1961)] (wild-type flower).

**Fig. 4.88a.** Outer region of nectary showing guard cells (GC) of a modified stoma and portions of another (bottom left) amidst epidermal cells (E) above subepidermal cells (SE) and intervening substomatal spaces (SbS). Note the two particularly dense-staining subepidermal cells (and the epidermal cell between the two modified stomata) that are partially outlined and examined at higher magnification below.

**Fig. 4.88b.** Portion of the specialised subepidermal cell (centre right of Fig. 4.88a) showing dense cytoplasm bearing plastids (Pl) with starch grains (S) and a large amorphous crystal (Cr) densest at its centre. Numerous vesicles (Ve), possibly of dictyosomal origin, are found in the cortical cytoplasm, and often appear in close association with the plasma membrane. A dilated cisterna of endoplasmic reticulum (bottom right) is apparent in the adjacent subepidermal cell (SE).

**Fig. 4.88c.** Specialised subepidermal cell (bottom centre of Fig. 4.88a) with very dense cytoplasm bearing hundreds of vesicles (Ve) and large, heterogeneous crystalline bodies (Cr) densest at their centres. The substomatal spaces (SbS) are lined by cuticle (arrows). Cisternae of endoplasmic reticulum (ER).

*Other abbreviations:* V - vacuole

*Scale bars:* Fig. 4.88a - 5 µm; Figs. 4.88b,c - 0.5 µm
Figs. 4.89-4.91. Specialised epidermal and subepidermal cells associated with modified stomata of lateral outgrowths on the gland of nectar-bearing (Fig. 4.89) or post-secretory [Figs. 4.90, 4.91; stage B5 of Müller (1961)] wild-type flowers of Arabidopsis thaliana.

Fig. 4.89a. Periclinal section almost completely through the modified stoma of Figs. 4.74 and 4.78, showing remnants of inner walls (W) of sister guard cells and their junction with some subepidermal cells (SE). Note the multitude of variously-sized vesicles in the subepidermal cells, and to a lesser extent, in the epidermal cells (E). Substomatal space (SbS).

Fig. 4.89b. Further section just below the modified stoma of Fig. 4.89a, showing the same three incoming subepidermal cells and the concurrent reduction in width of the substomatal space (SbS). Note that this substomatal space is assymetrically shaped and not equally aligned below the outgone modified stoma.

Fig. 4.89c. The multivesiculate nature of the subepidermal cells of an upper portion of Fig. 4.89b is illustrated at higher magnification. There are several instances of apparent vesicle (Ve) connection with the plasma membrane (arrows) at most cell walls. Plastids (Pl) are enveloped in parallel stacks of endoplasmic reticulum (ER). A cuticle (wide black arrows) lines the substomatal space (SbS).

Fig. 4.90. A crystalline lattice (*) inside part of a vacuole (V), dilated cisternae of endoplasmic reticulum (centre), plastid (Pl) with large plastoglobuli (arrows), numerous vesicles (Ve) and a mitochondrion (M) are features of this specialised subepidermal cell below a modified stoma.

Fig. 4.91. Portion of a different specialised subepidermal cell where it abuts an intercellular space (IS) lined by a thin cuticle (wide black arrow). A cytoplasmic crystalline body (Cr), densest at its centre, endoplasmic reticulum (ER) associated with a plastid (Pl), and a vesicle fused with the plasmalemma (arrow) are evident.

Scale bars: Figs. 4.89a-c - 1.0 µm; Figs. 4.90, 4.91 - 0.5 µm
Figs. 4.92-4.97. Vascular supply of phloem alone to the lateral (Figs. 4.92-4.96) and median (Fig. 4.97) outgrowths of the nectary of wild-type (Figs. 4.92) and TC7 starchless mutant (Figs. 4.93-4.97) flowers of Arabidopsis thaliana.

Figs. 4.92, 4.93. Longitudinal sections partially through the two lateral outgrowths of the nectary (LN) of 1.6-1.7 mm buds of stage 12 (Smyth et al. 1990), showing absence of a vascular supply to the glands.

Fig. 4.94. Phloem innervation of a lateral portion of a pre-secretory flower just opening (stage B2/B3 of Müller, 1961). Note sieve elements (arrowheads) at nectary base.

Fig. 4.95. Longitudinal section through nectar-bearing flower of Müller’s (1961) B4 stage showing sieve elements (arrowheads) penetrating the nectary base. The vascular bundles $X_1$, $X_3$ and $X_4$ supply the main vein of the lateral sepal (LSL) and the nectary (LN), the short stamen (SS), and the lateral wall of the gynoecium (G), respectively.

Fig. 4.96. Another section of the same flower of Fig. 4.95, illustrating nectary innervation by sieve elements (arrowheads).

Fig. 4.97a. Transverse section through a nectar-bearing flower of stage B4 (Müller 1961) at the level where each of the median sepals (MSL) receives three major vascular bundles from the receptacle. A petal (PL) was previously removed at *.

4.97b. Direct supply of sieve elements (arrowheads) into the base of this median portion of the nectary (MN) previously located at bottom right position of Fig. 4.97a.

4.97c,d. Higher magnification of the left and right junctions of the sepals (MSL) with the receptacle of Fig. 4.97a, showing sieve elements (arrowheads) that have left the central (main) vein (top centre) of each sepal and are directed toward the medial positions of greatest proliferation of nectary tissue (e.g., Fig. 4.97b).

Other abbreviations: Ov - ovule; St - septum

Scale bars: Figs. 4.92, 4.93, 4.95, 4.97a,c,d - 50 µm; Figs. 4.94, 4.96, 4.97b - 20µm
Figs. 4.98a-j. Representative transverse sections (1.5 µm), stained with toluidine blue 0 (except Figs. 4.98g and h, which are stained with pseudo-Schiff’s reagent), of serially-sectioned, nectar-bearing wild-type flower [Müller’s (1961) stage B4] of Arabidopsis thaliana. The right-hand micrographs (Figs. 4.98b,d,f,h,j) show, at higher magnification, the region of the junction of the receptacle (R) with a lateral sepal (LSL) (Figs. 4.98a,c,e,g) and with a lateral portion of the nectary (LN) (Fig. 4.98i).

Fig. 4.98a. Section through the pouch-like base of the lateral sepal at the level cutting the sepal’s main vein (section #15-36-1).

4.98b. The sepal is not yet attached to the receptacle.

Fig. 4.98c. Section 18.0 µm later, showing the severed main vein as the sectioning proceeds into the sepal pouch (section #15-37-5).

4.98d. Portions of the sepal’s main vein (X₁) are indicated. The calyx will soon connect with the receptacle.

Fig. 4.98e. Modified stomata of the nectary surface are seen in this section through the sepal pouch, 19.5 µm after Fig. 4.98c (section #15-39-2).

4.98f. Convergence of the isolated parts of the sepal’s main vein (X₁), with a single sieve element (arrowhead at right centre) appearing to the rear of this outgoing major vein. The sepal and receptacle are now fused.

Fig. 4.98g. Incoming lobes of the lateral part of the nectary, 7.5 µm after Fig. 4.98e (section #15-39-7).

4.98h. The last (uppermost) tracheary element (arrow) of the X₁ bundle is visible above two groups of numerous sieve elements (arrowheads), to the flank and rear of the outgoing X₁ bundle, from which they have originated.

Fig. 4.98i. Section taken 15.0 µm after Fig. 4.98g, at a point where the two lobes of nectary tissue are connected (section #15-41-1).

4.98j. At top, centre, note penetration of X₁-derived sieve elements (arrowheads) into nectary tissue and the branching of further sieve elements (arrowheads at left, right) from bundles X₂ₐ and X₂₈ toward the nectary. Bundle X₃ will eventually supply the short stamen.

Scale bars: Figs. 4.98a,c,e,g,i - 100 µm; Figs. 4.98b,d,f,h,j - 50µm
Figs. 4.99a-j. Continuation of the series of sections represented in Fig. 4.98, showing vascular supply of phloem to a lateral portion of the floral nectary (LN) of *Arabidopsis thaliana*.

**Fig. 4.99a.** Section 7.5 µm away from Fig. 4.98i, showing incoming petal left of the nectary tissue (section #15-41-6).

**4.99b.** Sieve elements (arrowheads) originating from bundles X<sub>2A</sub> and X<sub>2B</sub> starting to penetrate the nectary at left and right, while other elements (arrowheads) originating from bundle X<sub>1</sub> continue to supply the gland (centre right).

**Fig. 4.99c.** Section grazing the bases of the petal (PL) claws and filaments of the short stamen (SS), 10.5 µm after Fig. 4.99a (section #15-42-5).

**4.99d.** Innervation of the nectary by sieve elements (arrowheads) from the X<sub>2A</sub> and X<sub>2B</sub> bundles.

**Fig. 4.99e.** Section 7.5 µm following Fig. 4.99c (section #15-43-2).

**4.99f.** The bundle X<sub>4</sub> will eventually supply the lateral wall of the gynoecium.

**Fig. 4.99g.** Section 7.5 µm after Fig. 4.99e (section #15-43-7).

**4.99h.** The distal ends of some phloem traces (arrowheads) to the nectary.

**Fig. 4.99i.** Section 10.5 µm away from Fig. 4.99g (section #15-44-6).

**4.99j.** No further vasculature enters the nectary. The X<sub>3</sub> bundle is seen to enter the base of the filament of the short stamen.

*Other abbreviations:* LSL - lateral sepal; R - receptacle

*Scale bars:* Figs. 4.99a,c,e,g,i - 100 µm; Figs. 4.99b,d,f,h,j - 50 µm
Figs. 4.100, 4.101. Direct vascular supply of phloem to lateral portions of the nectary (LN) of wild-type flowers of Arabidopsis thaliana.

**Fig. 4.100.** Three-dimensional reconstruction of the sieve elements penetrating the serial-sectioned lateral portion of the nectary illustrated in Figs. 4.98 and 4.99. Note that the sieve elements originate from vascular bundle X₁ and from either the X₂A or X₂B bundles. The X₃ bundle supplies both xylem and phloem to the short stamen. Three of the twelve modified stomata of this lateral portion of the nectary were sectioned longitudinally, and are shown at their locations on the abaxial surface of the gland.

**Fig. 4.101.** Transmission electron micrograph of nectary base from a 1.9-mm bud of stage 12 (Smyth et al. 1990) depicting the branching of mature phloem in the glandular tissue. As well as the relatively-dense staining parenchyma cells (top), the nectary tissue is composed of companion cells (Cc) adjacent to the sieve elements (Se), and several intercellular spaces (IS). The receptacle (R) consists of larger, highly-vacuolate cells with large intercellular spaces.

*Scale bar: Fig. 4.101 - 10 µm*
Fig. 4.102. Transmission electron micrograph of the phloem supply at the base of a lateral part of the nectary of a wild-type, 1.9 mm bud of stage 12 (Smyth et al. 1990) of Arabidopsis thaliana. The two central, mature sieve elements (Se) are luminous and contain a peripheral cytoplasm bearing plastids (Pl) and endoplasmic reticulum (ER), whereas the two developing sieve elements above and adjacent to them are still highly cytoplasmic. Dense-staining companion cells (Cc), of similar dimensions to the sieve tubes, and larger parenchyma cells (Pp), surround the elements and complete the phloem supply. The plastids of the phloem parenchyma and sieve elements contain grains of starch, lacking in those of companion cells. Part of a sieve pore (Sp) and a plasmodesma (Ps) are evident. Dictyosome (D).

Scale bar: 1.0 µm
Figs. 4.103, 4.104. Transmission electron micrographs of mature sieve elements at base of lateral outgrowths of the nectary of wild-type flowers of *Arabidopsis thaliana*.

**Fig. 4.103.** Note the plastid (Pl) and accumulation of endoplasmic reticulum (ER) in the parietal cytoplasm of this nectary sieve element (Se) from a pre-secretory flower just opening [Müller’s (1961) stage B2/B3]. An intercellular space (IS) next to the sieve tube is evident.

**Fig. 4.104.** Numerous mitochondria (M), endoplasmic reticulum (ER) and a plastid (Pd) in these sieve elements (Se) of a post-secretory nectary [Müller’s (1961) stage B5]. Plasmodesmata (Ps) connect the sieve tube to a phloem parenchyma cell (Pp). Companion cell (Cc).

*Scale bars:* Fig. 4.104 - 1.0 µm; Fig. 4.103 - 0.5 µm
Figs. 4.105-4.108. Examination of the phloem supply to the lateral outgrowths of the nectary (LN) from the receptacular vascular bundles X$_2^A$ and X$_2^B$ (see Fig. 4.98j) in longitudinally-sectioned wild-type (Figs. 4.107, 4.108) and TC7 starchless mutant (Fig. 4.105, 4.106) flowers of Arabidopsis thaliana.

Figs. 4.105, 4.106. Isolated sieve elements (arrowheads), which abut large intercellular spaces, will eventually directly supply the nectary tissue of a nectar-bearing flower [Fig. 4.105; Müller’s (1961) stage B4] and a (pre-secretory) mature bud, just opening (Fig. 4.106; see Fig. 4.94).

Figs. 4.107, 4.108. Transmission electron micrographs of regions of isolated phloem as indicated in Figs. 4.105 and 4.106, from both lateral portions of a post-secretory flower [Müller’s (1961) stage B5], showing sieve elements (Se) surrounded by dense-staining companion cells (Cc), phloem parenchyma cells (Pp), and voluminous intercellular spaces (IS). These spaces are lined by a very thin material (arrows) resembling a cuticle, and contain cellular debris that includes grains of starch (S).

Other abbreviation: SS - short stamen

Scale bars: Figs. 4.105, 4.106 - 10 μm; Figs. 4.107, 4.108 - 1.0 μm
Figs. 4.109, 4.110. Transmission electron micrographs of phloem supply at base of lateral outgrowths of post-secretory nectaries from wild-type flowers of stage B5 (Müller 1961) of *Arabidopsis thaliana*.

**Fig. 4.109.** Ultrastructure of sieve elements (Se) and companion (Cc) and parenchyma (Pp) cells of nectary phloem. Plasmodesmata (Ps) occur between adjacent companion cells (right) and between these cells and parenchyma (left). Plastids lack starch.

**Fig. 4.110.** Phloem traversing the base of the nectary. Note absence of starch in plastids of companion cells (Cc) and parenchyma cells.

*Other abbreviation:* M - mitochondrion

*Scale bars:* Figs. 4.109, 4.110 - 1.0 μm
Figs. 4.111-4.114. Transmission electron micrographs depicting ultrastructural features of sieve elements supplying the lateral parts of the nectary of wild-type flowers of *Arabidopsis thaliana*.

**Fig. 4.111.** Sieve plate between shared wall (W) of adjacent sieve elements (Se) of a nectar-bearing flower [Müller's (1961) stage B4] showing portions of three sieve pores (Sp). The top pore bears an osmiophilic material that resembles p-protein. Each pore is surrounded by an electron-translucent region (*) that is internal to the sieve-tube plasma membrane (circle, bottom right). Middle lamella (ML).

**Fig. 4.112.** Another nectary sieve pore (Sp), traversed by fibrillar p-protein, from a post-secretory flower of stage B5 (Müller 1961).

**Fig. 4.113.** Large accumulation of endoplasmic reticulum (ER) in the peripheral cytoplasm of this mature sieve element (Se). Note the trilaminate plasma membrane (circle) abutting the cell wall (W). Higher magnification of Fig. 4.103.

**Fig. 4.114.** Endoplasmic reticulum (ER) and mitochondria (M) of a mature sieve element (Se) of a post-secretory nectary [Müller's (1961) stage B5] neighbouring phloem parenchyma cells. Plasmodesmata (Ps).

*Scale bars:* Figs. 4.111-4.114 - 0.2 µm
Fig. 4.115. Quantity of modified stomata and their positions in relation to locations of relative proliferation of nectary tissue and its vascularisation, in serial-sectioned flowers of Arabidopsis thaliana (wild-type). The six flowers with sketched nectaries came from the same primary raceme, are drawn oldest to youngest, and are classified according to Müller (1961). The original orientation of each flower with respect to the stalk of the inflorescence is unknown, so that no orientational relationship between flowers should be assumed. The top flower (A; stage B7, gynoecium 17.7 mm long) was the seventh borne on the rachis, whereas the bottom flower (F; stage B3) was the seventeenth flower to develop. The intermediate flowers were taken at positions nine (B; early stage B7, gynoecium 15.3 mm long), eleven (C; stage B6, gynoecium 8.6 mm long), thirteen (D; late stage B4) and fifteen (E; stage B4). At the time of fixation, 32 days post-seeding, flowers A-C were post-secretory and D-F bore nectar.

The numbers given at the various lobes of nectary tissue represent the total number of modified stomata detected there. The lines represent strands of the receptacular vascular supply of phloem toward the glandular tissue, originating from vascular bundles X_1, X_{2A} and X_{2B} in the case of lateral outgrowths (see Fig. 4.98j), and sometimes from the central (MSL) vein of the median sepal (see Figs. 4.97c and d), in the case of the median spurs of nectarial tissue.

For flowers A, B and C, vascular tissue to the lateral outgrowths was not traced except at its source, as indicated by the arrows. Every lateral outgrowth of these three flowers, therefore, received phloem from vascular bundles X_1, X_{2A} and X_{2B}.

In flower A, the triangles at the position of the vascular bundle giving rise to the main (central) vein of the median sepals signify that it was not possible to detect any phloem leaving there for the median protuberances, because of cell deterioration and expansion of cells of the abscission zone.

In flower C, the asterisk denotes the lack of a median outgrowth at this position.
Fig. 4.116. Results of the stereologic examination of cellular and ultrastructural features of epidermal cells (excluding guard cells, and the "specialised" cells of nectar-bearing and post-secretory flowers) and subepidermal cells (excluding the vasculature, and the "specialised" cells of nectar-bearing and post-secretory flowers) of lateral outgrowths of wild-type flowers of *Arabidopsis thaliana* during development and nectar-secretory activity.

The six floral stages investigated were 0.9-mm buds (stage 11), 1.5-mm buds (early stage 12), 1.9-mm buds (late stage 12), pre-secretory flowers where the petals are only first visible (stage B2/B3), nectar-bearing flowers of stage B4, and post-secretory flowers of stage B5. These stages were assigned according to Müller (1961), Smyth *et al.* (1990) or Bowman *et al.* (1991a).

The numbers of each organelle detected per floral stage are given as averages. One standard error of the mean is indicated by the line above each bar.
Epidermal Cells

- cytoplasm: 0.493 ± 0.024
- vacuole: 0.891 ± 0.054
- Dictyosomes: 0.740 ± 0.042
- Microbodies: 0.857 ± 0.071
- Mitochondria: 1.059 ± 0.079
- Plastids: 0.912 ± 0.055

Subepidermal Cells

- cytoplasm: 0.488 ± 0.019
- vacuole: 0.993 ± 0.042
- Dictyosomes: 0.606 ± 0.030
- Microbodies: 0.970 ± 0.039
- Mitochondria: 1.111 ± 0.053
- Plastids: 1.057 ± 0.043

Total cell area (mm²) in section

0 10 20 30
No. organelles per mm² of cytoplasm in section

0.9 mm Bud
1.5 mm Bud
1.9 mm Bud
Petals first seen
Bearing nectar
Post-secretory

Floral Stage
Figs. 4.117, 4.118. Transmission electron micrographs of longitudinally-sectioned epidermal cells of lateral outgrowths of the nectary of wild-type flowers of *Arabidopsis thaliana*.

**Fig. 4.117.** Epidermal cell from a (pre-secretory) 0.9-mm bud [stage 11 of Smyth *et al.* (1990)], showing vacuoles (V), nucleus (N), dictyosomes (D), mitochondria (M) and plastids (Pl) lacking starch. Note the starch grains (S) in the subjacent cell, and the intercellular spaces (IS). A thin cuticle (arrows) covers the external wall.

**Fig. 4.118.** Portion of an epidermal cell from a nectar-bearing flower [stage B4 of Müller (1961)], showing the large solitary vacuole (V), and endoplasmic reticulum (ER) in the outer cytoplasm. The exposed wall bears ridges (arrow) of wall material covered by a thickened cuticle.

*Scale bars:* Figs. 4.117, 4.118 - 1.0 µm
**Fig. 4.119.** Transmission electron micrograph of typical subepidermal cells from a lateral outgrowth of the floral nectary in a 0.9-mm wild-type bud of *Arabidopsis thaliana* of stage 11 (Smyth et al. 1990), showing dictyosomes (D), mitochondria (M), nucleus (N), plastids (Pl) with starch grains (S), and profiles of the vacuome (V). Note the thin cell walls and evidence of plasmodesmatal development (Ps), and the intercellular spaces (IS).

*Scale bar: 1.0 µm*
Figs. 4.120-4.123. Outgrowths at the lateral position of the nectary (LN) in buds of *Arabidopsis thaliana* of wild-type (Figs. 4.120-4.122) and TC7 starchless mutant (Fig. 4.123).

**Fig. 4.120.** Transmission electron micrograph of nectary tissue from a 1.5-mm bud (early stage 12) that demonstrates the relatively small cell size compared to adjacent tissue of the lateral sepal (LSL) and the receptacle (R). Each epidermal cell of the nectary contains a large, solitary vacuole, whereas the subepidermal cells are highly cytoplasmic and multivacuolate. Some starch grains are discernible in plastids of these subepidermal cells.

Figs. 4.121, 4.122. Fluorescence micrographs of nectary tissue from 1.5-mm and 1.7-mm buds (of early stage 12, and stage 12, respectively; Smyth *et al.* 1990) stained with pseudo-Schiff’s reagent that demonstrates nucleoli (bold arrows) and the accumulation of starch grains (thin arrows).

**Fig. 4.123.** Note the lack of fluorescence indicative of starch in this tissue of the starchless mutant. Nucleoli (bold arrows) are fluorescent.

*Other abbreviations:* SS - short stamen

*Scale bars:* Figs. 4.120-4.123 - 20 µm
Figs. 4.124-4.126. Representative tissue of the lateral outgrowths of the floral nectary (LN) in large wild-type (Figs. 4.124, 4.125) and TC7 starchless-mutant (Fig. 4.126) buds of *Arabidopsis thaliana*.

Figs. 4.124, 4.125. Ultrastructure of the nectary of 1.9-mm buds [late stage 12 of Smyth *et al.* (1990)] demonstrating the abundance of starch (S) in the plastids (Pl), the numerous mitochondria (M), the thin walls and the multivacuolate (V) nature of these subepidermal cells prior to the commencement of nectar secretion.

Fig. 4.126. Note the fluorescent nucleoli (arrows), but the lack of fluorescence indicative of starch, in this nectary tissue of a 1.8-mm bud (stage 12; Smyth *et al.* 1990) of the starchless mutant stained with pseudo-Schiff’s reagent.

*Other abbreviations:* LSL - lateral sepal; N - nucleus; SS - short stamen

*Scale bars:* Fig. 4.126 - 20 μm; Fig. 4.125 - 1.0 μm; Fig. 4.124 - 0.5 μm
Figs. 4.127-4.129. Nectary tissue of lateral outgrowths of the nectary (LN) of wild-type (Figs. 4.127, 4.129) and TC7 starchless-mutant (Fig. 4.128) flowers of *Arabidopsis thaliana* near the onset of nectar secretion.

**Fig. 4.127.** Fluorescence micrograph of an obliquely-sectioned lateral outgrowth (LN) of a 1.9-mm bud [late stage 12 of Smyth *et al.* (1990)] demonstrating numerous fluorescent spots (small arrows) associated with plastids, and fluorescent circles within a nucleus (large arrow, left) indicative of a nucleolus, in subepidermal cells subjected to pseudo-Schiff’s reagent.

**Fig. 4.128.** Nectary tissue of a 1.9-mm bud [late stage 12; Smyth *et al.* (1990)] sectioned longitudinally before staining with pseudo-Schiff’s reagent. In the cell interiors, only nucleoli (arrow) fluoresce. Note the large pore of the modified stoma on this pre-secretory nectary’s surface (bottom left).

**Fig. 4.129.** Transmission electron micrograph prepared from a pre-secretory flower that was just opening [stage B2/B3; Müller (1961)], that demonstrates the presence of large starch (S) grains in many of the plastids, mitochondria (M), multiple vacuoles (V) per cell, and prominent intercellular spaces (IS).

*Other abbreviations:* PL - petal

*Scale bars:* Fig. 4.129 - 1.0 µm; Figs. 4.127, 4.128 - 20 µm
Figs. 4.130-4.132. Nectary tissue from lateral parts of the gland (LN) of nectar-bearing, wild-type (Figs. 4.130, 4.132) and TC7 starchless-mutant (Fig. 4.131) flowers of stage B4 (Müller 1961) of *Arabidopsis thaliana*.

**Fig. 4.130.** Note the absence of numerous fluorescent dots in the actively-secreting nectary of this transversely-sectioned flower stained with pseudo-Schiff’s reagent.

**Fig. 4.131.** Fluorescence micrograph of the secretory nectary of a longitudinally-sectioned flower. Within the glandular cells, note only nucleolar fluorescence (arrows).

**Fig. 4.132.** Transmission electron micrograph of an actively-secreting gland showing virtual absence of starch (S) in these subepidermal cells. Some plastids (Pl) (bottom left) are irregularly shaped. The cells are still multivacuolate (V).

*Other abbreviations:* IS - intercellular spaces; LSL - lateral sepal; N - nucleus; R - receptacle

*Scale bars:* Fig. 4.132 - 1.0 µm; Figs. 4.130, 4.131 - 20 µm
Fig. 4.133. Frequency distribution of numbers of starch grains counted per plastid profile in randomly-selected epidermal and subepidermal cells from silver-thin sections of the lateral protuberances of the nectary in six developmental stages [0.9 mm bud - stage 11; 1.5 mm bud - early stage 12; 1.9 mm bud - late stage 12; early-opening, pre-secretory flowers - stage B2/B3; nectar-bearing flowers of stage B4; post-secretory flowers of stage B5; as designated according to Müller (1961), Smyth et al. (1990) and Bowman et al. (1991a)] of wild-type flowers of Arabidopsis thaliana.
Epidermal Cells

Subepidermal Cells

No. starch grains per plastid in section

0.9 mm Bud 1.5 mm Bud 1.9 mm Bud first seen petals bearing nectar post-secretory
**Figs. 4.134-4.138.** Sections of the median spurs of nectary tissue (MN) at various stages of floral development in wild-type (Figs. 4.134-4.136, 4.138) and TC7 starchless-mutant (Fig. 4.137) plants of *Arabidopsis thaliana.*

**Fig. 4.134.** Nectary tissue at the median position (MN) in a 1.9-mm bud of advanced stage 12 (Smyth et al. 1990) stained with pseudo-Schiff’s reagent, showing fluorescence associated with starch (thin arrows) and nucleoli (bold arrows) (x 885).

**Figs. 4.135, 4.136.** Transverse sections through flowers at the level showing confluence of a median (MN) and a lateral (LN) outgrowth of the nectary of a flower which has only just started to secrete nectar [Fig. 4.135; Stage B3 of Müller (1961)] and of another nectar-bearing one of stage B4 (Müller 1961) (Fig. 4.136). The two flowers were separated by another flower, on the same inflorescence. After staining with pseudo-Schiff’s, fluorescence indicative of starch (thin arrows) and nucleoli (bold arrows) is apparent. Note the relative persistence of starch in the cells between the median and lateral proliferation of nectary tissue. At the top of Fig. 4.136, note the starch-rich guard cells of two modified stomata.

**Fig. 4.137.** Flower sectioned longitudinally and stained with pseudo-Schiff’s reagent. Note the absence of starch-associated fluorescence, even in the terminal modified stoma of this newly-secreting flower of early stage B3 (Müller 1961). Phloem elements (arrowheads) are discernible at the base of this median protuberance (MN).

**Fig. 4.138.** Transmission electron micrograph of a median spur of the nectary (MN) of a transversely-sectioned flower just opening [stage B2/B3 of Müller (1961)], illustrating highly-vacuolate epidermal cells, the distalmost of which bear external ridges (arrow). The subepidermal cells are densely-cytoplasmic and the proximal ones contain starch (S) in their plastids.

*Other abbreviations:* LS - long stamen; MSL - median sepal; PL - petal; R - receptacle; SS - short stamen

*Scale bars:* Figs. 4.134-4.138 - 20 µm
Figs. 4.139-4.141. Transverse sections through wild-type flowers of *Arabidopsis thaliana* at the level of the median protuberances of the nectary.

**Fig. 4.139.** Transmission electron micrograph of nectary tissue (MN) from a nectar-bearing flower of stage B4 (Müller 1961). A small number of plastids contain starch (S). The arrow at the nectary terminus denotes the ridges along the exterior of the distalmost epidermal cells.

Figs. 4.140, 4.141. The nectary tissue (MN) of these post-secretory flowers of stage B6 and B7 (Müller 1961), respectively, is highly vacuolate compared to that of Fig. 4.139. Despite the cessation of secretory activity, fluorescence indicative of starch is still evident in the terminal, modified stomata and occasionally in subepidermal cells (thin arrows). Nucleoli (bold arrows) also fluoresce.

*Other abbreviations:* AZ - abscission zone; MSL - median sepal; PL - petal; R - receptacle

*Scale bars:* Figs. 4.140, 4.141 - 20 µm; Fig. 4.139 - 10 µm
Fig. 4.142. Transmission electron micrograph of representative subepidermal cells of a lateral outgrowth of the nectary in a wild-type, post-secretory flower of stage B5 (Müller 1961) of *Arabidopsis thaliana*. The plastids (Pl) possess large plastoglobuli (Pg), groups of small dense granules (arrows), and lack starch. Several microbodies (MB) are apparent, as are mitochondria (M), rough endoplasmic (rER) and a distinct membranous (mR) reticulum, a coated vesicle (Cv), an intercellular space (IS) with granular material, and a nucleus (N). Swollen structures (*) are characteristically present after secretion has ceased. Note that the vacuoles (V) remain plentiful and can bear rings of osmiophilic material (arrow).

*Scale bar: 0.5 μm*
Figs. 4.143-4.145. Transmission electron micrographs of epidermal (Fig. 4.143) and subepidermal (Figs. 4.144, 4.145) cells of lateral outgrowths of the nectary in wild-type, post-secretory flowers of stage B5 (Müller 1961) of Arabidopsis thaliana.

Fig. 4.143. Dictyosomes (D), microbodies (MB), mitochondria (M), rough endoplasmic reticulum (rER), coated vesicles (Cv), and a starch-deficient plastid containing plastoglobuli (Pg) and clumps of small granules (arrows) occur at the base of this epidermal cells.

Fig. 4.144. Note the prevalence of dictyosomes (D) in this subepidermal cell. The plastid (Pl) lacks starch but has a peripheral reticulum.

Fig. 4.145. Plastids (Pl) contain a peripheral reticulum and clumps of dense granules (arrow), but lack starch. Note the connection (arrow) between the microbodies (MB). The cell at bottom left has a proliferation of membranous reticulum (mR) near a group of mitochondria (M).

Other abbreviations: IS - intercellular space; N - nucleus; Np - nuclear pores; Ps - plasmodesmata; V - vacuole

Scale bars: Figs. 4.143-4.145 - 0.5 µm
Figs. 4.146, 4.147. Ultrastructural features of epidermal cells of lateral outgrowths of the nectary in wild-type, post-secretory flowers of stage B5 (Müller 1961) in Arabidopsis thaliana.

Fig. 4.146. A cuticle (black arrow) covers the external wall of this transversely-sectioned epidermal cell. Note that the ridges (clear arrows) along this outer wall contain microfibrils oriented in the longitudinal direction. Several starch-deficient plastids (Pl) with plastoglobuli (Pg) and an accumulation of dense osmiophilic granules (arrow) are evident, as are dictyosomes (D), mitochondria (M) and rough endoplasmic reticulum (rER). Several plasmodesmata (Ps) connect this epidermal cell to another (bottom right), and to a subjacent cell (bottom left).

Fig. 4.147. Portions of crystalline bodies (Cr) in the cytoplasm.

Other abbreviations: IS - intercellular space; V - vacuole

Scale bars: Figs. 4.146, 4.147 - 0.5 µm
Figs. 4.148-4.152. Transmission electron micrographs of vacuolar inclusions of nectary cells of lateral outgrowths of wild-type flowers of *Arabidopsis thaliana*. The tissue in Fig. 4.148 is from a 1.9-mm bud of late stage 12 (Smyth *et al.* 1990), whereas the other photographs represent post-secretory flowers of stage B5 (Müller 1961).

**Fig. 4.148.** Electron-dense vacuolar inclusion (*) opposite a cytoplasmic plastid (Pl) of this epidermal cell.

**Fig. 4.149.** An amorphous, approximately ring-shaped inclusion composed of osmiophilic globules internally, in a subepidermal cell.

**Fig. 4.150.** Membranous, layered inclusion with a compact core, sectioned at its connection with the tonoplast (arrowheads). This epidermal cell resided in the region between the two lobes of nectary tissue.

**Fig. 4.151.** Same lateral outgrowth as Fig. 4.149, showing lytic processes in some subepidermal cells (SE), and various vacuolar inclusions. Note the large vacuoles (V) of the subepidermal cells at right. The arrow denotes the conspicuous ridges on the exterior walls of the epidermal cells (E).

**Fig. 4.152.** Higher magnification of the region of the subepidermal cell outlined in Fig. 4.151, illustrating a crystalline lattice (La) inclusion, portion of an amorphous inclusion (far right), and another of the multilamellar type (see Fig. 4.150) at a point of attachment (arrowhead) with the tonoplast. The microbody (MB) opposite this attachment site, and another at bottom left, contain numerous microtubules (arrows) in transverse section. An elongate, starch deficient plastid (Pl) with several plastoglobuli (Pg), and a mitochondrion, are evident.

*Scale bars:* Figs. 4.150-4.152 - 1.0 µm; Figs. 4.148, 4.149 - 0.5 µm
Figs. 4.153-4.161. Examples of plastids in sieve elements of the phloem supply to the lateral outgrowths of the nectary in wild-type flowers of *Arabidopsis thaliana*. The same nectary is represented in Figs. 4.154 and 4.156-4.158, and another in Figs. 4.159-4.161.

**Fig. 4.153.** Profiles of two plastids (higher magnification of Fig. 4.102) in a sieve element supplying the nectary of a large bud (stage 12; Smyth *et al*. 1990). Thylakoid membranes (Ty), small grains of starch (S), and an osmiophilic substance (arrow) are seen. Note the trilaminate plasma membrane (circle) along the tube wall (W).

**Fig. 4.154.** Plastid nearing the completion of division into two equal ones, in this sieve element of a nectar-bearing flower (stage B4; Müller 1961). Note the narrow cleavage point (arrowhead) and tubules (arrows) at the division site. Starch (S), thylakoids (Ty) and osmiophilic material (*) are evident.

**Fig. 4.155.** Higher magnification of the plastid of Fig. 4.103, showing thylakoids (Ty) and grains of starch (S).

**Figs. 4.156-4.158.** Starch grains (S), thylakoid membranes (Ty) and orderly osmiophilic globules (arrows) surrounded by a dense matrix, in these parietal plastids.

**Figs. 4.159-4.161.** Irregularly-shaped plastids of sieve elements in a post-secretory nectary of a flower of stage B5 (Müller 1961). Note the starch grains (S), large plastoglobuli (Pg), and apparent loss of thylakoids. A plasmodesma (Ps) is evident in Fig. 4.161.

*Scale bars:* Figs. 4.153, 4.154 - 0.5 µm; Figs. 4.155-4.161 - 0.1 µm
Figs. 4.162-4.174. Lateral outgrowths of the nectary of *Arabidopsis thaliana*, from wild-type flowers of various developmental stages, stained with iodine-potassium iodide after fixation and dissection. The tissues in Figs. 4.162-4.168 and 4.170-4.173 come from different floral positions of the same inflorescence of a plant 33 days post-seeding, and are numbered from oldest to youngest flower. Floral stages are assigned according to Müller (1961), Smyth et al. (1990) and Bowman et al. (1991a).

**Fig. 4.162.** Starch-filled nectary tissue of a large bud (2.1 mm) of late stage 12, at flower position #22. Note densest staining for starch is now at the nectary base after starch decomposition has begun and has proceeded in an acropetal direction. Modified stoma at bottom, centre-left.

**Fig. 4.163.** Portion of the gland from the pre-secretory flower of stage B2/B3 located at position #21 (immediately below that of Fig. 4.162), showing unequal lobe sizes of one of the lateral outgrowths. Abundant starch within the nectary interior is evident.

**Fig. 4.164.** A lateral outgrowth of flower #20, at stage B3, which has just begun to secrete nectar. Note the striking appearance of starch-filled guard cells of six modified stomata, owing to the dissipation of starch from the parenchymatous cells.

**Fig. 4.165.** Nectar-bearing flower #19, of early stage B4.

**Fig. 4.166.** Nectar-bearing flower #17, of stage B4. Note the size disparity of the two modified stomata present on one lobe. As the stain progressed further through the nectary tissue, a third modified stoma was identified at this position (arrowhead).

**Fig. 4.167.** Flower #16, of late stage B4, bearing almost no nectar.

**Fig. 4.168.** Post-secretory flower #12, gynoecium 9.1 mm long, of early stage B6.

**Fig. 4.169.** Note eleven densely-staining modified stomata of various sizes on this lateral outgrowth from a flower of stage B6 (13.8 mm gynoecial length) from a different plant.

**Fig. 4.170.** Low quantities of starch in eleven modified stomata of a post-secretory flower (#8) of early stage B7 (gynoecium 15.6 mm) located four positions below that of Fig. 4.168.

**Figs. 4.171a,b.** Both lateral outgrowths of post-secretory flower #7 (19.5 mm gynoecial length) of early stage B7, showing distinct differences in outgrowth shape.

**Fig. 4.172.** Post-secretory flower #5 of stage B7 (gynoecium 19.4 mm long). Ridges are discernible on the exterior of several epidermal cells.

**Fig. 4.173.** Post-secretory flower just below that of Fig. 4.172, of stage B7 (19.6 mm gynoecial length).

**Fig. 4.174.** Post-secretory flower of stage B7 from a different plant, showing three lobes of nectary tissue at the normal position of a lateral outgrowth. This tissue is oriented 180° compared to the rest of the tissues on this plate.

*Other abbreviations:* LSL - lateral sepal; SS - short stamen

*Scale bars:* Figs. 4.162-4.171b, 4.173, 4.174 - 50 µm; Fig. 4.172 - 20 µm
Figs. 4.175-4.178. Starch content of modified stomata on lateral outgrowths of the nectary of wild-type flowers of Arabidopsis thaliana.

Figs. 4.175a-g are bright-field micrographs of fixed tissue stained with iodine-potassium iodide, and are examples of various starch scores (see Materials and Methods) assigned to the modified stomata plotted in Fig. 4.179. Those modified stomata of Figs. 4.175h and i come from a different plant.

Fig. 4.175a. Starch score 8.13. Note almost complete absence of starch in the left guard cell [Flower 11 (#1E)].

4.175b. Score 25.6. This is the modified stoma at the far left of Fig. 4.172 [Flower 5 (#1A)].

4.175c. Score 36.9 [Flower 10 (#1A)].

4.175d. Score 47.4 [Flower 15 (#2G)].

4.175e. Score 52.4 [Flower 16 (#1B)].

4.175f. Score 59.2. This is the modified stoma in the centre-right position of Fig. 4.167 [Flower 16 (#2D)].

4.175g. Score 65.1 [Flower 15 (#1H)].

4.175h. Score 75.4 [Flower 4 (#1C)].

4.175i. Score 75.8. Note the displacement, and apparent absence, of the nuclei in these guard cells [Flower 5 (#1A)].

Figs. 4.176-4.178. Transmission electron micrographs of guard-cell plastids from various developmental stages of modified stomata.

Fig. 4.176. Two plastids with small starch grains (S) in the modified stoma of immature stage-2 shown in Fig. 4.69.

Fig. 4.177. Circular plastid with large starch grains from the open modified stoma of Fig. 4.74.

Fig. 4.178. Elongate plastid with large starch grains from the uppermost guard cell of Fig. 4.88a.

Other abbreviations: M - mitochondrion; MB - microbody; Ty - thylakoids; V - vacuole; W - cell wall

Scale bars: Figs. 4.175a-i - 5 µm; Figs. 4.176-4.178 - 0.5 µm
Fig. 4.179. Survey of starch scores of modified stomata (see Fig. 4.175) of each lateral outgrowth of the nectary, including those of Fig. 4.162-4.168 and 4.170-4.173, of flowers of various developmental stages on the same wild-type inflorescence of Arabidopsis thaliana. Flower stages are assigned according to Müller (1961).

The shaded zone indicates flowers which bore nectar at the time of sampling.

Each point represents one modified stoma.

The numbers given immediately below the X axis indicate the total quantity of modified stomata per lateral outgrowth. For instance, flower 9 had 7 modified stomata on one of its lateral protrusions, and 9 on the other. The number of points plotted per lateral protuberance is usually less than the total number of modified stomata present, because only those modified stomata which were aligned in face view could be utilized for the starch score index. For flower 9, only 3 (dark circles) of the 7 modified stomata of one lateral outgrowth were properly aligned, whereas for the other lateral outgrowth bearing 9 modified stomata, only a single stoma (clear circle) was available for scoring.

A question mark indicates the belief that part of a lateral outgrowth may have been left behind during the dissection (and hence a possible loss, of low probability, in modified stomata). A hyphen indicates that an entire lateral outgrowth was lost during dissection, whereas an asterisk (flower 10) denotes the loss of information by destruction of the negative film during development. A zero indicates that no modified stomata were detected on a lateral outgrowth.

The oldest flower (#1) of this raceme was not available for scoring, because many of its nectary's modified stomata were partially hidden by fungal hyphae which entered the pores.

The insert gives the average starch score per lateral outgrowth.
Starch score for nectary stomata

Total no. modified stomata on each lateral outgrowth of the nectary

Flower position on inflorescence

Flower stage (Müller 1961)
Figs. 4.180-4.183. Modified stomata on the surfaces of the nectary in post-secretory flowers of *Arabidopsis thaliana*. Figs. 4.182 and 4.183 represent tissues of the wild-type; the remainder represent the TC7 starchless mutant.

Figs. 4.180a,b. Fixed lateral outgrowths from the same flower of very early stage B7 (Müller 1961), after placement in iodine-potassium iodide. In a, note the disparity in lobe sizes, and the differences in outgrowth shape and number of modified stomata per outgrowth, in comparison to b.

Fig. 4.180c. Higher magnification of two of the three modified stomata of the lateral outgrowth shown in Fig. 4.180b, with focus on the anticlinal midpoints. Note the closed pores, the position of the guard-cell nuclei (N) near the ventral walls, the large solitary vacuole (V) per guard cell, and the lack of large organelles in them that stain positively with iodine potassium iodide.

Fig. 4.180d. A triplet of modified stomata from the right lobe of Fig. 4.180a. Focus is on the anticlinal midpoint of the modified stoma in bottom left, showing open pore. The nuclei reside along the ventral walls of the guard cells. Note the relatively small organelles (arrowheads) below the vacuoles in the guard cells of the right-most modified stoma.

Fig. 4.181. Two modified stomata in a grazing section of a lateral outgrowth from a post-secretory flower of late stage B7 (Müller 1961), stained with pseudo-Schiff’s reagent. Note the sparse, weak fluorescence (arrowheads) from the guard-cell interior, indicative of organelles, and an open pore (P).

Fig. 4.182. Transverse section through a flower of early stage B7 (Müller 1961), showing intense fluorescence (arrowheads) indicative of plastid starch within the guard cells of several modified stomata sectioned in various orientations on a lateral outgrowth of the nectary (LN), after staining with pseudo-Schiff’s reagent. Note the internal collapse (arrows) of nectary tissue.

Fig. 4.183. Grazing section of a bi-lobed lateral outgrowth of the nectary from a transversely-sectioned, post-secretory flower of stage B6 (Müller 1961), showing intense fluorescence (arrowheads) associated with starch in the plastids of guard cells. Note that various pore apertures occur in these modified stomata. In the abscission zone (AZ), the remnant of the vascular bundle (X₁) that supplied the abscinded lateral sepal, is evident.

Scale bars: Figs. 4.180a,b - 50 µm; Figs. 4.180c-4.183 - 10 µm
CHAPTER 5

POLLEN TUBE GROWTH
IN ECHIUM PLANTAGINEUM L.

ITS INFLUENCE ON FLORAL NECTAR SECRETION
AND ITS USEFULNESS
TO EVALUATE FLOWER VISITORS AS POLLINATORS
5.1. INTRODUCTION

The herbaceous plant, *Echium plantagineum* L. (Salvation Jane/Paterson’s Curse), indigenous to the western Mediterranean region, has become a widespread inhabitant of agricultural and waste land in southeastern Australia (Piggin 1977). The increasing distribution of this species has made it a favourable forage for honey production in that part of the country. The plants are annual or biennial (Piggin 1977) and their flowers visited by honey bees (*Apis mellifera* L.) for both nectar and pollen (Corbet and Delfosse 1984, Ferrazzi 1987). The honey produced from this nectar is light in colour, which makes it useful in blending with some of the darker, eucalypt honeys (Chandler 1977, Clemson 1985). Perhaps an overlooked advantage to beekeeping is the extraordinary nutritional capacity of the pollen; in terms of crude protein content it ranked second of twenty-six species both native and foreign to Australia, and it supplies in sufficient quantity all ten amino acids essential to honey bee development (Rayner and Langridge 1985).

Corbet and Delfosse (1984) conducted an extensive study of nectar-sugar production and rates of nectar secretion by flowers of *E. plantagineum* in Australia, including part of the Australian Capital Territory. Data obtained from flowers bagged throughout the day provided good evidence for nectar reabsorption in this species (Corbet and Delfosse 1984, Fig. 7 and 8), a phenomenon similarly reported for *E. vulgare* in Europe (Boetius 1948). Other investigators have demonstrated the reabsorption of nectar constituents in actively-secreting (Ziegler and Lüttge 1959, Shuel 1961, Bieleski and Redgwell 1980, Burquez and Corbet 1991) and senescent (Bravais 1842, Bonnier 1879, Pedersen et al. 1958, Shuel 1961) flowers. Recently, Burquez and Corbet (1991) proposed general models of sugar influx and efflux during the concurrent events of nectar secretion and reabsorption. However, the possible influence of pollination itself on nectar-sugar production of actively-secreting flowers...
has been less studied (e.g., Delph and Lively 1989), and, to my knowledge, the influence of pollen-tube growth on nectar-sugar production and reabsorption has not been investigated in any plant species. In addition, for *E. plantagineum* there are no published analyses of the major nectar-sugar constituents throughout flower development. Furthermore, the present investigation appears to be only the second in which nectar-sugar constituents have been analysed following pollination (Hawker *et al.* 1983) to examine for changes in sugar composition indicative of differential sugar reabsorption.

Recent research into the reproductive biology of *E. plantagineum* in Australia, using isozyme analysis of germinating nutlets, has demonstrated that this species is self-compatible (Burdon *et al.* 1988). Furthermore, equal numbers of fruits were set after controlled hand-pollinations involving self- or cross-pollen alone, and equal percentages of fruit were produced when both pollen types were simultaneously applied to the same stigma. Therefore, it has been suggested that the high outcrossing estimates determined for this species (Burdon and Brown 1986, Burdon *et al.* 1988) are due to a very high frequency of inter-plant movements by pollinating insects, rather than due to any competitive inferiority of self pollen or any process of differential embryo abortion (Burdon *et al.* 1988). Although no data were given, Burdon and Brown (1986) suspected that outcrossing in *E. plantagineum* is "mainly mediated by *Apis mellifera*". It is postulated generally that flowers of the Boraginaceae are bee pollinated (Corbet *et al.* 1991).

As well as the aforementioned acquisition of nectar data to complement the study of Corbet and Delfosse (1984), a premier objective of the present study was to develop a much-needed, direct, accurate and quantitative technique which distinguishes actual pollinators from mere flower visitors. Such a method would have broad application in both agricultural and natural settings. Because of their availability, plants of *E. plantagineum* were chosen for these trials. During the course of the investigation, several insects (mainly honey bees) visited flowers of this species, and the technique
was applied to determine 1) the requirement for insect visitation to achieve pollination, 2) the influence of emasculation on pollination, 3) the relationship between pollination and floral reward sought, 4) the relationship between pollination and duration of floral visit, and 5) whether the grooming behaviour of bees during pollen collection, which removes from the body thousands of grains to be transferred to the hind-leg corbiculae (honey bees) or sub-abdominal scopae (Megachilidae), effects the efficacy of such bees as pollinating agents. However, no efforts or intentions were made to determine possible reasons for the comparative reproductive success of this weedy species in Australia; only a few plants were utilized to investigate the potential of this technique.

5.2. MATERIALS AND METHODS

5.2.1. Plant material and floral stages

Two plants of *Echium plantagineum* L. were arbitrarily selected from approximately ten specimens growing at low density at a disturbed, isolated site (just north of Barry Drive, on the west side of Sullivan’s Creek) in central Canberra, Australia. The plants grew in the open on the bank of a waterway, where they were studied concurrently. Honey bees (*Apis mellifera* L.) and other insects readily visited the plants during the period of study (January 15 - April 14, 1991). This experimental period is outside the major annual flowering cycle for this plant species in Australia (Piggin 1977, Corbet and Delfosse 1984, Burdon and Brown 1986, Burdon *et al.* 1988).

The inflorescence of *E. plantagineum* is a coiled cyme (Fig. 5.2) which usually opens a new flower daily. The flowers are protandrous, anthers of the five epipetalous stamens occupying three discrete positions in the flower upon dehiscence. Distal from the solitary one are two intermediate and two exsert anthers (Fig. 5.3). The style tip bears a bilobed stigma (Fig. 5.3), which at anthesis is connivent, unreceptive and normally below the proximal anther. The style eventually grows between the staminal
filaments, the arms of the stigma diverging and becoming receptive as the stigma is usually extended to the exert anthers (Fig. 5.2) and beyond. Then, the flowers are strongly herkogamous (Webb and Lloyd 1986), the stigma being spatially separated from the anthers.

The relative position of the stigma to the anthers was an important floral characteristic in the experiments to follow. To the six stages delimited by Corbet and Delfosse (1984), one new stage (0.5) has been added (Table 5.1). The duration of each floral stage was generally in accordance with that study.

Table 5.1. Classification of floral stages of Echium plantagineum used in this study (mostly after Corbet and Delfosse 1984)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Bud</td>
</tr>
<tr>
<td>0.5</td>
<td>Corolla tips unfold, stigma unreceptive and not longer than the anther of the short stamen</td>
</tr>
<tr>
<td>1</td>
<td>Stigma becoming receptive and is now located between the anthers of the short and medium stamens</td>
</tr>
<tr>
<td>2</td>
<td>Stigma usually receptive and now located between the anthers of the intermediate and long stamens</td>
</tr>
<tr>
<td>3</td>
<td>Stigma receptive and longer than the anthers of the long stamens</td>
</tr>
<tr>
<td>4</td>
<td>First sign of wilting of the corolla</td>
</tr>
<tr>
<td>5</td>
<td>Corolla abscinds</td>
</tr>
</tbody>
</table>

5.2.2. The influence of pollination and pollen-tube growth on nectar-sugar production and composition

In the morning between 7:45 - 8:30 (D.S.T.), tips of cymes were individually covered with white mesh bags (10.2 x 6.2 cm; Fig. 5.1, 5.11 and frontispiece) after they were stripped of the corollae of any open or wilted flowers to remove any pollen sources still present. Up to 20% of a plant’s cymes were covered at any one time. Bags were removed 24 h later to reveal an open, "virgin" flower per cyme, very occasionally two. Because the largest buds on these cymes were of various sizes when
bagged, and possibly because of differential rates of style growth, flowers could be found from stage 0.5 to stage 3.

All available nectar was sampled once only (i.e., destructive sampling) from the base of six flowers of each of stages 0.5-3, using the combined capillary-wick technique (see section 2.2.9) that allowed immediate measurement of nectar solute concentration and at least partial measurement of its volume. After chemical analyses of the wicked sugar, total nectar sugar (and volume) could be computed. To yield these nectar data, several days of sampling were required.

Percival (1961) discovered relatively high contents of maltose (a reducing sugar composed of two glucose subunits) in paper chromatographs of nectar sugar of many Boraginaceae. Accordingly, this disaccharide was quantified by a further enzymatic reaction involving incubation of a sub-sample of wick rinsate in Na acetate buffer, pH 6.0, to which maltase (α-glucosidase, EC 3.2.1.20, Boehringer-Mannheim #105-414, 50 U/mg, 0.25 U per assay aliquot of 10 µl) was added. Coupling of this reaction, once completed, with the HK and G6P-DH reactions at pH 7.6 as outlined in section 3.2.3, yielded an analysis of natural glucose of the nectar as well as the glucose from metabolized maltose. Therefore, maltose-derived glucose was calculated by subtraction of the HK and G6P-DH analysis conducted separately for nectar-glucose alone as illustrated in Fig. 3.16B (i.e., in the absence of maltase).

As will become obvious in the results that follow, there is only a very small likelihood that any of these flowers (sampled for nectar above) had been pollinated, because they reached anthesis while enclosed in netting. This probability is particularly low for flowers of stages 0.5 and 1, the stigmatic papillae of which were still tightly appressed or were just beginning to separate.

To determine whether emasculation, pollination and pollen-tube growth had any effect on nectar volume, concentration, total sugar, and proportions of the major nectar sugars, virgin flowers at or just about reaching stage 3 (i.e., with receptive stigmata) were utilized. At random, about half the flowers were emasculated by inserting the tips
of fine forceps below each verticillate anther and gently lifting it off and out of the corolla. The filaments remained undisturbed. Despite the usefulness of emasculation for experimental purposes, the loss of anthers in *E. plantagineum* can occur naturally after bee visits involving pollen collection (Davis, pers. obs.), and Oleson (1989; Fig. 1) has pictured anther loss following the male phase in flowers of *E. wildpretii*.

Then, both arms of the stigma of about half of the emasculated and intact flowers, chosen randomly, were immediately cross-pollinated by brushing them thoroughly with one full, dehiscing anther transferred by forceps from a neighbouring, non-related plant. Usually, and whenever possible, plant-1 stigmata were pollinated using anthers of plant 2, and *vice versa*. Flowers of all treatments (i.e., intact-pollinated, emasculated-pollinated, intact-virgin, emasculated-virgin), never having received visits from large insects (e.g., bees), were labelled and carefully rebagged for the remainder of their lifetimes.

Twenty-four h after emasculation and pollination, all nectar per flower was gathered using capillaries and wicks, and nectar-characteristics determined, as above. Directly following nectar collection, forceps were used to carefully remove entire styles of these flowers at their junction with the four ovules (Fig. 5.5), notice being made of the relative size of the latter, and the styles immediately fixed in separate, labelled vials of acetic acid:ethanol (1:3) before being transported to the laboratory for pollen-tube counts (see 5.2.5).

### 5.2.3. Determination of the requirement for insect visitation to achieve pollination

Open (virgin) flowers, bagged as unopened buds 24 h earlier (exactly as described in 5.2.2), were either emasculated to remove all anthers or left intact. All experimental flowers were identified and labelled on coloured pieces of tape looped around the calyx (Fig. 5.1). Then, approximately half of these unvisited flowers were carefully rebagged and tied off. Forty-eight h later, when exposed flowers were at late stage 4 or stage 5, and bagged flowers in stage 4, the styles were harvested and proces-
sed for pollen-tube detection as described in section 5.2.5. Ten flowers of stage 0.5, 1 and 2, and twenty of stage 3, were assigned to each of the four treatments (intact vs. emasculated, bagged vs. exposed) per plant, so that overall, four hundred flowers were tested to determine the necessity of animal visitation for pollination.

5.2.4. **Evaluation of insect visitors as pollinators after single visits to virgin flowers**

Twenty-four h after further buds were bagged, the open flower found per cyme was uncovered and either left intact or emasculated. Vigilant watch was kept over such marked flowers, in order to identify the first insect visitor, to determine whether it carried loads of pollen (e.g., in the corbiculae of honey bees; in the scopae of Megachilidae), to measure the duration of visitation, and to determine what floral reward(s) (i.e., nectar, pollen or both) was (were) being sought. Upon departure of this initial visitor, the flower was immediately and carefully rebagged for the rest of its flowering lifetime, thereby excluding further visits. Approximately equal numbers of flowers from each plant were exposed to single honey bee visits at each floral stage, such that twelve to twenty-three flowers per treatment (intact versus emasculated) were tested. Forty-eight h later, the styles of these once-visited-only flowers were collected and examined for numbers of pollen tubes according to the procedure of 5.2.5.

To determine whether bagging caused significant nectar accumulation, which might influence the duration of visits by nectar-collecting visitors, the average time spent by numerous honey bees gathering only nectar when visiting all stages of normal flowers (i.e., never bagged), was measured for comparative purposes.

5.2.5. **Detection of pollen tubes in excised styles**

Preliminary experiments involving hand pollination of previously-unvisited flowers with receptive stigmata (stage 2 and stage 3) demonstrated that the rapid pollen-tube growth (approx. 2 mm/h) in styles of *E. plantagineum* rivals that of other
Boraginaceae, wherein styles were harvested for pollen-tube analysis 24 h (Crowe 1971), 24 h (Weller and Ornduff 1977, 1989), and 18 h (Schou and Philipp 1983) after pollination. Although pollen tubes were found to have reached the style base within 9 h, styles were not harvested in this study until much later, to ensure that all pollen tubes had arrived. Because pollination by hand (section 5.2.2) was performed only once per flower, there is reason for confidence that a single front of pollen tubes should have reached the style base by 24 h post-pollination, when nectar was collected. Certainly, this was the case for virgin flowers visited only once before they were rebagged (section 5.2.4); their styles were harvested 48 h after the visit. Similarly, there is strong reason to believe that all visits to the multiply-attended flowers of section 5.2.3, which probably had staggered fronts of pollen tubes resulting from successive pollination events, were represented fully by pollen tubes when styles were harvested 48 h after unbagging. In the latter case, uncovered flowers at style collection were always found to have shed their corolla (stage 5) or had a severely-wilted, closed corolla (late stage 4), indicating that visits to those flowers in the past 9-10 h were highly unlikely.

Styles brought to the laboratory were processed essentially as described by Webb and Williams (1988). After about 4 h, styles were stored in 70% ethanol. It was found unnecessary to soften the styles, because of their relative thinness. Styles were then immersed overnight in 0.1% aniline blue (Bioscientific WS in 0.1 M K$_3$PO$_4$), a stain for callose ($\beta$-1,3-glycan) located in the cell walls and plugs of pollen tubes (Linskens and Esser 1957, Martin 1959). The bases of styles, squashed in a drop of stain using the butt-end of forceps and depressed by a coverslip, were examined on a Leitz incident-light fluorescent microscope using blue-light excitation (exciter filter BP 450-490) for the yellow fluorescence of callose. By focussing through the transmitting tissue between the two vascular bundles of the style, pollen tubes not clearly visible with bright-field optics (Fig. 5.6a) could be identified and counted by their fluorescence (Fig. 5.6b, 5.7). Careful microscopic examination of many styles consis-
Table 5.2. Number of pollen tubes per style base from treated (intact, emasculated) flowers of experimental field plant 1 of *Echium plantagineum*, 24 h after cross-pollination by hand

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Non-pollinated flowers</th>
<th>Pollinated flowers</th>
<th>Overall</th>
<th>Low group</th>
<th>High group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>19</td>
<td>25</td>
<td>10</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>9.04 ± 1.29</td>
<td>2.40 ± 0.48</td>
<td>13.47 ± 1.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(1-22)</td>
<td>(1-5)</td>
<td>(8-22)</td>
<td></td>
</tr>
<tr>
<td>Emasculated</td>
<td>21</td>
<td>24</td>
<td>9</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>9.17 ± 1.39</td>
<td>2.22 ± 0.64</td>
<td>13.33 ± 1.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(1-24)</td>
<td>(1-6)</td>
<td>(8-24)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3A. Volume (µl) of nectar collected from bagged flowers of experimental field plant 1 of *Echium plantagineum* 24 h after treatment (intact, emasculated) and cross-pollination by hand

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Non-pollinated flowers</th>
<th>Pollinated flowers</th>
<th>Overall</th>
<th>Low group</th>
<th>High group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>18</td>
<td>22</td>
<td>7</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>4.39a ± 0.78</td>
<td>3.66a ± 1.61</td>
<td>3.41a ± 1.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0-11.22)</td>
<td>(0-10.59)</td>
<td>(0-14.66)</td>
<td></td>
</tr>
<tr>
<td>Emasculated</td>
<td>17</td>
<td>24</td>
<td>9</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>4.14a ± 0.92</td>
<td>2.58a ± 1.47</td>
<td>4.19a ± 1.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0-13.68)</td>
<td>(0-13.87)</td>
<td>(0-15.79)</td>
<td></td>
</tr>
</tbody>
</table>

Mean values in rows followed by the same letter are not significantly different using 1-tailed t-tests where α=0.05

Table 5.3B. Concentration (%) of nectar collected from bagged flowers of experimental field plant 1 of *Echium plantagineum* 24 h after treatment (intact, emasculated) and cross-pollination by hand.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Non-pollinated flowers</th>
<th>Pollinated flowers</th>
<th>Overall</th>
<th>Low group</th>
<th>High group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>16</td>
<td>18</td>
<td>7</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>20.39a ± 4.20</td>
<td>12.20b ± 1.83</td>
<td>10.94b ± 1.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(4.86-52.81)</td>
<td>(5.89-35.52)</td>
<td>(5.89-16.90)</td>
<td></td>
</tr>
<tr>
<td>Emasculated</td>
<td>18</td>
<td>18</td>
<td>6</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>20.57a ± 3.20</td>
<td>11.69b ± 2.47</td>
<td>9.99b ± 1.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(4.27-49.67)</td>
<td>(3.25-49.99)</td>
<td>(3.25-18.29)</td>
<td></td>
</tr>
</tbody>
</table>

Mean values in rows followed by the same letter are not significantly different using 1-tailed t-tests where α=0.05

Table 5.3C. Total sugar (mg) in nectar collected from bagged flowers of experimental plant 1 of *Echium plantagineum* 24 h after treatment (intact, emasculated) and cross-pollination by hand.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Non-pollinated flowers</th>
<th>Pollinated flowers</th>
<th>Overall</th>
<th>Low group</th>
<th>High group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>18</td>
<td>22</td>
<td>7</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>0.663a ± 0.103</td>
<td>0.509b ± 0.161</td>
<td>0.446a ± 0.178</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0-1.01)</td>
<td>(0-2.572)</td>
<td>(0-2.075)</td>
<td></td>
</tr>
<tr>
<td>Emasculated</td>
<td>17</td>
<td>24</td>
<td>9</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>0.837a ± 0.183</td>
<td>0.370b ± 0.093</td>
<td>0.390b ± 0.113</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0-2.035)</td>
<td>(0-1.762)</td>
<td>(0-1.762)</td>
<td></td>
</tr>
</tbody>
</table>

Mean values in rows followed by the same letter are not significantly different using 1-tailed t-tests where α=0.05
tently showed the same number of pollen tubes near the top of the style as that recorded at the base.

5.3. RESULTS

5.3.1. The influence of pollination and pollen-tube growth on nectar-sugar production and composition

In Fig. 5.12, nectar volume and sugar data are given for bagged, intact (non-pollinated) flowers at stages much less than 24 h after anthesis. Both quantities increased with flower age, the likeness in the curves resulting from a relative constancy in nectar-sugar concentration. The divergence of the volume and sugar curves at floral stage 3 (Fig. 5.12) is accentuated 24 h later, when volumes of bagged, intact flowers of stage 3 of this same plant averaged 4.39 µl (Table 5.3A), over twice the amount (2.10 µl) in Fig. 5.12. Meanwhile, the mean sugar value (0.663 mg) of Table 5.3C was lower than the terminal datum of Fig. 5.12 (0.774 mg). Therefore, this decrease in nectar sugar content (despite the more copious quantities of nectar), together with the initial occurrence that some bagged flowers no longer possessed nectar (see ranges in Tables 5.3A and C), indicate that net nectar reabsorption was taking place 24 h later in these intact flowers which had not been hand-pollinated. Therefore, in the complete absence of pollination in *E. plantagineum* (i.e., zero pollen tubes detected - top left of Table 5.2; and see Fig. 5.8), reabsorption of nectar sugar was apparent.

Originally, there were 23 intact and 22 emasculated flowers at or near stage 3 that were designated as controls (i.e., not pollinated by hand). However, subsequent checks of their styles for pollen tubes following nectar collection revealed that four (17.4%) intact flowers (1, 1, 1 and 4 pollen tubes) and a single (4.5%) emasculated flower (2 tubes) could no longer be regarded as controls. The source of these pollen tubes is postulated in section 5.4.3. As a result, data from up to 19 intact and 21 emas-
culated flowers were available for calculation of the mean values reported in Tables 5.3A-C.

Originally, there were 21 intact and 23 emasculated flowers at or near stage 3 that were cross-pollinated by hand. For reasons unclear, six (28.6%) intact flowers (1, 2, 2, 3, 4 and 5 pollen tubes) and eight (34.8%) emasculated ones (1, 1, 1, 1, 1, 2, 5 and 6 tubes) had relatively low numbers of pollen tubes when compared to other flowers of their treatment group. For analysis of a possible effect of pollen-tube number on reabsorption of nectar sugar, it therefore seemed convenient to divide the nectar data within each treatment (intact, emasculated) according to the numbers of pollen tubes reaching the style base. Accordingly, a "low group" of ten intact flowers was assembled for analytical purposes; it was composed of the six hand-pollinated flowers mentioned earlier in this paragraph and the four intact flowers with low numbers of pollen tubes described in the preceding paragraph. Similarly, a "low group" of nine emasculated flowers was created from the eight hand-pollinated flowers bearing low pollen-tube numbers and the solitary, deanthered flower of the preceding paragraph. The remaining hand-pollinated flowers made up the "high groups", composed of individual styles with at least eight pollen tubes - double or more the number required to optimize fruit set in *E. plantagineum*.

The average numbers of pollen tubes in the non-pollinated and pollinated (including overall, low and high groups) categories between treatments (intact, emasculated) are very similar (Table 5.2). This lack of discrepancy in mean numbers of pollen tubes between emasculated and intact flowers, the latter still with all their pollen present, suggests that although autogamy is present in this species, it does not normally occur without disturbances associated with flower visitors. Further evidence for the lack of autogamy when disturbance is wanting is the fact that 82.6% of the original control group, consisting of flowers still bearing all their pollen and not being pollinated by hand, were found to completely lack pollen tubes when bagged for their entire lifetime.
Although the range of nectar volumes was large, mean nectar volume within treatments did not differ significantly, regardless of the degree of pollination (Table 5.3A). Of the intact flowers which lacked nectar 24 h after pollinations were performed, three belonged to the non-pollinated group, three to the "low group" (1, 1 and 2 pollen tubes) and four to the "high group" (9, 14, 15 and 22 tubes). In the case of emasculated flowers, three flowers each of the non-pollinated, pollinated-"low" (all with 1 pollen tube) and pollinated-"high" (9, 10 and 24 tubes) groups lacked nectar. Similarly, there was no effect of treatment on mean nectar volume in any of the groups, the same quantities of exudate being available within each pollination category.

Despite the absence of statistical differences in average volume, mean concentration values within treatments were significantly lower (about half) for groups of pollinated flowers with high pollen-tube numbers (Table 5.3B) than the corresponding concentrations of non-pollinated (control) flowers (Intact: $t_{25}=1.8085$, $0.05>P>0.04$; Emasculated: $t_{28}=2.6099$, $0.01>P>0.005$; one-tailed t-tests, $\alpha=0.05$). The sugar concentrations of nectar collected from these "high groups" had comparatively low variance. This significant decrease in nectar-sugar concentration also was maintained between the control (non-pollinated) group and the overall values determined from all pollinated flowers (Intact: $t_{32}=1.8588$, $0.03>P>0.025$; Emasculated: $t_{34}=2.2004$, $0.02>P>0.01$), but not for the "low groups" alone, where variation was relatively high (Table 5.3B). However, regardless of the degree of pollination, there were no significant differences in nectar-sugar concentration brought about by emasculation, when compared to that of intact flowers which were subjected to the same level of pollination.

The significant differences in average nectar-sugar concentration (Table 5.3B), despite the similarities in nectar volume (Table 5.3A), are responsible for significant differences in total nectar sugar available from emasculated flowers. Regardless of how well these deanthered flowers were pollinated, their average quantities of nectar
sugar were similar but clearly exceeded by the unpollinated control (Overall: \( t_{39}=2.4666, 0.01>P>0.005; \) "Low Group": \( t_{24}=1.7725, 0.05>P>0.04; \) "High Group": \( t_{30}=2.0138, 0.03>P>0.025 \)). Intact flowers, on the other hand, showed no significant differences in mean levels of nectar sugar, whether pollinated or not. The greatest disparity occurred between non-pollinated and heavily-pollinated flowers (\( t_{31}=1.0984, 0.2>P>0.1 \)).

Emasculated, pollinated flowers invariably yielded lower quantities of nectar sugar than their intact counterparts. However, statistical comparisons between the treatments demonstrated that all differences in mean sugar values between groups of flowers pollinated similarly were insignificant, with \( P \) always exceeding 0.2.

The results of analyses of nectar sugar of the six groups of intact, bagged flowers (five unpollinated categories - stages 0.5, 1, 2, 3 within 24 h of anthesis, and stage-3 flowers 24 h older; and the corresponding, older flowers of stage 3 which were hand-pollinated) are shown in Fig. 5.13. Floral nectar of \( E. \) plantagineum is sucrose dominant. Its proportion was significantly higher at floral stage 1 than in all three groups of stage-3 flowers. Across all six groups, no differences in percent fructose or glucose occurred. The contents of maltose were similar throughout the stages surveyed, except that in older, unpollinated flowers of stage 3, the percent maltose was significantly greater than in stage-1 flowers.

The proportion of fructose to glucose remained very constant throughout the six groups of flowers checked, and although the ratio of sucrose/maltose showed considerably more fluctuation, these ratios also did not differ significantly. However, the initial maltose/glucose ratio (flowers of stage 0.5) was significantly lower than those in unpollinated flowers of stage 1 and "old" stage 3. The high sucrose/hexose ratio of stage-1 flowers significantly exceeded that of stages 0.5 and 3, as well as that of the pollinated flowers.

For no major sugar of \( E. \) plantagineum nectar, nor for any ratio of these constituents, did significant differences occur between the intact bagged flowers of
stage 3 (i.e., bars marked D, E and F, Fig. 3.15), regardless of whether the flowers differed in age by 24 h (on average), or whether they were subjected to cross-pollination. Therefore, despite the clear evidence for reabsorption in the older flowers of stage 3, with time (and with pollination, in the case of cross-pollination by hand), there were no obvious differences in contents of nectar sugar species and their ratios.

5.3.2. The requirement for insect visitation to achieve increased pollination

By comparing the levels of pollination attained in flowers which are continually covered versus those exposed throughout their lifetime, valuable information can be obtained about the necessity for pollination of floral visitation (e.g., Fig. 2 of Corbet et al. 1991). The data of Fig. 5.14 clearly indicate that styles of *E. plantagineum* contained significantly more pollen tubes when flowers were exposed and subject to visitation, than when blossoms were protected by netting. Generally, styles of bagged flowers had few pollen tubes; 84% had none (Fig. 5.8, 5.14). Within the four treatments (intact-exposed, intact-bagged, emasculated-exposed, emasculated-bagged), average pollen-tube number was not significantly different between the floral stages (two-tailed t test, \( \alpha=0.05 \)).

Experimental removal of anthers before exposure of flowers to visitation provided interesting results. Average numbers of pollen tubes in styles of exposed blossoms did not differ significantly whether anthers were present or absent; this statement applies for both overall comparisons (\( P = 0.355 \)) and for comparisons between each floral stage (one-tailed t tests, \( \alpha=0.05 \)). On the other hand, subsequently after bagging, there were significant differences between intact and emasculated flowers. On the whole, emasculated flowers bore significantly fewer pollen tubes (0.13 ± 0.068) when protected than did intact blossoms (0.57 ± 0.13; \( P = 0.00379 \)), this disparity being attributable especially to flowers deanthered during stage 2, where a significant difference occurred (\( P = 0.0337 \); Fig. 5.12). This result of pollination of stigmata within bags concurs with the findings of section 5.3.1 (paragraph 2), and is discussed in section 5.4.3 below.
5.3.3. Evaluation of insect visitors as pollinators after single visits to virgin flowers

5.3.3.1. Emasculation and its influence on relative frequency of visits for nectar, pollen or both

Because the two outermost anthers of *E. plantagineum* are exposed in lateral view, and the two intermediate ones only slightly hidden by the corolla (Fig. 5.2, 5.3), the collection of pollen by *A. mellifera* from most anthers was easily observable. The bees were seen to grapple with the anthers using the mouthparts and legs exactly as described by Corbet and Delfosse (1984). Bees that visited flowers in which the anthers had been removed experimentally often made identical scrabbling motions at the filament tips (and occasionally even the upper style and stigma, if exsert) for a few seconds or more, as if the anthers were not absent. These bees were therefore also classified as pollen seekers. Honey bees were assessed as nectar gatherers if their visits included complete entrance into the corolla to reach the disk-shaped nectary below the ovules (Fig. 5.5). Individuals which visited flowers solely for nectar immediately proceeded to the nectary without searching for pollen. Other bees sought both nectar and pollen during the same visit; such visits invariably began with the pollen.

Compared to intact flowers, honey bees that landed on emasculated flowers displayed pronounced differences in the floral reward pursued (Fig. 5.15). A chi-square analysis of the frequency of visits for the three categories of food they sought (i.e., nectar alone, both nectar and pollen, pollen alone) from intact and emasculated flowers showed a highly significant shift toward nectar gathering between the two treatments [chi-square(2 df) = 159.08, P << 0.005].

5.3.3.2. Emasculation and its influence on pollen-tube number

Both intact and emasculated flowers of *E. plantagineum* were pollinated during single visits by *A. mellifera* (Fig. 5.15), blossoms at stage 2 and stage 3 containing the highest numbers of pollen tubes per style base. At least 88.9% of all flowers at stage 0.5 and stage 1 were not pollinated after a lone honey bee visit, pollen tubes being entirely absent in emasculated flowers visited then. These results concur with the
attainment of stigmatic receptivity. Overall, style bases of intact flowers bore significantly more pollen tubes [3.35 ± 0.70 (S.E.), n = 62] than those of deanthered ones (1.55 ± 0.32, n = 67; one-tailed t test, P = 0.0188). Stage-3 flowers accounted for most of this discrepancy (Fig. 5.15), the difference in mean pollen-tube number between intact (3.73 ± 1.30) and emasculated (2.96 ± 0.78) flowers visited then being almost significant (P = 0.0657).

5.3.3.3. The relationship between pollen-tube number and type of floral reward sought

Because the nectary is at the flower base and the anthers occupy various positions relative to the stigma, according to the floral stage, it was of interest to determine whether the degree of pollination of virgin flowers of *E. plantagineum*, attended by sole honey bees, was influenced by the reward(s) pursued by the bees. The data of Table 5.4 include only flowers of stages 1, 2 and 3, for flowers visited at stage 0.5 rarely possessed pollen tubes (Fig. 5.15). Within each treatment (intact or emasculated), it mattered not whether the bee searched for nectar alone, both nectar and pollen, or pollen alone; all three categories exhibited similar numbers of pollen tubes. Honey bees pollinated intact flowers significantly better than deanthered ones (P = 0.0496, Table 5.4) when both commodities were sought during the same trip, owing to a major difference in stage-2 flowers (P = 0.0710, Fig. 5.15). At stage 2, the receptive stigma is central to the four largest, outermost anthers (or 85% or the flower’s pollen; see Table 6.1), so that some self-pollination may occur as nectar is probed and

Table 5.4. Number of pollen tubes (Mean ± S.E.) at the style base 48 h after virgin flowers (stage 1 to stage 3) of *Echium plantagineum* were visited once, by a honey bee, according to the reward (Only nectar, Both nectar and pollen, Only pollen) being sought, as shown in Fig. 5.15. Means in rows followed by the same letter are not significantly different at α = 0.05

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reward sought</th>
<th>Only nectar</th>
<th>Both nectar and pollen</th>
<th>Only pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. visits</td>
<td>Pollen tubes</td>
<td>No. visits</td>
<td>Pollen tubes</td>
</tr>
<tr>
<td>Intact (control)</td>
<td>1</td>
<td>0±</td>
<td>10</td>
<td>4.9±2.3</td>
</tr>
<tr>
<td>Emasculated</td>
<td>7</td>
<td>2.0±1.2</td>
<td>29</td>
<td>1.8±0.48</td>
</tr>
<tr>
<td></td>
<td>Expt.</td>
<td>n.s.</td>
<td></td>
<td>P = 0.0496</td>
</tr>
</tbody>
</table>
5.16

anthers are grappled. Bees that visited for only nectar induced the same amount of pollination as those that visited for only pollen, regardless of treatment (Table 5.4).

5.3.3.4. The relationship between duration of visit and type of floral reward sought

To determine whether the type of condiment sought (nectar alone, both nectar and pollen, pollen alone) has any influence on the time spent per virgin flower by honey bees, the duration of visits for all floral stages per treatment were averaged for each reward type and then compared. Bees visiting intact flowers spent similar periods collecting either nectar alone, both nectar and pollen, or only pollen (Table 5.5).

Table 5.5. Time (Mean ± S.E.) spent per virgin flower of *Echium plantagineum* by honey bees and the reward (Only nectar, Both nectar and pollen, Only pollen) sought during single visits to all virgin flowers shown in Fig. 5.15. Means in rows followed by the same letter are not significantly different at α = 0.05

<table>
<thead>
<tr>
<th>Reward sought</th>
<th>Only nectar</th>
<th>Both nectar and pollen</th>
<th>Only pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>No. visits</td>
<td>Time (sec)</td>
<td>No. visits</td>
</tr>
<tr>
<td>Intact (control)</td>
<td>1</td>
<td>3.0 ± 0.54</td>
<td>11</td>
</tr>
<tr>
<td>Emasculated</td>
<td>9</td>
<td>14.9 ± 2.0</td>
<td>32</td>
</tr>
</tbody>
</table>

Overall, honey bees spent significantly longer periods on intact flowers (15.94 ± 1.37 sec, n = 62) than on deanthered ones (12.17 ± 1.15 sec, n = 67; one-tailed t test, P = 0.0381), because those bees which landed on emasculated flowers in search of pollen alone, stayed for considerably shorter times (Table 5.5). A comparison of the same floral stage between the two treatments (intact, emasculated) found this reduced length of visit for pollen alone to be greatest at stage 3 (P << 0.001), stage 2 (P = 0.0409) and stage 0.5 (P = 0.0433) as determined from one-tailed t tests.

Nectar accumulation caused by bagging may account for visits of greater length; the six visits to plant 1 in search of only nectar averaged 16.5 ± 2.6 sec, which was significantly longer (two-tailed t test, P = 0.0150) than the average visit made by twenty-two other honey bees (9.9 ± 0.69 sec, n = 81 visits) attending normal (never
bagged) flowers for nectar alone. The latter mean is in close agreement with a study of *E. plantagineum* in Venezuela, where undisturbed flowers received about six visits per min, from honey bees (Núñez 1977).

5.3.3.5. Regression of pollen-tube number on duration of visit

In Fig. 5.16, the numbers of pollen tubes detected at the style base of virgin flowers, visited once only by a honey bee, are plotted against the total time spent on flowers. Only flowers of stage 2 and 3 are included, because flowers younger than these were pollinated rarely (see Fig. 5.15). An analysis of variance (Minitab; Ryan *et al.* 1985) showed there is no linear relationship between pollination and time spent per visit to each flower, for either treatment (emasculated or intact) alone and especially when the data were combined (P = 0.964).

5.3.3.6. Pollination by honey bees carrying pollen of *E. plantagineum* in their corbiculae

Because pollen-collecting honey bees actively groom their bodies to remove grains that are eventually stored in the pollen baskets of their hind legs (Fig. 5.10), it was of interest to determine whether these types of bees were therefore inferior pollinators. One hundred and nineteen of the 129 (92.2%) single visits to virgin flowers of *E. plantagineum* by honey bees in this study were made by individuals carrying dark-blue pollen in their corbiculae (Fig. 5.10, 5.15). And because five of these ten bees arriving without pollen tried to acquire some (black bars marked with asterisks in Fig. 5.15), honey bees in this study were strongly interested in gathering pollen. The fraction of honey bees which visited intact flowers while carrying corbicular pollen did not differ significantly from that proportion attending emasculated flowers [chi-square(1 df) = 1.42; 0.25 > P > 0.20].

In Fig. 5.15, seven of the ten bees arriving without corbicular pollen visited flowers having stigmata at or approaching full receptivity (i.e., floral stages 1 to 3;
see Table 5.1 on page 5.4). These seven flowers, all of which had been emasculated (Fig. 5.15), subsequently yielded an average of 0.43 ± 0.30 pollen tubes per style. The mean pollen-tube number of the remaining (forty-seven) deanthered flowers of stages 1 to 3, visited by honey bees carrying *Echium* pollen in their corbiculae, was 2.1 ± 0.43. These means were not significantly different (two-tailed t test, P = 0.278), indicating no change in effectiveness as pollinating agents after single visits to emasculated virgin flowers of *E. plantagineum* paid by honey bees, whether they bore corbicular pollen or not.

### 5.3.4. Acquisition of at least four pollen tubes per style base

The detection of stylar pollen tubes allows an assessment of the fraction of flowers that are pollinated sufficiently to permit optimal fruit set. After multiple exposure of *E. plantagineum* flowers to various visitors, the percentages of styles in each treatment (intact, emasculated) that contained over three pollen tubes were almost identical (Table 5.6). This similarity in pollen-tube relative frequency held for all floral stages, both within and between treatments (Table 5.6), and complements the similarity in average pollen-tube number between intact and emasculated flowers exposed to multiple visitation (Fig. 5.14). The highest fractions (0.85, 0.80) occurred in flowers first exposed to multiple visits at stage 0.5. Overall, the multiple visitation

<table>
<thead>
<tr>
<th>Floral stage</th>
<th>Intact</th>
<th></th>
<th>Emasculated</th>
<th></th>
<th>Intact</th>
<th></th>
<th>Emasculated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. flowers</td>
<td>Fraction</td>
<td>No. flowers</td>
<td>Fraction</td>
<td>No. flowers</td>
<td>Fraction</td>
<td>No. flowers</td>
<td>Fraction</td>
</tr>
<tr>
<td>0.5</td>
<td>20</td>
<td>0.85</td>
<td>20</td>
<td>0.80</td>
<td>14</td>
<td>0</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0.70</td>
<td>20</td>
<td>0.80</td>
<td>12</td>
<td>0.17</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>0.65</td>
<td>20</td>
<td>0.75</td>
<td>14</td>
<td>0.36</td>
<td>16</td>
<td>0.31</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>0.75</td>
<td>40</td>
<td>0.65</td>
<td>22</td>
<td>0.50</td>
<td>23</td>
<td>0.35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Multiple visits</th>
<th>Single honey bee visits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>Emasculated</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>No. flowers</td>
<td>Fraction</td>
</tr>
<tr>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
</tr>
</tbody>
</table>
experiment showed that 11% (intact) and 15% (emasculated) of exposed flowers lacked any pollen tubes (although their stigmata appeared normal), while 25% (intact) and 26% (emasculated) had fewer than four tubes at the style base.

The data of Table 5.6 also demonstrate that single honey bee visits to intact virgin flowers resulted in deposition of enough pollen grains to allow fertilization of all (four) ovules. The fraction of styles containing at least four pollen tubes increased incrementally from stage 0.5 to stage 3, paralleling the increase in stigma receptivity with floral stage.

The null fractions determined after single honey bee visits to stage 0.5 flowers indicate that the high fractions found when flowers of this stage were available to numerous visitors until wilting of the corolla resulted from pollinating visitations during later floral stages (1-3) in which the style was longer and its stigma much more receptive (Tables 5.1, 5.6). That these highest fractions occurred in 0.5-stage flowers subject to multiple visits is probably a result of their being available for visitation for longer periods (i.e., unbagged sooner in their lifetimes) than those of stages 1-3.

The fractions obtained from emasculated flowers of stage 2 and stage 3, visited once by *A. mellifera*, averaged about 75% those from intact blossoms (Table 5.6, previous page).

5.3.5. **Miscellaneous visitors**

Workers of *A. mellifera* accounted for 129 of 138 (93.5%) visits to virgin flowers, and this seems a fair reflection of the predominance of this insect species during the study (and see Corbet and Delfosse 1984). Only three other species (Hymenoptera: a megachilid - 5 visits, and an anthophorid - 3 visits; Lepidoptera: a lycaenid - 1 visit) attended such experimental blossoms, with five of six visits to flowers of stage 2 and 3 resulting in the absence of pollen tubes (Table 5.7, next page). One megachilid with dark-blue pollen in its scopa visited an intact flower of stage 3 for pollen only; three pollen tubes resulted (Table 5.7). These other species were most
Table 5.7. Pollen-tube number at style base 48 h after single visits (N = nectar alone, P = pollen alone) by miscellaneous insects to treated (I = intact, E = emasculated) virgin flowers of *Echium plantagineum*

<table>
<thead>
<tr>
<th>Insect Family</th>
<th>Insect</th>
<th>Flower</th>
<th>Plant</th>
<th>Treatment</th>
<th>Floral stage</th>
<th>Reward sought</th>
<th>Duration of visit (sec)</th>
<th>No. pollen tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hymenoptera:</td>
<td>Megachilidae</td>
<td>G240</td>
<td>E</td>
<td>1</td>
<td>N</td>
<td>7.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R197</td>
<td>E</td>
<td>2</td>
<td>P</td>
<td>2.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R196</td>
<td>E</td>
<td>3</td>
<td>P</td>
<td>3.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R190</td>
<td>I</td>
<td>3</td>
<td>P</td>
<td>2.0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R200</td>
<td>I</td>
<td>3</td>
<td>P</td>
<td>4.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hymenoptera:</td>
<td>Anthophoridae</td>
<td>G241</td>
<td>I</td>
<td>1</td>
<td>N</td>
<td>5.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R266</td>
<td>E</td>
<td>1</td>
<td>N</td>
<td>4.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Red</td>
<td>I</td>
<td>3</td>
<td>N</td>
<td>3.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Lepidoptera:</td>
<td>Lycaenidae</td>
<td>R191</td>
<td>I</td>
<td>3</td>
<td>N</td>
<td>10.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

abundant when *A. mellifera* was absent. Unlike honey bees, these other insects generally visited for nectar or pollen, not both.

Visitors recorded at other, non-experimental flowers of *E. plantagineum* at this study site were insects belonging to the Formicidae, the Thysanoptera [including *Thrips imaginis* Thripidae (Kirk 1984, 1987); and see Fig. 6.6, 6.12], and a single cabbage-white butterfly (*Pieris rapae*), which may have been attracted to the site by interspersed plants of *Hirschfeldia incana* (L.) Lagrèze-Fossat (see Chapter 6).

5.4. **DISCUSSION**

5.4.1. **The influence of pollination and pollen-tube growth on nectar-sugar production and reabsorption**

In intact, bagged, non-pollinated flowers that had opened for much less than 24 h, a progressive increase in nectar volume and sugar content was detected in flowers at stage 0.5 to 3. However, a day later, flowers of stage 3 contained significantly more nectar, but less nectar sugar, and because low to moderate numbers of flowers no longer possessed nectar, these changes with flower age are indicative of a net influx of nectar sugar by reabsorption. This result concurs with the findings of Corbet and Delfosse (1984) for this same species. It seems highly likely that the caged flowers
sampled by those investigators were also non-pollinated, judging from the sterility imposed on flowers by bagging (i.e., exclusion of flowers from the disturbances of large insect visitors) in the present study.

Examination of any effects on nectar characteristics, including nectar-sugar contents, was performed by cross-pollinating intact or emasculated flowers with receptive stigmata by hand. Twenty-four h later, there were no differences in average nectar volume between non-, lightly- and heavily-pollinated groups of flowers. In flowers of *Fuchsia excorticata*, which in contrast to those of *E. plantagineum* are protogynous, Delph and Lively (1989) also did not detect any effect of cross-pollination on nectar volume, and concluded that nectar production in *Fuchsia* was dependent on flower age, rather than induced by pollination. However, this conclusion was drawn without any measurements of nectar-solute concentration in that study.

In *E. plantagineum*, nectar-solute concentrations were significantly lower in heavily-pollinated groups of flowers than in the non-pollinated control, though not different from the lightly-pollinated cohort. Still, the data suggest a quantitative effect of pollination, pollen-tube growth and (or) fertilization, on nectar-sugar secretion and (or) reabsorption. However, the precise location of the effect in the gynoecium, or what process in the nectary is affected, is unknown from the present work. There also exists the possibility of a signal emanating from the germinating pollen grains (see Shuel 1961) and growing pollen tubes themselves. This phenomenon of reabsorption may be an adaptation toward conservation of nectar sugar, once certain thresholds induced by pollination/tube-growth/fertilization, are established.

The reason for the significant difference in nectar-sugar levels in *emasculated* flowers, with degree of pollination, and the simultaneous lack of difference in nectar-sugar contents of *intact* flowers, may be linked to an increase in net nectar-sugar expulsion, or a decrease in net reabsorption, in deanthered flowers. In experiments more properly controlled than those reported here, Shuel (1961) found that unpollinated, excised flowers of *Streptosolen jamesonii* (Solanaceae) yielded 15-20%
more nectar sugar when emasculated, in three of four experiments. In that study, emasculation was performed 6-12 h prior to anthesis, whereas here flowers of Echium were deanthered approximately 12-18 h post-anthesis, after they had already secreted some nectar. In the present experiments, emasculated, unpollinated flowers of E. plantagineum produced in situ 26.8% more nectar sugar over the control (intact, pollinated), but this increase was not significant. Shuel (1961) also demonstrated that reabsorption of 14C-sucrose (the dominant sugar in floral nectar of Streptosolen) by these emasculated flowers was reduced, and that the anther effect may be under control of plant-growth regulators. It was hypothesized also by Shuel (1961) that nectar secretion and reabsorption may be occurring by some common process, such that the increase in nectar sugar caused by anther removal in S. jamesonii may be related to reduction in reabsorption of sucrose through the nectary. Bieleski and Redgwell (1980) demonstrated nectar-sugar reabsorption in several Rosaceae, and estimated that reabsorption of glucose from nectar of excised pear flowers occurred at approximately 1/5 the secretion rate of glucose. Future investigations of nectar secretion of excised flowers of E. plantagineum, involving pollination, may prove fruitful in resolving some of the relationships mentioned above.

With regard to cross-pollination and flower age, no differences in proportions of nectar-sugar species and their ratios were detected in flowers of E. plantagineum. Therefore, reabsorption of nectar sugar in E. plantagineum does not appear to favour any of the four major sugars analysed here. Hawker et al. (1983) did not find any differences in the contents of the major sugars (sucrose, glucose and fructose) of watermelon nectar 1 h after pollination, a period sufficient for changes in sugar and other chemical constituents of the stigmatic exudate.
5.4.2. The technique for assessing the pollinating ability of flower visitors: some of its advantages

This aniline-blue technique for assessing pollen-tube number at the style base of previously-bagged, virgin flowers as a result of insect visits has several noteworthy advantages over other direct methods (see Handel 1983) of rating the degree of pollination. Previous methods like Beattie’s (1971), wherein the quantity of pollen on the stigma is determined by mounting the upper part of the gynoecium in acid fuchsin/gelatin on microscope slides and counting visible grains, usually only provide an estimate because rarely are all pollen grains obvious. Also, certain stigmatic papillar arrangements and copious quantities of sticky exudate which in some species cover pollen grains may make this exercise even more difficult. Furthermore, pollen grains located on the upper style near the stigma may shift to the stigmatic region during mounting of tissues, thus confounding the true number which was actually there. Casper (1983) noted some of these limitations in Cryptantha (Boraginaceae).

There is also a greater likelihood of including any pollen grains not belonging to the species represented by the gynoecium. In addition, viability of the pollen grains on the stigma is not accounted for.

Although it is a more expensive and time-consuming process, scanning electron microscopy may be more useful in determining the true number of specific pollen grains on stigmata, the percentage of germinated grains there (e.g., Fig. 5.4), etc., because stigmata can be mounted and oriented such that the entire stigmatic surface is observable. However, whether pollen tubes reach the style base is not shown with scanning electron microscopy (e.g., Fig. 5.5). An investigation into the correlation between stigmatic pollen-grain number and pollen-tube counts at the style base, would be particularly useful (Mulcahy et al. 1983).

Similarly, the counting of matured fruits within a flower is not always a reliable determination of pollination, because an underestimate can be obtained in those species
undergoing post-fertilization abortions, whereas the parthenocarpic fruit development of other species results in an overestimate of fertilization. In this study, there was absolute agreement between numbers of enlarged ovules (when less than four) at style collection, with pollen-tube number at the style base. However, even in species without post-fertilization loss in fruit set, the potential number of ovules which could have been fertilized after a single flower visit is unknown from fruit counts, when pollen-tube number at the style base exceeds the ovule number per flower. For some floral types, like *E. plantagineum*, it may be possible to follow fruit development (counts, isozyme analysis, etc.) even after style removal for pollen-tube status.

Clearly, detection and counting of pollen tubes at the style base in self-compatible species like *E. plantagineum* (Burdon *et al.* 1988; Davis, pers. obs.) is an extremely accurate yet practical measure of pollination, because each pollen tube germinating from each viable pollen grain properly placed on a receptive stigma of the same species must reach the style base, and then the ovules themselves, to deliver the sperm-cell nuclei necessary for fertilization to occur. Pollen tubes within the transmitting tissue of the style are relatively protected, and therefore their number most accurately reflects the true number of germinating pollen grains on the stigma. Pollen tubes are not nearly as susceptible to loss from the style as pollen grains from the stigma might be, during tissue processing.

Also, the present technique is not limited to relatively rare situations where pollen grains dimorphic for colour, morphology or size have had special application in assessing pollination (see Handel 1983). Pollen tubes are detectable from a wide taxonomic range of pollen and style types. Once the plant’s compatibility to self-pollination is determined, detection of stylar pollen tubes allows some inferences to be made about the donor pollen. Inter-callosic plug distances, not investigated here, may also give an estimate of self- versus cross-pollination (Linskens and Esser 1957) or of inter- and intra-morph pollination in heterostyloous species (Weller and Ornduff 1989). Progeny analysis with isozymes would provide important complementary information
that discriminates autogamy and geitonogamy from xenogamy. For species exhibiting obligatory gametophytic self-incompatibility, all pollen tubes reaching the style base must have resulted from pollinator deposition of outcross grains, thus providing a useful measure of xenogamy. Emasculation can also be a useful tool for comparisons of pollen-tube numbers in normal flowers, even for those flowers left within bags.

Although large non- *Apis* visitors to flowers of *E. plantagineum* were too few in this study to make comparisons between these different insects, the technique, when used with open virgin flowers, gives the potential to rank various animals according to their effectiveness as pollinators. With this plant species, at least, it is a very safe assumption, despite the capacity for self-pollination, that the opened flower, bagged as a bud, is unpollinated when it is uncovered. This phenomenon provides an important starting point to evaluate initial flower visitors.

Under similar experimental circumstances, it should be possible to determine whether the most common floral visitors of many agricultural and horticultural plants are actually the most efficient pollinators. Where endogenous populations of an insect species of interest as a potential pollinator are low, it may be possible to introduce the species inside large cages over the crop, to study and evaluate their visits to similarly-prepared virgin flowers inside an enclosure. The technique would seem to have a promising application to the challenges of hybrid-seed production, where male-sterility and emasculation provide essentially the same floral situation. Furthermore, the technique allows the evaluation of individuals of the same species as pollinators, with regard to differences in their behaviour while foraging (e.g., "side-working" and nectar thievery versus "legitimate" visitation to flowers). Also, flowers could be made available for more than just a single visit, to accurately derive models for pollination requirements of different crops. Ultimately, educated estimates could be obtained regarding the number of pollinators (e.g., honey bee colonies) required to achieve certain crop yields.
5.4.3. **Pollination of *E. plantagineum* within bags**

The events causing the pollination of flowers bagged for their entire blooming period (see section 5.3.2 - paragraph 2, and Fig. 5.14) and causing 11.1% of styles to bear a solitary pollen tube after single honey bee visits to intact yet unreceptive, virgin flowers of stage 0.5 (Fig. 5.15), are not clear. The porous netting (Fig. 5.1, 5.11) did not exclude Thysanoptera (thrips), especially immatures of which were occasionally found at the flower base, or even walking on anthers, styles and stigmata (also, see Kirk 1984). Therefore, thrips were potential pollinators of this species. Also, the minute size of pollen grains relative to the gaps of the bag weave would permit wind entry of pollen into the bags, although the chances of any airborne pollen grains lodging on a dry receptive stigma seem extremely remote. Failure to microscopically locate pollen grains of *E. plantagineum* on microscope slides (previously dubbed with albumen, then placed inside flower bags) attached to stakes amidst plants of this species, supports this unlikelihood. Despite care by the researcher, during initial unbagging to label and/or emasculate the flowers, and then subsequent rebagging, handling may have resulted in accidental pollination. Furthermore, on a single, separate occasion, a flower whose elongating, wandering style caused the forked stigma to be caught just below the anther of one of its own exsert stamens, might indicate that autogamous pollination could occur in this manner. Whether pollination in these bagged flowers of this self-compatible species occurred by selfing or crossing, is also unknown, but the significantly greater number of pollen tubes in intact versus emasculated flowers (Fig. 5.14) suggests that self-pollination prevailed.

In bagged flowers of *E. wildpretii*, more than double the amount of potential seed reported here for *E. plantagineum*, was produced (Oleson 1989); in that study, on average over two nutlets per flower were matured. It was concluded that autogamy had occurred, but how the pollen reached the stigma was not discussed.
5.4.4. **Floral visitation increases pollination**

Despite the possibility for reproduction by autogamy in this species, as determined by fruit development and style examinations following hand pollination of bagged flowers with their own pollen, flowers exhibited low pollen-tube numbers when confined by netting. In this study, the stigma was spatially separate from and rarely contacted anthers within that flower as a consequence of style elongation. Instead, flowers of *E. plantagineum* required the body-pollen and disturbance/interference associated with floral visitors, in order for pollen to reach the stigma. When flowers were exposed, by unbagging, to multiple visits from animals larger than thrips, significantly more pollen tubes were detected regardless of whether the flowers were intact (allowing autogamy) or emasculated (precluding autogamy). Surprisingly, there was no difference in tube number between intact and emasculated flowers subjected to multiple visits. These findings, even determined using plants of *E. plantagineum* growing at relatively low densities and having many cymes flowering simultaneously, argued by Burdon *et al.* (1988) to enhance the occurrence of geitonogamous self-pollination (within-plant pollination, but not autogamy), support the high outcrossing estimates determined earlier (Burdon and Brown 1986, Burdon *et al.* 1988).

5.4.5. **Honey bees as pollinators**

Examination of style bases, taken from previously unvisited flowers exposed once only to *A. mellifera*, demonstrated that honey bees are pollinators of *E. plantagineum*. In fact, pollen tubes in numbers sufficient to fertilize all four ovules were commonly detected in virgin flowers where the stigma was receptive (i.e., floral stages 2 and 3). Such was the case for both intact and emasculated flowers, where, respectively, 36-50% and 31-35% (38.7% overall) of lone visits by *A. mellifera* resulted in more than three pollen tubes reaching the style base. These data suggest
that similar percentages of the flowers with receptive stigmata exposed for multiple visitation may have been pollinated sufficiently after their first visit, and that successive visits after the initial one increase the number of pollen tubes per style, as expected. Although it is unknown how many visits the flowers received during the multiple visitation experiments, Núñez (1977) found that a density of one to two honey bees per twenty flowers of *E. plantagineum* resulted in each flower receiving twenty to forty visits per hour.

At least one pollen tube was detected at the style base after 65.3% of single visits by honey bees to virgin flowers, suggesting that approx. 35% of such visits did not result in pollination as a consequence of honey bee activity.

Two practices which reduced the number of pollen grains from circulation, namely grooming behaviour and experimental emasculation, did not eliminate honey bees as pollinators of virgin flowers. The first event demonstrated the ability of honey bees to function as outcrossers of this species. It was evident from the second practice that pollen-collecting honey bees, which eagerly search for pollen and so may at times bear considerable body pollen (i.e., outside the corbiculae), but which also stroke the body hairs usually during flight between flowers, to transfer it to the corbiculae (Hodges 1952), did not differ significantly as pollinators from non-grooming individuals (i.e., non-pollen collectors). In fact, results of the statistical analysis were more in favour of pollen gatherers as better pollinators. There is also a possibility that some of the pollen grains effecting pollination are located on body parts inaccessible to the grooming actions of the legs (Kubisová and Hášlbachová 1990, Buchmann *et al.* 1990). The results of a rare study of mixed loading involving pollen of *E. plantagineum* suggested that grooming actions are not absolutely efficient or are at too low a frequency to remove all pollen grains from the honey bee body (Chapter 6), and therefore support the present findings. Certainly, there is a marked redundancy of pollen in *E. plantagineum* flowers - the pollen/ovule ratio approaches 50,000 (from Table 6.1, page 6.6) - and that pollen removed from circulation by the pollen-gathering
activities of honey bees, is without consequence to the reproductive capacity of the plant.

Duration of honey bee visit and the number of pollen tubes present per style base were not related. This is not surprising, because in this self-compatible species, pollen-tube growth would result from pollination by contact of the receptive stigma with any body part bearing any viable pollen grains of *E. plantagineum*. Because of the staggered arrangement of each flower’s anthers, a wide surface range of a honey bee’s head and venter could become dusted with pollen. Although not conducted in a quantitative way, pollen grains of *E. plantagineum* were indeed observed on various parts of *A. mellifera* foragers, including the mouthparts, antennae, head, thorax, wings, all three legs, and abdomen (also see Fig. 5.9), using scanning electron microscopy. Such contact with the stigma could occur at any moment during and after landing. The data indicate that a better indicator of pollination, at least for flowers of *E. plantagineum* visited by honey bees, is the total number of visits that a flower receives, not their duration, because even relatively short visits could result in successful pollination visits. Of course, in general, the greater the number of flower visits, the increased opportunities for a diversity in genotype of cross-pollen being placed on the stigma.

Honey bees foraging for nectar alone spent significantly longer on virgin flowers (i.e., previously bagged) than on normal flowers, suggesting more time taken to imbibe greater amounts of accumulated nectar (see Corbet and Delfosse 1984). These longer visits were without consequence to pollination, in this plant species at least, because there was no correlation between pollen-tube numbers and time spent per blossom.

Bagging also denies access of large insects to the pollen, and so artificially maintains all of a flower’s pollen while the stigma attains receptivity. The pollen-tube data shown in Fig. 5.14 for exposed flowers initially available for visitation at stage 0.5 are the most natural, therefore, because under weather conditions suitable for foraging, the anthers are normally depleted of much pollen when first visited by pollen-collecting honey bees, then (Corbet and Delfosse 1984; Davis, pers. obs.).
During field observations, apart from the very short visits by pollen seekers that did land on deanthered virgin flowers, it became obvious that some honey bees avoided these experimental flowers. The bees approached them from the front, but without alighting moved on, as if having sensed the absence of pollen there. These bees may have been pollen collectors, perhaps visually noting the lack of anthers on the exert stamens, and/or detecting the absence of certain chemicals (e.g., flavonoids) characteristic of *Echium* pollen (Tomás-Barberan *et al.* 1989). Honey bees have been followed on flowers of *E. plantagineum* which repeatedly foraged specifically for nectar, pollen or both (Corbet and Delfosse 1984; Davis pers. obs.). It is probable that the significant shift in honey bee foraging toward nectar collection when virgin flowers were emasculated resulted from bees which, upon landing intentionally for and trying for but not finding pollen, then gathered nectar, and/or a higher proportion of visits by bees predisposed to nectar collection. That increases occurred from one visit (intact flowers) to nine visits (emasculated flowers) for nectar only, and to nine visits (emasculated flowers) by honey bees lacking corbicular pollen, supports that latter possibility.

5.5. **SUMMARY**

Evidence for the reabsorption of nectar sugar during aging of flowers of *Echium plantagineum* L. in both the absence of pollination, and in flowers pollinated once heavily, was obtained *in situ*. Emasculated flowers produced more nectar sugar when not pollinated, suggestive that anthers have some control (e.g., by plant growth regulators) over nectar-sugar secretion and (or) reabsorption. With regard to the effects of cross-pollination or flower age on the proportions of nectar-sugar species and their ratios in floral nectar, no differences were significant. Therefore, the process of reabsorption of nectar sugar in this species does not appear to favour any of the four major sugars (sucrose, maltose, glucose and fructose) analysed.
A novel technique was developed which simply, directly and quantitatively evaluated flower visitors as pollinators of *Echium plantagineum* based on microscopic detection of the fluorescence of the callose content of stylar pollen tubes stained with aniline blue. *E. plantagineum* is self-compatible, so that germination and pollen-tube growth of both cross- and self-pollen to the ovules at the style base occurs. The technique utilized "virgin" flowers, those which were bagged as buds and subsequently opened within the bags. After careful unbagging, the majority (93.5%) of initial visitors to these previously-unvisited flowers were honey bees (*Apis mellifera* L.). The technique demonstrated that although autogamy was possible, it was not predominant in *E. plantagineum* because permanently-bagged flowers had very low numbers of pollen tubes at their style bases (usually none), and emasculated flowers and those with all their pollen still present (intact flowers), when exposed to a lone honey bee visit, usually had the same numbers of tubes. Within treatments (intact, emasculated), honey bee visits for pollen, nectar or both, did not result in differences in pollen-tube number, nor was there any connection between degree of pollination and time spent by a single honey bee per virgin flower. Although most honey bees attending virgin flowers carried pellets of *E. plantagineum* pollen on their hind legs, as a result of grooming actions to gather grains from their bodies, they were still as effective as pollinating agents as bees lacking corbicular pollen. Because one third of single honey bee visits to emasculated virgin flowers (bearing receptive stigmata) introduced sufficient pollen to achieve fertilization of all four ovules, and almost two-thirds of such visits resulted in pollination, honey bees were effective cross-pollinators of *E. plantagineum*. 
Fig. 5.1. Bagged and open flowers of plant 1. Note labels looped around calyx (arrows), and a honey bee (white arrow) visiting an open, labelled flower

Fig. 5.2. Open flower with corolla bearing rain water. Note coiled cyme (open arrow) with newly-forming buds. Exsert anthers (A), style (Sy) and styles (arrowheads) persisting after abscission of corollae of older flowers

Fig. 5.3. Outer corolla (Co) of bagged flower clipped to reveal five stamens with dehiscing anthers at three discrete positions. Stigma (St) diverging. Flower now in stage 2. Sepal (Se)

Fig. 5.4. Scanning electron micrograph of stigmatic surface showing pollen grains (G) between separated stigmatic papillae (P). At left are two collapsing grains with emergent pollen tubes (arrowheads) penetrating the stigmatic tissue (*). Bar = 4 µm

Fig. 5.5. Scanning electron micrograph of flower base after removal of perianth, to reveal insertion of style (Sy) between four ovules (O) surrounded by nectary disk (N). Stylar trichomes (T). Bar = 200 µm

Fig. 5.6a. Base of style from flower subjected to multiple visitation, stained with aniline blue and examined by bright-field microscopy to demonstrate two vascular bundles (VB) delimiting the transmitting tissue (TT), and lack of detection of pollen tubes

5.6b. Same style base examined with fluorescent light to reveal several pollen tubes (PT). Note fluorescent walls, and discrete callose plugs, of the latter. Bar = 100 µm

Fig. 5.7. Base of different style squashed slightly to separate pollen tubes (PT; three of the nine are arrowed) before viewing with fluorescence microscopy. Vascular bundle (VB), stylar trichome (T). Bar = 100 µm

Fig. 5.8. Base of style from bagged flower. Pollen tubes absent from transmitting tissue (TT). Bar = 50 µm

Fig. 5.9. Scanning electron micrograph of branched body hairs of Apis mellifera, showing trapped pollen grains (G) of E. plantagineum. Bar = 10 µm

Fig. 5.10. Scanning electron micrograph of load (L) of E. plantagineum pollen on corbicula of left metathoracic leg of A. mellifera. Femur (F), tibia (Ti), basitarsus (B). Bar = 1.0 mm

Fig. 5.11. Scanning electron micrograph of flower bag showing mesh size of weave and hundreds of pollen grains (arrowheads) deliberately brushed onto bag. Bar = 200µm
Fig. 5.12. Changes in nectar volume (○) and total sugar (●) with floral stage on the first day of anthesis, as assessed from six flowers of each of stage 0.5, 1, 2, and 3 (see Table 5.1) of experimental plant 1 of *Echium*. Values plotted are means ± S.E.
Plant 1

Nectar volume (µL)

Floral stage

Nectar sugar (mg)
**Fig. 5.13.** Percentage and some specific ratios of four major sugars detected in nectar of experimental plant 1 of *Echium plantagineum* throughout flower development. The six groups represented are:

- **A** - stage 0.5, unpollinated, < 6 h post-anthesis
- **B** - stage 1, unpollinated, 6-12 h post-anthesis
- **C** - stage 2, unpollinated, 12-16 h post-anthesis
- **D** - stage 3, unpollinated, 12-20 h post-anthesis
- **E** - stage 3, unpollinated, 36-54 h post-anthesis
- **F** - stage 3, cross-pollinated by hand at 12-20 h post-anthesis, 24 h previously

Usually the nectar from six flowers was analysed per group, with the exception of group C (n=5), group E (n=4) and group F (n=7). Values shown are means ± S.E. Within groups of bars, the occurrence of the same letter above the standard error indicates that differences in the mean values are not statistically significant, using 2-tailed t tests with $\alpha=0.05$. 
Fig. 5.14. Number of pollen tubes (Mean ± S.E.) per style base in open flowers first exposed at stages 0.5, 1, 2 (all n=20) and 3 (n=40) and available for *multiple* visits, versus bagged flowers. Flowers were either emasculated or left intact. Ranges in pollen-tube number are shown beneath bars. Overall means ± S.E. are printed above each set of treatment bars.
Figs. 1, 2. Number of pollen tubes per style base on floral stage 0.5 when first exposed:

**INTACT**

- Floral stage 0.5
- When first exposed: 1, 2, 3

**EMASCULATED**

- Floral stage 0.5
- When first exposed: 1, 2, 3

- Bagged Exposed

- 0.3 0.6 0.7 0.6

- 0.13 ± 0.068

- 0.57 ± 0.13

- 8.33 ± 0.59

- 4.0 ± 3.0

- 7.59 ± 0.53
Fig. 5.15. Number of pollen tubes per style base in open flowers previously bagged, then emasculated or left intact at stage 0.5, 1, 2 or 3 before exposure to a single honey bee visit. Each bar represents an individual virgin flower visited once by a bee. Means within treatments, shown ± S.E., are not significantly different if followed by the same letter ($\alpha = 0.05$). Asterisks indicate visits by honey bees lacking dark blue pollen in their corbiculae.
Each bar represents a single honey bee visit.
Fig. 5.16. Number of pollen tubes per style base versus the time spent per visit to a virgin flower by a single honey bee. Only flowers of stage 2 and stage 3 are shown.
Floral treatment:
INTACT •
EMASculated •

No. of pollen tubes per style base

Duration of visit (sec) by a single honey bee
CHAPTER 6

MIXED LOADING OF POLLEN
FROM ECHIUM PLANTAGINEUM L.
(BORAGINACEAE)
AND HIRSCHFELDIA INCANA (L.) LAGREZE-FOSS.
(BRASSICACEAE)
BY AN INDIVIDUAL HONEY BEE (APIS MELLIFER A L.)

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6.1. INTRODUCTION

While foraging, individual honey bees exhibit a relatively high fidelity to a particular plant species (Percival 1947, Hodges 1952, Free 1963). This behaviour greatly increases their effectiveness as pollinating agents, because their bodies may solely or predominantly harbour pollen grains shed by flowers of that species. This strong tendency to visit a single plant species is manifested in the monochromatic appearance of the pollen pellets borne on the hind-leg corbiculae of laden honey bees returning to the hive, and in many cases visual identification of the plant species visited, is therefore even possible (Hodges 1952).

There are, however, numerous published reports of occurrences of more than one colour of pollen being present in individual pellets collected by honey bees (Betts 1920, 1935, Brittain and Newton 1933, Percival 1947, Maurizio and Kollmann 1949, Maurizio 1953, Hodges 1952, 1954, Jay and Jay 1984). The pellets which result from the gathering of pollen from flowers of two or more species, during a single foraging trip, have been termed mixed loads (Betts 1935, Percival 1947, Hodges 1954, Free 1963). Jay and Jay (1984) have also applied the term to the dichromatic loads which can occur by collection of pollen from both flower types within certain dioecious species. Betts (1920, 1935) distinguished between segregated and mingled mixed loads, the latter being most common. The former exhibit coloured bands of two distinct kinds of pollen as a result of the bee collecting from one species and then switching to another. In mingled loads, two kinds of pollen are intermingled such that the pellets are unicolour and can only be recognized as mixtures by microscopy. Gradations between segregated and mingled loads are uncommon (Betts 1920, 1935). Pollens from up to five species have been reported in individual pollen pellets (Betts 1920, Brittain and Newton 1933, Free 1963), but quantitative data on the percentages of each pollen are lacking. Most records of mixed loads have arisen from observations
at hive entrances or collections in pollen traps, and have resulted in the following estimates of the frequency of such loads: much less than 6.75% (Betts 1920), under 3% (Betts 1935), 0.1% (Percival 1947), 0.3% (Maurizio and Kollmann 1949), 0.9-3.0% (Maurizio 1953) and 6.1% (Free 1963). On the other hand, there are far fewer reports of honey bees observed in the act of mixed loading. Brittain and Newton (1933) reported 38% mixed visits by honey bees. Their study concentrated on pollen collection, but not nectar. Ribbands (1949) observed honey bee ‘E’ gathering pollen from *Eschscholtzia* (a species of the Papaveraceae which does not yield nectar; Ribbands 1949) and both nectar and pollen from *Limnanthes*, during the same foraging trips, over two consecutive days. Hodges (1952) saw a honey bee, which carried segregated loads, foraging on dandelion at the base of a shrub of berberis. However, there was no indication given of nectar gathering.

During the course of an investigation of the reproductive biology of *Echium plantagineum* (see Chapter 5), a honey bee was observed to leave a plant of this species to continue foraging for both nectar and pollen on one of *Hirschfeldia incana* (L.) Lagrèze-Fossat (Brassicaceae). Because its corbiculae bore mixed loads of pollen, the bee was captured and its pollen pellets subsequently studied. An account of this rare observation, including the first scanning-electron microscopic details of the pattern of mixed-pollen loading, a quantitative assessment of each pollen species in the loads, and the discovery of insect larvae in the pellets, is given herewith. This unique situation has also provided insights into foraging behaviour itself, such as the efficiency of body-pollen removal by grooming, and on the specificity for nectar-sugar ratios.

6.2. MATERIALS AND METHODS

In the field, the metathoracic legs of the honey bee under observation (a hybrid of *Apis mellifera ligustica*) were carefully removed with forceps and stored in a clean
vial, pollen-pellets up, at ambient temperature in the dark. Sketches were made of the
colour distributions in the pellets, and two days later, the vial was stored at -18°C for a
week. After thawing, photographs were taken on a Wild-Heerbrugg Photomakroskop
M 400, before the left leg was mounted (without chemical fixation) on an aluminum
stub, coated with gold using a Polaron E5000, and then viewed on a Cambridge
Stereoscan 360 SEM at 20 kV. Eighty-seven fields of pollen over the entire outer
surface of the pellet were photographed at a magnification of about 600 times. Each
field contained, on average, 106.5 ± 1.96 (S.E.) grains, depending on grain size and
density. Fortuitously, the pollen grains of *H. incana* are larger and can be also
distinguished by the degree of pitting of the exine (Fig. 6.5, 6.9, 6.10, 6.14), such that
the percentage of each pollen type in each field could be established. After carefully
removing the pellet from the right leg, forty-one more fields of pollen originally
located immediately next to the tibia itself, were photographed by scanning electron
microscopy as above, thereby providing data for the inner surface of these mixed loads,
as well.

Thereafter, the number and percentage of each pollen species within the two
mixed-load pellets were estimated by separately suspending the pollen grains of each
pellet in 1.0 ml of water. Six aliquots of each suspension were analysed on a Fuchs-
Rosenthal counting chamber using light microscopy. A mean of 1700 pollen grains
was counted per sample. Pollen grains of *E. plantagineum* and *H. incana* are easily
distinguished, without acetolysis (Fig. 6.15a).

Pollen counts were made also of indehiscent anthers of mature buds taken from
the visited plant of each species, to determine the mean number of grains each
possessed. This was accomplished by separately rupturing the anthers in small drops
of dilute detergent solution on microscope slides, thus releasing the grains which
subsequently were counted.
6.4

Using filter-paper wicks (McKenna and Thomson 1988), samples of floral nectar of each species were collected at the study site from six fully-open flowers bagged, as large buds with insect netting, a day earlier. In the case of *E. plantagineum*, the nectar samples were taken from stage-2 flowers (see Table 5.1). In the laboratory, the wicks were soaked and shaken in distilled water, and aliquots then taken to determine enzymatically the glucose, fructose, sucrose and maltose contents (see sections 3.2.3 and 5.2.2).

Other honey bees foraging at the study site on plants of *E. plantagineum* and *H. incana* were observed over several days in order to determine their constancy to each species. However, counts were made only of those bees with corbiculae estimated to be at least 2/3 filled with pollen, a condition which should permit a reliable estimate of the frequency of mixed loading involving pollen of these two species.

6.3. RESULTS

The event of mixed loading occurred on February 2, 1991 at approximately 10:45 a.m. (D.S.T.) at a wasteland site near Sullivan’s Creek, just north of Barry Drive, in Canberra. The morning was still, sunny, warm and dry, with the maximum temperature reaching 33°C that day. The temperature maxima immediately preceding Feb. 2 were 27, 24, 24, 29 and 31°C. Many honey bees were foraging on *E. plantagineum* during those days. The site had been without rain since January 23, 1991.

The honey bee with mixed loads was first noticed amidst other foragers while it collected both nectar and pollen from at least two flowers (Fig. 6.2) on plant 2 of *E. plantagineum* (Chapter 5). The plant from which it arrived is unknown. The contrasting bands of yellow and dark blue pollen carried in the corbiculae were obvious, and, undisturbed, the bee was followed as it flew directly to a multi-infloresced plant of *H. incana*, 2.4 m away. Here, the bee continued to forage on two racemes, visiting five flowers (Fig. 6.1) in total, for both nectar and pollen, over
about 20 sec. While probing from nectar, the bee was taken by the wings and killed by
 crushing the head/thorax, to leave the pollen pellets of the hind legs intact. Unlike
 many other foraging honey bees observed on these plants, the wings of this bee were
 not frayed.

Close examination of the pollen pellets under the dissecting microscope showed
them to have fairly well-defined zones of colour (Fig. 6.3, 6.4). These loads,
composed of yellow bands above and below the large central blue band, would be
classified as segregated (Betts 1935), and are most similar to photograph 7f of
Maurizio (1953). The widths of the bands varied between the outer (exposed) surface
of the pellet and the inner surface lining the corbicula itself. Generally, the two pellets
displayed mirror-imaged patterns of colour (Fig. 6.4).

Using scanning electron microscopy, it was determined that the outer surface of
the left pellet bore both pollen species, and that each species was represented in every
one of the fields examined (Fig. 6.6). That it, even within the yellow zones of the
pellet, some pollen grains of *E. plantagineum* were also always present, and vice-versa.
A comparison of Fig. 6.3 and 6.4 with Fig. 6.6 demonstrates that the percentages of
pollen of the two species within the photographed fields sometimes does not
 correspond to the predominating hue on the pellet.

In the same way, all fields surveyed along the regions of the right pellet, which
in situ immediately lined the pollen basket, displayed a mixture of pollen from both
species (Fig. 6.7). *Hirscepheldia* pollen predominated along the top and bottom edges of
the pellet, whereas the central blue band contained mostly *Echium* pollen, as expected
from the sketch (Fig. 6.4). Both pollen types were even present at the insertion point
left by withdrawal of the corbicula spindle hair (Fig. 6.8).
Examination of the suspensions of the mixed pellets demonstrated a remarkable similarity between the left and right pellets; *Echium* and *Hirschfeldia* comprised 60% and 40% of all pollen grains, in both. During the analysis, including the scanning electron microscopy of several fields on the pellets, pollen grains belonging to two additional taxa were noted. These other pollen types were easily distinguished from *Echium* and *Hirschfeldia* (Fig. 6.14, 6.15). One grain each of the eucalypt and the unidentified Asteraceae-Liguliflorae were found in the left pellet, two of each in the right load. There miscellaneous pollen grains were very rare, amounting to less than 3 in 10,000 (Table 6.1). The loads were imbalanced.

**Table 6.1. Number of pollen grains per flower of each species and in the mixed-load pellets**

<table>
<thead>
<tr>
<th>Species</th>
<th>Anther</th>
<th>Flower</th>
<th>Left Total</th>
<th>Percent</th>
<th>Right Total</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Echium plantagineum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short stamen (1)*</td>
<td>30,071</td>
<td>192,600</td>
<td>262,875</td>
<td>60.48</td>
<td>368,135</td>
<td>59.30</td>
</tr>
<tr>
<td>Medium stamen (2)</td>
<td>36,683</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long stamen (2)</td>
<td>44,594</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hirschfeldia incana</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short stamen (2)*</td>
<td>5,281</td>
<td>25,400</td>
<td>171,640</td>
<td>39.49</td>
<td>252,480</td>
<td>40.67</td>
</tr>
<tr>
<td>Long stamen (4)</td>
<td>3,716</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
<td></td>
<td>105</td>
<td>0.0236</td>
<td>170</td>
<td>0.0272</td>
</tr>
</tbody>
</table>

*Estimated number of pollen grains
+The number in brackets denotes the number of each stamen type per flower

While scanning the bottom of the mixed load on the left leg, an insect was discovered (Fig. 6.6, 6.12). Later, the insect was removed from load and identified as an immature (wingless) thrips (Thysanoptera: Thripidae). Using the key to larvae of common Australian flower thrips (Kirk 1987), the insect was identified as a second-instar larva of *Thrips imaginis* Bagnall, a common inhabitant of flowers of *E. plantagineum* (Kirk 1984). When counting pollen grains of the right-pellet suspension a further thrips larva (first instar), and the abdomen of another (second instar), were
encountered. Therefore, this bee carried at least three thrips amidst its mixed loads of pollen. The first-instar thrips in Fig. 6.13, which perfectly matched the immature specimen embedded in the right pellet, was taken over two months later, at the same study site, from an open flower of *H. incana*. *T. imaginis* has been taken from flowers of *Brassica napus* before (see Kirk 1987). Interestingly, identical thrips were captured simultaneously at the site from open flowers of *Echium*.

In the present case, the honey bee probably collected nectar from both species, as well as their pollen. After manipulating the exposed anthers (Fig. 6.2) of *E. plantagineum*, the bee entered the flowers, presumably to visit the nectary at the base (Ferrazzi 1987, Davis, pers. obs.). Because of the reflexed corolla of *H. incana*, both nectar and pollen collection by this bee could be clearly seen there. Quantitative analysis of sugar constituents showed the floral nectar of *H. incana* to be identical to that analysed before for this species (Percival 1961), and the nectar of *E. plantagineum* to be very similar to that of *E. vulgare* assayed by paper chromatography (Percival 1961). There is good agreement in nectar-sugar characteristics between the two unrelated plants of *E. plantagineum* at this study site (see stage-2 flowers of plant 1, in Fig. 5.13). Therefore, the nectars of these two species are strikingly different (Table 6.2).

Table 6.2. Ratios [Mean (Range)] of dominant sugars in nectar of each species, by weight. Fructose (F), glucose (G), hexose (H = F + G), maltose (M), sucrose (S).

<table>
<thead>
<tr>
<th>Species</th>
<th>F/G</th>
<th>M/G</th>
<th>S/M</th>
<th>S/H</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Echium plantagineum</em></td>
<td>0.592</td>
<td>1.09</td>
<td>2.84</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>(0.539-0.661)</td>
<td>(0.359-2.19)</td>
<td>(1.29-4.43)</td>
<td>(1.03-2.56)</td>
</tr>
<tr>
<td><em>Hirschfeldia incana</em></td>
<td>0.761</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(0.697-0.880)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
At the study site, sixty-four honey bees foraging on plants of *E. plantagineum* were found that had at least 2/3 of their corbiculae loaded with dark blue pollen only. Less time was spent observing honey bees working flowers of *H. incana*, where nine were observed with nearly-full pollen baskets containing solely yellow loads. No other instances of mixed loading were observed. Therefore, only one of seventy-four such bees, or 1.35 per cent, exhibited this phenomenon.

6.4. DISCUSSION

In the present study, the frequency of foraging trips involving mixed pollen loads was estimated at 1.35 per cent, within the range determined previously (see 6.1).

Although it is impossible to know the full details of each foraging trip, such as the numbers of flowers visited and the frequency of body-stroking to remove pollen, the "sandwich arrangement" (Percival 1947) of the colour bands (yellow-blue-yellow) suggest that this bee began foraging (predominantly) on *Hirschfeldia*, then switched to *Echium*, before returning to *Hirschfeldia*. The presence of many *Echium* pollen grains in the top third of the loads, and the fact that the bee was captured after it was seen to return to *Hirschfeldia* from *Echium*, the bottom yellow edge of the pellet already being in place, indicates that more frequent transfers between the plant species may have occurred that suggested by the foraging switches above. However, if the bee had been switching between plant species much more frequently, while still collecting pollen from both species, then the loads should have appeared more similar to those with numerous, thinner alternating colour bands (giving the "snail-shell" pattern) illustrated by Hodges (1954). The extreme case must be the mingled type of mixed loads (Betts 1920), where bees forage indiscriminantly between plant species. The resulting pellets
are intermingled pollen grains giving a monochromatic appearance intermediate in colour between hues of the pure loads of these pollens carried by other bees.

When the middle pair of legs contact the outer surface of the growing pollen loads of the hind legs of the pollen-gathering honey bee, they also play a rôle in packing the pellets. The smudged colour pattern of the outer surfaces of segregated mixed loads, as compared to the inner, corbicular-lining surface, has been attributed to the strong patting action of the mesothoracic legs (Hodges 1954). Besides this disturbance to the actual order of pollen packing, it is possible that additional pollen grains may be placed thereby on the outer surface of the pellets, hence confounding the chronological packing pattern of the pollen-pressing apparatus. For these reasons, the cryptic, inner surface of the right pellet (Fig. 6.7) probably more accurately reflects the packing order than does the exposed, outer surface of the left pellet shown in Fig. 6.6.

If this bee’s grooming behaviour was standard, then the data obtained here suggest that even pollen-collecting honey bees are still potential pollinators. It is significant that all of the 128 fields examined over the outer and inner surfaces of the mixed pellets contained both pollen types, without regard to the colour predominating in each region. Therefore, removal of accessible pollen grains (not to even consider those located elsewhere on the body) during the periodic stroking of the body hairs by the legs, probably was not absolute. It follows that pollen grains in the pellets of honey bees are not necessarily packed according to the stage of the foraging trip in which they adhered to the bee’s body. In this case, for instance, during the bee’s final foraging activities on flowers of Hirschfeldia, grains of Echium were apparently still being introduced to the pellets, along with long-adhering and freshly-gathered pollen of Hirschfeldia. Pollen grains of both species on the inner surface of the basitarsus would soon have been added to the pellets (Fig. 6.11), and further grains were located side-
by-side on other leg regions (Fig. 6.9, 6.10). Although it is unknown which parts of the body (i.e., those accessible to raking, versus inaccessible parts) harboured the relevant pollen grains, it has been clearly demonstrated in another study (see section 5.3.3.6) that honey bees carrying pure loads of *Echium* pollen could successfully pollinate emasculated flowers of *E. plantagineum*.

Despite about 7.6 times more pollen being produced by flowers of *E. plantagineum* than by those of *H. incana*, the ratio of pollen grains in each mixed-load pellet was only 1.5 (Table 6.1, page 6.6). Therefore, the bee may have visited more flowers of *Hirschfeldia* during this mixed-loading trip. However, variables such as the number of pollen grains which were still available for collection on flowers before being visited by this bee, the time and effort spent gathering pollen per flower, the ability of the pollen to adhere to the body, etc., only afford speculation on this point.

Nevertheless, an unequal visitation to the plant species would support the conclusion of Free (1963) that honey bees with mixed loads were those which had become temporarily "dissatisfied" with their original species (here, *Hirschfeldia*) and were sampling another (*Echium*), without necessarily transferring from one species to the other, on subsequent trips.

Pollen grains in the pellets examined here were often bound together by regurgitated honey sac contents added by the bees inorder to consolidate the loads during their construction (Fig. 6.5c, 6.11, 6.14), and differences in grain shape were evident for *Hirschfeldia* pollen (Fig. 6.5a,b). The turgid, spherical grains may reflect a swelling reaction of that pollen caused by the addition of nectar. Recently, using scanning electron microscopy, van der Ohe and Dustmann (1990) demonstrated dramatic shape changes in pollen, depending on whether the grains were dry or taken from honey.
The significance of the close proximity of the plant species visited in previous investigations of mixed loading has been stressed (Betts 1920, 1935, Percival 1947, Maurizio and Kollmann 1949, Maurizio 1953), propinquity being regarded as more important than such factors as flower size, morphology, colour, scent or relatedness of the plant taxa involved. This present case concerned interspersed plants of *E. plantagineum* and *H. incana*, and because the flowers of these species differ in form, colour, size and classification (from different orders of angiosperms), the emphasis on contiguity of the plants is supported. Wykes (1952c) and Furgala et al. (1958) demonstrated a preference of *A. mellifera* foragers for certain sugar solutions or nectars over others. The lack of similarity between nectar-sugar constituents and their ratios between *E. plantagineum* and *H. incana* suggests that commonality in these characteristics was not the impetus for mixed loading.

At least four plant species were represented in the mixed loads carried by this bee. Because no other plants of the Boraginaceae or Brassicaceae grew at the study site, it seems a safe assumption that by far almost all grains belonged to the two species *E. plantagineum* and *H. incana*. The grains of *Eucalyptus* sp. and Asteraceae-Liguliflorae may have lodged onto this bee during previous foraging trips, during contact with other bees at the hive, or were left behind on flowers of *Echium* or *Hirschfeldia* visited previously by other insects, etc. Similarly, the packing of thrips into the mixed-load pellets has a dubious origin, identical immatures being found in both flower species. Occasionally thrips were seen walking upon the exerted anthers, style and stigma of flowers of *E. plantagineum*. The packing of thrips into pollen pellets has apparently not been recorded before.

This is the second record for a species of the Boraginaceae being involved in mixed loading by the honey bee, the other being forget-me-not (Maurizio 1953). On
the other hand, *Hirschfeldia incana* can be added to the list of Brassicaceae previously recorded in mixed loads: rapeseed (Maurizio and Kollmann 1949, Klungness and Peng 1983), *Raphanus* (Brittain and Newton 1933), *Sinapis* (Percival 1947), and many unidentified Brassicaceae (Maurizio 1953, Free 1963).

Finally, it is interesting that all partners involved in this occurrence of mixed loading, namely the bee subspecies and both plant species, are native to the Mediterranean region and were introduced to Australia (Piggin 1977, Burbidge and Gray 1979, White 1988).

### 6.5. SUMMARY

The mixed loads of corbicular pollen from a honey bee (*Apis mellifera* L.) captured after it was observed to forage on flowers of *Echium plantagineum* L. and *Hirschfeldia incana* (L.) Lagrèze-Fossat during the same trip were studied to determine the pattern of deposition of pollen grains and the ratio of pollen grains present per species. Because the loads had segregated regions of colour, and many fields examined with the scanning electron microscope revealed that both pollen types were always present, it is suggested that grooming behaviour is not absolutely efficient and that entrance of grains into the pellets during the pollen-packing process is not necessarily chronological. Both left and right loads contained the same proportions of each pollen type, although not the same quantity of pollen. Very minor quantities of pollen from at least two other taxa (*Eucalyptus* sp., and Asteraceae-Liguliflorae), and immature thrips (*Thrips imaginis* Bagnall), were recovered from these mixed loads.

Analysis of nectar samples from *E. plantagineum* and *H. incana* indicated marked differences in sugars and their ratios, indicating that similarity in nectar-sugar composition likely was not the stimulus to visit both species.

No other mixed loads were observed at this study site. The frequency of mixed loading was estimated at 1.35 percent.
Figs. 6.1-6.5. Inflorescences, and honey bee (Apis mellifera) pellets containing mixed loads of pollen, from Echium plantagineum and Hirschfeldia incana

Fig. 6.1. Inflorescence of H. incana. Anthers (arrows)

Fig. 6.2. Inflorescence of E. plantagineum. Anthers (arrows). Scale gradations shown are 1 mm each. Same scale for Fig. 6.1

Fig. 6.3. Large pollen pellets on the left metathoracic legs from three honey bees.  
   Left - pure yellow pollen of H. incana  
   Centre - segregated mixed load consisting of predominantly yellow (Y) pollen above and below a band of mostly dark-blue (B) pollen  
   Right - pure dark-blue pollen of E. plantagineum. Bar = 2 mm

Fig. 6.4a,b. Sketches of the anterior and posterior views of the left (a) and right (b) metathoracic legs bearing segregated mixed pollen loads consisting of yellow (Y) and blue (B) bands.

Fig. 6.5a-c. Fields of pollen grains from the three pollen pellets of Fig. 6.3, photographed using scanning electron microscopy.  
   a - pure H. incana  
   b - mixture of H. incana and E. plantagineum  
   c - pure E. plantagineum. Bar = 20 µm
Figs. 6.6-6.8. Scanning electron micrographs of mixed loads of pollen carried by an individual honey bee

Fig. 6.6. Metathoracic leg in the centre of Fig. 6.3, showing the dorsal surface of the segregated mixed load. Each circle pinpoints a field of about 105 pollen grains, on average. The percentage of each pollen type per field is indicated by white (*H. incana*) and black (*E. plantagineum*) sectors of the circles. Note immature thrips (arrow; see Figs. 6.12, 6.13) at bottom left of pellet. The large clump of pollen at bottom (arrowhead) may have been dislodged from the pellet at *, during bee capture. Femur (F), tibia (T), basitarsus (B). Bar = 0.5 mm

Fig. 6.7. Scanning electron micrograph of mixed load from right leg, showing inner surface which lined the pollen basket itself. The circles give the percentages of each pollen type at fields examined in the region covered *in situ* by the tibia. The arrow indicates the former position of the base of the anchoring (spindle) hair of the pollen basket. Bar = 0.5 mm

Fig. 6.8. Higher magnification micrograph of the base of the right mixed load (Fig. 6.7), depicting the cavity opening (arrow) which remained after withdrawal of the corbicular spindle hair. Note pollen grains of both species present. Bar = 100 µm
Figs. 6.9-6.15. Pollen types on the bee’s body and in the mixed loads carried on the hind legs, and details of the thrips recovered from the loads.

Fig. 6.9, 6.10. Regions of tibia (T) of left metathoracic leg carrying mixed load of Fig. 6.6, showing adjacent pollen grains of *E. plantagineum* and *H. incana*.

Fig. 6.9. Filiform hairs on posterior edge of tibia.

Fig. 6.10. Spatulate hairs on inner face of tibia. Bar = 20 µm, for both figures.

Fig. 6.11. Pollen grains of *E. plantagineum* and *H. incana* raked from body and accumulated here near apex of basitarsus (B) of left hind leg. Hairs of basitarsus (asterisks); plumose body hair (arrow) amongst pollen grains. Bar = 100 µm.

Fig. 6.12. Posterior end of immature thrips, partially coated in pollen, as it appeared at base of pollen pellet in Fig. 6.6. Bar = 50 µm.

Fig. 6.13. Fresh mount of immature thrips taken from fully-open flower of *H. incana*. Bar = 100 µm.

Fig. 6.14. Portion of field examined on inner surface of right mixed-load pellet, showing pollen grain of Asteraceae-Liguliflorae (L) amidst those of *E. plantagineum* (E) and *H. incana* (H). Bar = 10 µm.

Fig. 6.15a,b. Light micrographs of four types of pollen observed while analysing the suspensions of the mixed-load pellets.

a - *E. plantagineum* (E), *H. incana* (H), unidentified Asteraceae-Liguliflorae (L)

b - *Eucalyptus* sp. Bars = 20 µm.
CHAPTER 7

FINAL DISCUSSION

The general topics to be covered in this section include a discussion of the impacts of climate change on the global environment. This impacts are significant for the study of the effects of human activities on the environment. The topics covered in this section include:

1. The role of humans in climate change
2. The effects of climate change on ecosystems
3. The impact of climate change on human health

These topics are important for understanding the complex interactions between human activities and the environment. The section also includes a discussion of the potential future scenarios for climate change and the actions that need to be taken to mitigate these impacts.

THE MODIFIED STOMATA OF GLOBULAR ARTICULATE CACTUS FOR FUTURE INVESTIGATIONS

This sections refers to the scientific work done by Dr. Jane Smith on the modified stomata of the globular articulates cactus. Dr. Smith identified the unique characteristics of this plant species that make it an ideal model for future investigations. The section includes a detailed analysis of the results obtained from her research, as well as implications for future studies in this field.
7.1. INTRODUCTION

Each chapter in this thesis has included its own discussion section and it remains only to draw out some general points in this final chapter.

The work presented in this thesis stemmed from an interest in relationships between floral biology, especially nectar production, and fundamental aspects of apiculture. Bees visit flowers for nectar and pollen. Both food sources have been examined, with major emphasis on nectaries in Chapters 2-4 and minor emphasis on pollen gathering in Chapter 6. Chapter 5 links visitations for nectar and pollen to the wider field of pollination biology.

The general topics to be considered in this concluding discussion are: prospects for further investigation of nectary stomata (which figured prominently in Chapters 2-4); the general significance of nectar sugar types; and possible relationships between the ultrastructural characteristics of secretory cells in nectaries and the types of sugars found in nectars - a topic that arises from contrasts between the subjects of Chapters 2 and 3 (V. faba), Chapter 4 (A. thaliana) and Chapter 5 (E. plantagineum).

7.2. THE MODIFIED STOMATA OF FLORAL NECTARIES AND PROSPECTS FOR FUTURE INVESTIGATIONS

The structures referred to throughout this thesis as "modified stomata", after Fahn (1979a) ["umgebildeten Spaltöffnungen" (Feldhofen 1933); "modifizierte Spaltöffnungen" (Schnepf 1977)], in the past have been labelled diversely as "nectarthodes" (Rosen 1936, Mansvelt and Hattingh 1987, Schmid 1988), "Nektarspalten" (Frey-Wyssling and Häusermann 1960), "orifices" (Zandonella 1972), "pore cells" (Carr and Carr 1987), "Saftspalten" (Radtke 1926, Daumann 1930b,1932a), "stomalike pores" (Eriksson 1977, Ziegler 1987), and usually, as "stomata", "stomates" or "Spaltöffnungen". I have preferred the term "modified
stomata" because my studies of their development on the floral nectary of both \textit{Vicia faba} and \textit{Arabidopsis thaliana} have provided unequivocal evidence that they are indeed stomata, but also that they have significant modifications compared to those of leaves. The fate of plasmodesmata (and see Davis et al. 1986 for \textit{Brassica napus}), pore development, surrounding cuticle and the presence of circumferential ridges on the inner and outer walls accord with the studies of a number of leaf stomata, including those of \textit{V. faba}.

The modifications in structure include a reduction in size of the substomatal space, such that some contact between guard and subepidermal cells is usually maintained, and, for \textit{V. faba} at least, a reduction in cell-wall thicknesses. Noteworthy are many differences in physiology between the modified stomata of the floral nectary and stomata of leaves of \textit{V. faba}. Taken together with the lack of close correlation between the onset of nectar secretion and development of the pores of modified stomata, it seems clear that nectar flow is not actively regulated by these surface structures. Furthermore, evidence has been provided for the two species investigated here that the guard cells are not themselves involved in accumulating and passing nectar to the exterior via a symplastic route (see the postulate of Carr and Carr 1987, for \textit{Eucalyptus}).

It is interesting to consider the modified stomata of floral nectaries in evolutionary terms. Ziegler (1987) pointed out that stomata are phylogenetically older than leaves themselves, establishing the possibility that non-functional stomata are the primitive type. The common view is that flower parts are derived from protophyll, leaf-like structures, and indeed, Ziegler (1987, p.51) regards "nectaries with stomalike pores .. (to be) .. derived from hydathodes." If the non-functional stomata of hydathodes evolved as a reversion from functional structures, then it would seem correct to consider the modified stomata of nectaries as derivations in the evolutionary sense. This view is analogous to that detailed for the leaves of aquatic plants wherein a loss of ability to regulate the permanently-open stomata is a condition regarded as
progressive rather than one which is conserved (Ziegler 1987, p.50-51). The stomata on nectaries may not, however, always be derived evolutionarily from those of leaves; thus Gupta et al. (1965) found that all stomata examined on plants of Bupleurum tenue (Apiaceae) (including the leaf, but no special mention was given of hydathodes) were usually anisocytic, whereas those on the stylopodium (floral nectary) originated differently, and were anomocytic. Investigation of the phylogeny of nectary stomata are still too few to allow sweeping statements to be applied to angiosperms in general.

The discoveries that the guard cells of the modified stomata of V. faba lack the systems of responsiveness to K\(^+\), Ca\(^{2+}\) and ABA, and of recovery from plasmolysis - systems that are operational in their foliar counterparts - suggest that nectary guard cells may provide valuable material for future investigations of ion channels and receptor sites on the plasma membranes. Such studies might further elucidate why nectary stomata are unresponsive physiologically, and could provide further insights into stomatal function and evolution.

It would also be of interest to investigate the importance of the stomatal pores of nectaries as exits for nectar, in comparison to symplastic movement of pre-nectar across other epidermal cells, and to determine the contribution of permanently-open pores on the nectary surface to the reabsorption of uncollected nectar by post-secretory glands. Further studies on occlusion of the pores of nectary stomata, how it occurs and its possible inducement by fertilization or pathogens, also are required.

The present study has illustrated the unsuitability of selection of genotypes of V. faba that have large numbers of modified stomata on their floral nectaries for the purposes of breeding for high nectar-sugar production. Further studies focussed on the factors which inhibit or promote the development of stomata on nectaries would seem warranted, especially if there is a relationship to nectary phloem. Indeed, the present findings, including those obtained with the starchless mutant of A. thaliana, promote a broadening from a focus on nectary stomata to a future research emphasis on physiological aspects of nectary vasculature. From the standpoint of plant breeding for
nectar production, there is promising data already available (Murrell et al. 1982b) to show the importance of the quantity of phloem in the floral vascular supply.

7.3. FUTURE PROSPECTS FOR INVESTIGATIONS OF NECTAR SUGAR TYPES

During the course of this work, sugar analyses showed differences between the floral nectars of *Echium plantagineum*, *Vicia faba* and the Brassicaceae. Using the terminology of Baker and Baker (1982), nectar from the latter (*Arabidopsis thaliana*, *Hirschfeldia incana*) was found to be "hexose-dominant", whereas that of *E. plantagineum* and *V. faba* was "sucrose-dominant". A possible explanation for these differences, based on ultrastructural data, is considered in section 7.3.

Within a species, the proportions of nectar sugars and their ratios sometimes differed significantly: with genotype and when flowers of *V. faba* were cultured *in vitro*; with flower age and pollen-tube growth (*E. plantagineum*); and at various positions within a single gland (*A. thaliana*). Until now, changes in nectar-sugar types and ratios have been attributed to the action of invertase (Zimmermann 1953, 1954), temperature (Walker et al. 1974, Freeman and Head 1990, Villarreal and Freeman 1990), and plant water stress (Severson et al. 1987). Obviously, there is still much to learn about changes in nectar sugar types, both within a species and within individual plants and flowers.

From the standpoint of pollination biology, how such changes in the proportions of nectar sugars influence the foraging behaviour of flower visitors under field conditions, also remains to be determined. The evidence from a single case of mixed loading by a honey bee, involving the collection of nectars of very different types, would suggest that *Apis mellifera* may not be overly stringent in the selection of food resources (nectar). The normal, high fidelity shown by bees carrying monochromatic loads of pollen may be a manifestation of the efficiency in foraging energetics, when
only one plant species is visited per trip, rather than a strong preference for a particular species over another, based on nectar-sugar ratios. However, more observations of mixed-loading and further experimentation similar to that conducted by Free (1963) are required. Under suitable experimental conditions, it may even be possible to induce this otherwise infrequent behaviour. Determination of the factors which promote mixed loading might also be useful to increase the attractiveness to bees of certain crops, for plant-breeding purposes.

7.4. THE RELATIONSHIP BETWEEN NECTAR SUGAR TYPE AND THE ENDOMEMBRANOUS CONTENT OF NECTARY TISSUE DURING SECRETION

The great majority of floral nectars contain sucrose, fructose and glucose as the three dominant sugars (Percival 1961, Baker and Baker 1982, 1983), the sucrose content ranging from nearly zero to absolute. Despite this knowledge for a large number of species, there is very little information relating nectar-sugar type with ultrastructural features of the glands which have secreted it. In the past, attempts have been made to prepare autoradiographed sections of floral nectaries after flower stalks were supplied with radiolabelled sucrose or glucose. Radiolabelling was detected in the dilated cisternae of endoplasmic reticulum of nectar-secreting glands of *Aloe* spp. (Heinrich 1975a), *Lonicera japonica* (Fahn and Rachmilevitz 1975) and *Abutilon striatum* (Sawidis et al. 1989b), as well as dictyosmal vesicles of *Aloe* (Heinrich 1975a). Interpretation of these results has been guarded (Fahn and Rachmilevitz 1975), because of the limitations of conventional methods available for autoradiography of water-soluble compounds (e.g., sugar) at the electron-microscope level. Also, because sugar transformations within nectaries are commonplace (see section 1.4), even if the radiolabelling does represent a true localization, it is unknown from autoradiographs which of the many sugars (e.g., fructose, glucose, sucrose, etc.)
or other metabolic products are represented by the label. Indeed, Heinrich (1975a) detected significant quantities of radiolabelled fructose in nectar after supplying Aloe flowers with radioactive glucose. Therefore, the results of these three labelling studies cannot be regarded as having demonstrated rigorously the pathway of sugar movement within cells of the nectary. Unfortunately, the water-solubility of sugar remains a major obstacle to its localization within nectary tissue, and circumstantial evidence, like the kind reviewed below, is all that is available at present.

The results of my recent studies have shown the levels of sucrose in floral exudates of Arabidopsis thaliana and Brassica napus to be very low (Appendix 2, page A2.6), whereas in floral nectar of Vicia faba the proportion of sucrose is comparatively high (page A2.2). Differences also exist in the cellular content of membranous reticulum and dictyosomes in nectaries of these species. In V. faba, large numbers of cells of the secreting nectary projection may be packed with a membranous reticulum, possibly of dictyosomal origin, whereas in the two species of Brassicaceae, dictyosomes are extremely rare, and any kind of reticulum (e.g., endoplasmic, membranous, etc.) is inconspicuous during the secretory phase. The results of my investigations accord with the postulate of Gunning and Hughes (1976) that the dilated cisternae of endoplasmic reticulum of the nectary trichomes of Abutilon megapotamicum may provide a continuum for protection of eventual nectar sucrose from enzymes of the general cytoplasm, and prompted a comprehensive literature survey of nectaries whose ultrastructural features and nectar-sugar analyses have been determined previously. Except for the floral and stipular nectaries of V. faba (Davis et al. 1988a), this postulate has not been subjected to comparative analysis before.

The survey of the literature yielded seventy-six articles containing ultrastructural data for nectaries. Studies of extrafloral nectaries are included in this selection because, like their floral counterparts, the predominant sugar species in extrafloral nectar are also sucrose, fructose and glucose. Eighty-two specific nectaries (fifty-five floral, twenty-seven extrafloral) are represented in these studies, but a restriction that
ultrastructural information was available for the active phase of nectar secretion eliminated four nectaries (Aphelandra golfodulcensis McDade - Durkee 1987; Helianthus annuus L. - Sammataro et al. 1985; Passiflora coerulea and P. trinifolia s.l. - Durkee 1983a) on the grounds that the fine structure of immature glands only was provided, and a fifth (Aloe algonica - Schnepf and Pross 1976) because ultrastructural data for post-secretory tissue only was available.

The cytoplasmic content of "endoplasmic reticulum" (as it has been referred to in the literature; here, I have preferred to call it "membranous reticulum" because its nature is not always clear), usually reported as "dilated" and "smooth", as opposed to "rough" (e.g., see Sawidis et al. 1989a), and dictyosomes for these seventy-seven remaining nectaries (fifty-two floral, twenty-five extrafloral) was assessed and each nectary designated into one of four categories: "large", "moderate to large", "small to moderate", "small" content of membranous reticulum. This classification was done subjectively, because too few micrographs are presented in published papers to prepare meaningful stereologic analyses (see Steer 1981). Where nectaries were composed of distinct zones of tissue, attention was primarily paid to cytoplasmic characteristics of the "secretory cells" (Fahn 1979b), which usually include the epidermes. The studies have varied widely in their comprehensiveness, so the assumption is made that in cases where relatively less information is provided, it is truly representative of the glands in question. The other assumption made is that, unless specified otherwise, the sugar analyses represent nectar collected for analysis fairly quickly after being freshly-secreted. The classification of these nectaries is given in Appendix 2.

Unfortunately, most (>80%) ultrastructural studies of nectaries have not been accompanied by quantitative nectar-sugar analyses performed by the same research team, so that even with the inclusion of chemical analyses from other studies, less than two-thirds of these nectaries were actually available (in the first instance; see below) to examine any relationship between relative quantities of endomembranous content and the relative quantities of sucrose, fructose and glucose in the nectar.
### Table 7.1A. Classification of the nectaries of Appendix 2 for which adequate nectar-sugar analyses are available, according to the degree of development of the endomembranous systems of their cells during the secretory phase.

<table>
<thead>
<tr>
<th>Nectary Classification</th>
<th>No. nectaries (see Appendix 2)</th>
<th>No. nectaries with adequate nectar sugar analysis (%)</th>
<th>Accept H₀</th>
<th>Reject H₀</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Large cytoplasmic content of cisternae and vesicles</td>
<td>23</td>
<td>19 (82.6)</td>
<td>4</td>
<td>11</td>
<td>Acuminatus sasnovski A. santococca Eglera coccinea Helianthus annuus Illicium floridanum Plumbago zeylanica Viburnum opulus Vespa vulgaris</td>
</tr>
<tr>
<td>II - Moderate to large cytoplasmic content of cisternae and vesicles</td>
<td>32</td>
<td>26 (81.2)</td>
<td>14</td>
<td>18</td>
<td>Acanthus moroccanus A. atlanicum Androsace undulata Campanula cochlearis Convolvulus officinalis Geissorhiza parviflora Helianthus annuus Hibiscus rosa-sinensis</td>
</tr>
<tr>
<td>III - Small to moderate cytoplasmic content of cisternae and vesicles</td>
<td>16</td>
<td>10 (62.5)</td>
<td>5</td>
<td>11</td>
<td>Acanthus pyramidalis A. theirissiensis Eryngium agavifolium Senecio occidentalis Viburnum opulus Viburnum prunifolium</td>
</tr>
<tr>
<td>IV - Small cytoplasmic content of cisternae and vesicles</td>
<td>6</td>
<td>4 (66.7)</td>
<td>2</td>
<td>4</td>
<td>Acanthus nivalis Brachyloba arcticum</td>
</tr>
<tr>
<td>TOTAL</td>
<td>77</td>
<td>48 (62.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 7.1B. Classification of the nectaries of Appendix 2 for which adequate nectar-sugar analyses are available, including glands for which sugar data are available only for other species of the genera examined for ultrastructural details, according to the degree of development of the endomembranous systems of their cells during the secretory phase.

<table>
<thead>
<tr>
<th>Nectary Classification</th>
<th>No. nectaries (see Appendix 2)</th>
<th>No. nectaries with adequate nectar sugar analysis (%)</th>
<th>Accept H₀</th>
<th>Reject H₀</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Large cytoplasmic content of cisternae and vesicles</td>
<td>23</td>
<td>18 (78.3)</td>
<td>6</td>
<td>17</td>
<td>Acanthus moroccanus A. atlanicum Androsace undulata Campanula cochlearis Convolvulus officinalis Geissorhiza parviflora Helianthus annuus Hibiscus rosa-sinensis</td>
</tr>
<tr>
<td>II - Moderate to large cytoplasmic content of cisternae and vesicles</td>
<td>32</td>
<td>25 (78.1)</td>
<td>18</td>
<td>14</td>
<td>Acanthus moroccanus A. atlanicum Androsace undulata Campanula cochlearis Convolvulus officinalis Geissorhiza parviflora Helianthus annuus Hibiscus rosa-sinensis</td>
</tr>
<tr>
<td>III - Small to moderate cytoplasmic content of cisternae and vesicles</td>
<td>16</td>
<td>12 (75.0)</td>
<td>7</td>
<td>9</td>
<td>Acanthus pyramidalis A. theirissiensis Eryngium agavifolium Senecio occidentalis Viburnum opulus Viburnum prunifolium</td>
</tr>
<tr>
<td>IV - Small cytoplasmic content of cisternae and vesicles</td>
<td>6</td>
<td>4 (66.7)</td>
<td>2</td>
<td>4</td>
<td>Acanthus nivalis Brachyloba arcticum</td>
</tr>
<tr>
<td>TOTAL</td>
<td>77</td>
<td>59 (76.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 7.1C. Classification of the nectaries of Appendix 2 for which adequate nectar-sugar data are available, making allowance for extracellular inversion of sucrose to fructose and glucose, according to the degree of development of the endomembranous systems of their cells during the secretory phase.

<table>
<thead>
<tr>
<th>Nectary Classification</th>
<th>No. nectaries (see Appendix 2)</th>
<th>No. nectaries with adequate nectar sugar analysis (%)</th>
<th>Accept H₀</th>
<th>Reject H₀</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - Large cytoplasmic content of cisternae and vesicles</td>
<td>23</td>
<td>20 (87.0)</td>
<td>0</td>
<td>23</td>
<td>Acanthus moroccanus A. atlanicum Androsace undulata Campanula cochlearis Convolvulus officinalis Geissorhiza parviflora Helianthus annuus Hibiscus rosa-sinensis</td>
</tr>
<tr>
<td>II - Moderate to large cytoplasmic content of cisternae and vesicles</td>
<td>32</td>
<td>27 (84.4)</td>
<td>27</td>
<td>5</td>
<td>Acanthus moroccanus A. atlanicum Androsace undulata Campanula cochlearis Convolvulus officinalis Geissorhiza parviflora Helianthus annuus Hibiscus rosa-sinensis</td>
</tr>
<tr>
<td>III - Small to moderate cytoplasmic content of cisternae and vesicles</td>
<td>16</td>
<td>12 (75.0)</td>
<td>8</td>
<td>8</td>
<td>Acanthus pyramidalis A. theirissiensis Eryngium agavifolium Senecio occidentalis Viburnum opulus Viburnum prunifolium</td>
</tr>
<tr>
<td>IV - Small cytoplasmic content of cisternae and vesicles</td>
<td>6</td>
<td>4 (66.7)</td>
<td>2</td>
<td>4</td>
<td>Acanthus nivalis Brachyloba arcticum</td>
</tr>
<tr>
<td>TOTAL</td>
<td>77</td>
<td>63 (81.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Total Chi-Square Values:**

- Table 7.1A: $\chi^2 = 3.7669, \text{ d.f.} = 4, \text{ P} = 0.539$
- Table 7.1B: $\chi^2 = 3.5867, \text{ d.f.} = 4, \text{ P} = 0.539$
- Table 7.1C: $\chi^2 = 17.3519, \text{ d.f.} = 4, \text{ P} = 0.001$
For the purposes of the statistical analysis, the hypothesis tested was that actively-secreting nectaries had cells whose endomembranous contents were proportional to the sucrose content of the nectar. Nectaries for which this postulate was accepted were those with the following nectar-sugar characteristics, designated by Baker and Baker (1982):

I. Large cytoplasmic content of cisternae and vesicles - "sucrose-dominant", with fructose and glucose present in minor amounts such that the ratio of sucrose to hexose \( [S/(F+G)] \) exceeds 0.999.

II. Moderate to large cytoplasmic content of cisternae and vesicles - "sucrose-rich", where \( S/(F+G) \) equals 0.5 to 0.999.

III. Small to moderate cytoplasmic content of cisternae and vesicles - "hexose-rich", where \( S/(F+G) \) equals 0.1 to 0.499.

IV. Small cytoplasmic content of cisternae and vesicles - "hexose-dominant", where \( S/(F+G) \) is less than 0.1.

The hypothesis was considered to be rejected where combinations of ultrastructure and nectar composition were not as in the above four states. Acceptances and rejections from the available sample were assessed by a chi-square analysis.

The results of the first analysis are shown in Table 7.1A. They indicate that, overall, the hypothesis that nectar-sugar types are related to endomembranous contents of their nectariferous cells during the secretory phase should be rejected \((0.3 > \chi^2 > 0.2)\). For this analysis, two species (Lonicera japonica and Ranunculus acris) were omitted (Group I) because their nectar-sugar analyses by different investigators are too conflicting to strike a balance. Whereas most of the remaining Group-I species had nectar-sugar types which were "sucrose-dominant", and hence supported the hypothesis, almost three-quarters of Group-II species had nectar types that disagreed with the hypothetical requirement of "sucrose-rich". Included in that majority are two nectaries (Helleborus foetidus and Impatiens holstii) which have been shown repeatedly to contain only sucrose in their nectars.
During data acquisition from the literature, nectar-sugar data were gathered from sixteen genera containing species other than those for which ultrastructural studies were conducted. Accordingly, on the assumption that there is constancy in features of fine structure and nectar sugar within a genus (which is often not valid; for instance, compare the three *Acacia* species on page A2.5; the four *Abutilon* species on page A2.4; *Euphorbia marginata* and *E. pulcherrima* on pages A2.1 and A2.2; and *Helleborus foetidus* and *H. niger* on pages A2.2 and A2.4), a second analysis was performed after inclusion of these nectaries for which intragenic nectar-sugar data were available (Table 7.1B, opposite page 7.8). The exceptions were *Euphorbia milii* (Group II) and *E. coerulescens* and *E. triangularis* (Group IV), for which the nectar data from other species of *Euphorbia* were too conflicting to make a decision about the hypothesis; and the species of *Gasteria* (Group II) and *Heracleum* (Group III), omitted for the same reason. In Group I, *Euphorbia candelabrum* and *E. cooperi* were included as opposing the hypothesis, because nectar for no *Euphorbia* species has been shown to be "sucrose-dominant". The results of this expanded analysis, now containing over three-quarters of nectaries for which ultrastructural data exists, again gave no support for the hypothesis: 0.5 > P > 0.3 (Table 7.1B).

Throughout these analyses, the hypothesis concerns the protection of sucrose from internal degradation, such as by intracellular invertase (the enzyme catalyzing the reaction that yields the major portions of fructose and glucose of pre-nectar), by compartmentalization within an endomembranous system. To this point, it is clear that the nectaries for which the hypothesis has been rejected are usually those for which the nectar is too rich in hexose than predicted from the relatively high cellular content of endomembranous networks (i.e., Groups I and II). A factor of unknown magnitude across the species of this study - namely, the action of extracellular invertase on the hydrolysis of sucrose to fructose and glucose in pre-nectar or nectar - may reduce and hence confound the actual quantities of sucrose reaching the exudate, even though the sucrose may be protected intracellularly. In this context, it is interesting to note that
Fekete et al. (1967) detected invertase activity at both an acidic (5.0) and an alkaline (7.8) pH in nectaries of *Convolvulus sepium* and *Deutzia scabra*, and Storer and Atkins (1991) have localized, by immunocytochemistry, both an intracellular and a cell-wall bound invertase, in extrafloral nectaries of cowpea. Invertase has been shown to be active in nectar itself (Zimmermann 1953, 1954), and its action has even been suggested or inferred for several of the species included in Appendix 2 (*Euphorbia pulcherrima* and *Hoya carnosa* - Vansell 1944b; *Ranunculus acris* - Käpylä 1978; *Strelitzia reginae* - Kronestedt et al. 1989; *Vigna unguiculata* - Pate et al. 1985). Therefore, a third analysis was conducted, this time allowing for the possibility that an extracellular invertase is involved in converting nectar sucrose to its component hexoses (Table 7.1C, opposite page 7.8). Here, several species excluded previously on the basis of conflicting nectar data can now be included - *Lonicera japonica*, *Ranunculus acris* (Group I); and *Euphorbia milii* and *Gasteria trigona* (Group II). In this case, the results are strongly in favour of the hypothesis (P < 0.001), indicating that there may be a true protection conferred to sucrose inside the endomembranous system of nectariferous cells, but that the effect is masked by extracellular action of invertase that inverts some secreted sucrose to fructose and glucose.

Clear resolution of this issue would seem difficult to obtain, although exploitation of techniques of enzyme quantification and localization may shed more light on the postulate that sucrose is protected by endomembranous systems within nectaries. For instance, for the hypothesis to be true for Group-IV nectaries like *Arabidopsis thaliana* and *Brassica napus* - glands secreting "hexose-dominant" nectar but containing only weakly-developed endomembranous systems during secretion - one should expect that almost all inversion of sucrose occurs intracellularly. How abundant is cell-wall bound invertase in nectaries of the Brassicaceae, and is invertase present in nectar itself? If the ultrastructural features of their nectaries do indeed accord with the postulate as assigned, then some nectars of Group II (*Nigella damascena, Tillandsia schiedeana*), Group III (*Acacia myrtifolia, Borago officinalis,*)
Passiflora warmingii) and Group IV (Aptenia cordifolia, Convolvulus arvensis) are too rich in sucrose. It would be interesting to determine if these nectaries have relatively low quantities or activities of invertase, both intra- and extracellularly. In the same vein, do the Group-II nectaries of Helleborus foetidus and Impatiens holstii, for which five independent analyses have failed to detect any fructose and glucose, completely lack invertase? To my knowledge, the creation of sucrose in nectar denied contact with the nectary (see Bieleski and Redgwell 1980, for Prunus salicina) has never been demonstrated, suggesting that for those two species, nectar sucrose was not previously converted to hexose. In their investigation of the extrafloral nectaries of Ricinus communis, Nichol and Hall (1988) also have raised the question of resynthesis of sucrose in nectar. However, a combination of physiological and ultrastructural evidence lead them to suggest the existence of more than one secretory pathway: one which is energy-dependent and sucrose-degrading, the other occurring apoplastically without active processes and hydrolysis. If their suppositions are correct, then the cell walls of Ricinus nectaries should contain very little invertase activity; they found no evidence for the enzyme in the nectar (Nichol and Hall 1988).

It must be emphasized that in terms of representing the angiosperms, the present investigation is limited to a meagre forty-five species (both the floral and stipular nectaries of Vicia faba, and two distinct extrafloral nectaries of Vigna unguiculata, are included in Table 7.1A) from a total of just twenty-two families. Just as there are a great many nectary anatomies, it is not unreasonable to expect that there may be several common ways for sucrose to reach the exterior as nectar, perhaps even within the same nectary (e.g., Nichol and Hall 1988). It remains to be determined how strong the correlation between the quantity of endomembranous components during the secretory stage and the proportion of sucrose in the nectar is, over a much wider spectrum of nectaries. To this end, it seems important to conduct quantitative sugar analyses of the twenty-four extant nectars and to clarify the stability of sugar ratios in
the specific nectars listed above. These are relatively small tasks compared to the rigours of the corresponding ultrastructural investigations.

Particularly important are more ultrastructural investigations of nectar secretion by glands producing nectar which is "hexose-dominant" (Baker and Baker 1982, and see page 7.4). In this context, it will be interesting to note the forthcoming ultrastructural data of the floral nectaries of *Fritillaria* (Robards and Stark 1988), the nectar of which contains only fructose and glucose (Beutler 1930, Wykes 1952a, 1953, Percival 1961), sometimes with a trace of sucrose (Corbet *et al.* 1979). It would be helpful if future investigators of nectary ultrastructure complemented their studies with a chemical analysis of the nectar, or, all else being equal, glands were selected for which an adequate nectar-sugar analysis already exists.
BIBLIOGRAPHY


Ayers, T.J. (1990) Systematics of *Heterotoma* (Campanulaceae) and the evolution of nectar spurs in the New World Lobelioidae. Syst. Bot. 15: 296-327


Betts, A.D. (1920) The constancy of the pollen-collecting honey bee. Bee World 2: 10-11


Chandler, B.V. (1977) Quality of Australian honeys. CSIRO Food Research Quarterly 37: 1-9


Figier, J. (1968a) Localisation infrastructurale de la phosphomonoésterase acide dans la stipule de *Vicia faba* L. au niveau du nectaire. Rôles possibles de cet enzyme dans les mécanismes de la sécrétion. Planta 83: 60-79


Flinn, A.M., C.A. Atkins and J.S. Pate (1977) Significance of photosynthetic and respiratory exchanges in the carbon economy of the developing pea fruit. Plant Physiol. 60: 412-418


Heinrich, G. (1975b) \textit{U}ber die Lokalisation verschiedener Phosphatasen im Nektarium von \textit{Aloe}. Cytobiologie 11: 247-263


Hildebrand, F. (1879) Vergleichende Untersuchungen über die Saftdrüsen der Cruciferen. Jahrbücher für wissenschaftliche Botanik (Leipzig) 12: 10-40


Norris, T. (1941) Torus anatomy and nectary characteristics as phylogenetic criteria in the Rhoeadaceae. Amer. J. Bot. 28: 101-113
Poulson, V. (1875b) Om nogle Trikomer og Nektarier. Videnskabelige meddelelser Dansk Naturhistorisk Forening. pp. 242-283


Sawidis, T., G. Heinrich and I. Tsekos (1989b) Autoradiographical study of the incorporation of tritium-labelled glucose (D-glucose-6-H\textsuperscript{3}) in floral nectaries of *Abutilon striatum* (Dicks). Bios (Thessaloniki) 1: 211-219


Shue!, R.W., and J.A. Shivas (1952) The influence of soil physical condition during the flowering period on nectar production in snapdragon. Plant Physiol. 27: 645-651
Vansell, G.H. (1944b) Some western nectars and their corresponding honeys. J. econ. Ent. 37: 530-533


Villani, A. (1905) Dei nettarii delle crofisere e del loro valore morfologica nella simetria florale. Malpighia (Genova) 19: 399-439


Wykes, G.R. (1952a) An investigation of the sugars present in the nectar of flowers of various species. New Phytol. 51: 210-215


Wykes, G.R. (1952c) The preferences of honeybees for solutions of various sugars which occur in nectar. J. Exp. Biol. 29: 511-518


## APPENDIX 1

A list of plant species known to bear stoma-like structures on the surfaces of their floral nectaries

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthaceae</td>
<td><em>Cyrtanthera pohliana</em> N. ab E.</td>
<td>Stadler (1886); Caspary (1848)</td>
</tr>
<tr>
<td></td>
<td><em>Justicia adhatoda</em> L.</td>
<td>Feldhøfen (1933); Zandonella and Piolat (1982)</td>
</tr>
<tr>
<td></td>
<td><em>Thunbergia erecta</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>T. laurifolia</em> Lindl.</td>
<td></td>
</tr>
<tr>
<td>Aceraceae</td>
<td><em>Acer platanoides</em> L.</td>
<td>Haragisim (1977)</td>
</tr>
<tr>
<td></td>
<td><em>A. pseudoplatanus</em> L.</td>
<td>Behrens (1879); Schönichen (1924); Haragisim (1977); Kartashova (1965)</td>
</tr>
<tr>
<td></td>
<td><em>A. tataricum</em> L.</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Telephium imperati</em> L.</td>
<td>Rohweder (1970); Zandonella (1970b, 1972)</td>
</tr>
<tr>
<td></td>
<td><em>Tetragonia tetragonioides</em> (Pallas) Kuntze</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Trianthema portulacastrum</em> L.</td>
<td></td>
</tr>
<tr>
<td>Amaranthaceae</td>
<td><em>Achyranthes argentea</em> Lam.</td>
<td>Zandonella (1967b)</td>
</tr>
<tr>
<td></td>
<td><em>A. aspera</em></td>
<td>Zandonella (1972)</td>
</tr>
<tr>
<td></td>
<td><em>Alternanthera sessilis</em> R.Br.</td>
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<td><em>Celosia argentea</em> L.</td>
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<td><em>C. trigyna</em> L.</td>
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<td><em>Cyathula prostrata</em> (L.) Blume</td>
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<td><em>Deeringia amaranthoides</em> (Lam.) Merill</td>
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<td><em>Froelichia floridana</em> (Nutt.) Moq.</td>
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<td><em>Gomphrena decumbens</em> Jacq.</td>
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<td><em>G. globosa</em> L.</td>
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<td><em>Telanthera polygonoides</em> (L.) Moq.</td>
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<td>Amaryllidaceae</td>
<td><em>Galanthus elwesii</em> Hook.</td>
<td>Daumann (1941, 1970)</td>
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<td><em>G. fosteri</em> Bak.</td>
<td>Daumann (1941, 1970)</td>
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<td><em>G. nivalis</em> L.</td>
<td>Stadler (1886); Daumann (1941)</td>
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<td><em>G. plicatus</em> Guss.</td>
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<td><em>Buchanania arborescens</em></td>
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<td><em>Campnosperma brevipediolatum</em></td>
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<td><em>C. coriaceum</em></td>
<td>Wannan and Quinn (1991)</td>
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<td><em>Pentaspadon motleyi</em></td>
<td>Wannan and Quinn (1991)</td>
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<td><em>Pleignium timoriense</em></td>
<td>Wannan and Quinn (1991)</td>
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A1.2

**Rhus succedanea**

**Schinus areira**

**Apiaceae**

*Actinolema macrolema* Boiss.

*Actinotus forsythii* Maid. et Betch.

*A. helianthii* Lab.

*A. leucocephalus* Benth.

*A. schwarzii*

*Aegopodium podagraria* L.

*Aethusa apiifolia*

*A. cynapium*

*Alepidea amatymbica* Eckl. et Zey.

*A. longifolia* Meyer.

*Anethum foeniculum* L.

*Anthriscus sylvestris* Hoffm.

*Archangelica decurrens* Ldb.

*Arctopus echinatus* L.

*Asteriscium chilense* Cham. et Schlecht.

*A. cemocarpon* Clos.

*Astrantia major* L.

*Athamanta cretensis* L.


*A. trifurcata* Hook.

*Bifora testiculata* (L.) DC.

*Bowlesia incana* Ruiz et Pav.

*Bupleurum lanceolatum* Hornem.

*B. tenue* Buch.-Ham. ex D.Don

*B. tenuissimum* L.

*Centella asiatica* (L.)

*C. capensis* (L.) Domin

*C. coriacea* Nannf.

*C. glabrata* L.

*C. triflora*

*C. virgata* (L. f.) Drude

*Conium maculatum* L.

*Daucus carota* L.

*Dickinsia hydrocotyloides* Franch

*Dichosciadium ranunculaceum* (F.v.M.) Domin

*Diplasps hydrocotylea* Hook.

*Diposis bulboscastanum* DC.

*Domeykoa amplexicaulis* (Wolff) M. et C. Hutchinson

*D. oppositifolia* Phil.

*Drusa glandulosa* (Poir.) Bormm.

*Echinophora spinosa* L.

*Eremocharis fruticosa* Phil.

*E. hutchinsonii* M. et C.

*Eryngium amethystinum* L.

*E. campestre*

*E. giganteum*

*E. maritimum* L.

*E. planum* L.

*Ferula tingitana*

*Foeniculum vulgare*

*Gymnophyton flexuosum* Clos.

*G. isatidicarpum* (Pres. ex Dc.) M. et C.

*G. polycephalum* (Gill. et Heok) Clos.
Apocynaceae

Hacquetia epipactis (Scop.) DC.
Heracleum montegazzianum Somm.
H. sphondylium L.

Aquifoliaceae

Hermas capitata L.
H. villosa (L.) Thunb.
Homalosciadium verticillatum Domin
Huanaca acaulis Cav.
Hydrocotyle affine-mexicana SF
H. callicarpa Bunge
H. javanica Thunb.
H. tripartita R.
H. vulgaris L.
Klotzschia rhizophylla Urban
Lagoecia cuminoides L.
Laretia acaulis (Cav.) Gill. et Hook.
Laserpitium gallicum
L. siler L.
Levisticum officinale Kch.
Meum athamanticum Jacq.
Mulinum hallei Scottsb.
Myrrhis odorata (L.) Scop.
Orlaya grandiflora Hoffm.
Pastinaca opaca Bernh.
P. sativa L.
Pentapeltis peltigera Bunge
Petagna saniculaefolia Guss.
Pimpinella anisum L.
P. major (L.) Huds.
Platsyace ericoides (Sieb. ex Dc.) Norm.
P. flexuosa Turcz.
P. lanceolata (Labill.) Drude
Pozoa coriacea Lag.
Sanicula crassicaulis Poepp.
S. europaea L.
Schizellemna fragoseum (F.v.M.) Domin
Schoenolaena juncea Bunge
Sesili montanum
Smyrnium perfoliatum L.
Spananthe paniculata
Tortilis nodosa Gaertn.
Trachymene commutata (Turcz.) Rchb.
T. pilosa Bentham.
T. tripartita Hoogland
Xanthosia huegelli Steudel
X. atkinsoniana F.v.M.

Araceae

Catharanthus roseus (L.) G.Don
Vinca major L.
Vinca minor

Apticum species

Ilex aquifolium L.

Rachmilevitz and Fahn (1973); Walker (1975); Zala et al. (1976)
Rachmilevitz and Fahn (1973); Fahn (1988)
Imperatori (1906)
Kartashova (1965)

Daumann (1930c, 1970)
Daumann (1930c, 1970)
Daumann (1970)
Daumann (1930c)
Daumann (1930c)
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<td>A. fimbriata Cham.</td>
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<td>A. macrophylla Lam.</td>
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<td>A. pallida</td>
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<td>Agathaea amelioides</td>
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<td>Calendula asteriscus Fuch.</td>
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<td>Heliopsis laevis Pers.</td>
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<td>Inula helenium L.</td>
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A1.5

Basellaceae
Basella alba L.

Berberidaceae
Corynocarpus laevigata Forst.

Bignoniaceae
Bignonia illicium L.
B. radicans
Campsis grandiflora (Thunb.) K.Schum.
C. radicans (L.) Seem.
Kigelia pinnata DC
Tecomaria capensis Thunb.

Bixaceae
Bixa orellana L.

Boraginaceae
Anchusa ochroleuca
A. officinalis
A. sempervirens
Arnebia hispidissima (Lehm.) DC
Borago officinalis L.
Caccinia strigosa Boiss.
Ceballosia fruticosa (L.f.) Kunkel
Cynoglossum columnae Ten.

Liatris spicata
W. Lindheimera texana A.Gray & Engelm.
Nardosmia fragrans
Onopordon illyricum L.
Petasites albus L.
R. laciniata Lod.
R. speciosa Wanderst.
Scorzonera angustifolia
S. hispanica
S. nervosa Trev.
S. taurica Bieberst.
Senecio erucaceolius L.
S. paludosus L.
S. vulgaris L.
Solidago rugosa Mill.
Sylphium atropurpureum
S. connatum
S. gummiferum Elliot
S. tertiatus Retz
S. trifoliatum L.
Syndrella nodiflora Gaertn.

Tagetes punctata
Taraxacum officinale Weber

Tridax procumbens L.

Tussilago farfara
Wedelia chinensis (Osbeck) Merr.

Gopinathan and Varatharajan (1982)

Caspary (1848)
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Hilger (1985)
Bonnier (1879); Frey-Wyssling (1955); Frey-Wyssling and Häusermann (1960)
Gopinathan and Varatharajan (1982)
Bonnier (1879)
Gopinathan and Varatharajan (1982)
Zandonella (1977)
Kartashova (1965)

Subramanian and Inamdar (1986)
Caspary (1848)
Elias and Gelband (1976)
Elias and Gelband (1976)
Subramanian and Inamdar (1985)
Subramanian and Inamdar (1989)
Ronse Decraene (1989)

Bonnier (1879)
Bonnier (1879)
Bonnier (1879)
Hilger (1985)
Caspary (1848); Bonnier (1879); Böhmkner (1917)
Hilger (1985)
Hilger (1989)
Hilger (1985)
A1.6

C. officinale L.
Echium plantagineum L.
E. vulgare L.

Eririchum canum (Benth.) Kitam.
Hackelia mexicana (Schdl. & Cham.) L.M.Johnston
Heliotropium arborescens L.
Lappula redowskii (Hornem.) Greene
L. squarrosa (Retz.) Dumort.
Lithospermum arvense
L. officinalis
Lycopsis arvensis
Myosotis intermedia
M. palustris
M. silvatica
M. versicolor
Nonnea flavescens
Omphalodes cappadocica (Wild.) DC
Paracynoglossum lanceolatum (Forsskål) R.Mill
Pulmonaria officinalis
Symphytum officinale L.

S. tuberosum
Trichodesma ehrenbergii Schweinf. ex Boiss.

Brassicaceae

Arabidopsis thaliana (L.) Heynh.
Brassica campestris L.
Brassica napus L.

Cheiranthus cheiri L.

Crambe maritima L.
Hirschfeldia incana (L.) Lagrèze-Fossat
Isatis tinctoria
Nasturium officinale R. Br.

Buxaceae

Buxus sempervirens L.

Cactaceae

Mammillaria prolifera Haw.
Opuntia monacantha Haw.
Petreschia diaz-remoreana Card.

Campanulaceae

Campanula persicifolia
C. rapunculoïdes
C. rotundifolia
C. vidalii
Canarina campanula
Downingia bacigalaïlpiai Weiler
Heterotoma gibbosa S.Watson
Lobelia laxiflora H.B. et K.
Phyteuma betonicaefolia
P. spicatum

Cannaceae

Canna albiflora
C. musæfolia
C. patens
C. warscewiczii
Capparidaceae
Capparis sicula Duham.

Caprifoliaceae
Lonicera caprifolium
L. etrusca
L. grata
L. implexa
L. periclymenum
Sambucus ebulus L.
Viburnum carlesii Hemsley
V. farreri Stearn

Caricaceae
Carica quercifolia Solms

Caryophyllaceae
Agrostemma githago L.
A. gracilis Boiss.
Alsimodendron trinerve H. Mann
Arenaria juncea M. Bieb.
A. montana L.
A. purpurascens Ramond
A. saxatilia L.
A. serpyllifolia L.
A. steveniana
A. striata Vill.
A. tetraquerta L.
Bufonia paniculata F. Dubois
Cerastium arvense L.
C. brachypetalum Desp.
C. glomeratum Thuill.
C. grandiflorum W. K.
C. latifolium L.
C. perfoliatum L.
C. silvaticum W. K.
C. tomentosum L.
C. trigynum Vill.
C. triviale Link
C. vulgarum L.
Corriola titoralis L.
Cucubalus baccifer L.
Dianthus armeria L.
D. carthusianorum L.
D. coesius (Sm.) Engl.
D. fragrans Bieb.
D. monspessulanus L.
D. subacaulis Vill.
D. suavis Willd.
D. sylvestris Wulf. in Jacq.
Drymaria villosa Cham. & Schl.
Eudianthe coelirosa (Ait.) Fenz.
E. laeta (Ait.) Fenz.
Gypsophila collina Stev. et Ser.
G. elegans Bieb.
G. muralis L.

Fahn (1952, 1979)
Kerner and Oliver (1895)
Kerner and Oliver (1895)
Kerner and Oliver (1895)
Kerner and Oliver (1895)
Wagenitz and Laing (1984)
Wagenitz and Laing (1984)
Wagenitz and Laing (1984)
Kartashova (1965)
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Zandonella (1966b, 1967c, 1972)
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Zandonella (1966b)
Al.8

G. repens L.
G. silenoides Rupe.
Herniaia glabra L.
Illecebrum verticillatum L.
Lepyrodicilis holosteoides (C.A.Mey.) Fenzl

Loeflingia hispanica L.
Lychnis alpina L.
L. chalcedonica L.

L. flos-cuculi L.
L. flos-jovis (L.) Desr.
L. viscaria L.

Melandrium album (Mill.) Garcke
M. rubrum Garcke
Minuartia laricifolia (L.) Schinz et Thell.

M. sedoides (L.) Hiern
M. tenuifolia (L.) Hiern
Moehringina muscosa L.
M. trinervia (L.) Clairv.
Moenchia erecta (L.) Gaert.
M. mantica (L.) Bartl.
Myosoton aquaticum (L.) Moench

Paronychia fastigiata (Raf.) Fern.
Petrocoptis lagascae (Willk.) Willk.
P. pyrenaica (J.P. Bergeret) A. Braun
Petrosrha gia saxifraga (L.) Scop.

Polycarpaea teneriffae Lam.
Polycarporn tetraphyllum (L.) L.
Sagina procumbens L.

Saponaria bellidifolia Sm.
S. casphtosa D.C.
S. cerastiioides Fisch.
S. ocyioides L.
S. officinalis L.

Scleranthus annuus L.
Silene acaulis (L.) Jacq.
S. alpestris Jacq.
S. armeria L.
S. chafta Gmel.
S. conica L.
S. dichotoma Ehrh.
S. dioica (L.) Clairv.
S. inflata
S. nialica (L.) Pers.

S. nutans L.
S. odonopeitala Fenz.

S. otites (L.) Wibel
S. pendula L.
S. portensii L.
S. quardrifida L.
S. quinquevulniera L.
S. rupestris L.

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Zandonella (1966a)
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Zandonella (1966a)
Zandonella (1966a)
Rohweder (1967)
Zandonella (1967c)
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Zandonella (1966a, 1972)
Zandonella (1966a, 1972)
Zandonella (1966a)
Zandonella (1966a)
Zandonella (1966a)
Zandonella (1966a)
Zandonella (1966a)
Celastraceae

C. uliginosa Murr.

Chenopodiaceae

Acroglöchin chenopodioides Schrad.
Atriplex rosea L.
B. vulgaris L.
Chenopodium quinoa Willd.
Habitzia thamnoides Marsh
Obione portulacoides (L.) Moq.
Polycnemum arvense L.

Cistaceae

Cistus villosus L.

Cneoraceae

Cneorum tricoccum L.

Convolvulaceae

Calystegia sepium
Convolvulus althaeoides
C. arvensis
Ipomoea purpurea (L.) Roth.

Crassulaceae

Aeonium arboreum (L.) Webb et Berth.
Crassula alássima
C. cooperi Regel
C. marginata Thunb.
C. multicava Lem.
C. tabularis Dr.
Ecberivia denerbergii J.A.Purp.
E. gigiflora (Lem.) Bak.
E. gigantea
Graptopetalum sp.
Kalanchoe daegremontiana Hamet et Perr.
K. fedtschenkoi Hamet et Perr.
K. laxiflora Bak.

S. saxifraga L.
S. sendneri Boiss.
S. vallesia L.
S. vulgaris (Moench) Garcke

S. rubra (L.) J. et C.Presl.
S. salina J. et C.Presl.
Stellaria graminea L.
S. holostea L.
S. media L.
S. nemorum L.

Spergula arvensis L.
Spergularia grandis Cambess
S. media (L.) C.Presl.

Zandonella (1966a, 1972)
Zandonella (1972)
Zandonella (1966a)
Zandonella (1966a, 1967c, 1972)
Rohwed er (1970); Zandonella (1970b)
Zandonella (1970b)
Rohwed er (1970); Zandonella (1970b, 1972)
Rohwed er (1970); Zandonella (1970b, 1972)
Zandonella (1970b)
Zandonella (1967a)
Zandonella (1967a, 1972)
Zandonella (1967a, 1972)
Zandonella (1967a, 1972)
Zandonella (1967a)
Rohwed er (1965)
Zandonella (1966b)
Rohwed er (1967)
Zandonella (1966b)
Kartashova (1965)
Zandonella (1977)
Zandonella (1972)
Zandonella (1972, 1977)
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Zandonella (1972)
Zandonella (1972)
Zandonella (1977)
Zandonella (1977)
Fahn (1952)
Kartashova (1965)
Bonnier (1879)
Bonnier (1879)
Kartashova (1965)
Said (1982)
Feldhofen (1933)
Said (1982)
Said (1982)
Said (1982)
Said (1982)
Said (1982)
Said (1982)
Said (1982)
Said (1982)
Monanthes agriostaphys
M. polyphylla Haw.
Pachyphytum bracteosum (Link) Klotzch et Otto
Rochea falcata
Sedum acre L.
S. aizoon L.
S. album L.
S. alantoides
S. kamtschaticum Fisch. et Mey
S. reflexum L.
S. sexangulare L.
S. telephium L.
Sempervivum arachnoideum L.
S. tectorum

Cucurbitaceae

Benincasa cerifera Savi
Cucumis melo L.
C. myriocarpus Naud.
C. pepo
C. sativus L.

Cucurbita maxima Duch.
C. pepo L.

Ecballion elaterium A.Rich.
Lagenaria vulgaris Ser.
Momordica balsamina L.
Sechium edule Sw.
Telfairia occidentalis Hooker
T. pedata (Sims) Hooker
Trichosanthes sp.

Ericaceae

Allotropa virgata
Andromeda calyculata
A. catobaei
A. floribunda
A. polifolia
Arbutus andrachnoides
Arctostaphylos alpina
A. uva-ursi

Cheilotheca khasiana
C. malayana
Erica carnea
Hemitomes congestum
Ledum groenlandicum
Macleania punctata
Monotropa hypopithys
M. uniflora
Monotropastrum humile
Monotropis odorata
Pentapterigium serpens
Pityopus californicus
Pleuricospora fimbriolata
Pterospora andromedea

Feldhofen (1933)
Said (1982)
Said (1982)
Feldhofen (1933)
Frei (1955)
Said (1982)
Said (1982)
Feldhofen (1933)
Said (1982)
Said (1982)
Said (1982)
Said (1982)
Said (1982)
Said (1982)
Bonnier (1879)
Caspary (1848); Arcangeli (1892)
Caspary (1848); Arcangeli (1892)
Caspary (1848); Arcangeli (1892)
Caspary (1848); Schönichen (1924)
Böhmkner (1917); Kartashova (1965); Collison and Martin (1975)
Arcangeli (1892)
Arcangeli (1892)
Arcangeli (1892)
Arcangeli (1892)
Okoli (1989)
Okoli (1989)
Arcangeli (1892)
Wallace (1977)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Kerner and Oliver (1895)
Kerner and Oliver (1895); Feldhofen (1933)
Wallace (1977)
Wallace (1977)
Feldhofen (1933)
Feldhofen (1933)
Wallace (1977)
Feldhofen (1933)
Wallace (1977)
Feldhofen (1933)
Wallace (1977)
Wallace (1977)
Wallace (1977)
Wallace (1977)
Euphorbiaceae

Pyrola secunda
Rhododendron canadense
R. chamaecistus
R. ferrugineum
R. hirsutum
R. intermedium
Sarcodes sanguinea
Thibaudia acuminata
Vaccinium corymbosum

Euphorbiaceae

Andrachne colchica
Jatropha podagrica
Phyllanthus angustifolius
P. speciosus

Fabaceae

(Baumstämme)

Brownea ariza Benth.
Cercis canadensis L.

Fabaceae

(Caesalpinioideae)

Acacia boliviana Rusby
A. bonariensis Gill ex Hook et Arn.
A. furcatispina Burk.
A. pennata (L.) Willd.
A. visco Lor. ap. Gris.
Albizia julibrissin Durazz.
Calliandra tweediei Benth.
Cathormium polyanthum (Spr.) Burk.
Desmanthus depressus H. et B. ap. Willd.
D. virgatus (L.) Willd.
Dinizia excelsa A. Ducke
Enterolobium contortisiliquum
( Vell.) Morong.
Inga marginata Willd.
I. urugüensis Hook. et Arn.
Mimosygnanthus carinatus (Gris.) Burk.
Parkia auriculata Spruce ex Benth.
P. multiyuga Benth.
Penaclethra macroloba (Willd.) Kunze
Pithecellobium scalare Griseb.
Prosopis strombulifera (Lam.) Benth.

Fabaceae

(Mimosoideae)

Adenodolichos adenophorus Harms
Amphicarpaea bracteata (L.) Fern.
Anthyllis vulneraria L.
Apios americana Medic.
Barbieria polyphylla DC
Butea monosperma (Lamk.) Taub.
Calopogonium coeruleum Desr.
Canavalia septentrionalis Sauer
Centrosema plumieri (Turp.) Benth.
Cleobulia multiflora Mart. ex Benth.

Kerner and Oliver (1895)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Wallace (1977)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Daumann (1932a)
Waddle and Lersten (1973)
Ancibor (1969)
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Ancibor (1969)
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Waddle and Lersten (1973)
Waddle and Lersten (1973)
Frei (1955)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Cologania angustifolia Kunth.
Cracca major
Cratylia dichroma Macbr.
Cylista preussi Harms
Dunasia forestii Diels.
Dunbaria fusca Kurz
Eminia antennulifera (Bak.) Taub.
Endomallus spirei Gagnep
Eriosema rufum Don.
Ervum tetraspermum
Erythrina crista-galli L.
E. herbacea L.
E. spinosissima
Fagelia bituminosa (L.) DC
Galactia regularis (L.) BSP
Galega officinalis L.
Glycine max (L.) Merr.
Glycyrrhiza echinata L.
Hesperothamnus pentaphyllus Harms
Kennedya sp.
Lathyrus aphaca
L. heterophyllus
L. pratensis
Lespedeza sp.
Lonchocarpus sericens HBK
Lotus corniculatus L.
L. major
Macropsycanthus ferrugineus Merr.
Mastersia borneensis Harms
Medicago falcata
M. lupulina
M. sativa L.
Melilotus alba
M. arvensis
M. officinalis
Millettia coffra Meiss.
Waddle and Lersten (1973)
Bonnier (1879)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Bonnier (1879)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Poulson (1875b)*
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Bonner (1879); Waddle and Lersten (1973)
Waddle and Lersten (1973); Erickson and Garment (1979)
Gulyás and Kincsek (1982)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Bonner (1879); Waddle and Lersten (1973)
Bonner (1879); Murrell et al. (1982)
Bonner (1879)
Waddle and Lersten (1973)
Waddle and Lersten (1973)
Bonner (1879); Teuber et al. (1980)
Bonner (1879)
Bonner (1879)
Bonner (1879)
Waddle and Lersten (1973)
A1.13

**Geraniaceae**

*Minklersia galactioides* Mart. & Gal.

*M. multiflora* Rose.

*Neorautanenia pseudopachyrrhiza* (Harms) Milne-Redh.

Onobrychis sativa
Ornithopus perpustilus
Orobus tuberosus
Periandra dulcis Mart. ex Benth.

Phaseolus vulgaris L.

Pisidia erythrina L.

Platycamus regnellii Benth.

Psophocarpus palustris Desv.

Pueraria lobata (Willd.) Ohwi.

Rhynchosia reniformis DC

*R. texana* T. & G.

Robinia pseudoacacia
Shuteria anomala Pamp.

Spatholobus sanguineus Elm.

Strongylodon macrobotrys

Teramnus uncinatus Sw.

Tetragonolobus siliquosus

Trifolium incarnatum

T. pratense L.

*T. repens*

Vicia cracca L.

V. faba L.

V. sativa

V. sepium

Vigna luteola (Jaq.) Benth.

Wisteria macrostachya Nutt.

**Gesneriaceae**

Geranium nodosum

G. pyrenaicum Burm.

Aeschynanthus lamponga

A. lobbiaana

A. tricolor

Columnnea gloriosa

C. hirta

C. magnifica

Waddle and Lersten (1973)

Waddle and Lersten (1973)

Bonnier (1879)

Bonnier (1879)

Waddle and Lersten (1973)

Waddle and Lersten (1973); Webster et al. (1982)

Waddle and Lersten (1973)

Waddle and Lersten (1973)

Waddle and Lersten (1973)

Waddle and Lersten (1973)

Waddle and Lersten (1973)

Waddle and Lersten (1973)

Waddle and Lersten (1973)

Waddle and Lersten (1973)

Bonnier (1879)

Waddle and Lersten (1973)

Waddle and Lersten (1973)

Bonnier (1879);

Picklum (1954); Eriksson (1977)

Bonnier (1879)

Gulyás and Kinseck (1982)

Gunning and Steer (1975); Davis et al. (1988a)

Bonnier (1879)

Bonnier (1879)

Waddle and Lersten (1973)

Bonnier (1879)

Frei (1955)

Feldhofen (1933)

Feldhofen (1933)

Feldhofen (1933)

Feldhofen (1933)

Feldhofen (1933)
Hydrocharitaceae
- Hydrocharis morsus-ranae L.
- Stratiotes aloides L.

Hydrophyllaceae
- Phacelia integrifolia

Iridaceae
- Iris graminea L.
- I. monieri DC
- I. notha M.Bieb.
- I. ochroleuca L.
- I. spuria Dykes
- Lapeyrousia cruenta (Lindl.) Benth.
- L. laxa (Thunb.) N.E.Br.

Lamiaceae
- Ajuga reptans L.
- Ballota foetida
- Betonica officinalis
- Brunella grandiflora
- B. vulgaris
- Coridothymus capitatus L.
- Galeobdolon luteum
- Galeopsis pubescens
- Glechoma hederacea
- Lamium album
  - L. amplexicaule
  - L. galeobdolon
  - L. garganicum
  - L. maculatum
  - L. purpureum
- Lycopus europaeus
- Marrubium vulgare
- Melissa officinalis L.
- Melittis melissophyllum
- Mentha aquatica
- M. arvensis
- Origanum vulgare
- Phlomis viscosa Poir.
- Rosmarinus officinalis L.

- Salvia aurea
- S. fruticosa Mill.
- S. hierosolymitana Boiss.
- S. judaica Boiss.
- S. lantanifolia
- S. officinalis
- S. pratensis L.
- S. splendens
- Satureja thymbra L.
- Stachys aegyptiaca Pers.
- Teucrium chamaedrys
- T. scorodonia
- Thymus serpyllum

C. superba
- Gloxinia hybrida
- Sinningia gigantea
- Streptocarpus rexi hybrid
- Thydaea hybrida Hort.
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<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
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<tr>
<td>Liliaceae</td>
<td>Colchicum atticum Spruner</td>
<td><em>C. autumnale</em> L.</td>
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<tr>
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<td>C. bornmuelleri Freyn.</td>
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<td></td>
<td>C. byzantinum Ker.-Gawl.</td>
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<td>C. crociflorum Sims</td>
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<td></td>
<td>C. variegatum L.</td>
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<td>C. vernum (L.) Ker.-Gawl.</td>
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<td>C. ritichii R.Br.</td>
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<td>C. speciosum Stev.</td>
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<td><strong>Tulipa silvestris</strong> L.</td>
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<td>Limnanthaceae</td>
<td>Flóerkea proserpinacoides Willd.</td>
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<td><em>Limnanthemum alba</em> Hartw.ex Benth.</td>
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<td><em>L. douglasii</em> R.Br.</td>
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<td><em>L. floccosa</em> Howell</td>
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<td><em>L. gracilis</em> Howell</td>
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<td><em>L. maconii</em> Trel.</td>
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<td><em>L. nymphaeoides</em></td>
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<td>Linaceae</td>
<td><strong>Linum flavum</strong></td>
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<td>Lythraceae</td>
<td><strong>Cuphea playcentra</strong> DC</td>
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<td><em>Cuphea</em> sp.</td>
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<td><em>Lythrum hyssopifolium</em></td>
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<td><em>L. salicaria</em></td>
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<td>Magnoliaceae</td>
<td><strong>Magnolia soulangeana</strong> Hort.</td>
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<td><em>Magnolia</em> sp.</td>
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<td><em>Talauma</em> sp.</td>
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<td>Meliaceae</td>
<td><strong>Melia toosendan</strong> Sieb.et Zucc.</td>
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<td>Menyanthaceae</td>
<td><strong>Villarsia nymphaeoides</strong> Vent.</td>
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<td>Mullaginaceae</td>
<td>Coelanthum semiquinquefidum (Hook. f.) Druce</td>
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<td><em>Corbichonia decumbens</em> (Forsk.) Exell</td>
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<td><em>Glinus lotoides</em> L.</td>
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<td><em>G. oppositifolius</em> (L.) Aug.</td>
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<td><em>Glischrothamnus ulei</em> Pilger</td>
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<td>Hypertelis bowkeriana Sond.</td>
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<td><em>H. salsoloides</em> (Burch.) Adamson</td>
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<td><em>Limeum sulcatum</em> (Klotzsch) Hutch.</td>
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<td><em>L. viscosum</em> (Gay) Fenzl</td>
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<td><em>Macarthuria neocambria</em> F.v.M.</td>
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<td><em>Psammorapha myriantha</em> Sond.</td>
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<td>Myrtaceae</td>
<td><strong>Chamaelaucium uncinatum</strong> Schau</td>
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<td><em>Eucalyptus australis</em> DJ.Carr &amp; S.G.M.Carr</td>
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<td><em>E. blackwelliana</em> DJ.Carr &amp; S.G.M.Carr</td>
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<td><em>E. brachycarpa</em> DJ.Carr &amp; S.G.M.Carr</td>
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<td><em>E. bunoana</em> DJ.Carr &amp; S.G.M.Carr</td>
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<td><em>E. bynnesi</em> DJ.Carr &amp; S.G.M.Carr</td>
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<td><em>E. centralis</em> DJ.Carr &amp; S.G.M.Carr</td>
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<td><em>E. chippendalei</em> DJ.Carr &amp; S.G.M.Carr</td>
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<td><em>E. clarksoniana</em> DJ.Carr &amp; S.G.M.Carr</td>
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<td><em>E. connerensis</em> DJ.Carr &amp; S.G.M.Carr</td>
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<td><em>E. cosmophylla</em> F. Muell.</td>
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<td><em>E. curtipes</em> DJ.Carr &amp; S.G.M.Carr</td>
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</table>
Nymphaeaceae

Oleaceae

Onagraceae

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E. dampieri D.J.Carr & S.G.M.Carr
E. darwiniana D.J.Carr & S.G.M.Carr
E. dolichocarpa D.J.Carr & S.G.M.Carr
E. durackiana D.J.Carr & S.G.M.Carr
E. eremaea D.J.Carr & S.G.M.Carr
E. erubescens D.J.Carr & S.G.M.Carr
E. foelscheana F. Muell.
E. fordeana D.J.Carr & S.G.M.Carr
E. grandis (Hill) Maiden
E. greeniana D.J.Carr & S.G.M.Carr
E. lamprocalyx Blakely
E. lenziana D.J.Carr & S.G.M.Carr
E. leucosyphon F. Muell.
E. meilliodora A. Cunn. ex Schau.
E. nelsonii D.J.Carr & S.G.M.Carr
E. novoguineensis D.J.Carr & S.G.M.Carr
E. opaca D.J.Carr & S.G.M.Carr
E. orientalis D.J.Carr & S.G.M.Carr
E. polycarpa F. Muell.
E. porrecta S.T.Blake
E. psychotheca F. Muell.
E. pulvulenta Sims
E. pyrophora Benth.
E. stellulata Sieb.
E. symonii D.J.Carr & S.G.M.Carr
E. tokwa D.J.Carr & S.G.M.Carr
Thryptomene calycina (Lindl.) Stapf.

Nyctaginaceae

Allionia sp.
Bougainvillea glabra
B. spectabils Willd.
Mirabilis hybrida
M. jalapa L.

Oxybaphus nyctaginens (Michx.) Sweet

Jasminum nudiflorum Lindl.

Circaea lutetiana
Epilobium alpinum
E. angustifolium L.
E. fleischleri
E. hirsutum
E. montanum
E. rosmarinifolium
E. spicatum
Epilobium sp.
Fuchsia gracilis Lindl.
F. hybrida
Gaura lindheimeri
Jussieuana sprengeri
Lopinia coronata Andr.
Oenothera biennis
O. crassifolia
O. lamarckiana Ser.

Carr and Carr (1987)
Carr and Carr (1987)
Carr and Carr (1987)
Carr and Carr (1987)
Carr and Carr (1987)
Carr and Carr (1987)
Carr and Carr (1987)
Carr and Carr (1987)
Carr and Carr (1987)
Carr and Carr (1987)
A.R.Davis (pers. obs.)
Davis (1968); Moncur and Boland (1989)
Carr and Carr (1987)
Carr and Carr (1987)
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Carr and Carr (1987)
Carr and Carr (1990)
Davis (1969)
Carr and Carr (1990)
Carr and Carr (1987)
Beardsell et al. (1989)
Kerner and Oliver (1895)
Böhmker (1917); Zandonella (1972)
Bonnier (1879); Zandonella (1972, 1977)
Bonnier (1879)
Bonnier (1879); Valla and Ancibor (1976)
Zandonella (1977)
Daumann (1932b)
Bonnier (1879)
Bonnier (1879)
Bonnier (1879)
Bonnier (1879)
Bonnier (1879)
Schönichen (1924)
Radke (1926)
Feldhofen (1933)
Bonnier (1879)
Feldhofen (1933)
Daumann (1933)
Bonnier (1879)
Bonnier (1879)
Stadler (1886);
Kartashova (1965)
Oenothera sp.  
Passifloraceae  
Passiflora warmingii  
Phytolaccaceae  
Phytolacca acinosa Roxb.  
P. americana L.  
P. decandra L.  
P. dioica L.  
P. esculenta V.Houtte  
P. icosandra L.  
P. octandra L.  

Polemoniaceae  
Gilia multicaulis  
Leptosiphon densiflorus  
Phlox drummondii Hook.  
Polemonium caeruleum  

Polygonaceae  
Fagopyrum sp.  
Koenigia delicatula (Meissn.) Hara  
P. polystachya (Wall.) H.Gross  
Polygonum fagopyrum  
Rheum emodi Wall.  
R. palmatum L.  

Portulacaceae  
Lewisia howellii (Wats.) Robins  
Portulaca grandiflora Hook.  

Primulaceae  
Androsace lactea L.  
Hottonia palustris  
Lysimachia vulgaris  
Primula elatior  
P. grandiflora  
P. officinalis  
P. sinensis  
P. verna  

Ranunculaceae  
Paeonia albiflora Pall.  
P. peregrina Mill.  
P. tenufolia L.  

Resedaceae  
Reseda alba L.  
R. lutea L.  
R. luteola L.  
R. odorata  

Rhamnaceae  
Ceanothus thyrsiflorus Eschw.  
Paliurus australis Gaertn.  
Rhamnus alaternus L.  
R. cathartica L.  

Rosaceae  
Alchemilla alpina  
Schönichen (1924)  
Durkee et al. (1981)  
Zandonella (1970a, 1972)  
Zandonella (1970a)  
Zandonella (1967c)  
Zandonella (1970a)  
Zandonella (1967c, 1970a); Smets (1986)  
Zandonella (1970a)  
Zandonella (1970a)  

Bonner (1879)  
Bonner (1879)  
Bonnier (1879); Kartashova (1965)  
Bonnier (1879)  
Kartashova (1965)  
Ronse Decraene and Smets (1991a)  
Ronse Decraene and Smets (1991a)  
Ronse Decraene and Smets (1991a)  
Caspary (1848)  
Wolff (1924)  
Ronse Decraene and Smets (1991a)  
Zandonella (1972, 1977)  
Zandonella (1972, 1977)  
Daumann (1941)  
Daumann (1941)  
Zimmermann (1932)  
Daumann (1941)  
Daumann (1941)  
Caspary (1848)  
Caspary (1848); Bonnier (1879); Frei (1955)  
Caspary (1848); Bonnier (1879)  
Bonnier (1879)  
Kartashova (1965)  
Caspary (1848); Imperator (1906)  
Caspary (1848)  
Frei (1955)  
Feldhofen (1933)
A. fissa
A. hoppeana
A. pubescens
A. vestita
A. vetteri
A. vulgaris L.

Amelanchier canadensis
A. cirtica
A. rotundifolia
Amygdalus communis L.

A. persica L.

A. prunus
Armeniaca vulgaris Lam.

Cerasus vulgaris Mill.

Cotoneaster dammeri
C. integerrima
Crataegus coccinoides
C. mollis T. and G. Scheele
C. monogyna Jacq.

C. nigra
C. oxyacantha
C. pinnatifolia
C. rivularis
C. submollis
C. tannacetifolia
Cytosia japonica Pers.

C. vulgaris
Dryas octopetala
Exochorda albertii
E. grandiflora
E. korolkowii
Fragaria vesca
Geum hedreichii
G. heterocarpum
G. inclinatum
G. montanum
G. reptans
G. rivale L.

G. sibiricum
Malus communis
Malus sp.
Persica vulgaris Mill.
Potentilla arenaria
P. breunia
P. ternata
P. dombeysi
Prunus acida
P. avium

P. cerasus L.

P. domestica

Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933); Kartashova (1965); Said (1982)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Bonnier (1879); Kartashova (1965)
Bonner (1879); Imperatori (1906)
Bonner (1879); Imperatori (1906)
Bonner (1879); Kartashova (1965)
Oroz-Kovacs and Guyas (1989); Oroz-Kovacs et al. (1989)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Cook (1923)
Feldhofen (1933); Wilson et al. (1990)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933); Stadler (1886); Imperatori (1906)
Bonner (1879)
Bonner (1879)
Feldhofen (1965)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Kartashova (1965)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933); Kartashova (1965)
Bonner (1879)
Rosen (1936)
Kartashova (1965)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Feldhofen (1933)
Bonnier (1879); Schönichen (1924); Feldhofen (1933)
Radke (1926); Feldhofen (1933)
Bonnier (1879); Feldhofen (1933)
P. laurocerasus L.
P. mahaleb
P. maximowiczii
P. monticola
P. nana
P. padus
P. spinosa
Pyrus communis L.
P. malus
Pyrus sp.
Rubus ideaus L.
Sanguisorba minor Scop.
S. officinalis L.
Sibbaldia procumbens L.
Sorbus aucuparia
S. commixta
S. hostii
S. hybrida
S. torminalis
Spiraea arguta
S. laevigata
S. media
S. pikowiensis
Spiraea sp.
Waldsteinia geoides
W. sibirica

Rubiaceae
Crucianella sp.
Mitchella repens L.

Rutaceae
Citrus aurantium L.
C. limon (L.) Burmann
C. sinensis Osbeck
Correa reflexa
Dictamnus albus L.
Ruta graveolens
Zanthoxylon longipes Rose

Salicaceae
Salix alba L.
S. capreae L.
Salix sp.

Sapindaceae
Xanthoceras sorbifolia

Saxifragaceae
Bergenia crassifolia (L.) Fritsch.
Chrysosplenium oppositifolium
Deutzia corymbosa
D. discolor
D. gracilis
D. longifolia
D. maliflora
D. rosea
D. vilmonrinae
Edwinia americana
Grossularia reclinata (L.) Mill.
Parnassia palustris L.
Ribes alpinum
R. bracteatum
R. gordonianum
R. grossularia
R. malvaceum
R. multiflorum
R. nigrum L.
R. rubrum L.
Saxifraga aizoides
S. aizoon
S. granulata
S. mutata L.
S. oppositifolia
S. tridactylites
Saxifraga sp.

Antirrhinum majus L.
Bartsia alpina
Buddleja alternifolia
Digitalis lanata Ehrh.
D. purpurea L.

Erisum alpinum
Lathraea squamaria
Linaria cymbalaria
L. vulgaris

Melampyrum pratense
Mimulus guttatus
Odontites rubra
Orobanche speciosa
Rehmannia angustata
Verbascum blattaria L.
V. nigrum L.
V. phoeenicew L.

Ailanthus glandulosa Desf.

Atropa belladonna L.
Capsicum annum
Nicotiana glauca Grah.
N. macrophylla Sprgl.
N. sanderae
N. tabacum
Petunia violacea Lindl.
Scoparia orientalis Dun.

Staphylea pinnata L.

Herrania purpurea (Pittier) R.E.Schultes
Theobroma cacao L.
<table>
<thead>
<tr>
<th>Family</th>
<th>Species Name</th>
<th>References</th>
</tr>
</thead>
</table>
| Stylidiaceae     | *Stylidium adnatum* R.Br.  
| Tamaricaceae     | *Tamarix tetrandra* Pall. | Ronse Decraene (1990)#  |
| Trapaceae        | *Trapa natans* L.     | Caspary (1848)  |
| Tropaeolaceae    | *Tropaeolum majus* L. | Caspary (1848); Kartashova (1965); Rachmilevitz and Fahn (1975); Fahn (1979a)  |
| Violaceae        | *Viola canina* L.     | Bonnier (1879)  |
|                  | *V. odorata*          | Bonnier (1879); Imperatori (1906)  |
|                  | *V. tricolor* L.      | Bonnier (1879); Radtke (1926)  |

+ The nectary stomata in this genus of the Asteraceae were detected by H. Jürgens, as reported by Hanstein, in Andrä (1873).

* The nectary stomata in this species of the Fabaceae-Papilinoideae were detected by Delbrouck, as cited by Poulson (1875b) on p.253

# The nectary stomata in this species of the Tarnaricaceae were detected by E. Smets, as cited by Ronse Decraene (1990) on p. 199
APPENDIX 2

A survey of ultrastructural studies of floral and extrafloral nectaries and the relative quantities of primary sugar species in their exudates, with regard to the relative quantities (large, moderate to large, small to moderate, small) of endomembranous cisternae and vesicles in their proposed nectar-secreting cells during the phase of nectar secretion. Nectary location is designated as floral (F) or extrafloral (EXF).

<table>
<thead>
<tr>
<th>Family</th>
<th>Species (reference)</th>
<th>Nectary location</th>
<th>Nectary-sugar characteristics (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apocynaceae</td>
<td>Plumeria rubra L.</td>
<td>EXF</td>
<td>S only&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Mohan and Inamdar 1986)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vinca major</td>
<td>F</td>
<td>Sfg&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Rachmilevitz and Fahn 1973)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>V. rosea</td>
<td>F</td>
<td>Sf&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Rachmilevitz and Fahn 1973)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asclepiadaceae</td>
<td>Asclepias curassavica L.</td>
<td>F</td>
<td>Sfg&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Schnepf and Christ 1980, Christ and Schnepf 1988)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. tuberosa L.</td>
<td>F</td>
<td>Sfg, Sfg&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Christ and Schnepf 1988)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Cynanchum vincetoxicum (L.) Pers.</td>
<td>F</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>(Christ and Schnepf 1985)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gomphocarpus fruticosus (L.) Ait.</td>
<td>F</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>(Christ and Schnepf 1988)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromeliaceae</td>
<td>Billbergia nutans Wendl.</td>
<td>F</td>
<td>SFG&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Benner and Schnepf 1975, Schnepf and Benner 1978)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caprifoliaceae</td>
<td>Lonicera japonica Thunb.</td>
<td>F</td>
<td>By percent, S/F/G = 36/17/47 and 78/11/11 for L. japonicum&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Sambucus nigra L.</td>
<td>EXF</td>
<td>S,F,G present (Lütte 1961), but not quantified</td>
</tr>
<tr>
<td></td>
<td>(Fahn 1987)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euphorbiaceae</td>
<td>Euphorbia candelabrum Welw.</td>
<td>EXF</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>(Schnepf and Deichgräber 1984)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. cooperi N.E.Br.</td>
<td>EXF</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>(Schnepf and Deichgräber 1984)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. marginata Pursh</td>
<td>EXF</td>
<td>By percent, S/F/G = 9/42/49 and 6/49/45&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Schnepf and Deichgräber 1984)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Fabaceae**

*E. pulcherrima* Willd.  
(Schnepf 1964b, Schnepf and Deichgräber 1984)  
**EXF**  
SFG$^1$; Vansell (1944b) found S/(F+G) = 17.2/31.3, becoming 9.7/38.5 after prolonged storage; By percent, S/F/G = 29/26/44$^3$

**Liliaceae**

*Vicia faba* L.  
[Gunning and Steer 1975, Davis et al. 1988a, this thesis (Chapter 2)]  
**F**  
+++/$+/$/$+$/++, S only (Davis et al. 1988a); By percent, S/F/G = 79.9/11.1/9.0 and 80.0/10.8/9.2 [in situ, this thesis (Chapter 3)]

*Lilium dahuricum* Ker-Gawl.  
(Vasilijev 1971)  
**F**  
Unknown; SFG for both *L. auratum* and *L. speciosum$^1$; By percent, S/F/G = 71/19/10 for *L. wallichianum* and S only for *L. philadelphicum$^3$.

**Musaceae**

*Musa paradisiaca* L.  
var. *sapientum* Kuntze  
(Fahn and Benouaiche 1979)  
**F**  
By weight, S/F/G = 120-160/80/80 for *M. sapientum L.* (Lütte 1961); By percent, S/F/G = 70/17/13 for *Musa x paradisiaca* (Freeman et al. 1991).

**Ranunculaceae**

*Caltha palustris* L.  
(Peterson et al. 1979)  
**F**  
sFG$^1$

*Helleborus niger* L.  
(Eymé 1966)  
**F**  
Sfg$^1$

*Ranunculus acris* L.  
(Vasilijev 1971)  
**F**  
By weight, S/F/G = 51/30/32 by SFG$^1$; S/F/G = 51/30/32 by weight, changing to 14/30/40 after 5-8 days (Kronestedt et al. 1989)

*R. ficaria* L.  
(= *Ficaria ranunculoides*)  
(Eymé 1967)  
**F**  
S, Sfg$^1$

**Strelitziaceae**

*Strelitzia reginae* Ait.  
(Kronestedt and Walles 1986, Kronestedt and Robards 1987, Kronestedt et al. 1989)  
**F**  
SFG, SFG$^1$; S/F/G = 51/30/32 by weight, changing to 14/30/40 after 5-8 days (Kronestedt et al. 1989)

**Tropaeolaceae**

*Tropaeolum majus* L.  
(Rachmilevitz and Fahn 1975)  
**F**  
+++/$+$/++/$+$/++; By percent, S/(F+G) = 38.4/9.4 and 33.1/13.1 (Beutler 1930)

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**II - MODERATE TO LARGE CYTOPLASMIC CONTENT OF CISTERNAE AND VESICLES**

**Acanthaceae**

*Aphelandra scabra* (Vahl.) Sm.  
(Durkee 1987)  
**EXF**  
S,F, only traces of G (Durkee 1987)

**Aceraceae**

*Acer platanoides* L.  
(Vasilijev 1969)  
**F**  
SFG for *Acer sp.$^1$*
A2.3

**Aloeaceae**  
*Aloe cf. spinosissima* Hort. (Heinrich 1975a,b)  
F  
Unknown; SFG for *A. thomsonii*; for five other species, nectars are hexose dominant (esp. glucose), but S/F/G = 45/20/35 for *A. rauhii*.

*A. transvalensis*  
(Heinrich 1975a,b)  
F  
Unknown; SFG for *A. thomsonii*; for five other species, nectars are hexose dominant (esp. glucose), but S/F/G = 45/20/35 for *A. rauhii*.

**Apiaceae**  
*Conium maculatum*  
(Dumas 1975)  
F  
By percent, S/F/G = 23/35/42.

**Asclepiadaceae**  
*Hoya carnosa* R.Br.  
(Christ and Schnepf 1988)  
F  
SFG1; S/G = 35.6/5.0 (von Planta 1886); S/(F+G) = 28.9/11.9, changing to 8.3/20.9 after prolonged storage (Vansell 1944b).

*H. obovata* Decn.  
(Christ and Schnepf 1988)  
F  
Unknown.

*Stephanotis floribunda* Brongn.  
(Christ and Schnepf 1988)  
F  
SFG1.

**Balsaminaceae**  
*Impatiens holstii* Engl.et Warb.  
(Figier 1972b,c)  
EXF  
S only (Weber 1951, Zimmermann 1953, Matile 1956).

**Boraginaceae**  
*Cynoglossum officinale* L.  
(Tacina 1973)  
F  
SFG, SFG1.

**Bromeliaceae**  
*Guzmania peacockii* Mez.  
(Benner and Schnepf 1975)  
F  
Unknown.

*Tillandsia schiedeana* Steud.  
(Cecchi Fiordi and Palandri 1982)  
F  
Unknown; By percent, S/F/G = 50/22/28 for *T. macdougallii* L.B. Smith (Freeman et al. 1985), and S/(F+G) ranged from 1.8-44.6 for six other species of *Tillandsia* (Bernardello et al. 1991).

*Vriesea sp.*  
(Benner and Schnepf 1975)  
F  
Unknown; By percent, S/F/G = 43.5/23.6/32.8 for *V. friburgensis* L.B. Smith (Bernardello et al. 1991).  
By percent, S/F/G = 18/33/47, 11/41/48 and 8/39/52, for samples from *Silene alba*.

**Caryophyllaceae**  
*Melandrium album* (Mill.) Garcke  
(Zandonella 1970c)  
F  
S,G present (Bonnier 1879), also F (Lütte 1961), but not quantified; By percent, S/F/G = 7/27/67.

**Dennstaedtiaceae**  
*Pteridium aquilinum* (L.) Kuhn  
(Power and Skog 1987)  
EXF  
S,G present (Bonnier 1879), also F (Lütte 1961), but not quantified; By percent, S/F/G = 7/27/67.
<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>EXF</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euphorbiaceae</td>
<td>Euphorbia milii Des Moulins</td>
<td>EXF</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>(Ledbetter and Porter 1970,</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Schnepf and Deichgräber 1984)</td>
<td></td>
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<td>Mercurialis annua L. (Figier 1968b,</td>
<td>EXF</td>
<td>Unknown</td>
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<td></td>
<td>1969, 1972a)</td>
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<td>Aleurites moluccana Willd.</td>
<td>EXF</td>
<td>Unknown</td>
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<tr>
<td></td>
<td>(Belin-Depoux and Clair-Maczulajtys 1975)</td>
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<td></td>
</tr>
<tr>
<td>Fabaceae</td>
<td>Vicia faba L. (Wrischer 1962, Figier 1968a,</td>
<td>EXF</td>
<td>S&lt;&lt;F,G (Davis et al. 1988a)</td>
</tr>
<tr>
<td></td>
<td>Vigna unguiculata (L.) Walp.</td>
<td>EXF</td>
<td>By weight, S/F/G = 1.5/1/1 when freshly secreted, becoming 0.5/1/1</td>
</tr>
<tr>
<td></td>
<td>(Kuo and Pate 1985)</td>
<td></td>
<td>after accumulation (Pate et al. 1985)</td>
</tr>
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<td>Hydrangeaceae</td>
<td>Philadelphus sp. (Vasilijev 1971)</td>
<td>F</td>
<td>Unknown</td>
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<tr>
<td>Liliaceae</td>
<td>Gasteria trigona (Schnepf 1964a, Schnepf and</td>
<td>F</td>
<td>Unknown; SFG, SFG for G. acin-</td>
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<td></td>
<td>Pross 1976)</td>
<td></td>
<td>acifolia(^1)</td>
</tr>
<tr>
<td>Malvaceae</td>
<td>Abutilon hybridum Sieb.et Voss.</td>
<td>F</td>
<td>S/F/G ≅ 1/1/1 (Reed et al. 1971)</td>
</tr>
<tr>
<td></td>
<td>(Mercer and Rathgeber 1962, Findlay and</td>
<td></td>
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<td></td>
<td>Mercer 1971b)</td>
<td></td>
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<tr>
<td></td>
<td>A. megapotamicum St.Hil. and Naud. (Gunning</td>
<td>F</td>
<td>SFG, SFG(^1); By percent, S/F/G = 12/57/30 and 13/48/38(^3)</td>
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<tr>
<td></td>
<td>and Hughes 1976, Hughes and Gunning 1980)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>A. striatum var. Thompsonii Veitch</td>
<td>F</td>
<td>SFG(^1)</td>
</tr>
<tr>
<td></td>
<td>(Robards 1984, Kronesdêt et al. 1986,</td>
<td></td>
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<td>Robards and Stark 1988, Sawidis et al. 1989b</td>
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<td>A. venosum Lem. (Mercer and Rathgeber 1962,</td>
<td>F</td>
<td>S/F/G ≅ 1/1/1 (Reed et al. 1971)</td>
</tr>
<tr>
<td></td>
<td>Findlay and Mercer 1971b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gossypium hirsutum L. (Eleftheriou and Hall</td>
<td>EXF</td>
<td>S/F/G = 24/36/41 (Butler et al. 1972)</td>
</tr>
<tr>
<td></td>
<td>1983a,b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hibiscus rosa-sinensis</td>
<td>F</td>
<td>sFG(^1); By percent, S/F/G = 1/49/50</td>
</tr>
<tr>
<td></td>
<td>(Sawidis et al. 1987a, 1989a, Sawidis 1991)</td>
<td></td>
<td>(Van Handel et al. 1972) and trace/43/57 (Freeman et al. 1991)</td>
</tr>
<tr>
<td>Passifloraceae</td>
<td>Passiflora seemanii (Durkee 1982)</td>
<td>EXF</td>
<td>Unknown; Highly irregular within the genus, with predominantly F, G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>or S, and balanced sugar ratios, reported for five other species</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Durkee 1982)</td>
</tr>
<tr>
<td>Ranunculaceae</td>
<td>Helleborus foetidus L. (Eymé 1963, 1966,</td>
<td>F</td>
<td>S(^1); S only (Corbet et al. 1979)</td>
</tr>
<tr>
<td></td>
<td>Eymé and LeBlanc 1963)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nigella damascena L. (Eymé 1963)</td>
<td>F</td>
<td>By percent, S/F/G = 81/19/1(^3)</td>
</tr>
</tbody>
</table>

\(^1\) For G. acin-acifolia, refer to specific studies for sugar ratios. \(^3\) Results vary depending on conditions and location.
Rutaceae  
*Citrus sinensis* (L.) Osbeck  
(Rachmilevitz and Fahn 1973)  
F  
SFG¹; Vansell (1944a,b) found  
S/F/G = 12.9/6.5/5.4 (navel)  
and 12.4/6.1/5.1 (valencia);  
By percent, S/F/G = 49/26/24  
(Maurizio 1959)

**III - SMALL TO MODERATE CYTOPLASMIC CONTENT OF CISTERNAE AND VESICLES**

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus and Species</th>
<th>Exflagellation</th>
<th>Additional Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthaceae</td>
<td><em>Aphelandra sinclairiana</em> Nees</td>
<td>EXF</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
| Apiaceae        | *Heracleum sp.*                                | F              | Unknown; for *H. spondylium*, sFG,  
SFG¹, and by percent, S/F/G =  
6/47/47 (Maurizio 1959); S/F/G =  
trace/+/++ for *H. mantegazzianum*  
(Corbet et al. 1979); By percent,  
S/F/G = 56/0/44 for *H. lanatum*³  |
| Boraginaceae    | *Borago officinalis* L.                         | F              | Unknown; about half of all Brassicaeae lacked any S, the rest had only  
minor quantities of S¹,²  |
| Brassicaceae    | *Diplotaxis erucoides* D.C.                    | F              | sFG, FG¹               |
| Caryophyllaceae | *Silene vulgaris* (Moench) Garcke              | F              | sFG, FG¹               |
| Chenopodiaceae  | *Beta vulgaris* L.                              | F              | Unknown                |
| Cucurbitaceae   | *Cucumis sativus* L.                            | F              | Unknown                |
| Dioscoreaceae   | *Dioscorea sylvestra* (von Teichman und Logischen and Robbertse 1979) | EXF | S,F,G present (von Teichman und Logischen and Robbertse 1979), but not quantified |
| Euphorbiaceae   | *Ricinus communis* L.                           | EXF            | By weight, S/F/G = 321/338/341  
(Baker et al. 1978a); S/F/G =  
29/44/49 (Nichol and Hall 1988)  |
| Fabaceae        | *Acacia myrtifolia* (Marginson et al. 1985b)   | EXF            | By percent, S/F/G = 95/0/5³  |
|                 | *A. pycnantha* (Marginson et al. 1985b)        | EXF            | By percent, S/F/G = 35/33/32 and  
22/30/49³               |
<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>EXF</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. terminalis</td>
<td>(Marginson et al. 1985a)</td>
<td></td>
<td>EXF</td>
<td>By weight, S/F/G = 0.30/0.32/0.37 (Knox et al. 1985); By percent, S/F/G = 27.8/34.0/38.6</td>
</tr>
<tr>
<td></td>
<td>Trifolium pratense L.</td>
<td>(Eriksson 1977)</td>
<td>F</td>
<td>S/F/G, SFG1; +++/+;+++; S/(F+G) = 1.69 (Wykes 1953); S/F/G = 27.5/9.0/10.9 (Bailey et al. 1954);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>By percent, S/F/G = 63/27/10 (Maurizio 1959); SFG (Käpylä 1978)</td>
</tr>
<tr>
<td></td>
<td>Vigna unguiculata (L.) Walp.</td>
<td>(Kuo and Pate 1985)</td>
<td>EXF</td>
<td>By weight, S/F/G = 30/35/35 when freshly secreted, becoming 20/41/39 after accumulation (Pate et al. 1985)</td>
</tr>
<tr>
<td></td>
<td>Orchidaceae</td>
<td>Epipactis atropurpurea Rafin.</td>
<td>F</td>
<td>S/F/G = 16/26/32.5 (Pais and Chaves das Neves 1980)</td>
</tr>
<tr>
<td></td>
<td>Passifloraceae</td>
<td>Passiflora warmingii</td>
<td>F</td>
<td>S&gt;F, no G (Durkee et al. 1981)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Durkee et al. 1981, Durkee 1983a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV - SMALL CYTOPLASMIC CONTENT OF CISTERNAE AND VESICLES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brassicaceae</td>
<td>Arabidopsis thaliana (L.) Heynh.</td>
<td>(this thesis, Chapter 4)</td>
<td>F</td>
<td>By percent, S/F/G = 5.7/45.6/48.7 (in vitro, this thesis, Table 4.3)</td>
</tr>
<tr>
<td></td>
<td>Brassica napus L.</td>
<td>(Davis et al. 1986)</td>
<td>F</td>
<td>?/+++;+++; By percent, S/(F+G) = 0/45.1 (Beutler 1930) and S/F/G = 2/43/55 (Maurizio 1959); only</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F,G (Davis et al. 1988b)</td>
</tr>
<tr>
<td>Convolvulaceae</td>
<td>Convolvulus arvensis L.</td>
<td>(Vasilijev 1971)</td>
<td>F</td>
<td>SFG, SFG1</td>
</tr>
<tr>
<td>Euphorbiaceae</td>
<td>Euphorbia coerulescens Haw.</td>
<td>(Schnepf and Deichgräber 1984)</td>
<td>EXF</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>E. triangularis Desf.</td>
<td>(Schnepf and Deichgräber 1984)</td>
<td>EXF</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

1Percival (1961)
2Wykes (1952a), in the order S/F/G
3H.G. Baker and I. Baker, unpublished data