A STRUCTURAL AND FUNCTIONAL STUDY OF STOMATA

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SUMMARY

Substomatal ion-adsorbent bodies are reported on here for the first time*. A brief survey of the plant kingdom suggests that the structures are mainly restricted to the Commelinaceae and Filicales although analogous structures may occur in other plant groups. Microscopical studies indicate that the bodies have a solid external aspect, a hollow lumen, and are situated extracellularly. In Polypodium, the bodies are narrowly attached to the lower periclinal walls in the polar regions of the guard cell complex, whilst in Tradescantia they are located in the intercellular space between the poles of the complex and the adjacent subsidiary cells. In both genera, the body is covered by the endocuticle which can be distended into a substomatal sac by the body pressing against it. The endocuticle, in the immediate vicinity of the bodies, is modified into a series of hollow trabeculae which are considered to be important apoplastic pathways. The bodies are formed at an early stage of stomatal ontogeny from the migration of outer elements of the lower periclinal wall of the guard-cell mother-cell to both poles of the eventual guard cell complex. The walls of the body are believed to be highly pectinaceous and capable of adsorbing a wide variety of ions non-selectively. Preliminary X-ray microanalyses suggest that the bodies may be involved in potassium fluxes associated with stomatal movements.

Ultrastructural studies of immature Polypodium guard cells resulted in the erection of a hypothetical model for stoma formation.

Ontogenetic studies revealed a major anomaly in existing stomatal classifications which is rectified in a proposed new classification of stomatal types which is explicit at both ontogenetic and morphological levels. Previously unrecorded ontogenetic and morphological stomatal types are reported from Polypodium.

* Preliminary communications arising out of this work have been published:

Stevens, R.A. & E.S. Martin. New structure associated with the stomatal complex of the fern Polypodium vulgare. Nature (Lond.), 265, 331-334 (1977a)

Stevens, R.A. & E.S. Martin. The ion-adsorbent substomatal structures in Tradescantia pallidus. Nature (Lond.), 268 364-365 (1977b)

GLOSSARY OF TERMS

Cell Walls. Cell walls which are subparallel to the leaf surface are referred to as periclinal, whilst those which are subvertical to the leaf surface are anticlinal. Those periclinal walls which contribute to the structure of the outer surface of the leaf are referred to as upper periclinal walls, and those which contribute to the inner suface of the epidermis are referred to as lower periclinal walls. The anticlinal guard cell walls which are adjacent to the stoma (vide infra) are referred to as inner anticlinal walls, whilst those anticlinal guard cell walls which are distal to the stoma and in contact with the subsidiary and/or neighbouring cells (vide infra) are referred to as outer anticlinal walls. Those inner anticlinal walls which are common to both guard cells are referred to as common anticlinal walls. These terminologies are illustrated diagrammatically in Fig. G.1.

Guard cell complex. This expression is used to refer to the pair of guard cells by themselves.

Neighbouring cells. This term is used to describe all epidermal cells which directly abut onto the guard cell complex, but not the subsidiary cell(s) (vide infra). Neighbouring cells are not ontogenetically related to the guard cells and are no more differentiated than other ordinary epidermal cells.

Stoma. This expression is used only in its restricted sense, and applies only to the pore formed by the guard cells.

Stomatal complex. This expression is used to cover the guard cell complex plus all the subsidiary cells (vide infra) and neighbouring cells.

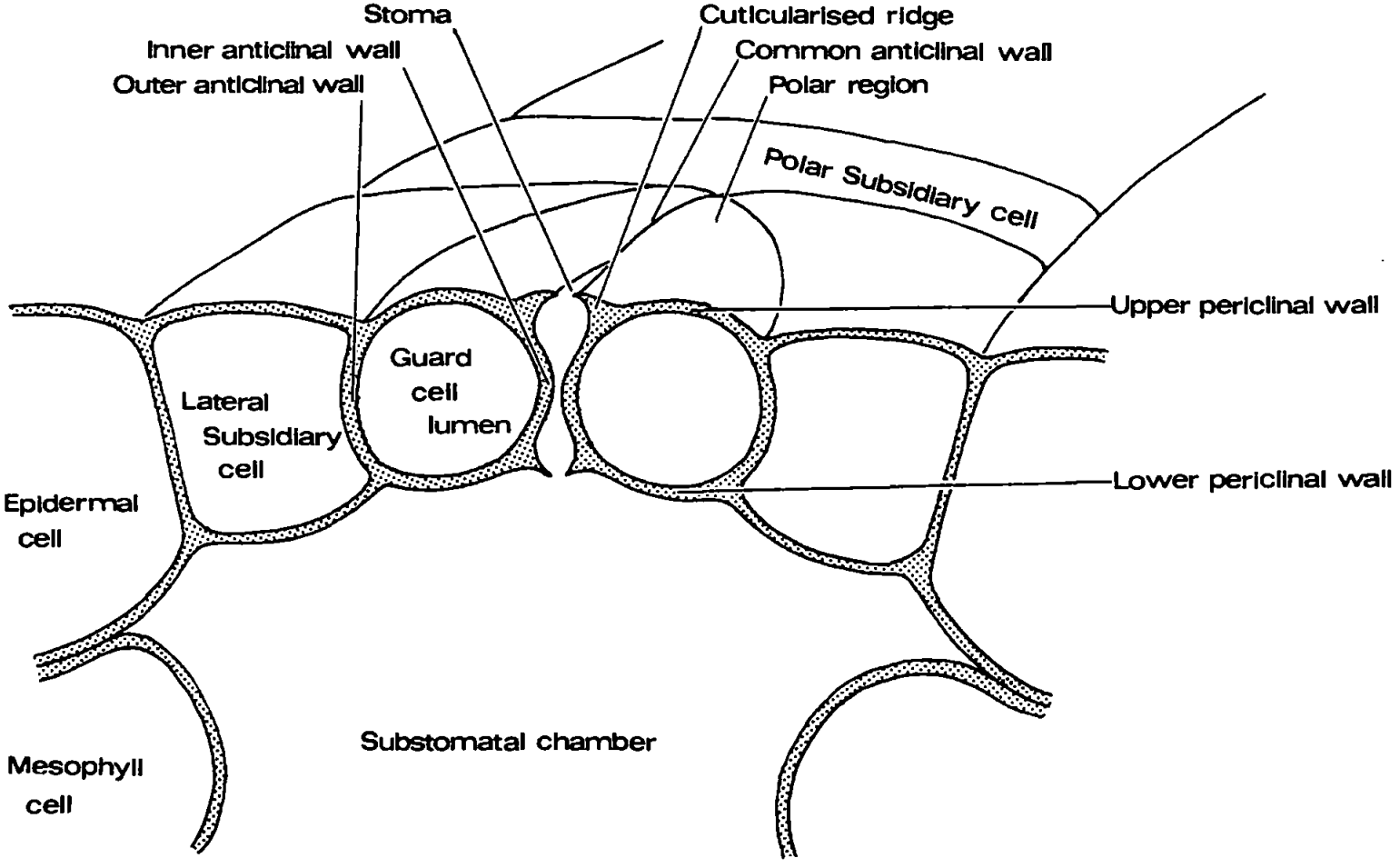
Subsidiary cells. These cells are ontogenetically related to the guard cell complex and are usually morphologically distinct from other epidermal cells. Subsidiary cells need not be in direct contact with the guard cell complex but, if they are not, they will be in indirect contact via other subsidiary cells which are in direct contact with the guard cell complex. They are usually, but not always, further characterised by not being in contact with the underlying mesophyll tissue.

Substomatal chamber. This expression refers to the air chamber which is invariably formed in the mesophyll below the guard cell complex.

Fig. G.1.

Stomatal terminology.

A diagrammatic representation of a transverse vertical section through a stomatal complex.


Stoma
Cuticularised ridge
Inner anticlinal wall
Common anticlinal wall
Outer anticlinal wall
Polar region
Polar Subsidiary cell
Upper periclinal wall
Guard cell lumen
Lateral Subsidiary cell
Lower periclinal wall
Epidermal cell
Substomatal chamber
Mesophyll cell

CHAPTER 1

GENERAL INTRODUCTION

Stomata are pores, generally found in leaf surfaces, bordered by a pair of inflatable guard cells whose movements effect variations in the size of the pore. The prime function of stomata is the regulation of gaseous exchange associated with photosynthesis, but concomitantly they provide portals through which large amounts of water vapour are lost from the plant in transpiration. Plant physiologists are particularly interested in the precise mechanism(s) of stomatal movements, since a clearer understanding of their modus operandi may open the way to controlling stomatal apertures exogenously. In this way, the potential yields of many crops could be realised in areas of high evapo-transpiration/low rainfall, by reducing unnecessary water loss without impairing photosynthesis.

Stomatal movements are believed to result from osmotically-regulated changes in the guard cell volume (Heath,1938). Thus, in typical mesophytes, solutes accumulate in the guard cell protoplasts early in the photoperiod which results in the osmotic uptake of water, and the guard cells gaining an osmotic advantage over their adjacent epidermal cells. Due to the physical characteristics of the guard cells, the resultant expansion of these cells is directed laterally and the guard cell pair part mesially to open the stoma (De Michele & Sharp, 1973; Aylor et al., 1973; Meidner & Willmer, 1975). Two major hypotheses have been put forward to account for the turgor

movements of guard cells; namely the starch - sugar interconversion theory (Lloyd, 1908; Sayre, 1926) in which starch is hydrolysed to sugars in the light and converted back to starch in the dark, and the ion-transport hypothesis in which the major cationic participant is potassium (reviewed by Raschke, 1976; Hsiao, 1976).

THE ROLE OF POTASSIUM IN STOMATAL MOVEMENTS

Macallum (1905) detected potassium in tulip guard cells during an appraisal of his histochemical reagent for the detection of this ion in tissues, and subsequently this technique has been basic to much of the early work on the involvement of potassium in the stomatal mechanism. Imamura (1943) was able to correlate stomatal aperture with endogenous levels of potassium in the guard cells. Fujino (1967) proposed that potassium was actively transported into the guard cells during stomatal opening and out of the guard cells during stomatal closure. Independantly, Fischer (1968) concluded that active potassium accumulation in the guard cells occurred during stomatal opening and that the potassium uptake (estimated with $^{86}Rb^{+}$) was sufficient to cause the observed changes in stomatal aperture, if it were counter-balanced by an anion.

The involvement of potassium in stomatal ion fluxes has been corroborated by ^{86}Rb tracer studies (Fischer & Hsiao, 1968), X-ray microprobe analyses (Sawhney & Zelitch, 1969; Humble & Raschke, 1971; Raschke & Fellows, 1971; Willmer & Pallas, 1974; Dayanandan & Kaufman, 1975), flame photometry of isolated guard cells (Allaway & Hsiao, 1973), and potassium-sensitive microelectrodes (Penny & Bowling,

1974). These direct studies on the role of potassium in the stomatal mechanism are further substantiated by indirect studies. Carbon dioxide concentration effects on stomatal aperture produce a change in the guard cell potassium levels (Pallaghy, 1971), as does water stress (Hsiao, 1973). Fusicoccin, which induces abnormal stomatal opening, also induces abnormally high potassium accumulation in the guard cells (Turner, 1973; Squire & Mansfield, 1974). The fungal toxin from Helminthosporium maydis induces stomatal closure and the guard cells from infected plants have been shown to have very low potassium levels (Arntzen et al., 1973). Abscisic acid induces stomatal closure, and the results of Mansfield and Jones (1971), Horton & Moran (1972), and Arntzen et al. (1973), indicate that the potassium levels of the guard cells decrease under its influence. Abscisic acid is of particular interest since it is a hormone whose endogenous levels increase under stress conditions, such as water deficits (Wright, 1969). It may, therefore, operate as a feedback mechanism, causing stomatal closure at the onset of water stress and thus reduce further transpirational losses (Raschke, 1975).

A wide variety of plant types and species (50+ spp.) have been shown to exhibit guard cell potassium fluxes associated with stomatal movements (Willmer & Pallas, 1973; Dayanandan & Kaufman 1973, 1975).

STOMATAL SINK-SOURCE RELATIONSHIPS

Most plant cells are symplastically connected to adjoining cells by plasmodesmata, but these have only been rarely

demonstrated as occurring between mature guard cells and adjacent cells (Litz & Kimmins, 1968; Pallas & Mollenhauer, 1972; Fujino & Jinno, 1972). Moreover, Penny & Bowling (1974) have demonstrated that stomatal potassium fluxes are maintained against a potassium activity gradient which suggests that the guard cells are not in symplastic continuum with adjacent cells. If they were, the electrochemical gradients found (Penny & Bowling, op. cit.) could not be maintained. For these reasons, the movement of ions between the guard and immediately adjacent cells, at least, is considered to be apoplastic.

Work on ion fluxes in the stomatal complex of maize by Pallaghy (1971) and Raschke & Fellows (1971) indicates that the fluxes are contained within the complex (i.e. restricted to the guard and subsidiary cells). Thus, during opening, the guard cells act as the sink and the subsidiary cells as the source of the ions whilst, during closure, the roles are reversed. This localised ion flux may be restricted to graminaceous stomatal types whose guard cell walls are particularly thin (0,1 μm, Brown & Johnson, 1962), thus minimising the diffusion of the ions as they pass through the cell walls.

The work of Penny & Bowling (1974) and Willmer & Pallas (1974) on Commelina communis shows that the ion fluxes, in this species, extend to the epidermal cells outside the stomatal complex. The sink of the ions during stomatal opening is the guard cell protoplast where it is tacitly assumed that they are accomodated within the vacuole (Heller et al., 1971). No discrete sink/source has been identified outside the guard cell protoplasts, although Milthorpe (1969) has suggested that ions could be stored within the the differentially-thickened walls of the guard cells. However,

calculations by Raschke (1976) indicate that the epidermal apoplast in Vicia faba is too small by a factor of between 10 and 20 to store sufficient potassium for stomatal opening. This is confirmed by Penny & Bowling (1974) whose data relate to intercellular (vacoular ?) potassium levels. Despite this evidence, it is possible that the apoplast in the immediate vicinity of the guard cells contains sufficient potassium to initiate stomatal opening.

BASIS AND OBJECTIVES OF THE PRESENT INVESTIGATION

The present study arose out of an investigation into the effects of certain phenolics on the stomatal mechanism, during which attempts were made to correlate guard cell potassium levels with stomatal aperture. Whilst subjecting epidermal strips to the Macallum histochemical stain for potassium (Macallum, 1905), it was noticed that stain localisations occurred consistently at the poles of the guard cells in this species. Furthermore, the stain localisations were precipitated in very discrete masses in a superficial position on the face of the guard cell complex adjacent to the mesophyll. It was also obvious that the localisations were not simple artefacts such as might be caused by topographical features or the coverslip pressing against the cells in these localised areas. No reference could be found relating to these polar localisations in the literature.

It was decided to investigate the polar stain localisations in detail since their superficial nature indicated that they could be extracellular and, therefore, the possibility arose that they could be a sink/source of potassium ions associated with stomatal movements.

Because of the complete novelty of the localisations, the ensuing investigations were directed towards elucidating their physical and chemical nature, their distribution in the plant kingdom, their morphogenesis, and their functional role(s).

CHAPTER 2

MATERIALS AND METHODS

PLANT MATERIAL

The main plant species used in this investigation were the common polypody, Polypodium vulgare (3n) (Polypodiaceae : Filicales), Commelina communis, Tradescantia pallidus, and the spiderwort or trinity flower, Tradescantia x andersoniana (= T. virginiana) (Commelinaceae : Monocotyledones).

Polypodium vulgare was originally collected from a variety of habitats (rupicolous, epiphytous, and terricolous) and sites in and around the Plym Forest, Devon. This stock material was subsequently cultivated in a heated greenhouse.

Commelina communis was cultivated under greenhouse conditions (vide infra) from seeds originally supplied by Dr. T.A. Mansfield, Lancaster University. Tradescantia pallidus was propagated, by root cuttings, from material obtained originally from the greenhouses of Exeter University. Tradescantia x andersoniana was similarly propagated vegetatively from parent stock, of unknown origin, in the Polytechnic's greenhouse and from var. 'Isis' supplied by Plymouth Park's Department.

A wide variety of other plant material has been examined during the investigation and includes indigenous species collected

primarily from the environs of Plymouth, horticultural species and varieties obtained from private gardens, and exotic species obtained from the greenhouses of Plymouth Park's Department, Exeter University, Newcastle University, and the Herbert Whitley Trust, Paignton Zoological Gardens, Devon.

PLANT CULTIVATION

Initially plant material was maintained in a greenhouse under a minimum photoperiod of 14 h which was extended naturally during the summer months. During late Autumn, Winter, and early Spring, natural daylight was augmented by fluorescent sodium lights for three hours between 06.00 and 09.00 h and for up to five hours in the late afternoon and evening before cutting out at 20.00 h G.M.T. The fluorescent sodium lights (G.E.C., 180 W, cat. no. SOX FL) were mounted in Camplex reflectors (Simplex of Cambridge Ltd., Cambridge) between 1,0 and 1,5 m above the plant material being cultivated. Attempts were made to regulate the ambient temperature of the greenhouse to ca. 20°C. During Summer, the temperature frequently rose to above 30°C on clear sunny days despite ventilation. During Winter, the temperature was maintained by a 2 KV 'Thermomatic Glasshouse Fan Heater' (Simplex of Cambridge Ltd., Cambridge, cat. no. HD1970T) although on very cold nights the temperature did drop as low as 12°C. This fan heater was kept running during the Summer months to increase ventilation.

Commelinaceous plants were grown on slatted bench tops and were watered once a day in the evening for most of the year although two waterings were required on occasion during very hot weather.

Fern material was grown under the slatted benches and was watered every two or three days except during hot weather when it was necessary to water it more frequently. No fertilisers, liquid or solid, were used.

The seeds of Commelina communis were planted in batches of ca. 20 in a 7 cm square plastic pot of commercial 'John Innes Seed Compost' (D.O. Hunt Ltd., Bishopsteignton, Devon) and covered with ca. 2 mm of the same soil. The individual plants were pricked out as soon as they had emerged to a height of ca. 1 cm after 7 - 10 days, and replanted individually in commercial 'John Innes No. 2 Compost' (same supplier as above) in 7 cm square plastic pots. New stocks were established weekly. Root cuttings of Tradescantia pallidus were established and maintained in commercial 'John Innes No.2 Compost' in 8 cm square plastic pots; Tradescantia x andersoniana was likewise established and maintained, but in 21 x 33 cm plastic storage boxes. New stocks of Tradescantia spp. were established as and when the parent stocks flowered. Polypodium vulgare stocks were cultivated in either their natural substrate which was collected at the same time as the plant material, or else sub-cultures were established in a 1:1:1 mixture of leaf mould, 'John Innes No. 2 Compost', and water-washed silver sand.

All plant material was acclimatised to the eventual experimental conditions of photoperiod and/or temperature for a minimum of 7 days. This was done in either a Fisons growth cabinet (Fisons Scientific Apparatus Ltd., Loughborough; type 280G-CT/DEG-2) maintained at 60% relative humidity, 12 or 14 h photoperiod (10.00 - 22.00/24.00 h B.S.T.) at 10 760 lx / 37 J m^{-2} s^{-1} and 20°C, if the material was to be used for porometry or, for all other experimental purposes, in a Fisons growth cabinet (same supplier as above; model

no. 140G2) with a synchronised photoperiod/thermoperiod (10.00 - 22.00 h B.S.T.) at 16 000 lx / 56 J m^{-2} s^{-1} and 25 $\pm$ 1°C (light) / 19 $\pm$ 1°C (dark).

The age of the plant material used in the investigation varied considerably. For porometry and all *in vitro* physiological studies, the third and fourth fully expanded true leaves of *C. communis* were used (ca. 4/5 weeks old) whilst any recently fully expanded leaves of *Tradescantia* spp. were used. Isolated epidermes of *P. vulgare* used in physiological experimentation were taken from fronds which were just turning from the light green of immaturity to the dark green of maturity. Shortly after the pinnae turned dark green, the stomata start to die off and the tissue rendered unsatisfactory for such studies.

For studies in stomatal ontogeny and morphogenesis, very young tissues were required. This was obtained from the still circinately rolled pinnae of *P. vulgare*, much of which was collected in the field since insufficient material was produced by greenhouse maintained stocks. In *Tradescantia* spp., the material was dissected out of young shoots.

Plant material used in the scanning electron microscope studies and X-ray microanalyses was difficult to prepare for the relevant studies because the plant material was transported to London by car in the early hours of the morning prior to examination or analyses. Dark treated material was transported in a light-tight dustbin. In London there were no facilities available to maintain the plants under any constant quantifiable environmental conditions. Consequently, for a light treatment, the plants were placed on the

nearest window sill, and for dark treatments, the plants were placed in a photographic darkroom.

HISTOCHEMICAL AND CYTOCHEMICAL TECHNIQUES

The various stains and enzymes employed at various times during the investigation, and referred to in this thesis, are listed, and their formulations given in Appendix 1. For ease of reference, each stain and enzyme is coded with two numerals, the first of which relates to the chapter in which it is first mentioned, and the second of which signifies the order of appearance of the various stains in a particular chapter.

The Macallum histochemical stain for potassium

This stain, formulated by Macallum (1905), has been used extensively in stomatal physiology to demonstrate potassium fluxes associated with stomatal movements (vide Chapter 1). Its popularity derives from its reputed specificity (Macallum, 1905, Crout & Jennings, 1957), sensitivity, ease of application, speed of action, distinctness of results, and absence of suitable alternative histochemical techniques. An alternative technique, the tetraphenylboron method (Collewijn, 1963), has been attempted but found to be unsatisfactory due to the lack of a distinct positive reaction in epidermal tissue.

The original Macallum formulation is given in Appendix 1. It was found, during the present study, that no advantage was gained by making the sodium cobaltinitrite (hereinafter referred to as the double

salt) from a combination of the single salts since the results were apparently identical.

Theoretically, when the double salt is applied to tissues, it combines with any free potassium to produce sodium potassium cobaltinitrite (hereinafter referred to as the triple salt) which is precipitated. After a suitable staining period, the tissue is washed in ice-cold water which results in the soluble double salt being washed out whilst the insoluble triple salt (potassium-complexed element) is left in situ. When the washed tissue is then treated with ammonium sulphide, the resultant black precipitate should correspond to the sites of potassium localisation.

The critical stage in the procedure is clearly the ice-cold wash since, if this is insufficient, traces of the double salt will remain in the tissue and react with the ammonium sulphide in a manner indistinguishable from the triple salt. No set duration has been laid down for this critical wash. Lloyd (1925) employed a wash of 15 - 90 minutes, Mansfield & Jones (1971) one of 30 minutes, Willmer & Pallas (1973) continued the wash until no further yellow double salt diffused out of the tissue, whilst Dayanandan & Kaufman (1975) used only a 2 minute wash. During the present investigation it was found that the treated epidermal strips continued to visibly lose what is presumed to be the double salt after 20 minutes. Consequently a half hour wash was employed (c.f. Mansfield & Jones, 1971). It was found that after 2 h all traces of all cobalt had been washed out of the tissue (Commelina communis) which, consequently, gave no positive reaction to the ammonium sulphide.

Whilst these preliminary studies into the Macallum stain

were directed to perfecting a technique to demonstrate the polar stain localisations in the guard cells of certain stomata (vide Chapters 1 and 3), certain problems came to light which should be considered when using this stain for quantifying potassium localisations in tissues. The time of penetration of the tissue by the double salt will depend on the anatomy of the tissue; clearly heavily cutinised tissues will require a much longer treatment than poorly cutinised ones. Allied to this, the time of the subsequent wash in ice-cold water must be at least as long as the treatment period with the double salt. There must be a critical time when a maximal amount of the double salt has been washed out and a maximal amount of the triple salt has been retained. It is difficult to interpret this in the laboratory, but it could be elucidated by X-ray microprobe analyses. The basic formulation of Macallum produces a 4% double salt solution (w/v). This will clearly have an osmotic effect on the treated tissues. This would result in water loss and possibly the leaching of potassium salts, especially since membrane integrity will be affected. Coupled with this, as the stain diffuses in, water and salts must diffuse out. It seems unlikely, therefore, that much, if any, of the unbound potassium will be precipitated at its in vivo location. Evidence was also found that a considerable body of cobalt, from the double salt, becomes adsorbed onto the cell surfaces (c.f. Lloyd, 1925) which is not dislodged by the ice-cold water wash (vide legend of Plate 2.1). This cobalt is subsequently precipitated as the sulphide, and unless washed off after the ammonium sulphide treatment, will give spurious results. The importance of this second wash may not be appreciated by some workers using this stain and the adsorbed cobalt element must be considered when interpreting the results of those workers who mount

their epidermal strips directly into a glycerine/ammonium sulphide mixture after the ice-cold water wash.

In attempting to modify the Macallum technique for an improved demonstration of the nature of polar stain localisations (Chapters 1, and 3), note was taken of the fact that absolute alcohol pretreatment before staining with the double salt ensures more consistent results (Lloyd, 1925). Consequently a variety of permutations of the Macallum stain were carried out, an example of one being illustrated in Plate 2.1. In this experiment, five concentrations of the double salt (40, 20, 10, 5, and 2% w/v) were used in conjunction with four different washing schedules, explained in the legend of Plate 2.1. It can be seen that the polar stain localisations are demonstrated most clearly with the 2% cobaltinitrite treatment, and the best washing sequence involved a 10 s absolute ethanol prewash, followed by a 60 s agitated wash in absolute ethanol after staining, and completed by another 60 s wash in absolute ethanol after the ammonium sulphide treatment. This washing sequence was considered to be the best after experimentation on several plant species (it is clearly not the best for T. pallidus), and similarly a 7,5% (w/v) cobaltinitrite solution was considered to be better suited for the demonstration of the polar stain localisations in most plant species. It was also found that the speed of precipitation of the cobalt at these sites was temperature dependant and can be most conveniently carried out at room temperature. The speed of precipitation at 20°C is illustrated in Plate 2.2 where it can be seen that it commences within 5 s, and is virtually complete after 60 s. A suitable formulation of this modified Macallum stain is given in Appendix 1 together with the staining procedure for use with most types of epidermal tissue; the 10 minute treatment with the double salt allows adequate penetration

time in the more heavily cuticularised species.

With hindsight, it should be pointed out, that many heavy metal salts could be used to demonstrate polar stain localisations in stomata (vide Chapter 11) in conjunction with ammonium sulphide. It should also be noted that the modified Macallum stain employed here is not designed to demonstrate potassium, although it may also serve this function.

MICROSCOPY

Light microscopy

The majority of histochemical observations and microphotography was carried out on a Carl Zeiss Photomicroscope II (Oberkochen, W. Germany) using a neutral filter. A Wild M20 (Heerbrugg, Switzerland) was used for much of the morphogenetic and ontogenetic studies and fluorescence microscopy was carried out using a Vickers Patholux (Coulsdon and York). Measurements of stomatal aperture in the physiological studies were made with a graduated eyepiece on a Domo MBR-1E (Leningrad, U.S.S.R.) at a magnification of x 400. Unless otherwise stated, all observations on isolated epidermes were carried out with the side adjacent to the mesophyll facing the objective lens.

Microphotographs were taken on Ilford Pan F film (Ilford Ltd., Basildon, Essex), developed in Acutol FX-14 (Paterson Products Ltd., London), fixed in Fix-Sol (Photo Technology Ltd., Potters Bar, Hertfordshire), and printed on Rapidoprint FP 1-2 Projection Paper

(Agfa-Gevart, W. Germany). For the plates in this thesis, Rapidoprints were arranged as required on plain white card, re-photographed with Ilford FP4 film (Ilford Ltd., Basildon, Essex) and printed on Ilfospeed Photographic Paper (Ilford Ltd., Basildon, Essex).

Transmission electron microscopy

Fixation, staining, embedding, and post-staining techniques in the purely ultrastructural studies reported on in the first half of Chapter 4, are to be found in Appendix 2. Thin sections (silver or gold) were cut on either an LKB Ultrotome III (LKB Instruments Ltd., Croydon, Surrey), or on a Porter Blum MT2-B Ultra-microtome (Ivan Sorvall Inc., Newtown, Connecticut, U.S.A.). The sections were mounted on carbon and Formvar-coated 100 μm grids (Taab Laboratories, Reading) and examined in a Phillips EM300 microscope at 80 KeV in the case of normally prepared tissue, and at 40 or 60 KeV in the case of unstained tissues (vide infra).

In attempting to elucidate the structures associated with the guard cell polar stain localisations (Chapters 1, and 3), tissues pretreated with the Macallum stain were fixed, stained, and embedded using the standard procedures described in Appendix 2. It was found that the cobalt precipitates largely disappeared from the tissues during this process and it was assumed that the cobalt was associated with some lipid structure which was denatured by the chemicals used and leached out of the tissue. Consequently, material was fixed in glutaraldehyde and osmic acid in the normal way (Appendix 2) and, after washing in cacodylate buffer, it was embedded directly into water soluble Durcupan resin (Fluka A.G., Switzerland). Despite following the manufacturer's recommendations, it was not possible to cure the

blocks sufficiently for ultramicrotomy (This may have been because the resin used was of unknown antiquity).

To overcome these problems, tissue was pretreated with 7,5% (w/v) modified Macallum's stain in the normal way, fixed and dehydrated in absolute ethanol (completed in 10 minutes), before being embedded directly in Spurr resin. This modified programme resulted in the cobalt precipitations being retained in the tissue. Ultramicrotomy of the resultant blocks proved extremely difficult because the cobalt crystals in the tissue blunted the glass knives after very few passes so that any sections obtained were badly scored. A diamond knife was used in an attempt to avoid this problem but was equally unsatisfactory as the knife dislodged the cobalt crystals in the tissue and tore them through the sections. When sections from this material were examined in the microscope, a low beam voltage had to be used because the cobalt inclusions in the sections heated up extremely quickly and caused tears in the sections. This tearing under the electron beam was accentuated by the poor fixation of the cell walls with the ethanol treatment. Alternative techniques will have to be developed to cope with the problems in any further work of this nature.

Scanning electron microscopy and X-ray analysis

The largely unsatisfactory results of the transmission electron microscope studies prompted consideration of the scanning electron microscope as an alternative means of investigation. In the absence of local expertise in this field, the evaluation of the various techniques available was worked out from first principles. It was decided that if scanning electron microscopy was to be used, X-ray microanalyses might also be possible as an invaluable aid to physiological

studies on stomata. The prime requirement, therefore, was that the tissue should be treated as little as possible before examination and analysis. There were three possibilities: freeze-drying, critical point drying, and the use of a cryostage. It was felt that that the last option offered the most satisfactory solution, since the treatments involved in the first two options were considered undesirable since they would result in a certain amount of ion-leakage, especially of potassium, which is notoriously mobile. JEOL are, currently, the only company producing a commercially-available cryostage. Their London office was approached and agreed to the work being carried out on one of their demonstration machines.

The operation of a cryostage is extremely simple. Leaf/ epidermal tissue was attached with double-sided adhesive tape to a brass or aluminium stub which was then capped and plunged into liquid nitrogen. Once the nitrogen had ceased boiling, the sample was introduced into the pre-evacuation chamber where the cap was knocked off with a manipulator before being mounted on the cryostage of a JSM 35 scanning electron microscope. The sample was then immediately ready for viewing, and could be left there for hours since the cryostage is maintained at ca. -150^{o}C by liquid nitrogen. Leaf tissues were examined at between 8 and 15 KeV, and it was found that the tissue was conductive enough not to require either coating or earthing with silver paint. Although both adjuncts were used on occasion, they did not result in markedly superior results. Occasionally ice deposits proved to be so extensive as to occlude much of the tissue surface, but this was easily remedied by removing the stub from the cryostage and exposing the iced surface to a heating element in the pre-evacuation chamber where the ice was sublimated before the stub was returned to the cryostage.

The procedure had to be modified slightly when the examination of leaf sections was carried out. Normally, a block of tissue is frozen in liquid nitrogen and, after de-capping in the pre-evacuation chamber, the section is obtained by slicing with a remote-controlled scalpel. This is not straightforward, since the frozen material is very brittle and tends to fracture irregularly. In addition, although the pre-evacuation chamber is cooled with liquid nitrogen, the manipulators are not, with the result that they tend to melt water on the surfaces they come into contact with. This re-freezes on the cryostage and makes observations more difficult. Consequently a different approach was attempted which requires two people. The block of tissue was mounted in a grooved stub, fixed in its holder, and held immediately above a wide necked vacuum flask filled with liquid nitrogen for quenching. Then, whilst one person held the tissue immediately adjacent to the stub with forceps, the other cut the tissue in the desired plane of section with a scalpel as quickly as possible. At almost the same time the sectioned tissue was plunged into the liquid nitrogen and capped. This operation produces first class results providing it is carried out extremely quickly so that the section, or large parts of it, are frozen before the cell sap begins to exude.

Energy dispersive X-ray microanalyses were performed on the cryostage of the same instrument. A Kevex silicon crystal detector of 145 eV resolution coupled to a Link system was used with a 600 µm aperture at between 15 and 18 KeV for 100 s at ca. 0,1 nA absorbed current. Although X-ray distribution images of single elements, notably potassium, were attempted, the elements concerned were present in such low concentrations that, to achieve satifactory images, prolonged exposures would have been required. Since time was always a limiting

factor, the majority of analyses attempted were of a point type, where the electron beam was reduced to a maximum of 6 μm^2 and focussed onto a specific area of the tissue. Full spectral analyses of K emissions were obtained in this way.

PHYSIOLOGICAL TECHNIQES

Isolated tissue techniques

A purpose-built water bath with controlled lighting, temperature, and air supply was constructed. It is illustrated and described in Fig. 2.1 and Plate 2.3.

Epidermal strips, semi-leaf discs, leaf discs, and parts of excised leaves were incubated in experimental solutions contained in the phials. The apparatus accomodates 12 phials and, generally, 5 treatments were employed at one time with a control. In this way the experiment could be replicated each time it was performed. The leaf tissue was prepared in subdued light, one hour before the commencement of the photoperiod, when initially-closed stomata were required, and the tissue bulked in a petri dish containing the control medium. Once sufficient material had been prepared, it was randomly distributed into the phials containing the experimental incubatory media, which had been randomly distributed themselves. If initially-opened stomata were required, the source material was enclosed in a 250 cm^3 conical flask whose neck was them plugged with cotton wool. The source material was then left for 90 minutes in a Fisons growth cabinet (Fisons Scientific Apparatus Ltd., Loughborough; model no. 140G2) at 16 000 lx / 56 J m^{-2} s^{-1}. This procedure causes the stomata to

open fully under the combined influence of the light treatment, increased relative humidity, and reduced carbon dicxide concentration.

All incubations were carried out at 20 $\pm$ 1°C, and in the presence of carbon dioxide-free air unless stated to the contrary. The numbers of stomatal apertures measured, and the number of replications employed in each experiment are given in Chapter 11.

All the chemicals used throughout this study were of analar grade and supplied by British Drug Houses Ltd., Poole, Dorset, with the exception of the ($\pm$) cis-trans abscisic acid and chlorogenic acid (Sigma Chemical Co., Kingston-upon-Thames, Surrey; cat. nos. A.1012, and C.3878, respectively), and ferulic acid (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire; cat. no. 2622h).

Porometry

A four channel automatic porometry system, which incorporates both a Wheatstone bridge circuit (Heath & Russell, 1951) and Gregory and Pearse circuits (Gregory & Pearse, 1934), was constructed to monitor stomatal behaviour. The system is illustrated in Figs. 2.2, and 2.3, and Plate 2.4. The stomatal behaviour of four separate leaves can be investigated simultaneously for up to ten days with this system, with each leaf being monitored for 233 s in every 20 minutes. The porometer has, latterly, been used exclusively on Commelina communis. Due to the rather delicate nature of the leaves in this species, an applied pressure of 300Pa is used which does not appear to physically damage the leaf, even after 10 days of continuously applied pressure. A special porometer cup was designed and constructed for C. communis which is illustrated in Fig. 2.4. The 300 Pa is applied continuously to the

adaxial epidermis. This is because it is believed that the stomata on this surface always offer the greatest resistance to air being passed through the leaf. There are ca. 2.3 times more stomata per unit area on the abaxial surface than on the adaxial surface, and scanning electron microscopy (Chapter 5) indicates that the mesophyll resistance will always be less than that of the adaxial epidermis.

Details of capillary resistance construction are given in Appendix 3, together with a sample resistance calibration curve.

Fig. 2.1.

Water bath used in physiological investigations.

The water-cooled light bath contains five 150 W tungsten bulbs which are individually controlled; there are also eight 8 W warm white fluorescent light tubes (two above and two below the water bath and one on each side of the bath). The temperature of the water bath was maintained with a Gallenkamp Thermo Stirrer 2 (Gallenkamp, London; cat. no. BJH 400). The sample phials are supported in a perspex holder.

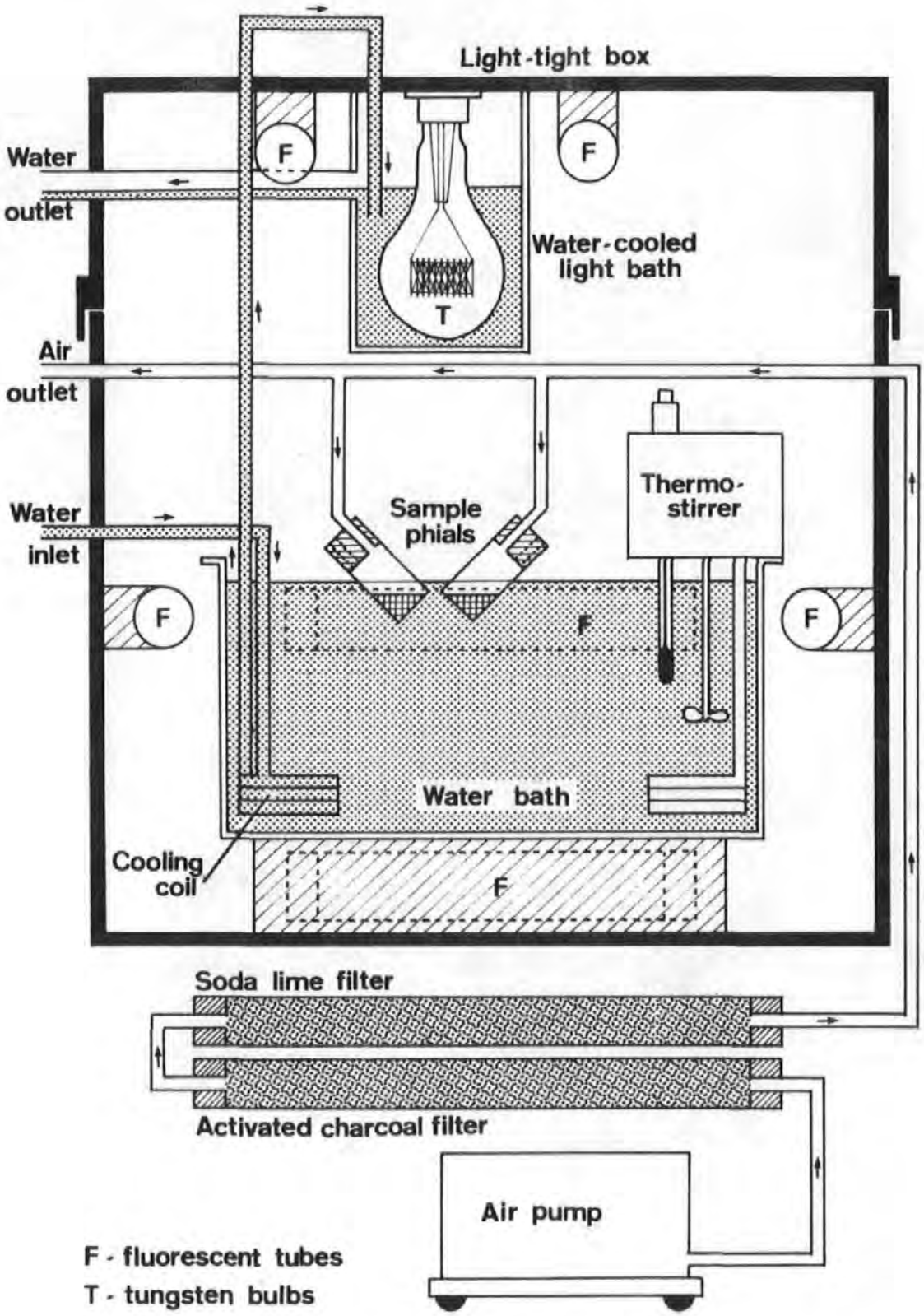

Light-tight box
Water
outlet
F
F
Water-cooled
light bath
T
Air
outlet
Thermo-
stirrer
Water
inlet
Sample
phials
F
F
F
Water bath
Cooling
coil
F
Soda lime filter
Activated charcoal filter
Air pump
F - fluorescent tubes
T - tungsten bulbs

Fig. 2.2.

Diagram of the porometry circuit.

A. Air pump (Charles Austen Pumps Ltd., Byfleet, Surrey; DYMAX mk. II).

B. Winchesters used to even out the air flow.

C. Simple screw valve to adjust the applied air pressure to 300 Pa.

D. Activated charcoal filter.

E. Silica gel filter.

F. Glass wool filter.

G. Simple manometer to monitor applied air pressure.

H. Individual glass valves used during calibration.

K. Nylon gas valves used during calibration.

L. Fine control needle valves (Edwards High Vacuum, Crawley, Sussex; model LB1B). L_{cal} used to calibrate the capillary resistances (R); L_W used in conjunction with the Wheatstone bridge circuit.

M. Concordia solenoid valves operated by the cam-timer (Elremco, Harlow, Essex; type D63). The valves connect each porometer cup to the manometer (N) for 233 s in every 20 minutes, and short circuit the system for 100 s, once every 20 minutes to provide a base line on the print out.

N. Electromanometer (Mercury Ltd., Glasgow; type 7M, F.S.D. 30 mm water)

P. Glass valve connecting Wheatstone bridge circuit to the resistances.

Q. Null point manometer filled with Kreb's fluid and liquid paraffin. This compound manometer is 5,15 times more sensitive than a simple manometer. R_W - Wheatstone bridge resistance.

R. Capillary resistances approximating 0,1 (a), 1 (b), and 10 (c) Gregory and Pearse resistance units.

S. Glass valve used during operation of the Wheatstone bridge circuit.

T. Reservoir of Krebs fluid used to adjust height of meniscus.

U. Glass valve used to connect flow meter (V) to the circuit.

V. Flow meter constructed out of a 100 cm^3 burette.

W. Perspex porometer cups.

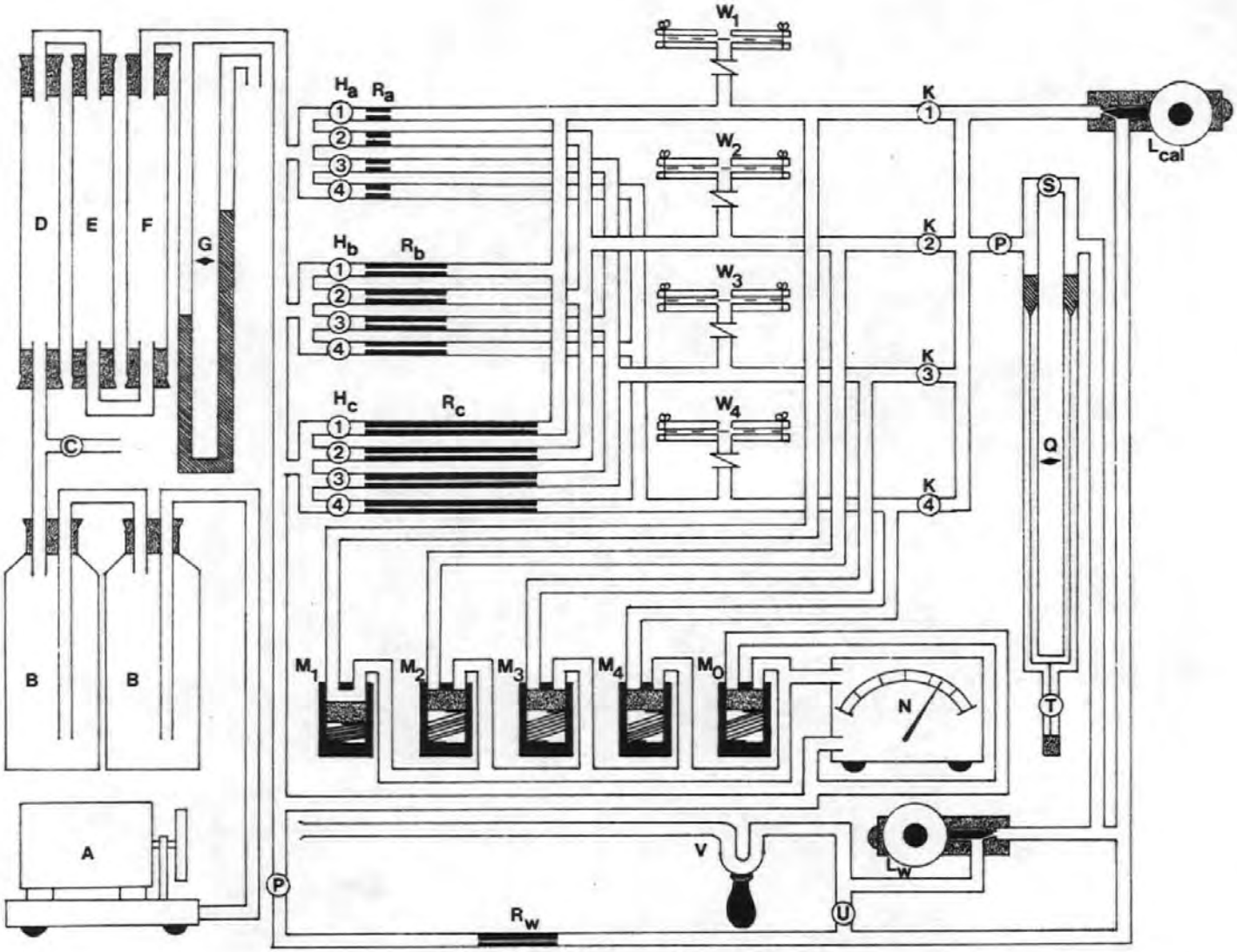

D
E
F
G
C
B
B
A
P
H_a R_a
H_b R_b
H_c R_c
1
2
3
4
W_1
W_2
W_3
W_4
K
1
2
3
4
L_{cal}
S
P
Q
T
M_1
M_2
M_3
M_4
M_0
N
V
L_w
U
R_w

Fig. 2.3.

Circuit diagram of the amplifier unit used in porometry.

The electromanometer (Mercury 7M, Glasgow; F.S.D. 30 mm of water) linked to the Minicomp DN/192 pen recorder (Hartmann & Braun, Frankfurt, W. Germany) via an amplifier powered by a A15 stabilsed power supply unit (Farnell Instruments Ltd., Wetherby, Yorkshire).

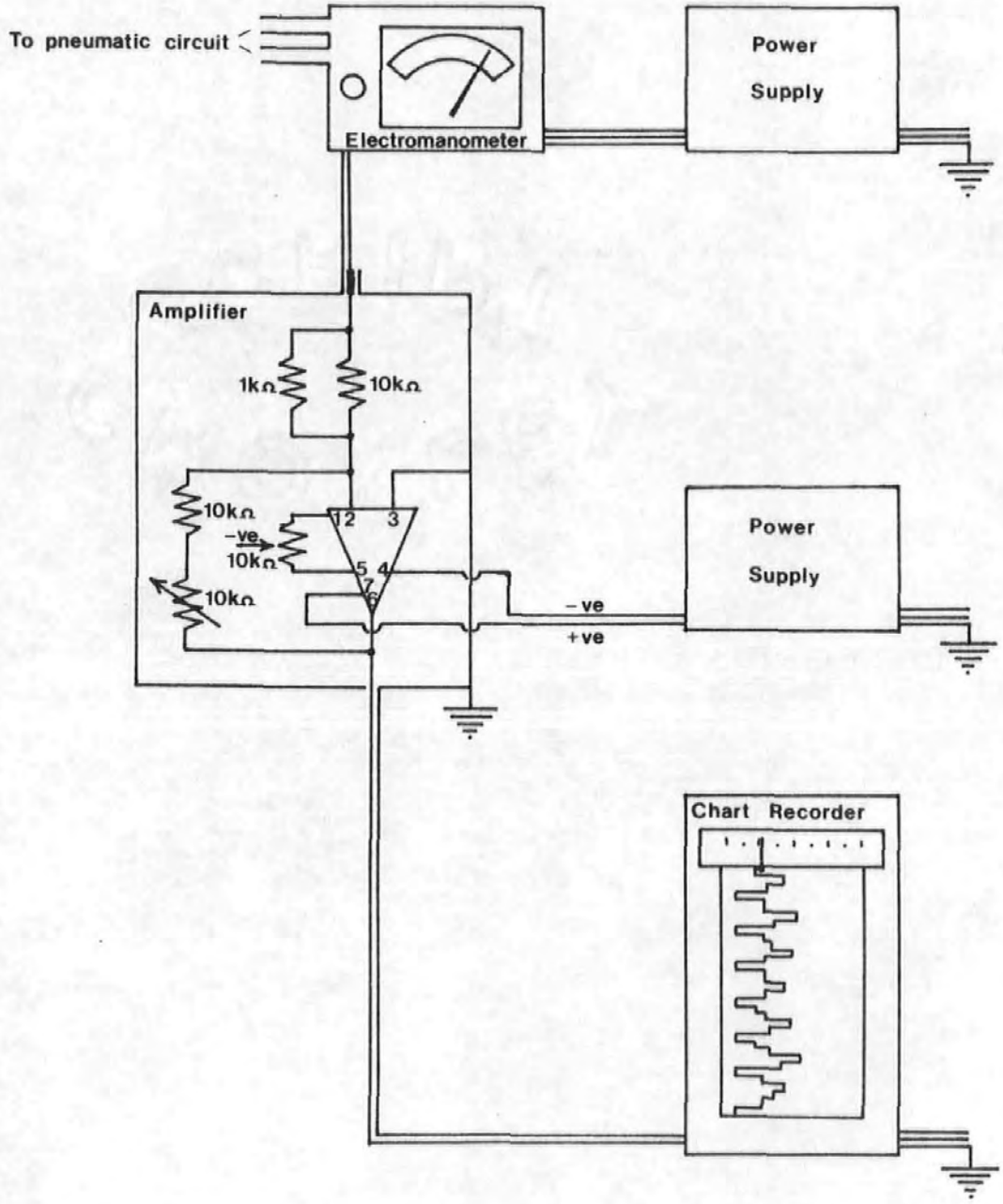

To pneumatic circuit
Electromanometer
Power
Supply
Amplifier
1kΩ
10kΩ
10kΩ
-ve
10kΩ
10kΩ
12
3
5
4
7
6
Power
Supply
-ve
+ve
Chart Recorder

Fig. 2.4.

Diagram of porometer cup used on Commelina communis.

a. Vertical section through cup; actual size.

b. Plan view of upper detachable-half of porometer cup; actual size.

c. Plan view of lower fixed-half of porometer cup; actual size.

Note how the air is passed through the adaxial epidermal surface with the midrib of the leaf accomodated in a groove set in the silicon rubber* seal which forms an air-tight porometer cup/leaf interface. The wing nuts are spring-loaded to minimise leaf damage. The two halves of the porometer cup chamber are interconnected in the upper (adaxial) half of the porometer cup which is sealed externally with a glass cover slip. The lower (abaxial) chambers of the cup are not sealed off.

*'Lastic 55' (A. Kettenbach, Eschenburg, W. Germany.)

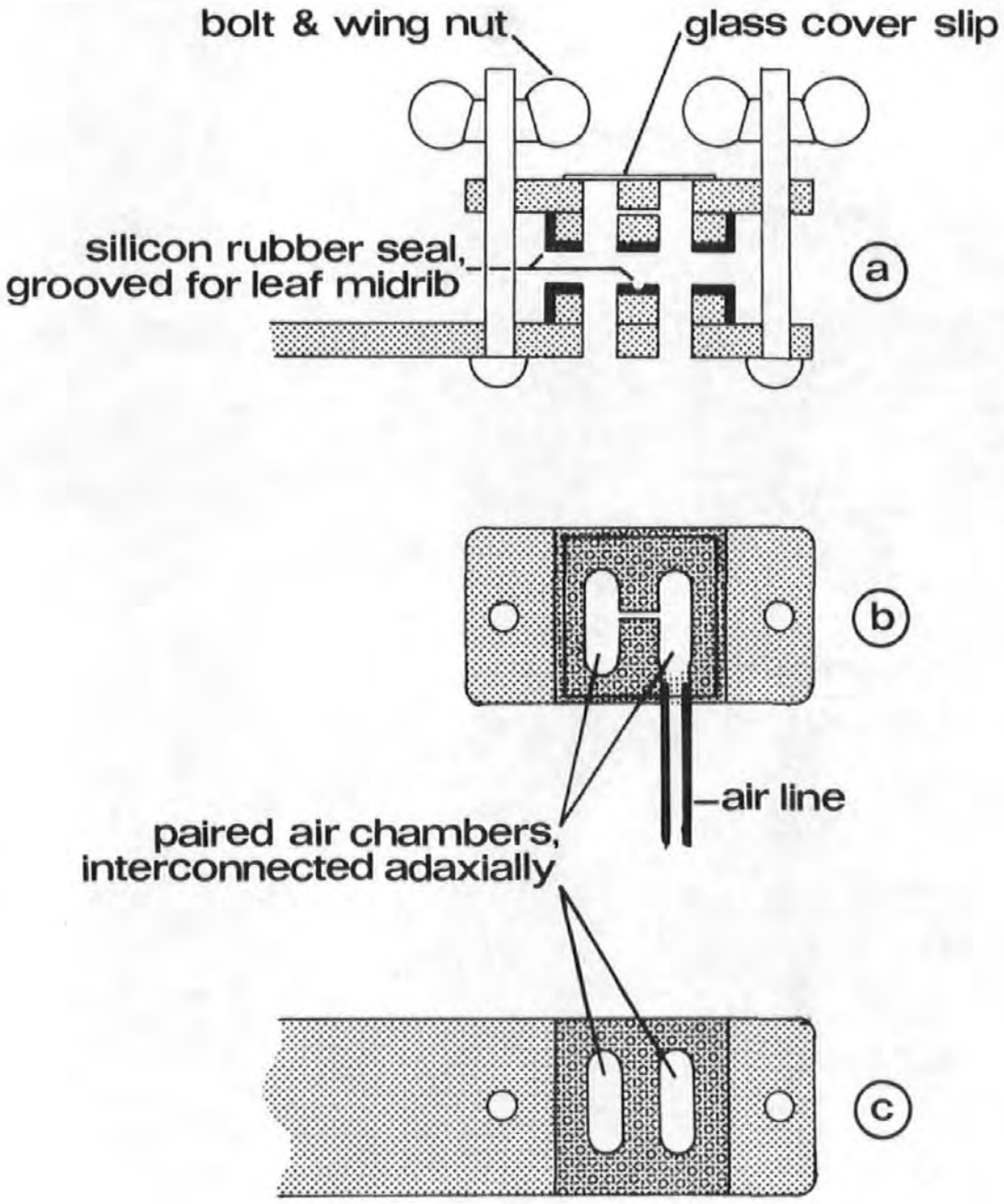

bolt & wing nut
glass cover slip
silicon rubber seal,
grooved for leaf midrib
a
b
air line
paired air chambers,
interconnected adaxially
c

Plate 2.1.

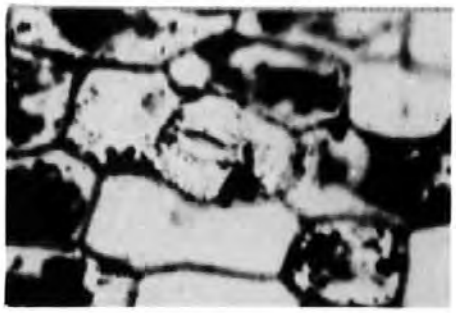
The effect of different washing programmes and sodium cobaltinitite concentrations on epidermal tissue of Tradescantia pallidus.

The tissues in vertical row A were prewashed in ice-cold water for 30 s, followed by a 0,5 h wash in ice-cold water after the cobaltinitrite treatment, and completed with a 60 s agitated wash in ice-cold water after the ammonium sulphide treatment. The tissues in vertical row B were treated in the same way except that the final wash was done in absolute ethanol. In vertical column C, the first two washes were in absolute ethanol and the final one in ice-cold water. In vertical row D, all the washes were carried out in absolute ethanol.

The tissues in horizontal row 1 were treated with 40% cobaltinitrite, those in row 2 with 20%, those in row 3 with 10%, those in row 4 with 5%, and those in row 5 with 2%. Staining time was 0,5 h.

It is noticeable that those tissues whose final wash was carried out in absolute ethanol show very heavy superficial deposits of cobalt precipitate whilst those whose final wash was in water do not. This suggests that the final wash in water removed superficial cobalt which may become adsorbed onto the surface of the tissue during the cobaltinitrite treatment.

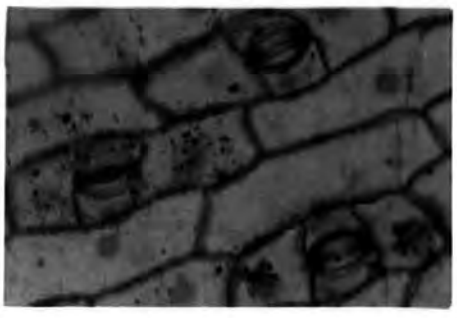
Light micrographs, x 160.



1

2

3



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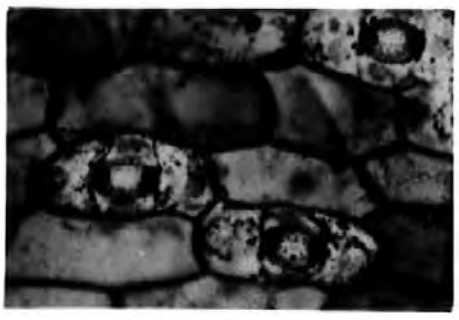

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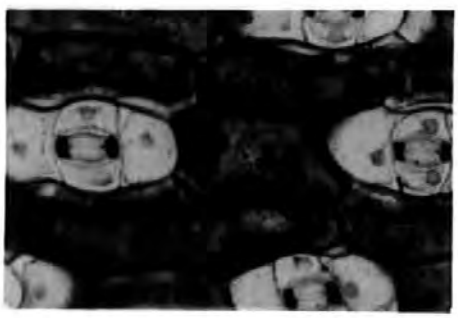
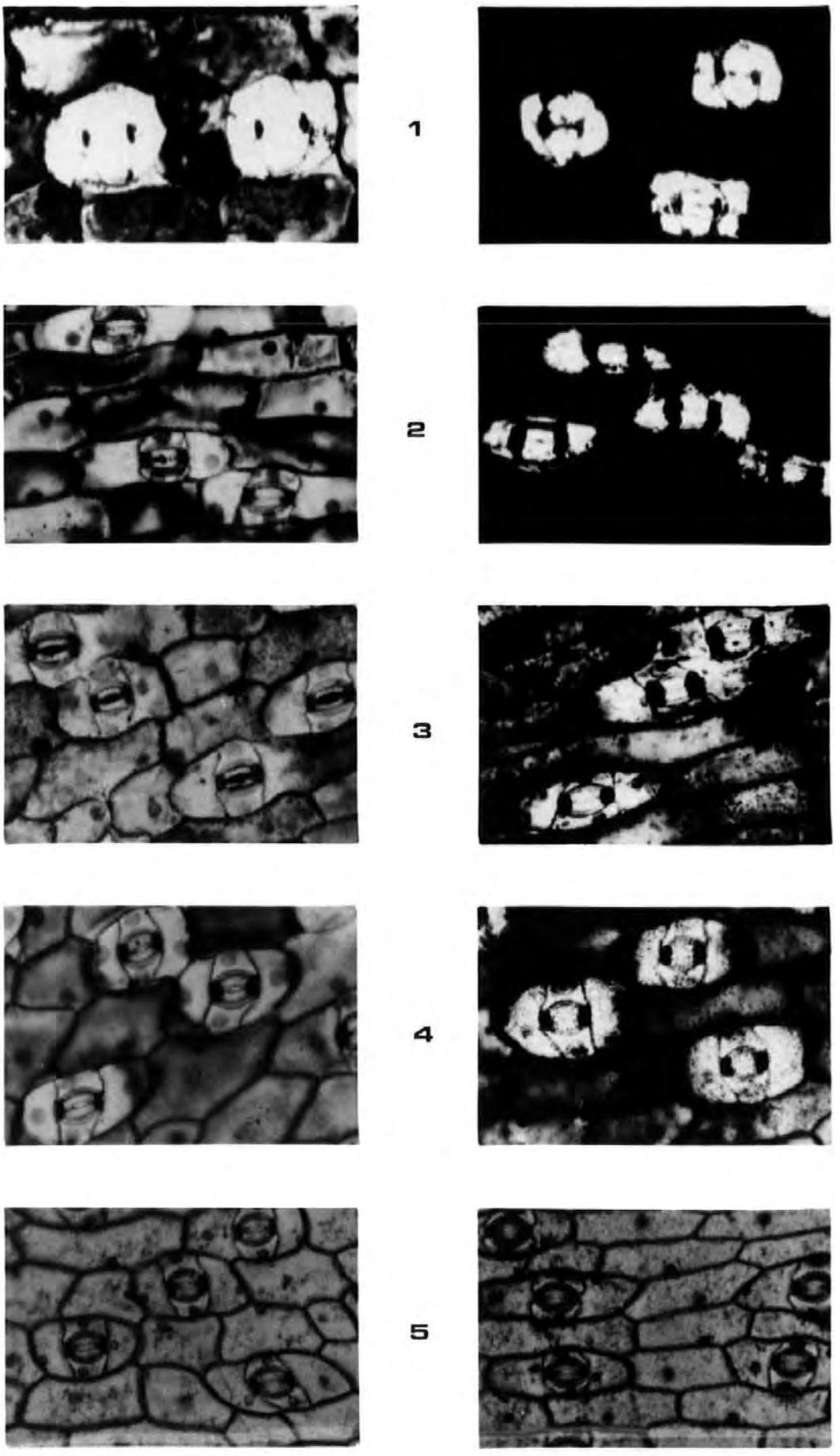
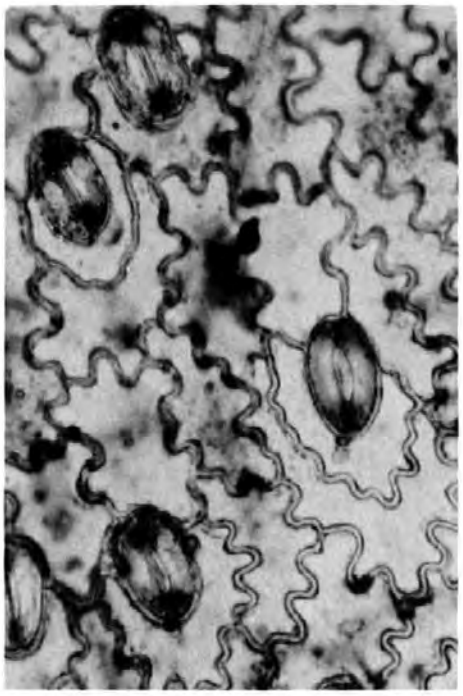


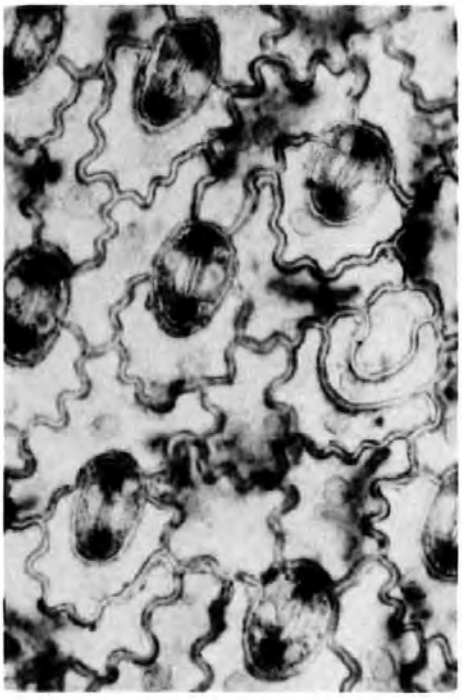
A

B

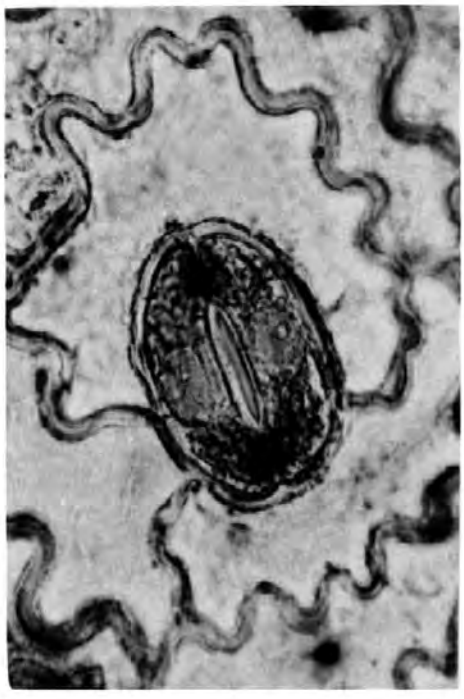

1
2
3
4
5
C
D

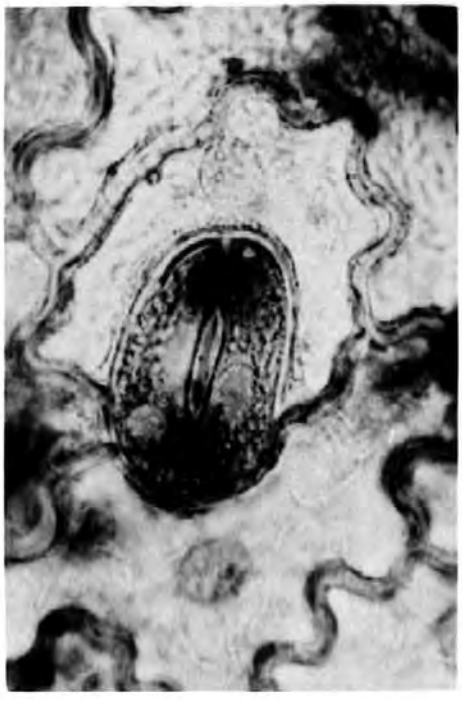


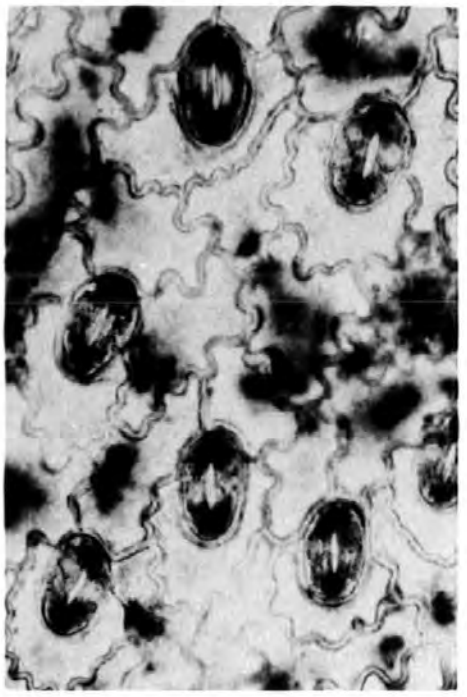
A



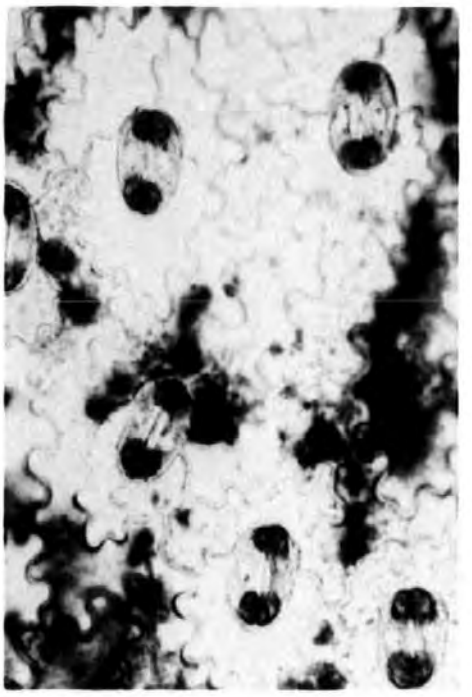
B



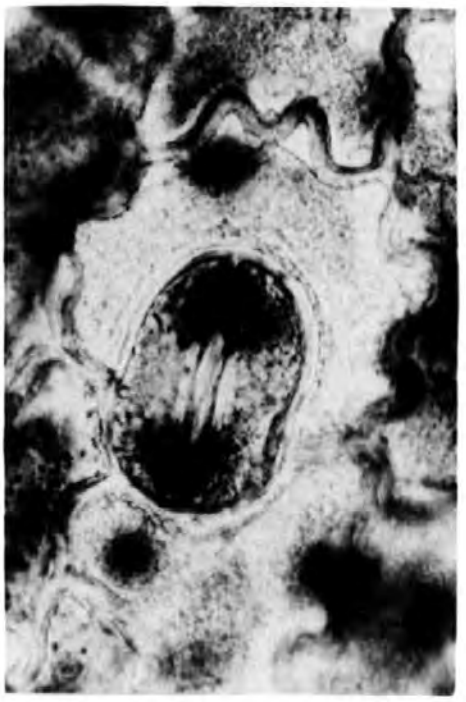




C



D



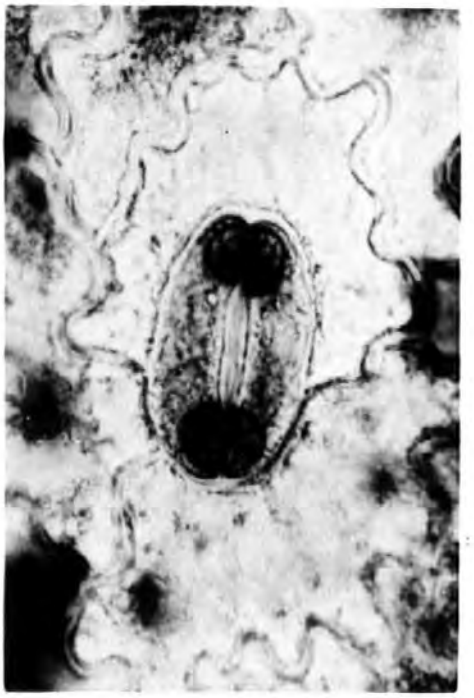
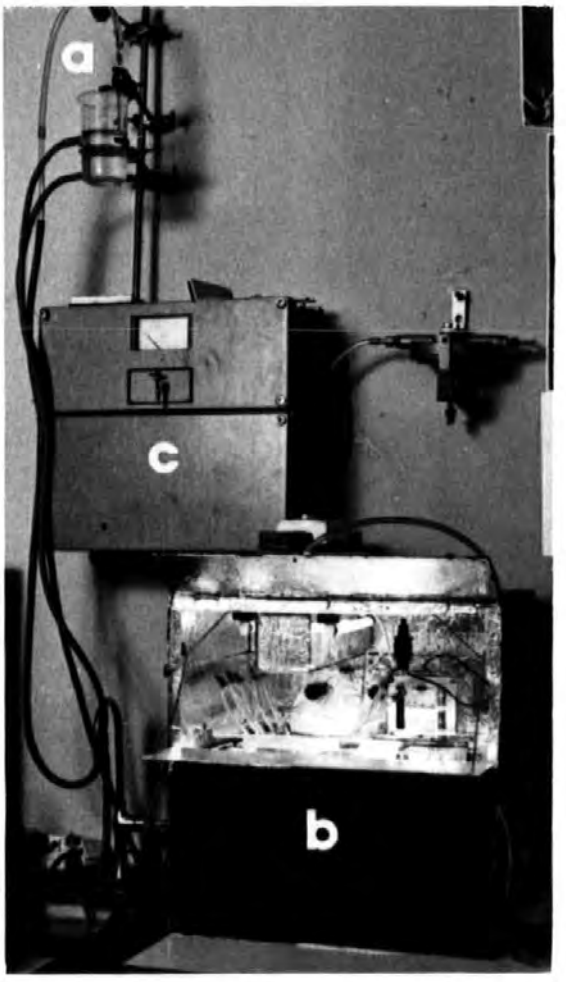


Plate 2.3.

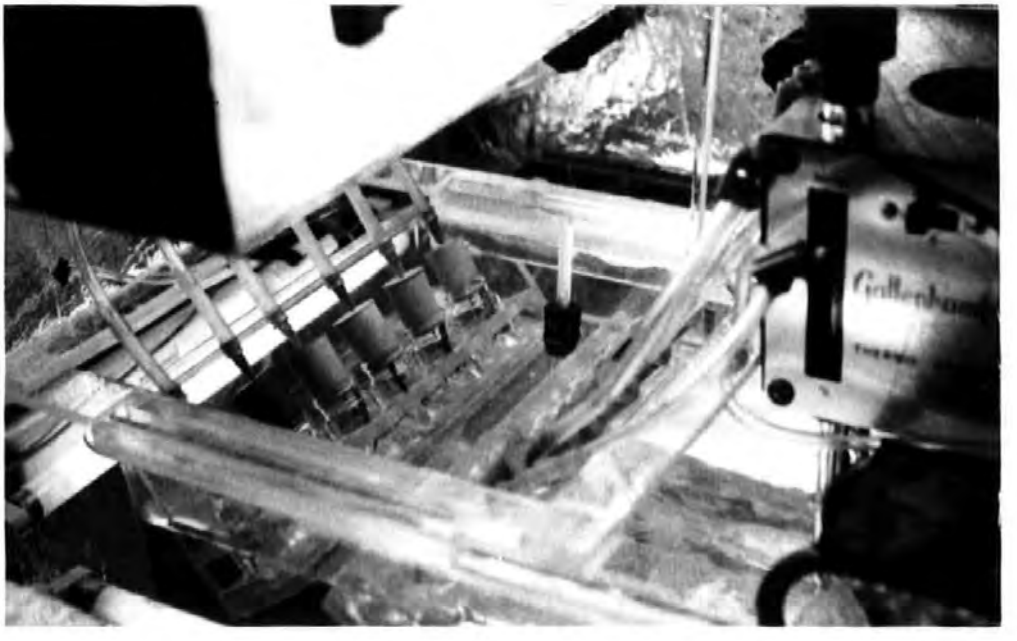
Water bath used in physiological investigations.

A. The equipment in use in the laboratory showing the constant head device (a) supplying water at a constant rate to the cooling coil and tungsten light bath housed in the main unit (b) which contains the water bath in which the experimental phials are incubated. The infra-red gas analyser (c) is incidental.

B. Close up of the water bath illustrating the perspex holder containing the experimental phials.



A



B

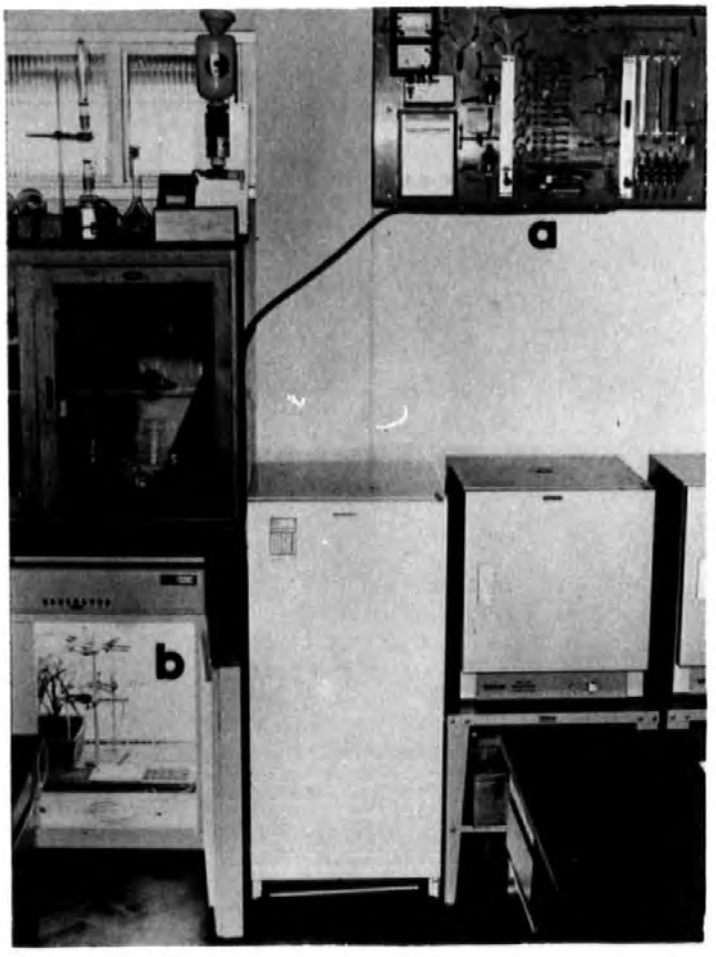
Plate 2.4.

Viscous flow porometry.

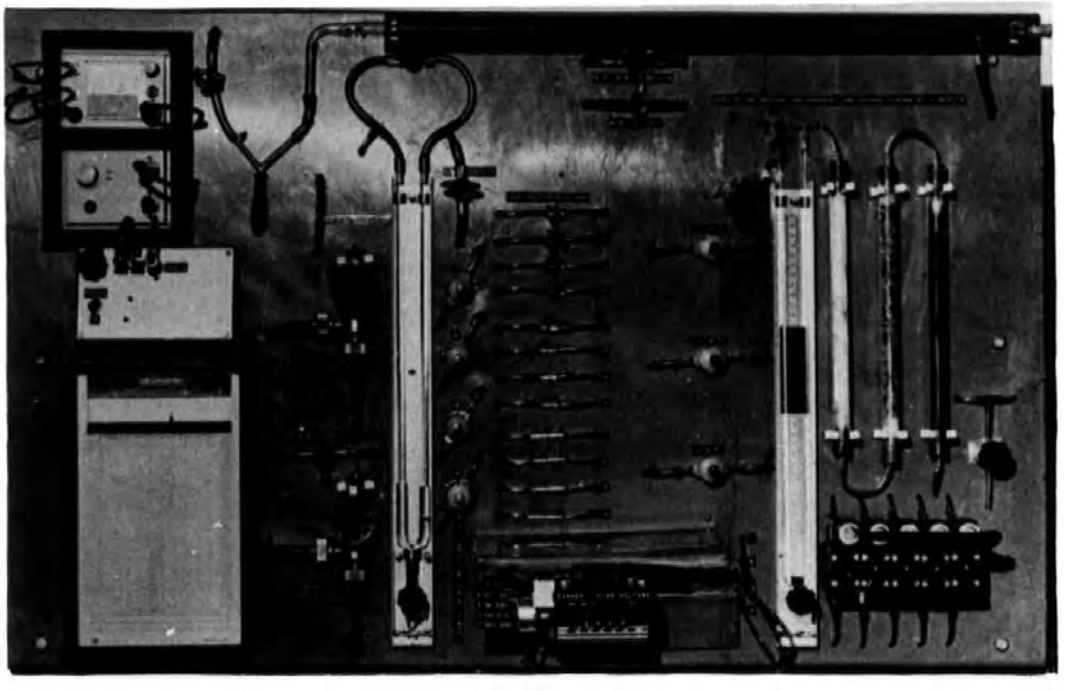
A. The equipment in use in the laboratory showing the porometry circuit board (a) connected to the porometer cups attached to Tradescantia x andersoniana plants in a Fisons growth cabinet (model no. 140G2) (b).

B. The porometry circuit board. The transducer and recording systems are housed on the left hand side of the board; the capillary resistances (3 x 4) are in the centre of the board above the cam-timing unit. The Wheatstone bridge manometer and needle valves are sited between the resistances and the chart recorder. The solenoid cut-out valves are at the bottom right hand corner of the board.

This photograph is of a prototype circuit which was later modified.



A



B

CHAPTER 3

LIGHT MICROSCOPE STUDIES

INTRODUCTION

This study arose from a chance observation that Macallum's histochemical stain for potassium (Macallum, 1905) gave a polar stain localisation (hereinafter referred to as p.s.l.) at both ends of guard cell complexes in Polypodium vulgare. The p.s.ls. were characterised by their discreteness and unique site of precipitation within the guard cell complex. Repeated treatments of P. vulgare epidermal strips, from a variety of sources within the environs of Plymouth, with Macallum's stain indicated that the p.s.ls. were consistent features of the stomatal complexes in this species. However, the original stain, as formulated by Macallum (1905), produced rather variable results and was consequently modified, as detailed in Chapter 2, to give more consistent results.

OBSERVATIONS IN Polypodium vulgare

Modified Macallum's histochemical stain

Epidermal strips were removed from the abaxial surface of pinnae, treated with Macallum's histochemical stain for potassium (Macallum, 1905), and observed under the light microscope, normally

from the side adjacent to the mesophyll.

The stain precipitates in all the ordinary epidermal cells, especially in the supra-fascicular region, but only reacts weakly, if at all, in the subsidiary cells and other epidermal cells which abut onto the guard cell complex. Within the guard cell complex, the stain is precipitated as a near-black granular deposit into discrete subcircular localisations at either pole of the complex (Plates 3.1a & b). The individual p.s.ls. are of variable size but are commonly up to 25 μm in diameter. This variation in size occurs between the pair of p.s.ls. associated with a single guard cell complex, between the different complexes of a single plant, and between those of different plants. Within a single guard cell complex, the p.s.l. adjacent to the mesogenous subsidiary cell (vide Chapter 9) is usually larger than that at the opposite pole of the complex; a feature which will be discussed more fully in Chapter 10.

Close examination of individual p.s.ls. reveals that the stain has a bimodal distribution with a small darker central core surrounded by a more extensive paler outer region (Plate 3.1c). The difference between the two elements of a single p.s.l. is often very indistinct (Plate 3.1b), or even occluded (Plate 3.3a), by the density of the stain itself. This may be compounded by the dense aggregations of chloroplasts in the guard cell protoplast. The darker central body is sited immediately under the common anticlinal cell walls of the guard cells on the inner face of the complex, half-way between the polar extremities of the anticlinal cell walls and their bifurcation at the stoma. The central body has a diameter of ca. 10 μm which remains fairly constant between the different complexes of a single plant as well as between those of different plants. The less intensively

staining periphery of the structure is very variable in size, and it is this element of the p.s.l. which is responsible for the great differences observed in the sizes of the p.s.ls.

The p.s.ls. appear to be in a very superficial position on the inner face of the guard cell complex as evidenced by fine focussing of the microscope, and the fact that they conceal the underlying anticlinal cell walls when observed from the side adjacent to the mesophyll. Further evidence of their superficial nature is found in a small proportion of complexes where the margins of the p.s.ls. extend beyond the polar limits of the guard cell complex (Plates 3.2A & B). In the cases illustrated, the p.s.ls. clearly subtend the common anticlinal cell walls of the complex and extend beyond its polar cell walls by ca. 5 um.

A very large proportion of the stained complexes are characterised by densely-staining strands radiating out from the p.s.ls. and traversing the peripheral anticlinal walls of the guard cell complex (Plate 3.3A). These strands are not unique to the stomatal complex and can be found in very reduced numbers between ordinary epidermal cells. The strands are ca. 0,7 µm in diameter and variable in length. The strands, like the p.s.ls., appear to be situated in a very superficial position below the guard cell complex.

In a small proportion of guard cell complexes observed, the paler peripheral element of the p.s.l. appears to take the form of an inflated sac-like structure (Plate 3.3B). This is invariably the case in those complexes in which the margins of the p.s.ls. extend beyond the guard cell walls at the poles.

The delimitation of p.s.ls. is most clearly demonstrated in

unstained epidermal strips. In such tissue, the polar structures appear to be bi-lobed so that a single p.s.l. is divided longitudinally into two halves in a vertical plane below the common anticlinal cell walls of the guard cell complex (Plate 3.4A). The discreteness of the two halves of each p.s.l. is illustrated in Plate 3.4B, in which one of the guard cells has been damaged, probably during preparative procedures, with the result that the delimiting 'membranes' of its polar structures have been ruptured and their contents dissipated, whilst the structures associated with the undamaged guard cell have maintained their integrity. This characteristic is further illustrated in Plate 3.4C, in which the guard cell complex is made up of both a living and a dead guard cell; the polar structures are associated only with the living half of the complex.

It is interesting that the p.s.ls. found in living guard cell complexes are also commonly found in dead complexes. Typically, p.s.ls. in dead complexes are similar to those in living ones in that they possess both the darker central element of the structure, the paler peripheral element, and the characteristic strands traversing the outer anticlinal walls of the complex (Plate 3.5A). The bimodal pattern of the p.s.ls. is more clearly demonstrated in dead complexes since the stain appears to be precipitated in a translucent brown sheet which is enhanced by the absence of chloroplasts in the underlying dead cell. The extent of the pale outer element of the p.s.ls. is even more variable in dead complexes than in living ones and can be extremely extensive. The structures of one pole can fuse with those of the opposite pole (Plate 3.6A), or can be completely lacking (Plates 3.5B & C), although in the latter cases, the darker central element often persists. As in living complexes, the p.s.ls. are bilobed with each lobe being

independant of each other (Plate 3.5B). The superficial nature of the structures in relation to the guard cell complex is further evidenced by their clear extension into the lower cuticular lips of the stoma and further on down into the stomatal throat in many dead guard cell complexes (Plate 3.6B).

Unstained epidermal studies

Fresh unstained epidermal strips of Polypodium vulgare were dry-mounted, without a coverslip, on double-sided transparent adhesive tape on a microscope slide. The material was than examined, from the side adjacent to the mesophyll, using a combination of epi-illuminescence and transmitted light to obtain the best possible contrast in surface features.

Epidermal tissues prepared in this way consistently exhibit swellings on the undersurface in the polar regions of the guard cell complexes (Plate 3.7A). In certain complexes, and especially those which have become partially dehydrated on the microscope stage, the polar swellings are much less pronounced and often reveal an underlying discoid body. The body is ca. 10 μm in diameter and lies in a position which corresponds to that of the darker central core of the p.s.ls. formed as a result of treatment with Macallum's stain (Macallum, 1905) (Plate 3.7B). The potassium-rich strands demonstrated with the Macallum technique appear as membranous folds on the inner face of the complex (Plate 3.7B).

OBSERVATIONS IN *Tradescantia* spp

Modified Macallum's histochemical stain

Two species of *Tradescantia*, *Tradescantia x andersoniana* and *Tradescantia pallidus*, have been used extensively in the present study. *T. pallidus* exhibits the clearest and most consistent p.s.ls. of any plant species examined (*vide* Chapter 8).

In both *Tradescantia* spp., the p.s.ls. conform to those found in *Polypodium vulgare* with the exception that the stain precipitates in a more uniform manner and rarely exhibits a bimodal staining pattern (Plates 3.8 & 3.9A). The darker-staining central area is apparently absent. Another difference is that the potassium-rich strands found to radiate out from the p.s.ls. in *P. vulgare* are not present in *Tradescantia* spp., although non-staining strands extend between the polar extremities of the p.s.ls. and adjacent polar subsidiary cells (Plate 3.9B). As in *P. vulgare*, the p.s.ls. are of a very superficial nature on the inner face of the guard cell complex and can extend beyond and below the polar cell walls of the complex (Plate 3.9B). An occasional feature noted in some submature stomatal complexes of *T. pallidus* is an additional amorphous element of the p.s.ls. lying immediately between the standard element of the p.s.l. and the guard cells, which extends laterally into the intercellular region between the polar and lateral subsidiary cells (Plate 3.9C).

The p.s.ls. show a variable reaction to the Macallum stain (Macallum, 1905) if the guard cells or their associated subsidiary cells have been damaged during removal of the epidermis from the leaf. Damaged regions are almost always confined to torn or cut peripheral areas

of epidermal peels. Usually damaged stomatal complexes fail to develop p.s.ls., but in some cases they will even when the guard cell complex has been mutilated prior to staining (Plate 3.10A). Once the epidermis has been stained, mutilation of guard cell complexes did not result in the disappearance of the p.s.ls., even when the p.s.ls. were directly affected (Plates 3.10B & C).

An interesting feature observed in Tradescantia, but not in Polypodium vulgare, is the development of multi-laminate p.s.ls. (Plate 3.11A), in which the stain appears to have been precipitated in up to five superimposed sheets. The individual sheets vary in extent with the result that staggered margins are visible at that end of the p.s.l. proximal to the stoma. Close examination of the margin adjacent to the polar subsidiary cell shows that the edges of the individual lamellae have a stacked vertical relationship (i.e. they are not staggered), and that those associated with the localisation in one guard cell are not contiguous with those in the adjacent guard cell; the break occurring below the common anticlinal guard cell walls (Plate 3.11B).

An interesting feature observed in Tradescantia spp. is the occurrence of cobalt-rich vesicles in the subsidiary cells of some tissues. The vesicles are more numerous in the polar subsidiary cells and are generally situated deep within the protoplast. They are usually associated with fine cobalt-rich strands which interconnect adjacent vesicles, radiate out into the surrounding protoplast, and occasionally can be seen to communicate directly with the general region of the p.s.ls. (Plate 3.12). These structures were the only evidence obtained that cobalt, from the Macallum stain, can be precipitated in a non-granular form in unequivocably intracellular

locations, in what appear to be small vacuoles.

Heavy metal staining in Tradescantia x andersoniana

A physical characteristic of the polar structures is their ability to adsorb a variety of ions from the transpiration stream (vide Chapter 11). The following observations were made on epidermes removed from excised leaves of Tradescantia x andersoniana which had been fed with 7,5% aqueous solution of cupric sulphate through their cut ends for 16 hours at 5 400 lx. The copper was visualised optically by a post treatment of 5% ammonium sulphide.

Cupric ions fed into the leaves generally accumulated in discrete localisations (as in the left hand localisations illustrated in Plate 3.13). The p.s.ls. differ somewhat from those obtained with the Macallum technique (Macallum, 1905) in that a bimodal staining pattern, similar to that in Polypodium vulgare is present. The darker-staining element is restricted to the extreme pole and lies between the paler-staining element and the guard cell. The polar structures can extend laterally into the region between the polar and lateral subsidiary cells as found in submature Tradescantia pallidus (Plate 3.13A, c.f. Plate 3.9C). Each p.s.l. in T. x andersoniana appears to be composed of a pair of elements, as in P. vulgare, each of which is associated with its adjacent guard cell. An extreme case of dimorphism between the two elements of a single pole is illustrated in Plate 3.13B. Not infrequently one, or even both, of the elements in a single p.s.l. fails to adsorb the heavy metal as in Plate 3.13C, where one element of the structure has adsorbed the cupric ions whilst the other has not, although it is possible to determine the rather vague outlines of the latter.

DISCUSSION

Relationship of present findings to previous studies

The light microscopical studies indicate that an unique localisation of potassium occurs in the polar regions of the guard cells in certain plants. The p.s.ls. are believed to be a manifestation of an hitherto unknown stomatal structure. There are no reports in the literature concerning the potassium localisations reported on here despite extensive studies on potassium fluxes within the stomatal complex carried out using the Macallum's histochemical stain (Macallum, 1905). Careful examination of the numerous illustrations of epidermes treated with Macallum's stain in the literature often reveals the presence of the p.s.ls., in relevant species (vide Chapter 8), although they are frequently hidden by more general precipitation. The best published illustration is probably that of Commelina communis (Squire & Mansfield, 1972, Plate 2.1a). However, the earliest reference to p.s.ls. in guard cells is that of Drawert (1942) who reported on, and illustrated, the accumulation of toluidine blue at the poles of the guard cells complexes in Tradescantia virginica (sic.[1] = virginiana = x andersoniana). Neither he, nor later workers, pursued these findings (vide Chapter 7).

Yamada et al. (1966) reported on the presence of ion-binding sites on isolated cuticular surfaces of astomatous epidermes of ripe tomato fruit and stomatous epidermes of onion leaves. They found that the inner cuticular surface had a much greater ion-binding capacity than the outer surface and, in onion epidermes, ions were bound preferentially to a sac-like structure on the inner cuticular

surface subtending the stomata. Their findings must be treated with some caution since it would appear that they assumed they were dealing with a single cuticular sheet. In fact, they were probably comparing the outer surfaces of two cuticles - one being the classical cuticle which commonly covers the outer epidermal surfaces in most plants (= their outer surface), and the other being the endocuticle (= their inner surface) which is, perhaps, not fully recognised by some workers. Evidence of an inner cuticle, or endocuticle, will be discussed in Chapters 4 & 5. The substomatal bag they refer to on the inner surface of the cuticle is probably referable to a sac-like structure which is contiguous with the endocuticle below the stomata, and could be formed from the endocuticular lining of the substomatal chamber. In any case, the single bag they report on as being associated with each stomatal complex is not compatible with the paired polar structures associated with each stomatal complex found in the present study. Further confirmation that the structures reported on by Yamada et al. (op. cit.) are not analogous with those discussed here, is the absence of p.s.ls., or for that matter substomatal bags, when onion epidermes were treated with the Macallum stain.

The presence of 'polar vesicles' in the guard cell complexes of Casuarina were reported on by Rehfous (1917), and similar structures from the stomata of Psilotum by Zimmermann (1926). Casuarina has recently been re-examined by Pant et al. (1975) who consider the structures to result from differential cutin deposition in the polar regions of the guard cells, forming distinct lamellae. A similar conclusion was reached in the present study during the examination of Bowenia stomata which would also appear to have distinct polar structures formed by lamellar protruberances on their internal faces.

Differential thickening of cell wall / cutin elements at the poles of guard cells may be a common feature of the more primitive woody plants, as they were also found in Ginko biloba. In the case of Psilotum, it is believed that Zimmermann's report (op. cit.) of stomatal anomalies in the polar regions may be a case of misinterpretation caused by the dumbell-shaped lumen of the guard cell. Sawyer (1932) reports that the thickened anticlinal walls of the guard cell complex in Vaccinium spp. gives rise to the appearance of polar pouches. A re-examination of this feature in V. myrtillus indicates that the pouches are formed by the subsidiary cells completely subtending the guard cell complex except in the polar regions. Differentially-thickened polar guard cell walls have been reported from a variety of species by Raju et al. (1975), but whilst the present study confirms their existence under the light microscope, transmission electron microscopy does not support this interpretation. It is believed that the cell walls concerned are not actually thickened, but that they appear so under the microscope due to the refraction of light as it passes through the length (depth) of the vertical cell walls.

Formulation of models of polar structures

From the light microscope studies on the p.s.ls. in Polypodium vulgare and Tradescantia spp., it is possible to draw up hypothetical models of the polar structures.

There can be little doubt that the polar structures are extracellular since evidence obtained here precludes the possibility of their inclusion within the guard cell protoplast. The p.s.ls. must, therefore, be formed within, or on the face of, the lower periclinal guard cell walls, and within any space that might exist

between these walls and the underlying endocuticle, or else they must be directly incorporated into the endocuticle.

The evidence obtained in this study strongly suggests that two differentially-staining elements are involved, a smaller dark-staining body of comparatively uniform size, and a paler-staining peripheral element which is very variable in size. The evidence in the case of T. pallidus is rather weak but it is suggested that the density of the reaction obtained with the Macallum stain (Macallum, 1905) may have masked the presence of the smaller body. The fact that the darker-staining element is of constant size suggests that it may be either a solid structure or a discrete specialised area of the cell wall. Careful focussing with the microscope indicates that this body lies between the paler-staining element and the guard cell wall. The above features are common to both P. vulgare and Tradescantia spp.

The nature of the more extensive paler element of the structures varies between Polypodium and Tradescantia. In the case of P. vulgare, the evidence indicates that this element may take the form of a swollen sac, a feature which has not been observed in Tradescantia. If this element of the structure does take the form of a substomatal sac, and there is strong evidence for this supposition (Plate 3.7), it must almost certainly be contained in an elastic element of the guard cell. This material requirement for the structure must preclude the cell walls themselves, since although they do have an elastic capability, it could hardly seem possible that they could accomodate such a distension as indicated in Plate 3.3B. A more logical location for this sac-like structure is a space between the endocuticle and the lower periclinal cell walls of the guard cell complex.

In the case of Tradescantia spp., evidence of substomatal swellings is lacking. However, a multilaminate staining pattern was revealed in T. pallidus (Plate 3.11). To maintain a multilaminate structure, it is necessary to separate the successive layers in some way, and the only known structures in the polar regions of the guard cells which could perform this function are the lower periclinal cell walls. To accomodate a series of plate-like structures it would be necessary for the cell wall in this region to have a laminate structure itself. Although there are no reports in the literature of cell walls containing intra-mural cavities, it is established that cell wall elements are laid down sequentially during cell wall formation (Esau, 1965). However, Chafe & Wardrop (1972) have shown that the periclinal walls of epidermal tissue from a variety of angiosperms are composed of successive layers of longitudinally-orientated microfibrils alternating with transversely-orientated microfibrils with the latter layers in continuum with the microfibrillar orientation of the anticlinal walls. The microfibrillar layers were considered to be laid down in an appositional manner so that successive lamellae were not bonded together as they would be if the layers were laid down intususceptively. Chafe (1970) noted that the longitudinal lamellae in collenchyma walls contained a higher pectin content than the transverse lamellae, but Chafe & Wardrop (op. cit.) were unable to confirm this feature in epidermal cell walls although they did detect a weak lamellar deposition of pectin within the walls.

The other main difference between the polar structures in Polypodium and Tradescantia concerns the darker-staining central body. In the case of P. vulgare, the evidence suggests that there is a single central body whereas, in Tradescantia, the evidence supports

there being a pair of bodies. However, it would seem reasonable to assume that there is an intimate relationship between the darker- and lighter-staining elements of the structure. In both P. vulgare and Tradescantia, there are examples where one half of a single p.s.l. is developed to greater extent than the other, which may well suggest that the darker central body actually consists of a pair of bodies in both genera. In Polypodium, the two bodies may be very intimate causing them to appear as a single unit under the light microscope.

Hypothetical model of polar structures in Polypodium

The hypothetical model for the polar structures in Polypodium vulgare is based on the discussions of the preceding section and is illustrated, diagrammatically, in Fig. 3.1. The darkly-staining central element is considered to be a solid discoid binding site situated on the outer face of the lower periclinal cell walls at the poles of the guard cell complex. The paler-staining peripheral element of the structure is believed to represent a substomatal polar sac, which is fluid-filled and capable of distension in nature, located between the lower periclinal guard cell walls and the endocuticle. The peripheral strands which traverse the outer anticlinal walls of the guard cell complex are interpreted as hollow cylindrical endocuticular trabeculae.

Hypothetical model of polar structures in Tradescantia

The proposed model for the structures in Tradescantia spp. (Fig. 3.2) is more tentative than that for Polypodium vulgare. The major problem is to determine the location of the darker-staining element of the structure. It has to be accepted that the darker-

staining elements (binding sites) in Tradescantia spp. lie between the guard cell protoplast and the paler-staining elements as observed by fine focussing of the microscope, and yet the only structure which can accomodate the multilaminate elements are the cell walls. This can only mean that the binding sites are subtended by the lower periclinal cell walls of the complex. Thus, the relative positions of the two elements of the structure are reversed in Tradescantia in respect of those in P. vulgare. However, the binding site in P. vulgare is undoubtably in a very superficial position on the outer surface of the cell wall. It is assumed that the binding sites in both Polypodium and Tradescantia serve similar functions, and it would consequently be more plausible if the sites were located similarly in both genera. The binding sites in Tradescantia have, accordingly, been provisionally located in 'pockets' in the lower regions of the polar anticlinal walls of the guard cells.

The only other major difference between the polar structures in the two genera is that Tradescantia lacks the endocuticular sacs of Polypodium. The paler-staining element of the structure is located in multilaminate intramural spaces in the proposed model for Tradescantia spp.

Fig. 3.1.

Hypothetical polar structures in Polypodium vulgare,

a. Plan view of the proposed structures. The binding sites are stippled heavily, the endocuticular sacs are stippled lightly. Axes b and c refer to the planes of section illustrated in Figs. b and c respectively. The polar structures on the left-hand side represent the inflated condition of the substomatal sacs, whilst those on the right-hand side represent the deflated condition.

b. Longitudinal vertical aspect of Figure a. The cell walls are stippled, and the binding sites are in solid tone.

c. Transverse vertical aspect of Fig. a, through the polar region. The cell walls are stippled, and the binding sites are in solid tone.

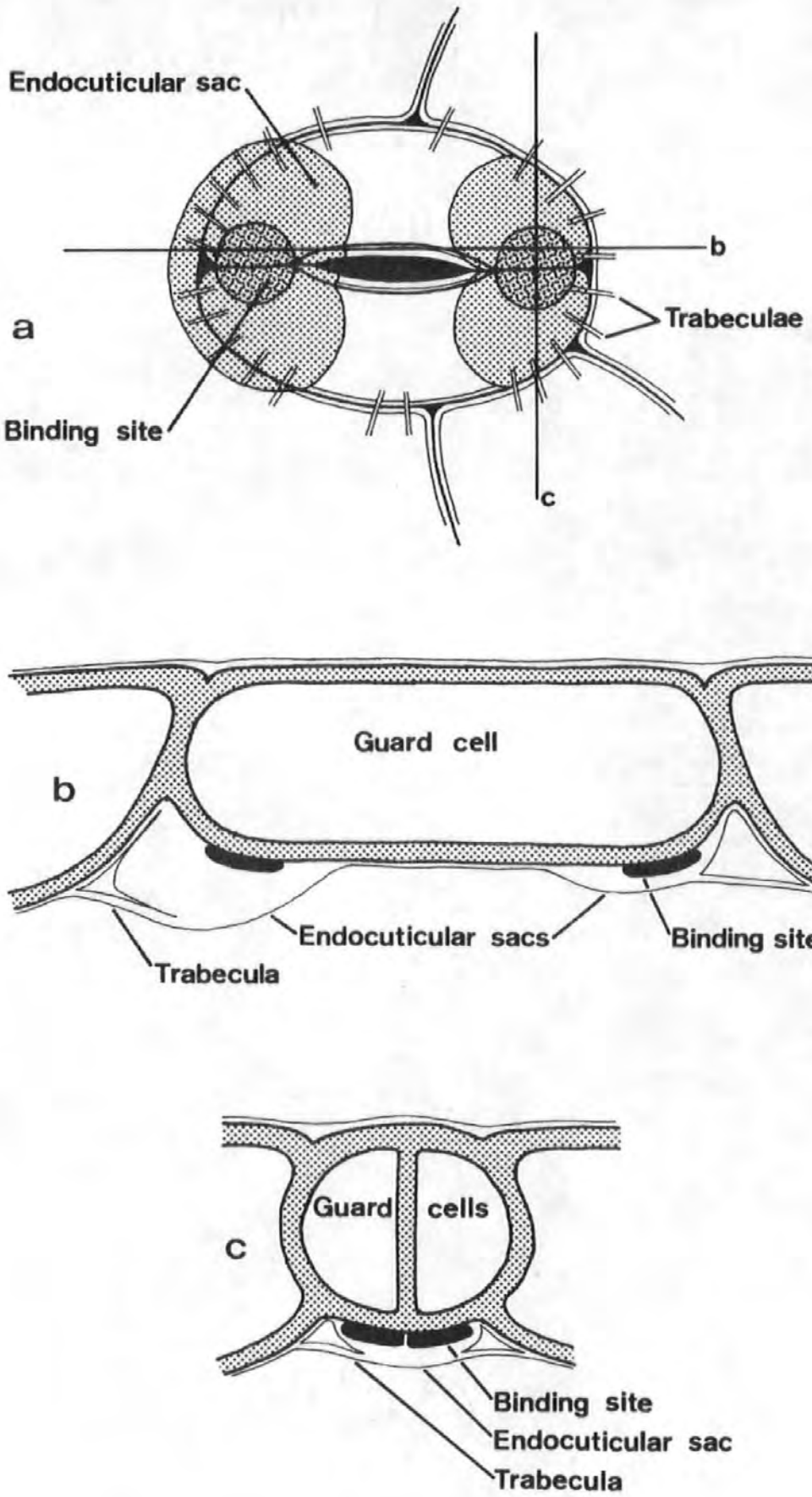

Endocuticular sac
b
a
Trabeculae
Binding site
c
Guard cell
b
Endocuticular sacs
Binding site
Trabecula
Guard cells
c
Binding site
Endocuticular sac
Trabecula

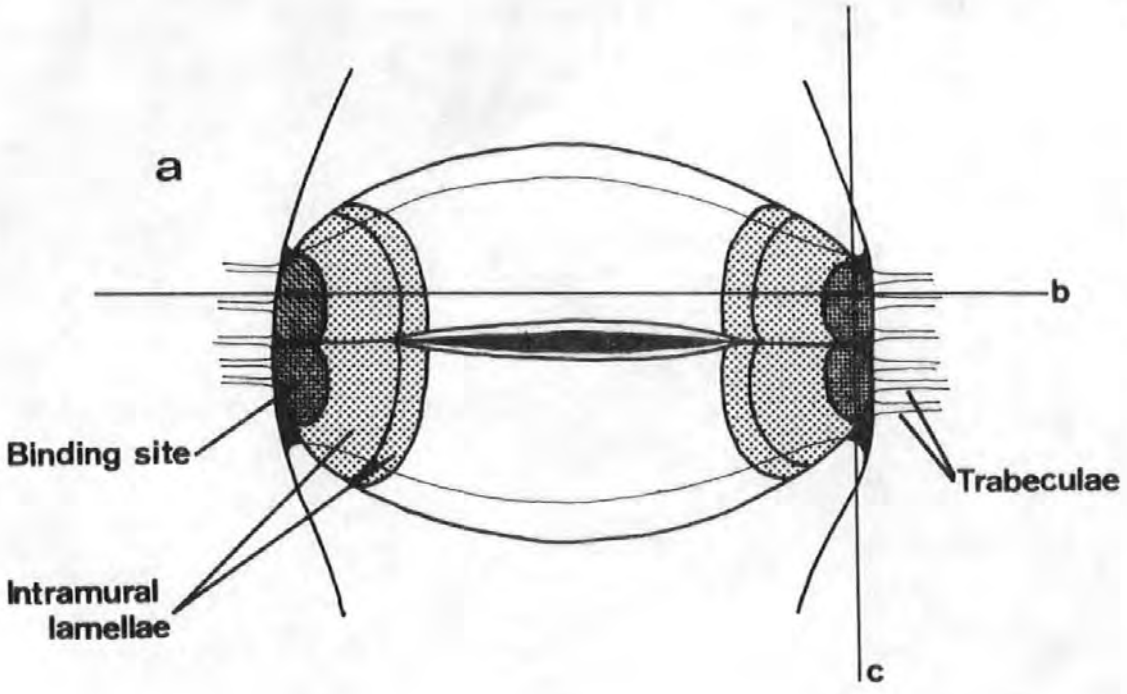
Fig. 3.2.

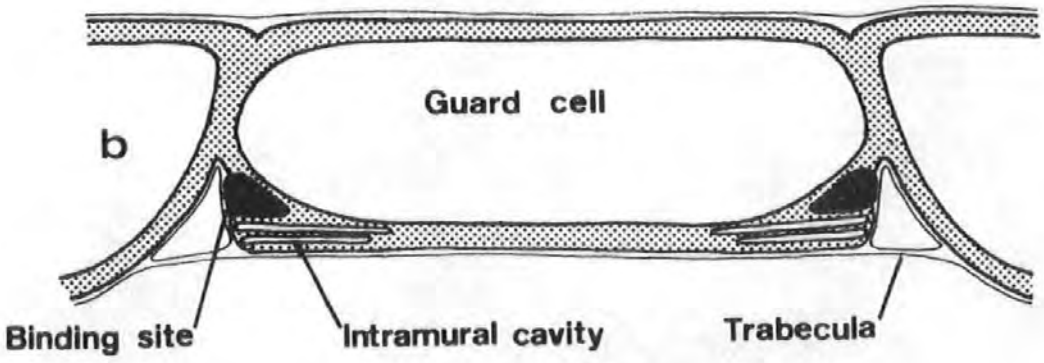
Hypothetical polar structures in Tradescantia spp.

a. Plan view of the proposed structures. The binding sites are stippled heavily, the intramural lamellae are stippled lightly. Axes b and c refer to the planes of sections illustrated in Figs. b and c respectively.

b. Longitudinal vertical aspect of Fig. a. The cell walls are stippled, and the binding sites are in solid tone.

c. Transverse vertical aspect of Fig. b, through the polar region. The cell walls are stippled, and the binding sites are in solid tone.


a
b
c
Binding site
Intramural lamellae
Trabeculae


b
Guard cell
Binding site
Intramural cavity
Trabecula

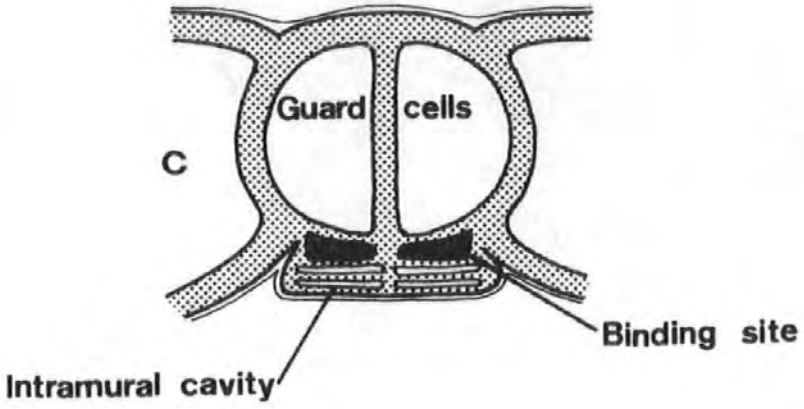

c
Guard cells
Binding site
Intramural cavity

Plate 3.1.

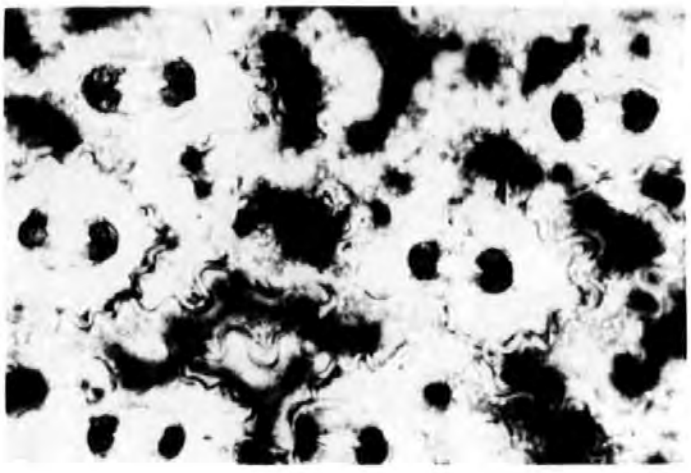
Polar stain localisations in Polypodium vulgare, I.

Epidermal strips stained with the basic Macallum stain and viewed from the side adjacent to the mesophyll.

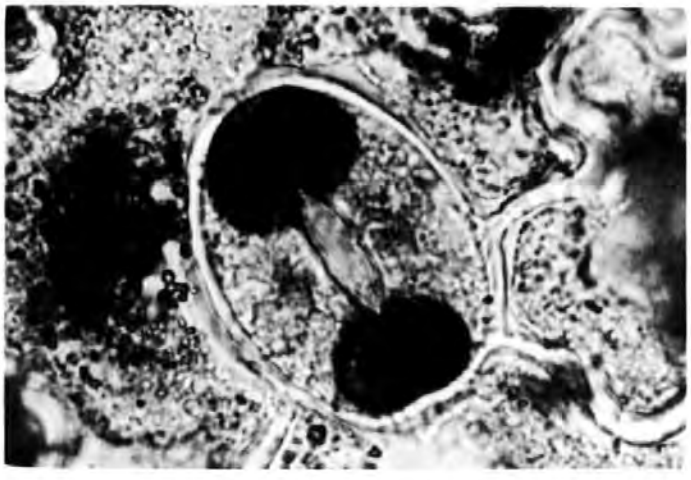
A. Low power view, x 250. Micrograph illustrates the typical distribution of the stain with the polar stain localisations in the guard cell complexes.

B. Polar stain localisations in a guard cell complex, x 1 000.

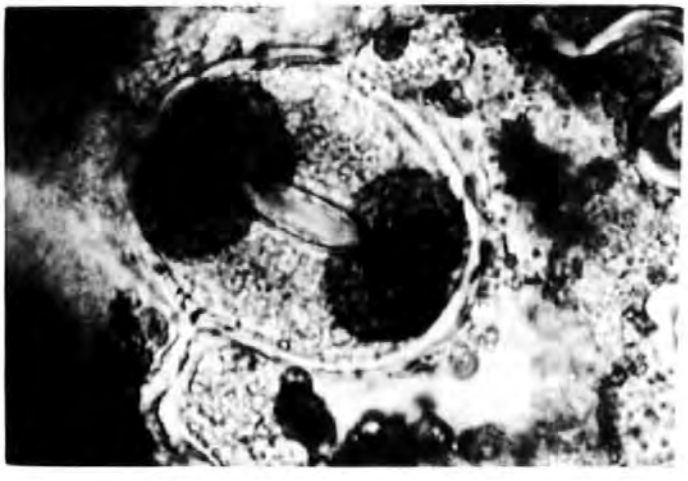
C. Single guard cell complex, x 1 000. The bimodal distribution of the stain can be seen within each polar stain localisation.



A



B



C

Plate 3.2.

Polar stain localisations in Polypodium vulgare, II.

Light micrographs of guard cell complexes treated with the basic Macallum stain, x 800.

A. Viewed from the external surface. The polar stain localisations can be seen to subtend and extend beyond the polar cell walls of the guard cell complex.

B. Viewed from the side adjacent to the mesophyll. The polar stain localisations can be seen to extend beyond the polar walls of the guard cell complex.

A

B

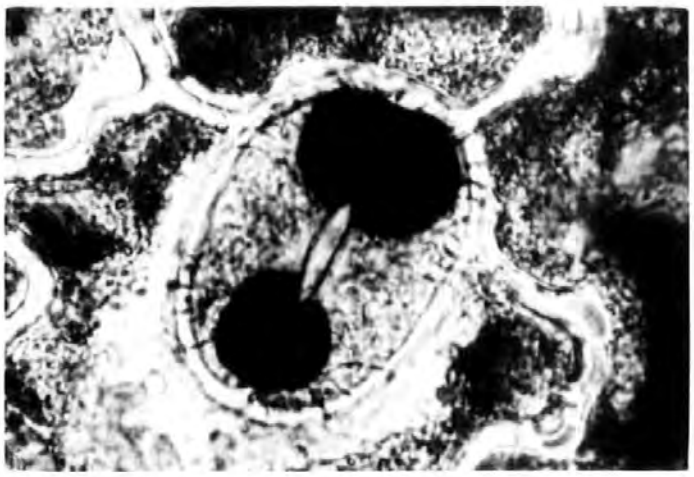
Plate 3.3.

Polar stain localisation in Polypodium vulgare, III.

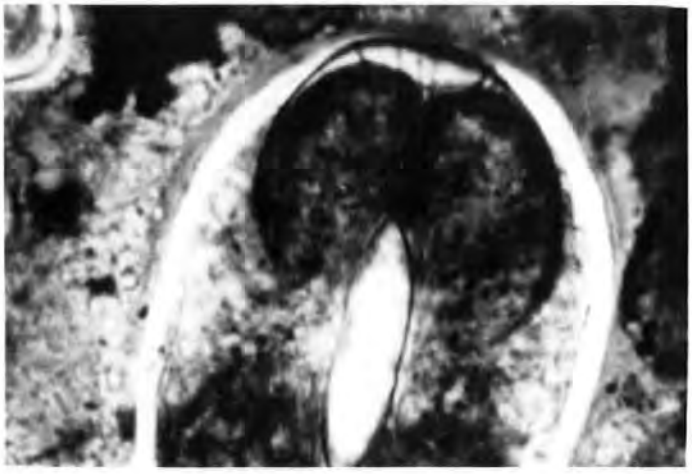
Light micrographs of stomatal complexes treated with the basic Macallum stain and viewed from the side adjacent to the mesophyll.

A. Guard cell complex, x 1000. Cobalt-rich strands can be seen to radiate out from the polar stain localisations and traverse the peripheral anticlinal walls of the complex.

B. Single polar stain localisation, x 1,700. This micrograph illustrates the swollen sac-like nature of the localisation.



A



B

Plate 3.4.

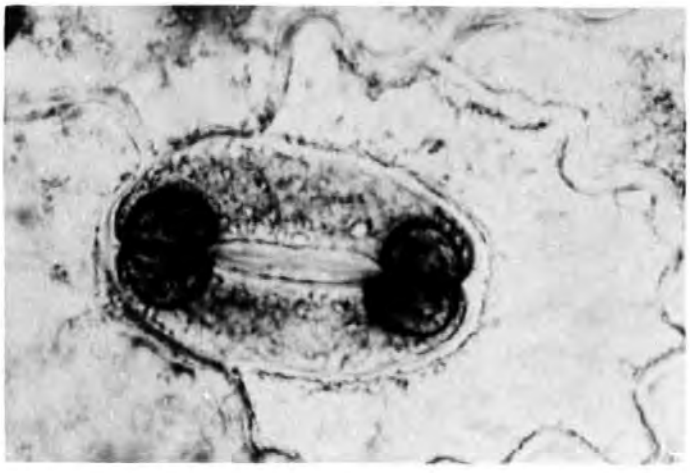
Polar stain localisations in Polypodium vulgare, IV.

Light micrographs of guard cell complexes treated with 7,5% (w/v) modified Macallum technique and viewed from the side adjacent to the mesophyll, x 1 000.

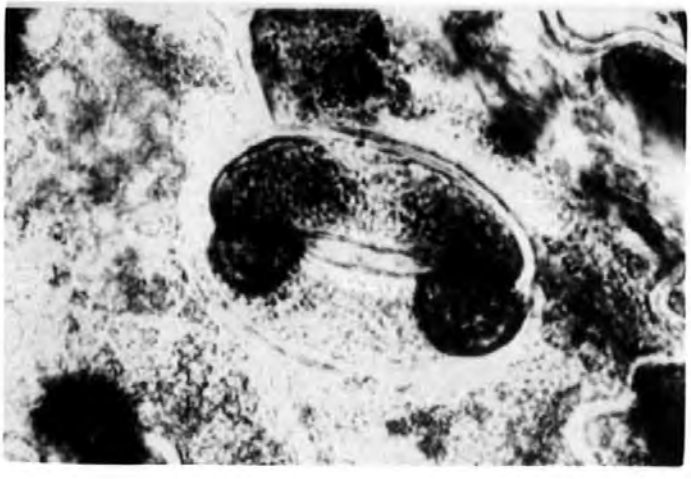
A. A guard cell complex showing the bilobed appearance of the polar localisations.

B. A guard cell complex which has apparently been damaged during preparation illustrating the discreteness of the individual polar localisations. The elements containing the localisations in the upper guard cell have been ruptured and their contents dissipated. The localisations in the lower guard cell have retained their integrity.

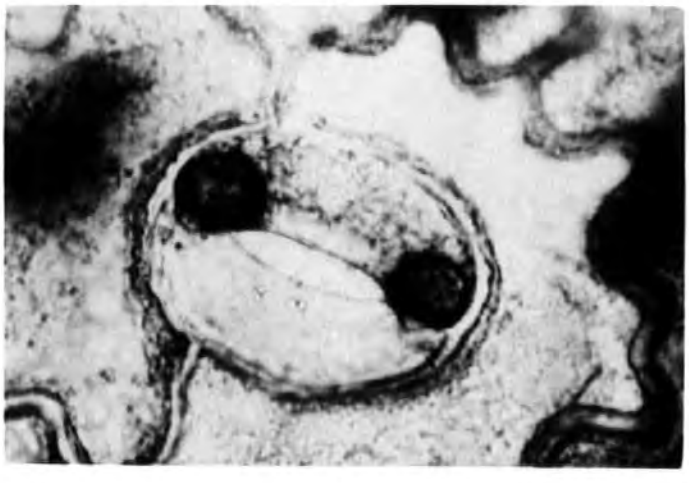
C. A guard cell complex in which the lower guard cell is apparently dead (i.e. it lacks a protoplast). The polar localisations are only associated with the living half of the complex.



A



B



C

Plate 3.5.

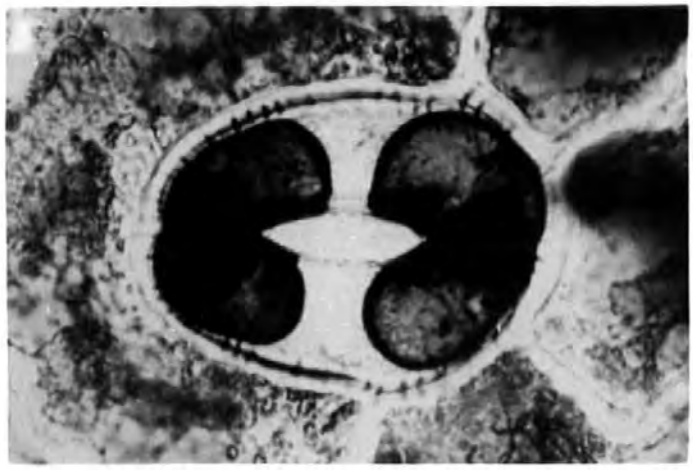
Polar stain localisations in dead guard cells of Polypodium vulgare, I.

Light micrographs of dead stomatal complexes stained with the basic Macallum stain and viewed from the side adjacent to the mesophyll, x 1 000.

A. A guard cell complex exhibiting very extensive polar stain localisations which have a distinct bimodal stain distribution. Note the cobalt-rich strands traversing the outer anticlinal walls of the guard cell complex.

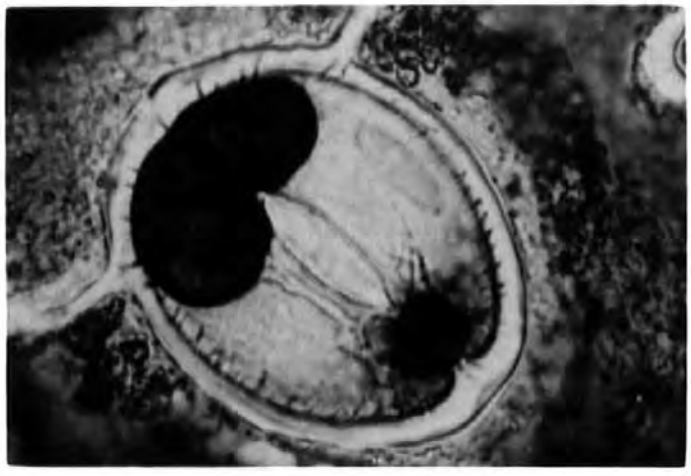
B. A guard cell complex illustrating a normal polar stain localisation at one end of the complex. The polar stain localisation at the other end shows the presence of only a single element.

C. A guard cell complex with a normal polar stain localisation at one end only. The small localisation at the other end is believed to represent the darker staining central element as found in localisations which exhibit a bimodal staining pattern as in A, above.



A

B



C

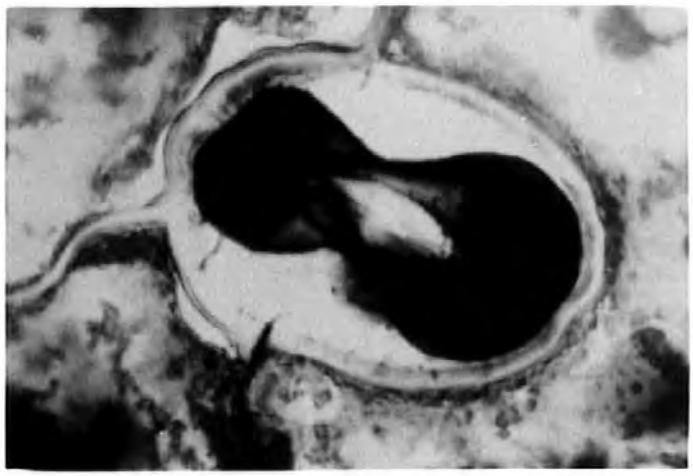
Plate 3.6.

Polar stain localisation in dead guard cells of Polypodium vulgare, II.

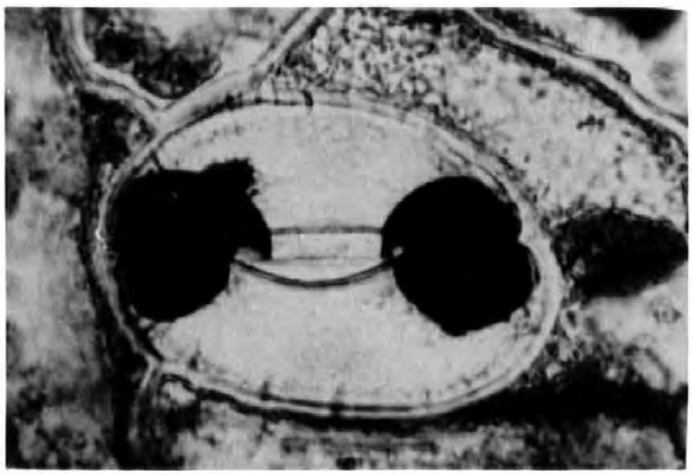
Light micrographs of dead guard cell complexes stained with 7,5% (w/v) modified Macallums reagent, viewed from the side adjacent to the mesophyll, x 1 000.

A. Guard cell complex with the polar stain localisations attached to each other in the region immediately adjacent to the stoma.

B. Polar stain localisations extending into the throat of the stoma.



A



B

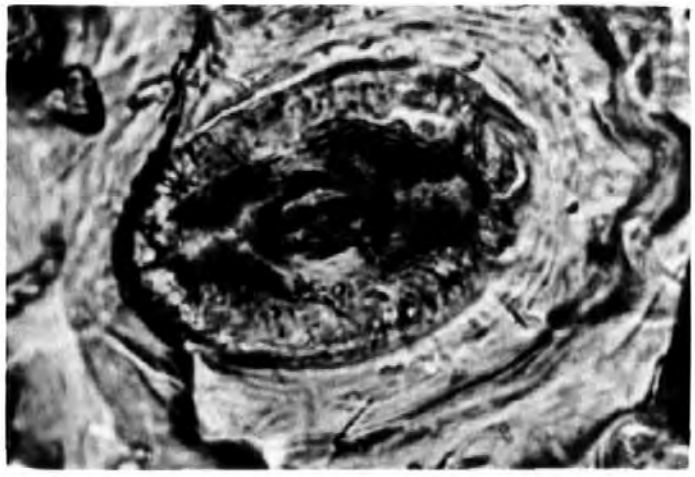
Plate 3.7.

Substomatal polar swellings in Polypodium vulgare.

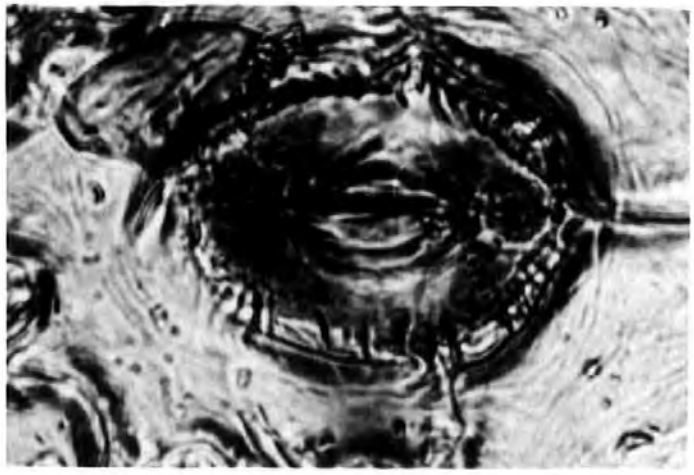
Light micrographs of epidermal tissue dry-mounted onto a slide with double sided adhesive tape. The tissue is unstained and viewed from the side adjacent to the mesophyll, x 850.

A. The guard cell complex exhibiting sac-like structures which correspond in position to the polar stain localisations.

B. This complex illustrates the strands radiating out across the outer anticlinal walls of the guard cell complex to the surrounding epidermal/subsidiary cells. As in A, above, the common anticlinal walls of the complex are not visible. Note the discoid body lying at the right hand pole of the complex.



A



B

Plate 3.8.

Polar stain localisations in Tradescantia pallidus, I.

Light micrograph of epidermal tissue stained with 7,5% (w/v) modified Macallum's solution and viewed from the side adjacent to the mesophyll, x 380.

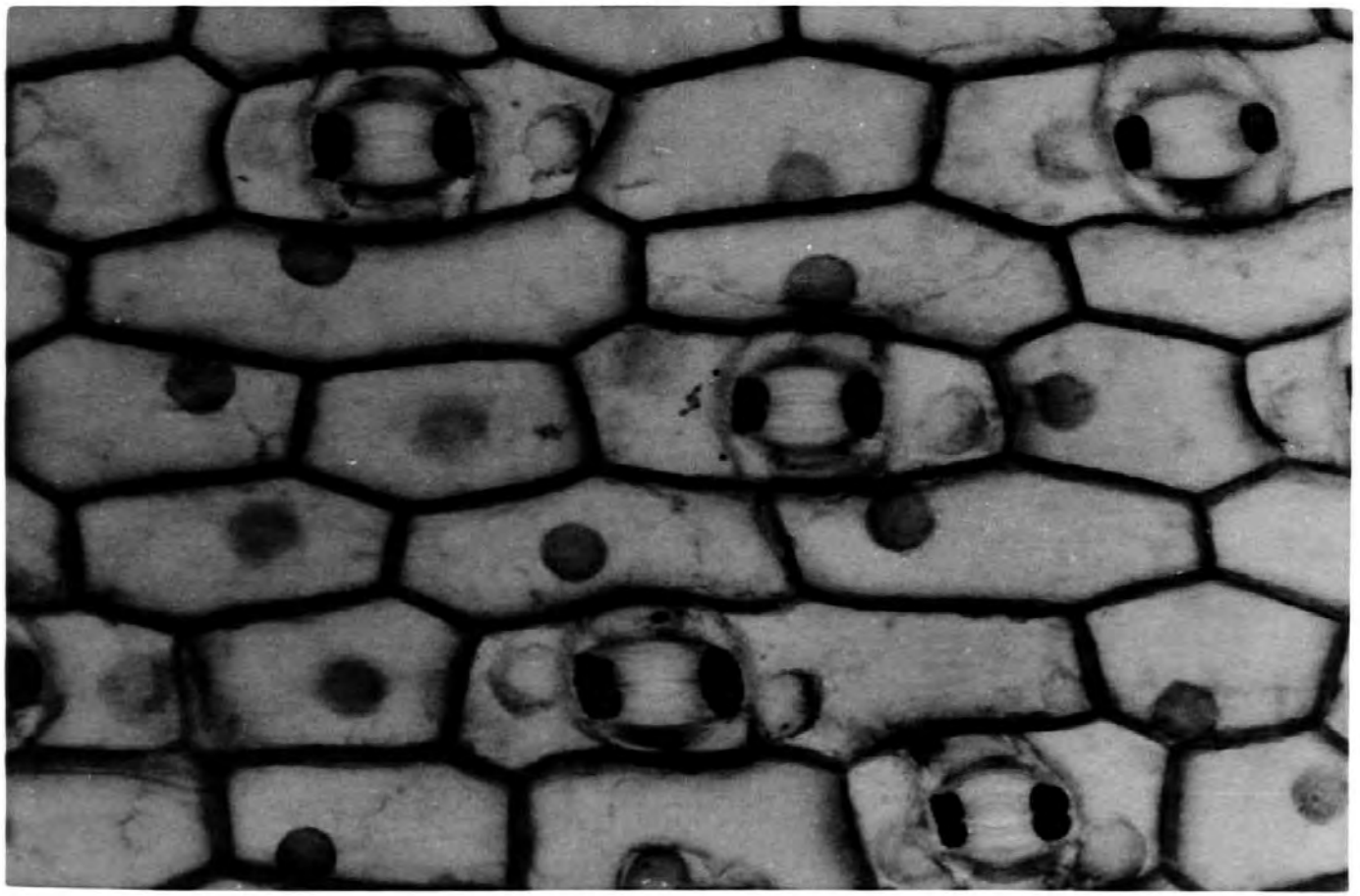


Plate 3.9.

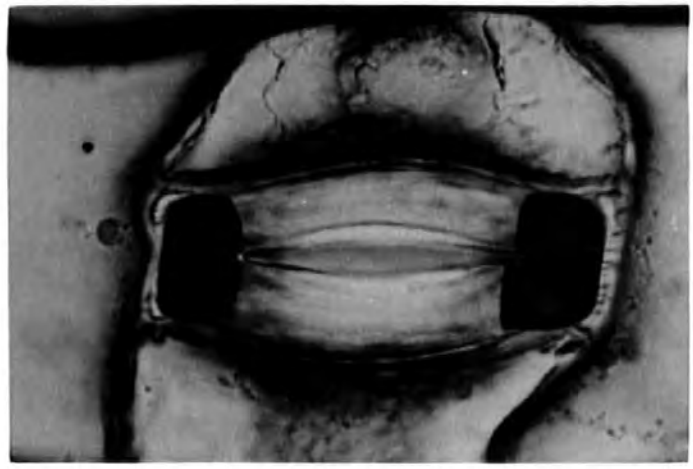
Polar stain localisations in Tradescantia pallidus, II.

Light micrographs of guard cell complexes atained with 7,5% (w/v) modified Macallum's reagent and viewed from the side adjacent to the mesophyll, x 1 000.

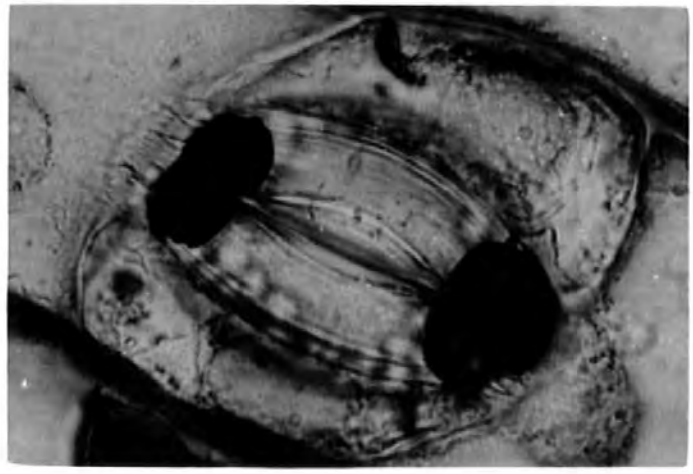
A. Typical polar stain localisations.

B. Polar stain localisation showing how they can extend beyond the polar anticlinal walls of the complex (right hand side localisation). Trabeculae can be seen extending from the localisations onto the polar subsidiary cells, but, unlike those of Polypodium vulgare, thay have not stained.

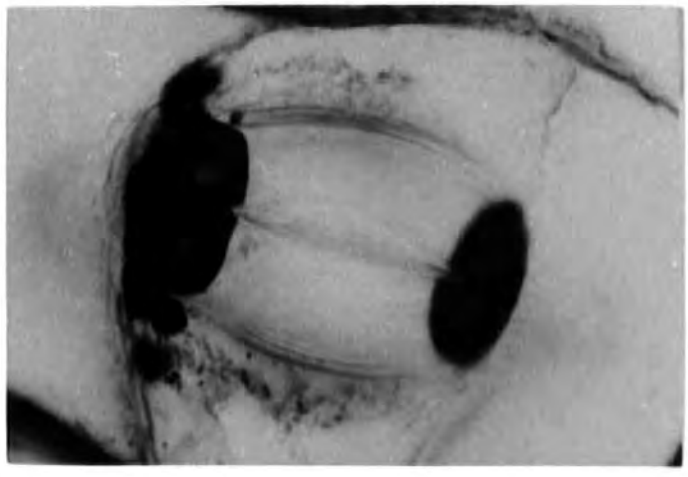
C. The left hand localisation is made up of two elements, one of which extends laterally between the polar and lateral subsidiary cells.



A



B



C

Plate 3.10.

Polar stain localisations in Tradescantia pallidus, III.

Light micrographs of damaged guard cell complexes stained with 7,5% (w/v) modified Macallum's reagent and viewed from the side adjacent to the mesophyll.

A. Stomatal complex which was damaged before the Macallum treatment, x 1 200. Note how, although the right hand polar localisation is not stained, it is represented by a structural 'shell'.

B. Stomatal complex damaged after it had been treated with the Macallum stain, x 1 000. Note how the undamaged localisation retains it integrity despite the chloroplasts having been squeezed out.

C. Stomatal complex damaged after it had been treated with the Macallum stain, x 900. Note how the remaining part of the damaged polar stain localisation retains its integrity.

A

B

C

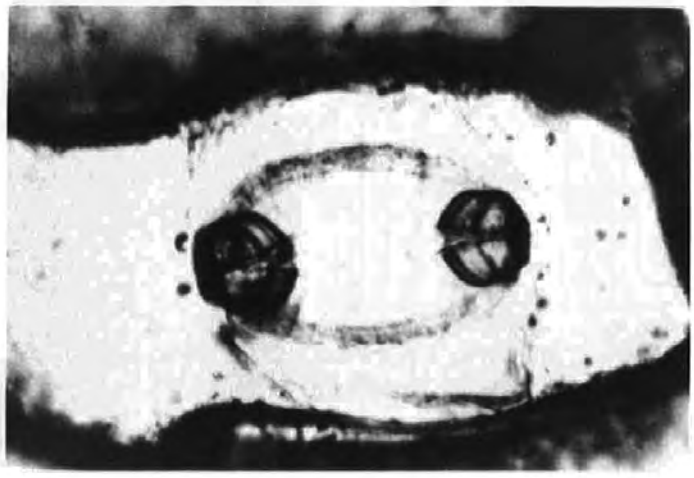
Plate 3.11.

Polar stain localisations in Tradescantia pallidus, IV.

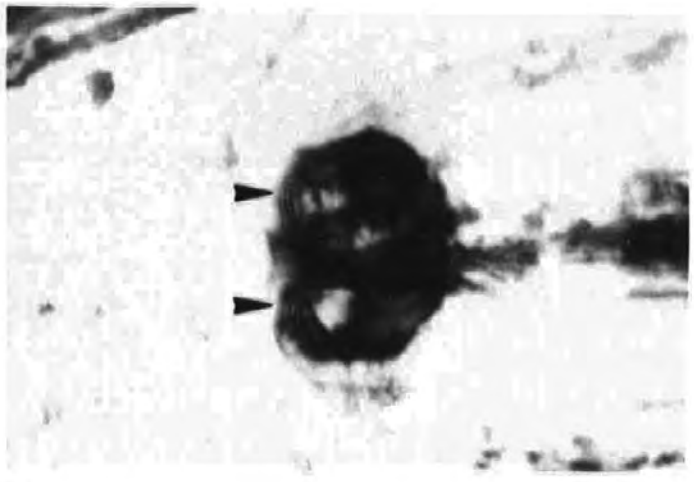
Light micrographs of guard cell complexes stained with 7,5% modified Macallum's reagent and viewed from the side adjacent to the mesophyll.

A. Polar stain localisations illustrating their laminate appearance, x 900.

B. A single polar stain localisation illustrating how the localisation is made up of to 5 superimposed laminae. The individual laminae can be seen at the extreme polar end of the localisation (arrowed).



A



B

Plate 3.12.

Polar stain localisations in Tradescantia pallidus, V.

Stomatal complexes stained with 7,5% (w/v) modified Macallum's reagent and viewed from the side adjacent to the mesophyll, x 700.

A. A typical example of polar stain localisations.

B. The same complex but focussed in the subsidiary cells. The arrows point to cobalt-rich vesicles from which fine strands emanate.

C. Another complex showing cobalt-rich vesicles in both the polar and lateral subsidiary cells. The vesicles in the polar subsidiary cell are connected to each other by fine strands and the lowermost vesicle of this group of three appears to be connected to the polar stain localisation by a similar strand.

A

B

C

Plate 3.13.

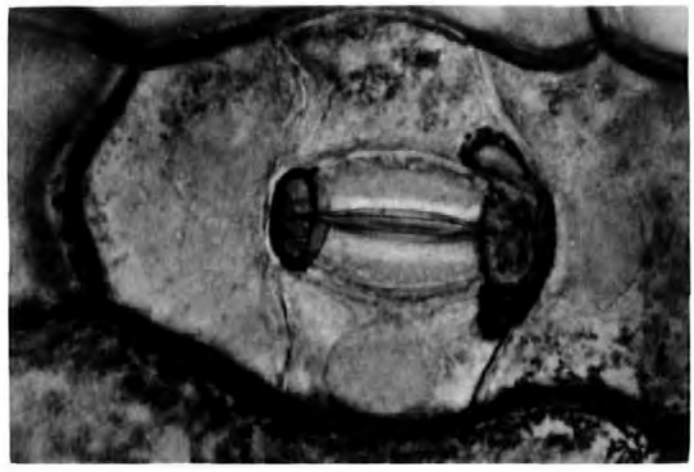
Polar stain localisations in Tradescantia x andersoniana.

Light micrographs of stomatal complexes which have been stained with 7,5% aqueous cupric sulphate in their transpiration stream. The complexes are viewed from the side adjacent to the mesophyll, x 570.

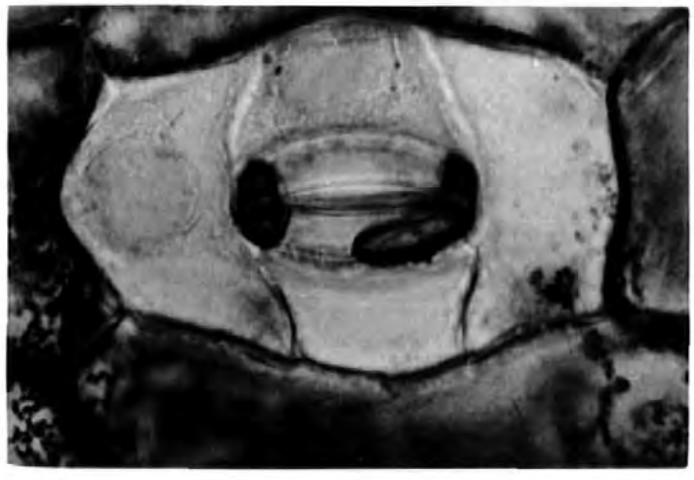
A. The right hand polar stain localisation is extended laterally into the region between the polar and lateral subsidiary cells.

B. The right hand polar stain localisation in the lower guard cell is extended into the region between the guard and lateral subsidiary cells.

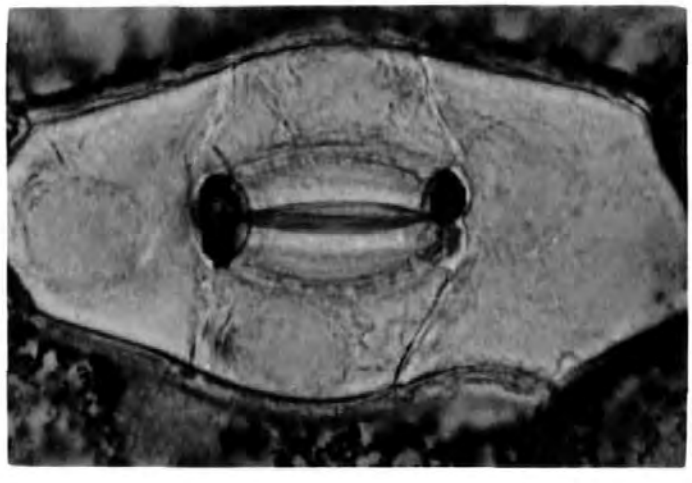
C. Only one element of the right hand polar stain localisation has developed, although the undeveloped one is just visible.



A



B



C

CHAPTER 4

TRANSMISSION ELECTRON MICROSCOPE STUDIES

INTRODUCTION

The light microscopy studies of the previous chapter provided evidence that previously unreported polar structures occurred on the inner face of certain stomatal complexes. Analyses of these findings were informative enough to construct provisional hypothetical models of these structures which were based on the surmise that they consisted of an extracellular darker-staining central binding site surrounded by a more extensive peripheral element whose size was probably osmotically or imbibitionally controlled.

Evidence concerning the exact structure of the two elements of the polar structures was not forthcoming from light microscopy studies however, and it was decided to pursue this aspect of the investigation by transmission electron microscopy. The basic aim of these studies was to identify the physical nature of the binding sites and to establish their exact location within the stomatal complex, both in relation to the peripheral elements of the structures and to other established stomatal features.

Before continuing the studies on the structure of the binding sites later in this chapter, the ultrastructural features of the guard cells in Polypodium vulgare and Tradescantia pallidus

will be considered. A comparison between these two species is particularly relevant because it highlights the differences in stomatal ultrastructure in ferns and mesophytes.

ULTRASTRUCTURAL STUDIES

The following studies were carried out on whole leaf segments fixed in glutaraldehyde and osmic acid, embedded in Spurr epoxy-resin, and post-stained with uranyl acetate and lead citrate, as detailed in Chapter 2.

Observations in Polypodium vulgare

The most distinctive feature of the guard cells is the large number of chloroplasts present and their rich starch content (Plate 4.1, et seq.). It is estimated, from electron micrographs, that a typical guard cell contains between 80 and 100 chloroplasts which are each ca. 4,5 µm by 3,0 µm. Those of the mesophyll are ca. 7,5 µm by 3,5 µm (c.f. Plates 4.2 and 4.3). The grana of the guard cell chloroplasts had an average of some six thylakoids in each granal stack, and are rather poorly developed when compared with those of the mesophyll. Numerous osmiophilic granules of ca. 0,1 µm diameter are present within the stroma of the chloroplasts and are apparently more numerous in those of the guard cells than in those of the mesophyll.

Chloroplasts are present in the guard-cell mother-cell (Plate 4.7A) and contain relatively well-developed granal stacks. By the time the young guard cells are fully differentiated, but still

submature, the starch content of the chloroplasts is extremely high and exceeds that of fully mature guard cells proportionately (Plate 4.9). The relative area of the sections occupied by the chloroplasts appears to be greater in submature than mature guard cells, Vide Table 4.1.

Table 4.1.

The percentage areas of the guard cell protoplasts (excluding the nucleus) occupied by chloroplasts in Polypodium vulgare.

Plate number	Age of tissue	% area of protoplast (excluding the nucleus) occupied by chloroplasts
4.1A	mature	56%
4.1B	mature	51%
4.6A	mature	45%
4.6B	mature	39%
4.9A	submature	63%
4.9B	submature	61%

The cell walls of mature guard cells vary in thickness from ca. 1,2 µm (0,8 - 1,4 µm) in the periclinal plane, to ca. 0,5 µm (0,4 - 0,7 µm) in the anticlinal plane with greatly thickened regions occurring as ridges protecting the upper and lower limits of the stoma (Plates 4.4 and 4.5A). An interesting feature demonstrated by some polar sections is the laminate nature of the lower periclinal guard cell walls in which the stoma is subtended by an unbroken cell wall element which arises in the lower periclinal wall of one guard cell,

subtends that of its partner, and merges into that of the adjacent subsidiary cell (Plate 4.5A).

The inner anticlinal walls of the guard cells are formed immediately after the guard-cell mother-cell divides to form the pair of daughter guard cells. The first indication of their formation, after nuclear division, is the aggregation of endoplasmic reticular vesicles in the plane of the phragmoplast (Plate 4.7B). The vesicles enlarge, and eventually fuse together, as cell wall components are laid down in the anticlinal plane (Plates 4.8A and 4.9). The development of the cuticularised ridges is of particular interest since it is brought about by cell wall depositions on the side of the wall adjacent to the protoplast which has the effect of considerably distorting the developing guard cell lumen (Plate 4.8). During the development of the thickened ridges, the external cell wall surface of the ridge is depressed below that of the rest of the guard cell (Plate 4.8B). The regions of the periclinal walls immediately adjacent to the developing ridges remain particularly thin. The common anticlinal cell wall of the immature guard cell complex separates to form the stoma from the mesial regions outwards so that the last to part are those cuticular elements at the apices of the ridges (Plate 4.8).

The cuticle is poorly defined in this species, but a very thin cuticle can be observed, in both mature and immature tissue, covering all the external faces of the epidermis, including that of the stoma, and the exposed surfaces of the mesophyll tissue. In only one instance was evidence found to support the presence of endocuticular trabeculae, as predicted from light microscopy studies in Chapter 3, and this is in a submature complex (Plate 4.9B). This evidence is

rather weak since close examination of the lumen of one of the trabeculae indicates that it is filled with a matrix of identical composition to that of the adjacent epidermal cell wall and is, indeed, contiguous with this structure. It is possible, therefore, that the 'tabecula', in this case, is actually a cell wall element as illustrated in the mature complex in Plate 4.5A.

No evidence was found for the presence of plasmodesmata in mature guard cell walls although they are numerous in the surrounding epidermal and subsidiary cell walls (Plate 4.5B). Similarly, no identifiable ectodesmata were found; the ectodesmata-like structures illustrated in Plate 4.4 are artefacts probably caused by the sections folding in the cell wall regions as they dried out on the grids. Plasmodesmata-like artefacts have been similarly produced in Plate 4.1B.

Numerous gaps are often visible in the common anticlinal cell walls of recently formed guard cells and also in the anticlinal walls between the guard-cell mother-cell and adjacent protoderm cells. These gaps are inter-vesicular spaces which are present during the early stages of cell wall formation but gradually disappear as the cells mature. Although they may superficially resemble plasmodesmata, shortly before they disappear, they cannot be regarded as such because of their transient nature, and their lack of associated pit fields.

Vacuoles are of particular interest in guard cells since they are thought to act as the sink for osmotically-active ions which accumulate during stomatal opening. They are difficult to analyse from electron micrographs in this species since they are polymorphic (Plate 4.1 et seq.). There is apparently no single large

vacuole, the vacuolar element being made up of numerous, often widely-separated, smaller components. The vacuolar elements in some of the sections illustrated at the end of this chapter were analysed in respect of the area of the protoplast occupied by the vacuoles as well as the nature of their matrix in Table 4.2. Only those sections which exhibit unambiguous vacuoles are considered in Table 4.2.

Table 4.2.

Analysis of vacuolar elements in sections of Polypodium vulgare.

Plate number	Age of tissue	Vacuolar element as % of total protoplast area (excluding nucleus)	Vacuolar matrix	Inclusions
4.1A	mature	7%	electron translucent	many membranous
			electron opaque	absent
4.1B	mature	11%	electron translucent	absent
		18%	electron opaque	many membranous
4.6A	mature	23%	electron dense	absent
4.6B	mature	34%	electron translucent	osmiophilic granules
4.9A	sub-mature	24%	electron translucent	absent
4.9B	sub-mature	22%	electron translucent but grainy	some large osmiophilic elements

Mitochondria are more numerous in the guard cells than in other epidermal or mesophyll cells. They exhibit well-developed cristae and contain osmiophilic granules of ca. 25 nm diameter.

The guard cell cytoplasm is particularly rich in endoplasmic reticulum and myelin figures (Plate 4.2A) are not uncommon. Osmiophilic lipid bodies with darker staining cores, identified as spherosomes (Pallas & Mollenhauer, 1972), were found in the peripheral regions of the guard cell protoplast in some sections (Plates 4.4 and 4.5A). No confident identification of peroxisomes or dictyosomes were made. In some sections, unidentified spherical organelles were observed around the periphery of the protoplast (Plate 4.6) which were variable in size (0,4 - 0,7 µm in diameter) with a comparatively electron transparent core surrounded by a denser marginal area.

Observations in the Commelinaceae

Guard cell structure in Tradescantia pallidus will only be briefly compared with that of Polypodium vulgare, since it is of a typical mesophyte-type which have been described previously in the literature (Allaway & Setterfield, 1972; Pallas & Mollenhauer, 1972; Pearson & Millthorpe, 1974; Fujino & Jinno, 1972).

The upper periclinal and all the anticlinal walls of the guard cells are between 0,3 and 0,5 µm thick. However, the lower periclinal walls are greatly thickened to ca. 1,5 µm near the poles of the complex (Plate 4.10B), increasing to nearly 3,0 µm in regions adjacent to the stoma (Plate 4.10A). There is tentative evidence that the outer regions of the lower periclinal walls near the pole of the complex are distinct from the inner regions in that they merge

insensibly into the cell walls of the adjacent lateral subsidiary cell (Plate 4.10B). An interesting cell wall feature, not present in P. vulgare, is the crenulated outer surface of the lower periclinal cell walls immediately adjacent to the lateral subsidiary cell (Plate 4.10A). The cuticle, whilst having a similar distribution to that of P. vulgare, is better developed and particularly heavy on the protective ridges of the stoma. No plasmodesmata were observed in the guard cell walls although they are widespread in other epidermal cell walls.

The epidermal cells of T. pallidus differ from those of P. vulgare in that chloroplasts are virtually absent in these cells and only occur as rudimentary bodies which are frequently associated with the nuclei. The most significant difference between the guard cell complexes of the two species is that in T. pallidus there are only 15 to 25 (av. 20) chloroplasts in each guard cell although, as in P. vulgare, they contain many sites of starch deposition and many osmiophilic lipid bodies (Plate 4.10). The guard cells of T. pallidus also contain many mitochondria with well-developed cristae but no positive identifications of dictyosomes, spherosomes, or peroxisomes were made and, if present, can only exist in very reduced numbers.

The vacuolar element in T. pallidus is very extensive but this may be partly due to the dearth of chloroplasts. The vacuolar volume is made up of numerous smaller irregularly-shaped units interspersed with cytoplasmic strands. Membranous vesicles and osmiophilic granules of up to 0,5 µm in diameter appear to be common vacuolar inclusions.

STUDIES ON TISSUE PRETREATED WITH MACALLUM'S STAIN

Standard fixation and embedding procedures failed to reveal any specialised structures associated with the polar regions of the guard cells with the possible exception of the anomalies noted in the lower periclinal walls of the guard cell complex in this region. However, the light microscope studies in Chapter 3 strongly implicated the presence of a binding site on the outer face of the lower periclinal cell walls near the poles of the guard cell complex as visualised by the precipitation of the cobalt stain.

Cobalt is very electron-dense and should appear as an electron-dense mass at the site of deposition under the transmission electron microscope. Consequently, the ultrastructural studies were repeated on epidermal strips which had been pretreated with the modified Macallum stain (7,5% w/v) and subsequently processed in the usual way with the exception of the post-staining sequence detailed in Chapter 2.

Observations

The cobalt depositions were almost completely leached out of the epidermal strips of Polypodium vulgare, as has been described and discussed in Chapter 2. In Commelina communis, small areas of the tissues retained appreciable amounts of the cobalt precipitate although, unfortunately, no guard cells from these areas were sucessfully sectioned through their polar regions.

A section through the central region of a guard cell of

C. communis is illustrated in Plate 4.11A. Very dense cobalt deposits occlude the greater part of the cytoplasm around the nucleus and can be seen to be associated with what is assumed to be the plasmalemma on the inner face of the upper parts of the outer anticlinal cell wall. The present study indicates that cobalt is not deposited to any great extent within the vacuoles or onto organelles. A layer of precipitate covers the endocuticular region of the lower periclinal cell wall on the immediate outside of an electron translucent outer layer of the cell wall which is ca. 0,15 µm thick. This outer cell wall element is infiltrated by more recognisable cell wall material (Plate 4.11B). Interestingly, no evidence of cobalt can be found within the cell walls to indicate how it might be taken up from the Macallum's stain into the protoplast.

The cobalt was also found precipitated into small deposits in the peripheral regions of the epidermal cells (Plate 4.12). Close examination of these deposits show that the bulk of the cobalt is incorporated into the cytoplasm with smaller amounts associated with the adjacent plasmalemma (Plate 4.12A). Cobalt-rich pinocytotic-like vesicles occur on the plasmalemma and cobalt-rich strands extend between the plasmalemma and the cytoplasm. Examinations of high magnifications of the deposits (Plate 4.12B) indicate the presence of a large number of circular (=spherical?) aggregations of cobalt in the cytoplasmic mass. Very fine electron transparent threads of ca. 5 nm thickness run through these aggregations both in the cytoplasm as well as in the plasmalemma and its pinocytotic vesicles. In the few regions where the cobalt deposit is not so dense as to mask the threads completely, the electron translucent threads can be seen to be sandwiched between pairs of electron dense layers. The width of the

whole structure is ca. 12 nm, which is compatible with that of an unit membrane.

MODIFIED TRANSMISSION ELECTRON MICROSCOPE TECHNIQUES

From the foregoing studies, it became clear that the hypothetical polar structures were either completely electron translucent or became completely denatured in fresh tissue which had been fixed and dehydrated in a standard embedding procedure. Furthermore, when the tissue was pretreated with Macallum's stain, the cobalt depositions on the polar structures disappeared during the protracted preparative procedures prior to embedding. It was decided, therefore, to by-pass these procedures by embedding the tissues directly into epoxy-resin after the Macallum treatment and fixation in ethanol (vide Chapter 2 for details).

Observations in Polypodium vulgare

The guard cells were instantly recognisable by their starch content and in every case, where the section passed through the polar region of the guard cell complex, by a cobalt deposit immediately underlying the common anticlinal walls of the complex (Plates 4.13 & 4.14). All these deposits were characterised by their being attached to the outer surface of the lower periclinal walls of the complex and, in the majority of cases (typically as in Plates 4.13A & 4.14), by the lateral extremities of the structure hanging free of the overlying cell wall. From a study of the sections obtained, the structures appear to be disc-shaped with the central area being attached to the

overlying cell wall. The structure is delimited on its free sides by a 'membrane' of between 30 and 40 nm thickness. The structure is between 0,5 and 0,7 µm in total thickness and has a lumen containing crystalline aggregations of cobalt. The diameter of that part of the structure attached to the guard cell wall varies from 5,5 to 8,0 µm whilst the diameter of the total structure is up to 20 µm across (Plate 4.14). In some sections, the structure is apparently bi-lobed with a distinct break occuring between the two halves immediately below the common anticlinal walls of the guard cell complex (Plate 4.14).

The cell walls immediately above the cobalt-rich structures are frequently rich in cobalt crystals which results in the section tearing under the electron beam (Plate 4.13A). The corners of the guard cell protoplasts immediately above the structures are also cobalt-rich but the quality of the sections does not permit the exact locations of these deposits to be identified (Plate 4.13). Another very interesting feature is that there is very often a cobalt-rich trace extending from beneath the endocuticle, immediately below the centre of the outer anticlinal walls of the guard cells, which extends obliquely through this cell wall to reach the internal face, and presumably the protoplast, about half to three-quarters of the way up this wall (Plate 4.13A).

Plate 4.15 is of an exceptional pale gold section (ca. 70 nm) through the centre of a guard cell. This micrograph gives a fair representation of the distribution of the cobalt within this region of the guard cell. The cobalt appears to be associated with the plasmalemma, which has become pulled away from the cell wall, and the cytoplasm. More interestingly, the cobalt has become adsorbed onto the starch granules in the chloroplasts, but is not associated with

either the thylakoids or the stroma.

Observations in the Commelinaceae

Plate 4.16A is a micrograph of a typical section through the polar region of the stomatal complex of Commelina communis. The micrograph shows very heavy cobalt deposits in the walls of the complex but, like others, is characterised by a peculiar localisation in the lower periclinal cell walls. The localisation is contained chiefly within this cell wall, but also extends partially into the common anticlinal cell walls and, laterally, into the corner of the protoplast of the adjoining lateral subsidiary cell. It appears to be delimited by a membrane-like structure which is ca. 60 nm thick and extends along the faces of the cell walls concerned and also passes through the common anticlinal walls and lower periclinal walls of the complex as well as through the subsidiary cell protoplast.

The cobalt deposits within the guard cell walls vary greatly in size from 12 nm to 1,3 μm in diameter and, as in Polypodium vulgare, have also become adsorbed onto the starch grains of the chloroplasts. The fixation procedure employed precludes the identification of the cobalt distribution within the protoplast, but it appears probable that it is associated with the cytoplasm rather than the vacuoles. Cobalt precipitates associated with the internal faces of the walls of the guard and subsidiary cells appears as pinocytotic-like vesicles arising from the plasmalemma.

A rather different type of cobalt precipitation is illustrated in Plate 4.16B which is of a transverse section through a region adjacent to the stoma. In this section, the cobalt has precipitated

as monoclinic crystals within the cell walls. The crystals are orientated very regularly within the periclinal and upper anticlinal walls of the guard cells. This regular orientation does not persist in the cobalt-rich sites of the protoplast where, again, it seems to be restricted to the cytoplasm and starch grains.

The involvement of the anticlinal walls of the complex in the pathway of cobalt into the protoplast is also shown in Plate 4.17 which is of a transverse section through the corner of the stoma. In this region, the periclinal walls show little sign of cobalt precipitate whilst the anticlinal walls are particularly rich. The cytoplasm adjacent to the anticlinal walls is also cobalt-rich and exhibits pinocytotic-like vesicles. The cuticle is fairly well defined in this section and it can be seen that there is a distinct trace of cobalt between the endocuticle and the lower periclinal cell wall which may persist as a trace up the middle lamellar region of the outer anticlinal wall of the guard cell.

LIGHT MICROSCOPY OF RESIN-EMBEDDED SECTIONS

The problems encountered during the ultramicrotomy of the Macallum-treated tissue led to the examination of thick sections (1,0 to 1,5 um thick) under the light microscope. It is difficult to determine ultrastructural detail in such sections but the general distribution of the cobalt is easily observed.

Observations in Polypodium vulgare

Sections of tissue which had not been pretreated with the

Macallum stain rarely showed any polar structures. A few sections, however, revealed evidence of substomatal sacs as indicated by the small arrows in Plate 4.18. The sacs vary from 9,5 µm deep by 21,6 µm long and 5,8 µm deep by 18,0 µm long in Plate 4.18A, to 8,5 µm deep by 13,3 µm long in Plate 4.18B. The sacs are clearly restricted to the polar regions of the complex and do not impinge on the actual stoma although they do extend, in the other direction, onto the region below the adjacent epidermal cell next to the poles of the guard cells. An internal structure is clearly visible within one of the sacs in Plate 4.18A which is 13 µm long by 8,5 µm deep. This structure is not considered to be an artefact, such as an air bubble, since as the inset to Plate 4.18A shows, its lumen is filled with resin as evidenced by the presence of the knife marks.

Sections, pretreated with the Macallum stain prior to the modified embedding procedure, consistently showed that all the guard cell complexes sectioned through their polar regions were associated with a plate-like cobalt precipitation immediately below their common anticlinal walls on the outer surface of their lower periclinal cell walls (Plate 4.19). The width of the deposition varies with the exact location of the section but can extend to as much as 28 µm, as in Plate 4.19, and is generally 1,5 to 2,0 µm deep.

Occasionally, the cobalt depositions show a very different form, appearing as hollow sacs below the poles of the complexes. Plate 4.20 illustrates three sections from the same complex. Plate 4.20A is from a region towards the extreme pole of the complex and the structure is 13,6 µm wide by 7,0 deep; Plate 4.20B is rather further towards the stoma and the structure measures 20,0 µm wide by 6,5 µm deep, whilst Plate 4.20C is a section even further towards the stoma

and its structure is 25,0 μm wide by 5,0 μm deep. Each structure is apparently made up two separate elements; a thin element of 0,5 μm thickness along its upper edge (i.e. adjacent to the periclinal wall of the complex), and a thicker element extending down the sides of the structure and along its lower edge of ca. 1,7 μm thickness. There is not any appreciable cobalt precipitation within the structures. These micrographs also provide additional evidence for a cobalt 'pathway' up the outer anticlinal walls of the guard cells.

Observations in the Commelinaceae

Epidermes of Tradescantia pallidus, pretreated with Macallum's stain prior to the modified embedding procedure, exhibit dark cobalt precipiations in the lower halves of their guard cell complexes. Serial sections (from different stomatal complexes) are illustrated in Plate 4.21. In Plate 4.21A, the section passes through the extreme pole of the guard cell complex so that only elements of the polar anticlinal guard cell walls are present abutting into the polar subsidiary cell. The cobalt precipitate in this section is discretely contained in a sac-like structure which measures 13,0 μm across by a maximum of 2,5 μm deep. The exact location of the precipitate is difficult to determine although it does appear to be superimposed over the lower periclinal wall of the subsidiary cell. The section in 4.21B is through the central area of the polar region. The cobalt precipitate is extremely dense and clearly extends into the protoplast of the guard cells as well as into that of one of the lateral subsidiary cells. Its location is more definite in this case and the deposition is sharply delimited both intra- and extracellularly and appears to be in a continuous phase between the protoplasts and the cell walls.

Plate 4.21C is probably directly comparable, both in its plane of sectioning and the nature of its cobalt depositions, to that of Plate 4.16A, and needs no further elaboration here. Sections through the stoma of the guard cell complex (Plate 4.21D) significantly lack the dense cobalt precipitation associated with the lower periclinal cell walls of the polar regions. However, the section demonstrates what appears to be cobalt depositions around what are assumed to be the periphery of vacuoles.

Plate 4.22 illustrates a section through the polar region of the guard cell complex in Tradescantia pallidus. The accumulation in this section is clearly contained primarily within a body, 15 μm wide by 3.3 μm deep, which extends laterally onto the walls of the subsidiary cells from a position which is definitely below the lower periclinal walls of the guard cells.

DISCUSSION

Ultrastructure of guard cells

The most characteristic feature of the guard cells is their relative abundance of chloroplasts when compared with other epidermal cells. The very large numbers of chloroplasts found in the guard cells of Polypodium vulgare seems to be typical of the filicopsid ferns and have been reported on by Mansfield & Willmer (1969) in Phyllitis scolopendrium, Stuart (1968) in Polypodium polypodioides, and Humbert & Guyot (1972) in Anemia rotudifolia. Unlike the epidermal cells of Tradescantia pallidus, those of P. vulgare are rich in chloroplasts, a feature which is considered to be a typical feature of shade-plants

(Esau, 1965), and whilst this is true of filicopsid ferns in general, it is worth noting that much of the fern material used in this study was collected from open sun-drenched banks in the environs of the Plym Forest. If the guard cell chloroplasts and the surrounding epidermal cell chloroplasts are assumed to have an identical metabolism and if part of the osmoticum utilised during stomatal opening is a product of this metabolism, it could be that the large number of chloroplasts found in fern guard cells are required to overide osmotic effects of the metabolites formed by epidermal cell chloroplasts. This complication would not arise in T. pallidus and other mesophyte-type epidermes in which there are usually very few, often vestigial, chloroplasts in the epidermal cells (Shaw & Maclachlan, 1954; Allaway & Setterfield, 1972). However, it is unlikely that the chloroplasts of guard cells and those of other epidermal cells behave in the same way since it has been noted in the present study that there are usually fewer starch inclusions in the epidermal cell chloroplasts, a feature also noted by Pallas & Mollenhauer (1972) who also commented on the differences in respect of the grana from the two locations. Another peculiarity of guard cell chloroplasts is the possession of a peripheral reticulum (Pallas & Mollenhauer, 1972; Allaway & Setterfield, 1972), which has not been recorded in epidermal chloroplasts. There was no evidence of a peripheral reticulum in any of the species studied in the present investigation although it is not unknown in filicopsid ferns and has been observed in the guard cell chloroplasts of Phyllitis scolopendrium (Martin, unpublished data), although somewhat less extensive than that described for Vicia faba and tobacco. The function of the peripheral reticulum is not understood although it is established as a common feature of

the mesophyll and bundle sheath chloroplasts of C_4 plants (Laetsch, 1968). It can be inferred, therefore, that it plays a role in the C_4-dicarboxylic acid pathway of carbon dioxide fixation (Hatch & Slack, 1970). Laetsch (1971) also suggested that it is a likely site for phosphoenolpyruvate carboxylase, whilst Pallas & Mollenhauer (1972) suggested that it could be involved in sugar transport. Therefore, it may well be that the guard cells act as isolated C_4 metabolic islands in the epidermis and that their chloroplasts could be the source of organic anions, such as malate and aspartate, which balance the influx of potassium cations during stomatal opening (Allaway, 1973; Pearson, 1973; Pallas & Wright, 1973; Pearson & Milthorpe, 1974).

The present study confirms earlier findings that the mature guard cell is isolated from the symplast of the epidermal cells since plasmodesmata have not been observed between them. Plasmodesmata have usually been demonstrated in mature guard cell walls using light microscopy after suitable staining techniques (Kienitz-Gerloff, 1891; Litz & Kimmins, 1968; Inamdar et al., 1973; Dave & Patel, 1976). These findings must remain controversial especially since the endocuticular trabeculae reported in the previous chapter could easily be mistaken for plasmodesmata (c.f. Plate 3.3A and Plate 5J of Dave & Patel, 1976). Similarly the plasmodesmata depicted by Fujino & Jinno (1972) and Pallas & Mollenhauer (1972) are not entirely convincing as there are no indications of pit fields associated with the structures. The presence of plasmodesmata in developing guard cells has been fully established (Kaufman et al., 1970; Pallas & Mollenhauer, 1972; Singh & Srivastava, 1973). This is confirmed in the present study but these structures are not considered to be plasmodesmata (sensu stricta) since they are of a transient nature formed by incomplete fusion of

cell wall elements during cell wall deposition. The presence of large gaps in the common anticlinal cell walls of guard cell complexes has only been reported in graminaceous species, and are probably only found in this group of plants (Brown & Johnson, 1962; Miroslavov, 1966; Srivastava & Singh, 1972; Kaufman et al., 1970; Pallas & Mollenhauer, 1972). Ectodesmata have been shown to be abundant in the guard cell complex after specific staining under the light microscope (Franke, 1960, 1961a, 1961b, 1962, 1964a, 1964b, 1967; Sievers, 1959; Schonherr & Bukovac, 1970a, 1970b) but no definite evidence has been forthcoming from electron microscope studies. The structures illustrated by Pallas (1966) are almost certainly artefacts (Pallas & Mollenhauer, 1972) which occur in certain regions of the cell walls that contain accumulations of waxes, cutins, and similar compounds. Such inclusions could cause differential drying out of the sections on the grids to produce the observed folds in the cell walls, and which give the appearance of ectodesmata under the electron microscope.

The vacuolar element of typical guard cells is relatively small for their size when compared with other epidermal or mesophyll cells, but this is probably a reflection of the volume of the protoplast occupied by the chloroplasts and nucleus. As Allaway & Milthorpe (1976) suggest, it is probably impossible to fix cells so that the size of their vacuole remains unaltered. This observation must cast some doubt on the findings of Humbert & Guyot (1972) who found an increase in the vacuolar volume of the fern, Anemia rotundifolia, during stomatal opening. However, it has been established that chloroplasts shrink on exposure to light (Nobel, 1968; Heber, 1969; Raghavendra et al., 1976) and this, in itself, could induce an increase in the relative volume of the guard cell vacuole irrespective of possible

increases due to guard cell extension during stomatal opening. Raghavendra et al. (1976) report huge decreases in chloroplast size during stomatal opening in Commelina benghalensis, which indicates that the shrinkage is in excess of 100 fold. Whilst this might produce only a relatively small increase in the vacuolar volume in typical mesophytes, it could, when applied to the guard cells of Polypodium vulgare, with its large complement of chloroplasts, result in very significant increases in vacuolar volume.

The relatively large numbers of mitochondria observed in the guard cells and their well-developed cristae agrees with the reports of other workers (Pallas & Mollenhauer, 1972; Allaway & Setterfield, 1972, etc.) and suggests that they may play an important role in stomatal movements. The relative dearth of other organelles found in the present study is confirmed by the findings of Vassilyev & Vassilyeva (1976).

The only observations to emerge from the ultrastructural studies which could be related to the polar structures reported on in the previous chapter are anomalies associated with the lower periclinal cell walls in the polar regions of the guard cell complex. The outer region of this cell wall appears to be contiguous with the lower periclinal cell wall of the adjacent subsidiary cell, which imparts a bi-laminate appearance to this guard cell wall. This unusual cell wall arrangement was found in both P. vulgare and T. pallidus, but its significance cannot be assessed at this time.

The other aspect of the cell walls which merits discussion is the plicated nature of the outer surfaces of the lower periclinal walls of the guard cells adjacent to the subsidiary cells. They are perhaps indicative of the flexibility of the cell wall and could

accomodate some radial distortion of this cell wall during stomatal opening.

The formation of the common anticlinal walls of young guard cells and development of the thickened ridges which protect the mature stoma give some insight into early developmental events and will be discussed more fully in the following section.

Hypothetical model to account for the formation of the stoma

The short study into the ultrastructure of immature guard cells in Polypodium vulgare provided new information about the deposition of the cell walls and, in particular, the differentially-thickened protective ridges. The most important finding, as illustrated in Plate 4.8, is the deposition of the wedge-shaped ridges on the inside of the cell wall in such a manner as to severely distort the guard cell lumen. It is also notable that the periclinal walls immediately adjacent to the thickened ridges are particularly thin.

No hypothesis has been put forward to explain how the stoma appears between the pair of guard cells and it is assumed that they merely pull apart from each other. Whilst broadly agreeing with this, the peculiar distortion of the guard cell lumen, and differential thickening of the periclinal guard cell walls could play an important role in the process. There is evidence that the split in the common anticlinal walls is initiated medially and that the cuticularised apices of the ridges are the last to part (Plate 4.8). Similarly there is evidence that the cross-section of the mature guard cell is subcircular (Plate 4.4) whilst that illustrated in Plate 4.8 is considerably distorted. It can therefore be assumed that the

formation of the stoma is concommitant with a change in the cross-sectional shape of the guard cell.

It is suggested that the formation of the stoma is brought about by turgor changes in the guard cell protoplast. It appears that the submature guard cells are particularly rich in starch (Plate 4.9, & Table 4.2) and starch hydrolysis could provide the necessary osmoticum and, therefore, turgor potential to cause swelling of the protoplast which will then exert a force against the cell walls. Since the anticlinal walls of the complex will be fairly inflexible, the inner ones pressing against each other, and the outer ones constrained to some extent by the adacent subsidiary cells, the greater part of this turgor pressure will be directed against the periclinal walls which will consequently swell outwards. However, as has already been mentioned, the periclinal walls adjacent to the thickened ridges are particularly thin and will effectively act as hinges for the ridges to tilt under the turgor pressure. This tilting will cause the common anticlinal walls of the complex to part, as in Plate 4.8, whilst the apices of the ridges will remain attached to each other and act as a fulcrum. As the common anticlinal walls part from each other, they will be forced against each other again because of the turgor pressure, so that eventually the apices of the cuticularised ridges will be torn apart. This hypothesis is summarised diagrammatically in Fig. 4.1. It is not suggested that the whole process is turgor-operated since it is probable that the middle lamellar region of the common anticlinal walls are pre-digested enzymatically.

Polar structures

In only a few specimens of unstained tissue were any traces

of polar structures noted. It is thought that either the structures are electron translucent, or else they are denatured by the lengthy preparative procedures. Their presence, however, was confirmed by the studies on tissue which had been pretreated with Macallum's stain prior to a very abbreviated embedding procedure.

The observations of the binding sites in Polypodium vulgare show that they are in an extracellular position below the lower periclinal walls at the poles of the complex. Furthermore, these bodies are clearly delimited by an inflatable membranous envelope. In the previous chapter, it was hypothesised that the bodies were ca. 10 μm in diameter and were enveloped in an extensible endocuticular substomatal sac which could be up to 25 μm across. The present findings are not entirely in agreement with these measurements. The electron microscope studies indicate that the body can be almost 30 μm across whilst its neck by which it is connected to the overlying periclinal walls is between 5 and 8 μm across. Additionally, the bodies are hollow structures capable of considerable distension.

It therefore appears that the darker central core, which was previously identified as the binding site, actually corresponds to the neck of the structure and that the peripheral paler element, which was thought to represent the the extent of the endocuticular sac, is in fact the marginal limits of the binding site. It seems advisable, at this stage, to retain an endocuticular substomatal sac as the outer element of the substomatal structure although little evidence of its involvement was found in the present transmission electron studies (vide Plate 4.18A). It was also suprising that no indication was found of the endocuticular trabeculae, but it is feasible that the

preparative procedures possibly dissolve these structures (vide Chap. 7). This is substantiated by unstained thick sections observed under the light microscope (Plate 4.18) in which an indistinct, but definite, membranous structure subtends the body. Another reason for retaining the concept of an endocuticular substomatal sac is that, if there were no such structure, the binding sites would hang down below the poles of the complex and would appear as such when viewed from the side adjacent to the mesophyll. That is not the case however, as indicated in Plate 3.7. In this micrograph, the polar structures appear to be covered by a membranous sheet which is believed to be the endocuticle.

Consequently, it is necessary to revise the model for the polar structures in P. vulgare, as proposed in Chapter 3, and take into account the distensible and more extensive nature of the binding sites. In the revised model, represented diagrammatically in Fig. 4.2, the small discoid binding site of the original model is replaced by a much larger distensible body with a hollow lumen. The limiting membrane of the body cannot be identified from the present study but is most likely composed of some element(s) associated with the outer regions of the cell wall since there is a degree of continuum between the two in Plate 4.14. Little supporting evidence was found for the contention that each polar structure consisted of a pair of elements. The binding site appears to be bilobed in Plate 4.14 and in other sections studied but not illustrated here. Conversely, in studies on thick sections (Plates 4.19 and 4.20), there is no sign of the bodies being bisected in the region below the common anticlinal walls.

Studies on the structures in the Commelinaceae provide little additonal information about their nature. There is clearly a binding site

which is a structure ca. 15 μm across by 3 μm deep (Plates 4.21A and 4.22) located beneath the lower periclinal walls of the guard cell complex between the pole of the complex and the polar subsidiary cell. Similarly, the involvement of the lower periclinal guard cell walls in the peripheral element of the localisation is confirmed by transmission electron microscopy (Plate 4.16).

Localisation of Macallum's stain in cell walls and protoplasts

The cobalt precipitate seems to be restricted to the plasmalemma, and chloroplastic starch grains of the guard cells. The evidence obtained from cobalt precipitations within ordinary epidermal cells of Commelina communis, strongly suggests that cobalt is initially adsorbed onto the plasmalemma from which it migrates into the body of the cytoplasm as pinocytotic vesicles. This is confirmed by the fact that what appear to be unit membranes of ca. 12 nm thickness thread through the pinoctotic vesicles and separated vesicles aggregated within the protoplast. Rather suprisingly there is little evidence of cobalt within the vacuoles of either the epidermal or guard cells.

As has been discussed in Chapter 2, the specificity of Macallum's stain for potassium is in doubt and therefore it is uncertain if it is the distribution of potassium or cobalt that is being observed in the sections. This point will have to be resolved using other electron-dense elements which are not potassium-specific. If, however, cobalt is being precipitated at the sites of potassium localisation, the present observations must question the assumption that potassium is accumulated within the vacuoles.

The presence of cobalt crystals within the starch grains of guard cell chloroplasts was unexpected but not considered to be very significant to stomatal functioning, since cobalt may be adsorbed non-specifically onto starch as are certain other heavy metals (Lane, pers. comm.).

There was little evidence of cobalt in guard cell walls of Polypodium vulgare except as localised traces running up the middle lamellar region of the outer anticlinal walls, and also in the cell wall immediately adjacent to the binding sites. It is thought that the traces up the outer anticlinal walls may reflect the presence of ion-channels in this region. This will be discussed more fully in Chapter 6. Cobalt depositions within the guard cell walls of the Commelinaceae were more general but particularly prominent in the anticlinal walls although the traces reported on from P. vulgare are only weakly present in some sections (vide Plate 4.17).

Fig. 4.1.

Hypothetical model of stoma formation in Polypodium vulgare.

a. Young guard cell complex in vertical transverse section after common anticlinal cell wall initiation.

b. Transverse vertical section of the guard cell complex as the thickened ridges are laid down on the inside of the cell wall. The arrows adjacent to the thickened areas indicate cell wall areas which are particularly thin. The line down the centre of the common anticlinal wall indicates preparatory enzymatic breakdown of the middle lamellar elements in this region.

c. As turgor builds up within the guard cells it will cause the cell walls to bulge out in the direction of the small arrows. Because of the thin areas in the periclinal walls (vide c), the greatest cell wall movement will occur in these cell walls which will result in the thickened ridges rotating (curved arrows) about their apices which will act as fulcra (hollow arrows). This rotation of the thickened ridges will result in the common anticlinal walls pulling apart from each other. The turgor pressure within the guard cells will, at the same time, force the anticlinal walls against each other so that eventually the turgor pressure will be directed solely at the cuticularised apices of the ridges which will eventually part to form the stoma.

d. Transverse vertical section through a fully differentiated guard cell complex. Note how the cross-section of the guard cell lumen changes during these events.

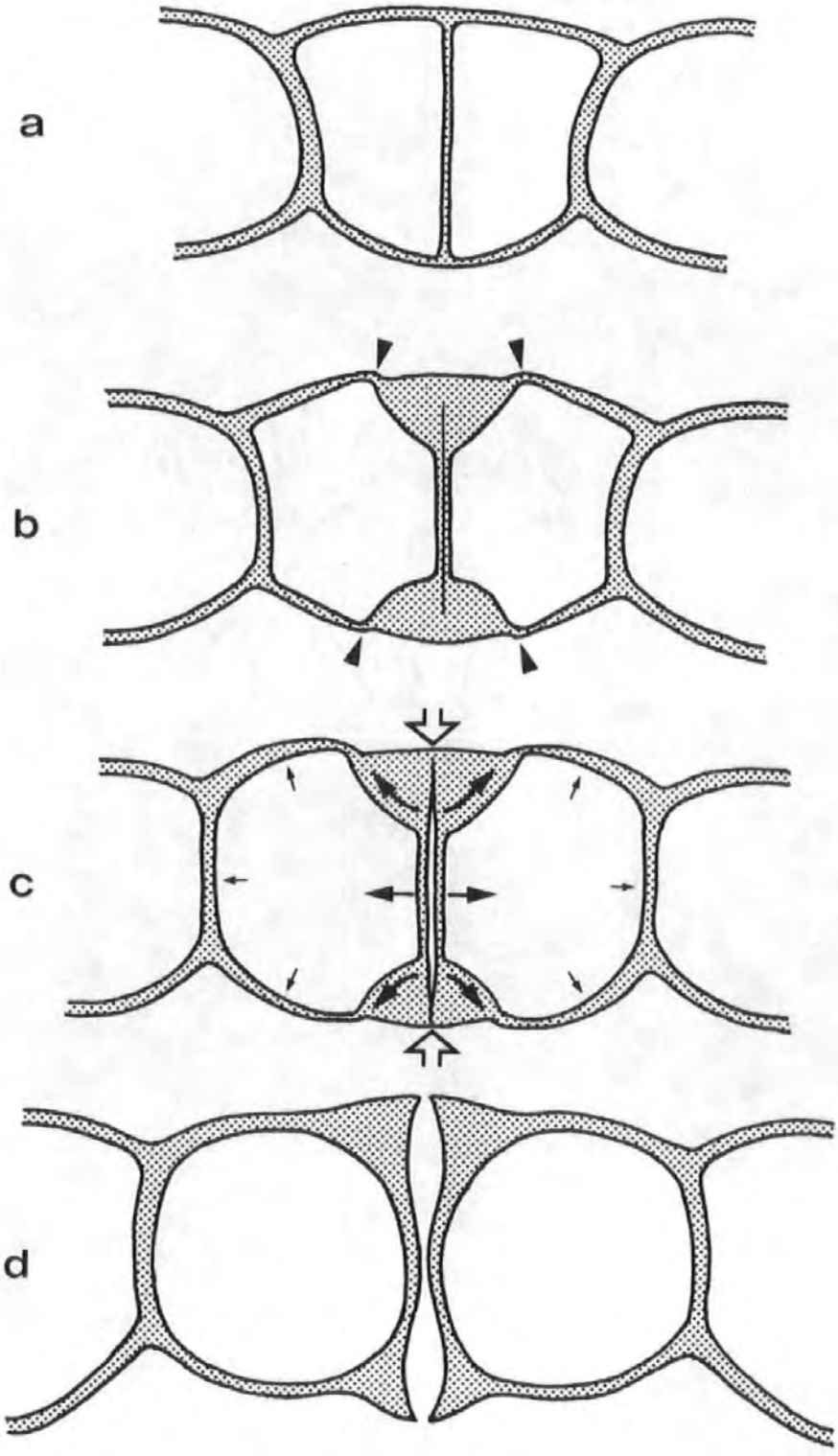

a
b
c
d

Fig. 4.2.

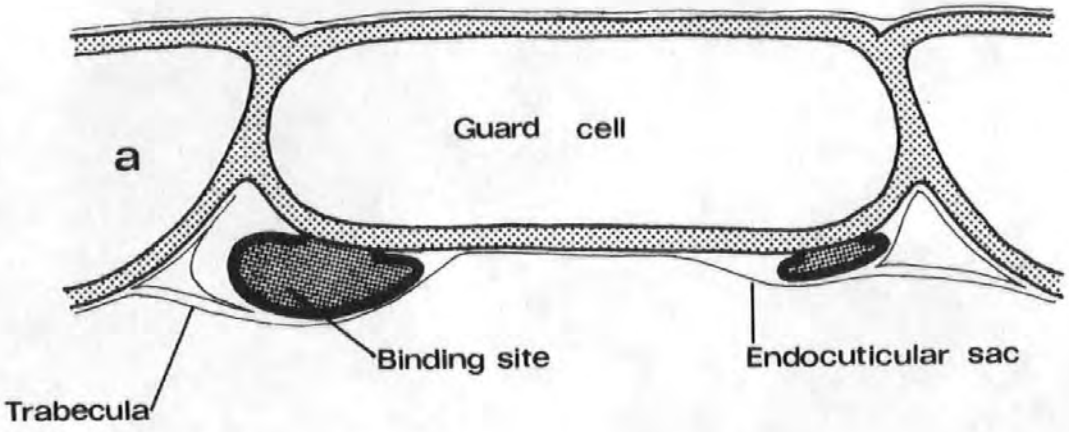
Hypothetical model of the polar structures in Polypodium vulgare.

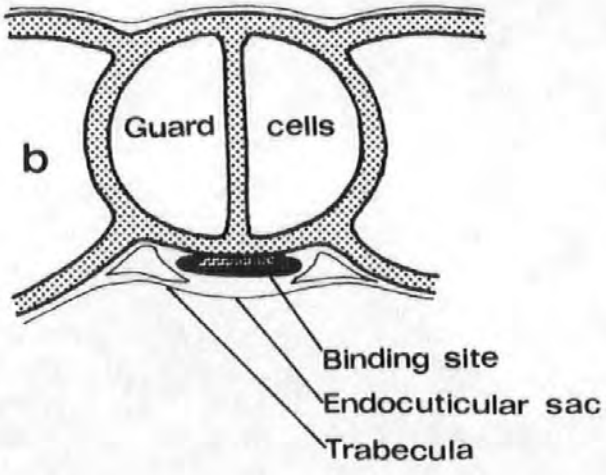
Light stippling = cell walls, heavy stippling = lumen of the binding site, and heavy solid line = walls of the binding site.

a. Longitudinal vertical section through an inflated polar structure (left hand side) and a deflated polar structure (right hand side).

b. Transverse vertical section through the polar region showing the binding site in its deflated state.

c. Transverse vertical section through the polar region showing the binding site in its inflated state.


a
Guard cell
Binding site
Endocuticular sac
Trabecula


b
Guard cells
Binding site
Endocuticular sac
Trabecula

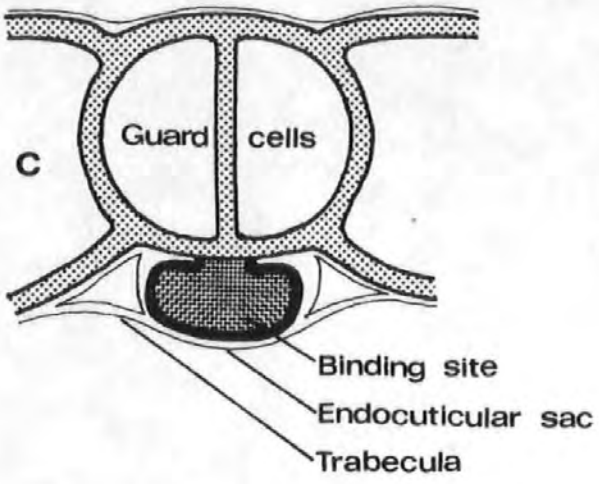

c
Guard cells
Binding site
Endocuticular sac
Trabecula

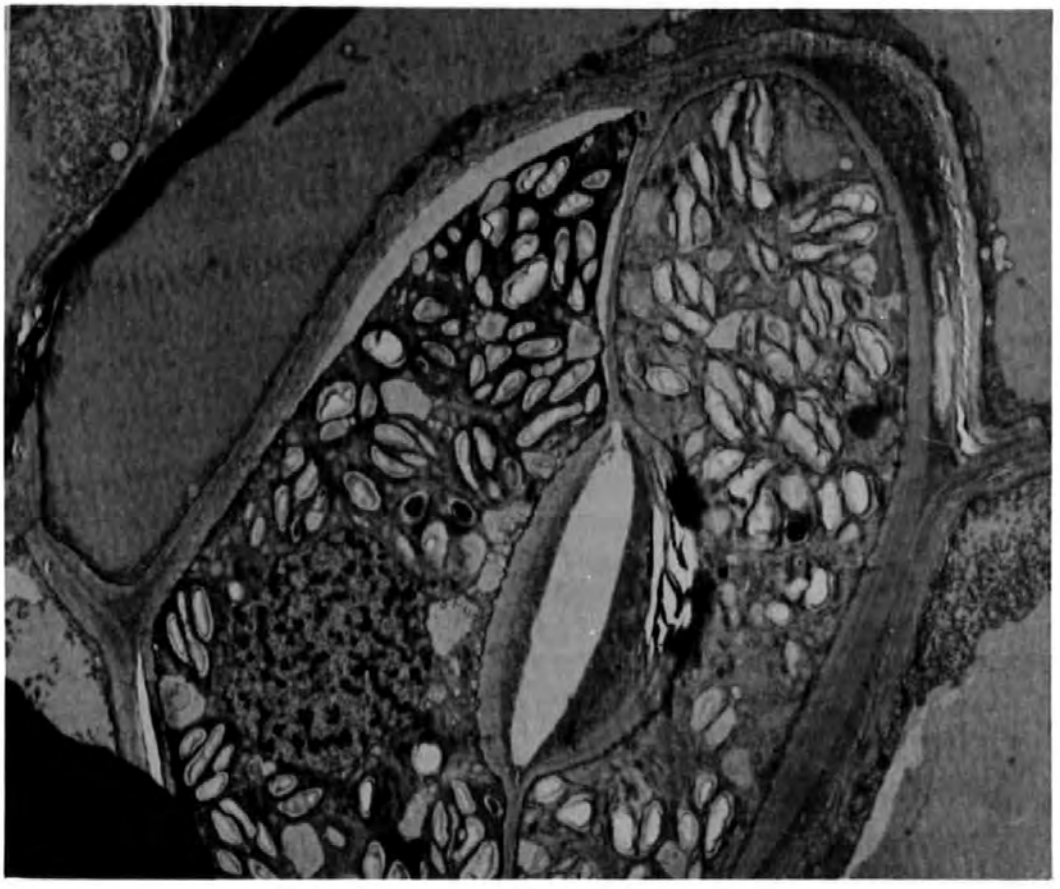
Plate 4.1.

Ultrastructure of mature Polypodium vulgare guard cells, I.

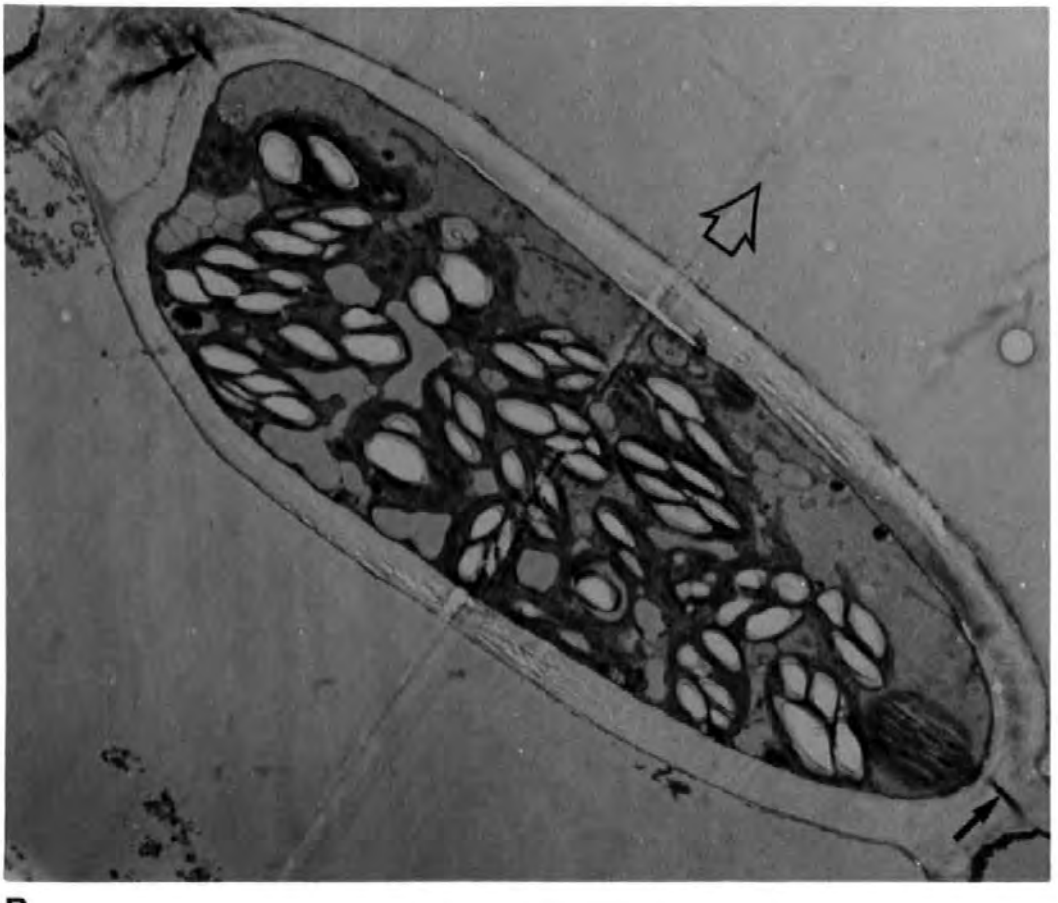
Transmission electron micrographs of tissues fixed in glutaraldehyde and osmic acid; post-stained with uranyl acetate and lead citrate.

A. Oblique paradermal section, x 3 750. The section illustrates the density of the chloroplasts found in the guard cells of this species and their rich starch inclusions, the relative paucity of guard cell vacuoles, and the reticulate nature of the nuclear chromatin. The adjacent subsidiary/epidermal cells show peripheral cytoplasm enclosing very extensive vacuoles.

B. Longitudinal vertical section, x 4 400. The hollow arrow indicates abaxial orientation. The section illustrates the rich chloroplastic starch inclusions, and a more extensive vacuolar system than A. The extensive vacuole of the upper half of the cell is rich in membranous inclusions and is more electron dense than those of the lower half. The plasmodesmata-like structures (small solid arrows) are believed to be artefacts caused by the sections becoming folded at these points.



A



B

Plate 4.2.

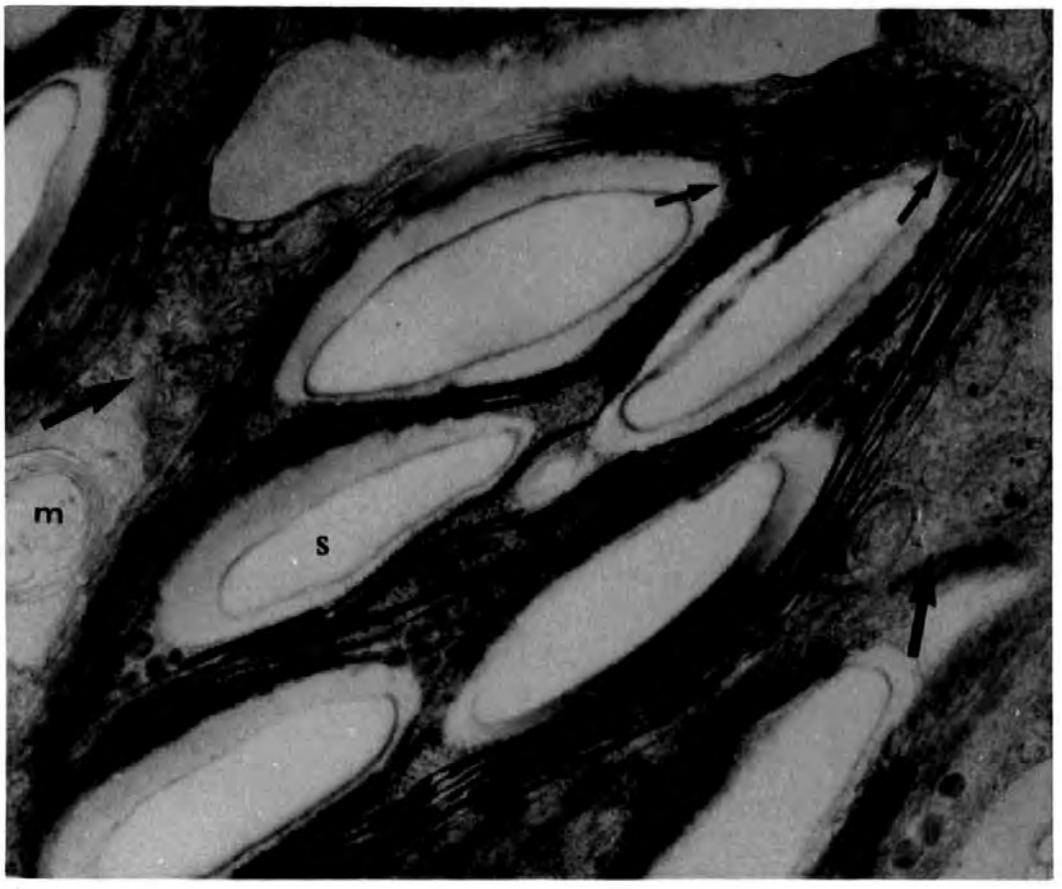
Ultrastructure of mature Polypodium vulgare guard cells, II.

Transmission electron micrographs of tissues fixed in glutaraldehyde and osmic acid; post-stained with uranyl acetate and lead citrate.

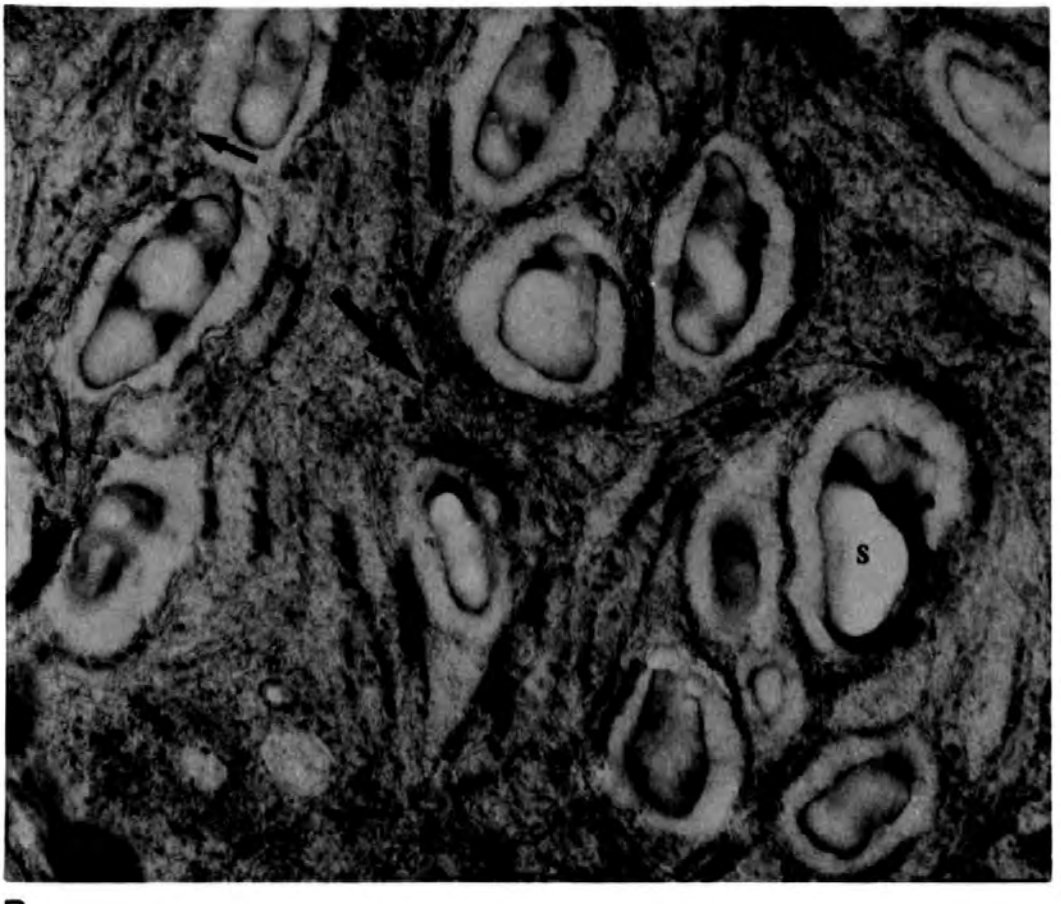
A. Longitudinal vertical section of single chloroplast, x 36 500.

B. Transverse vertical section through group of chloroplasts, x 20 700.

Both sections show the rich starch inclusions (s) and also the presence of osmiophilic lipid bodies (small arrows). The interstices of adjacent chloroplasts are rich in mitochondria (large arrows) which usually contain small osmiophilic bodies (Plate 4.2.A). A myelin body (m) is also illustrated in Plate 4.2.A.



A



B

Plate 4.3.

Mesophyll chloroplast from Polypodium vulgare.

Transmission electron micrograph of tissue fixed in glutaraldehyde and osmic acid; post-stained with uranyl acetate and lead citrate, x 12 700.

The section shows two mesophyll chloroplasts; the upper one is of typical structure, whilst the lower one shows dilated thylakoids which may indicate that it is degenerating. The typical chloroplast shows well developed granal stacks, relatively fewer osmiophilic bodies than guard cell chloroplasts, and only a single starch inclusion (arrowed).

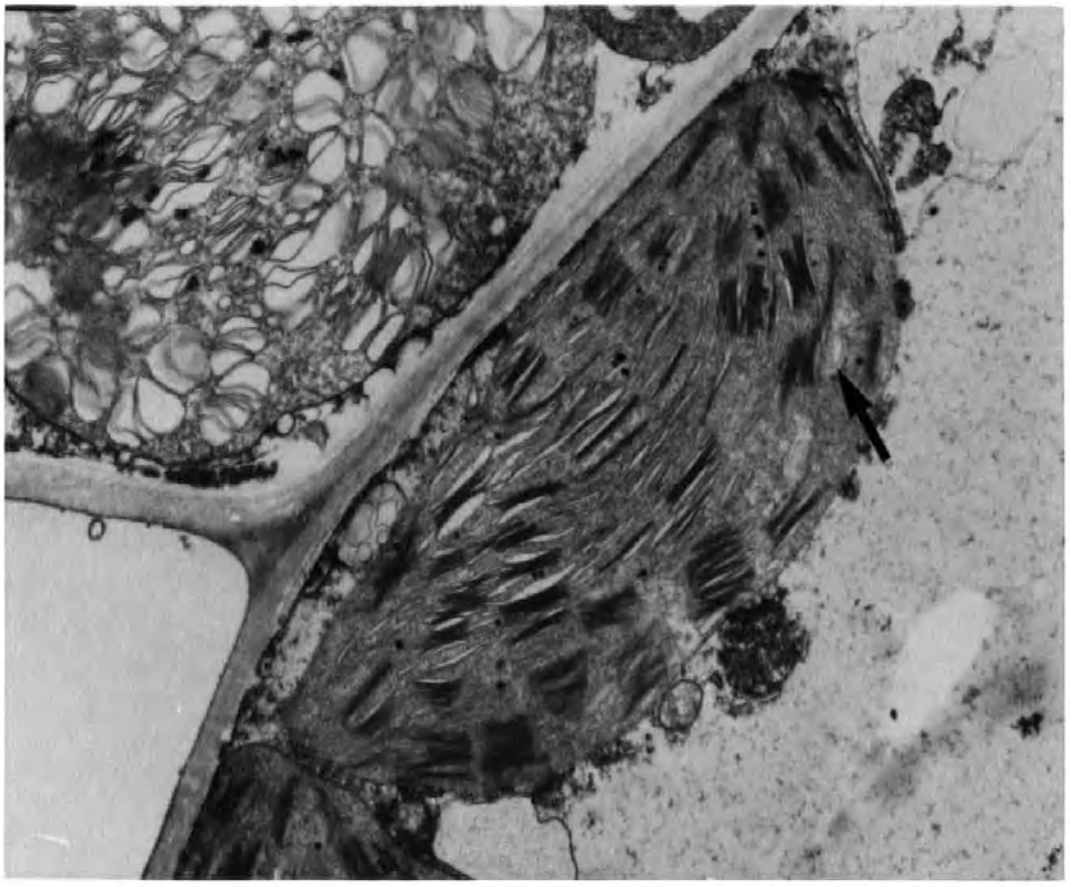


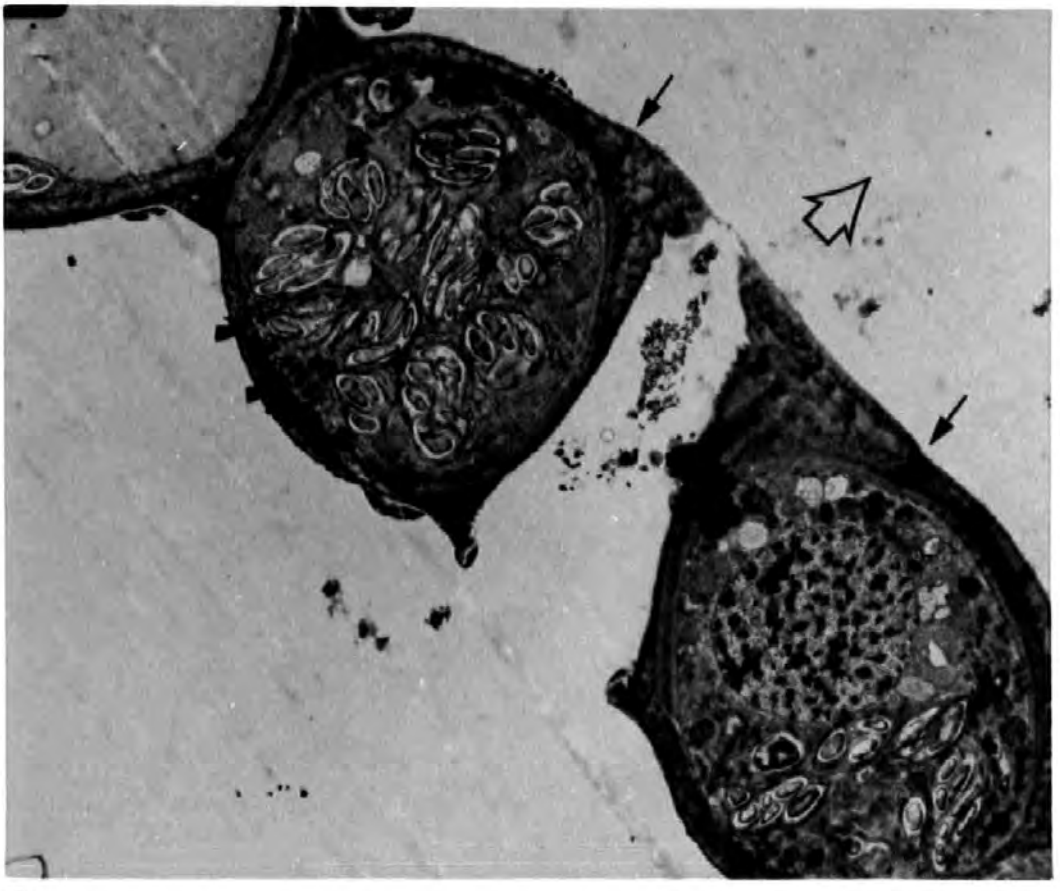
Plate 4.4.

Ultrastructure of mature Polypodium vulgare guard cells, III.

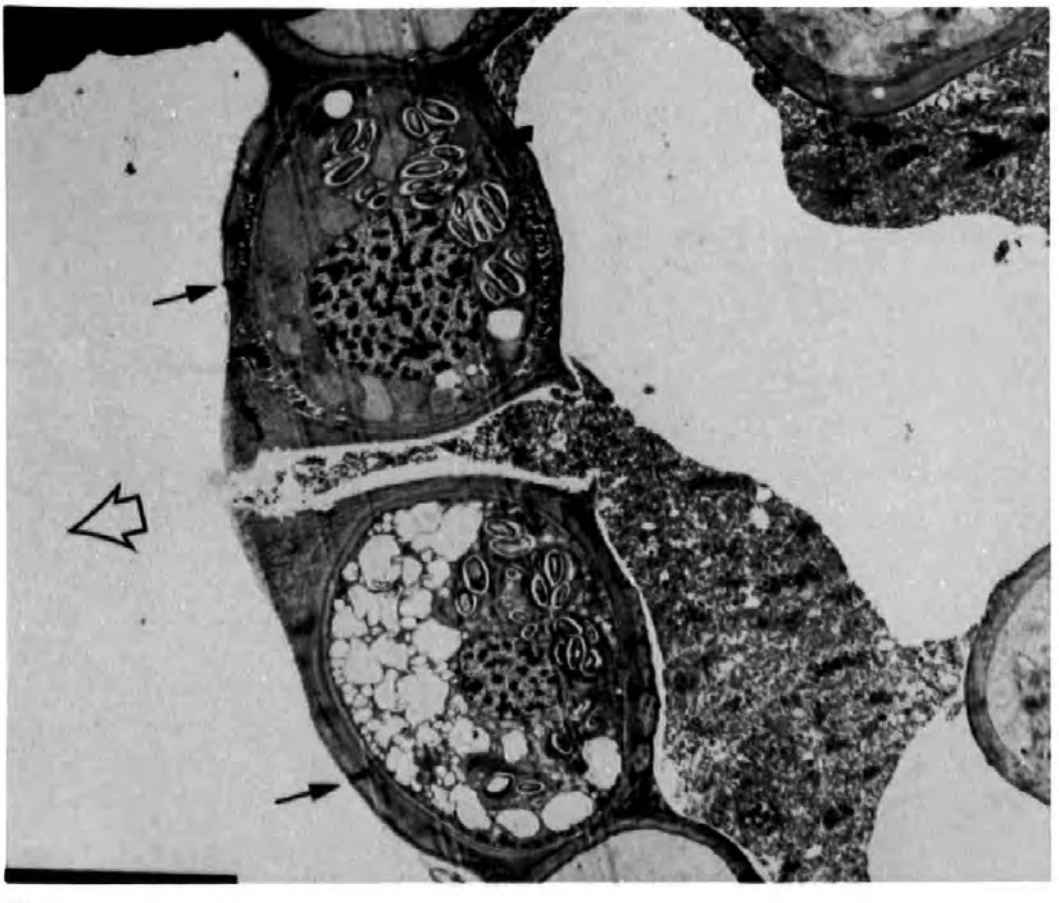
Transmission electron micrographs of tissues fixed in glutaraldehyde and osmic acid; post-stained in uranyl acetate and lead citrate. The hollow arrows indicate abaxial orientation.

A. Oblique vertical section, x 3 500. The section shows the densely packed guard cell protoplast. The vacuolar elements are very variable in nature ranging from electron translucent to relatively electron dense. Other features illustrated include the greatly thickened ridges protecting the stoma, ectodesmata-like artefacts (straight arrows) which probably result from the tissue folding on the grid as it dried out, and the presence of osmiophilic peripheral bodies (curved arrows) which are tentatively identified as spherosomes. Starch inclusions are present in the epidermal cell chloroplast at the upper left hand corner of the micrograph.

B. Transverse vertical section, x 2 900. The section shows similar details to that of A. The difference between the vacuolar elements of the two guard cells is notable. That in the upper guard cell is electron opaque and compartmented into subequal units whilst that in the lower guard cell is electron translucent and highly compartmented. The source of the debris in the substomatal chamber is uncertain but contains very large quantities of membranous material. The underlying mesophyll cells are covered with an endocuticle.



A



B

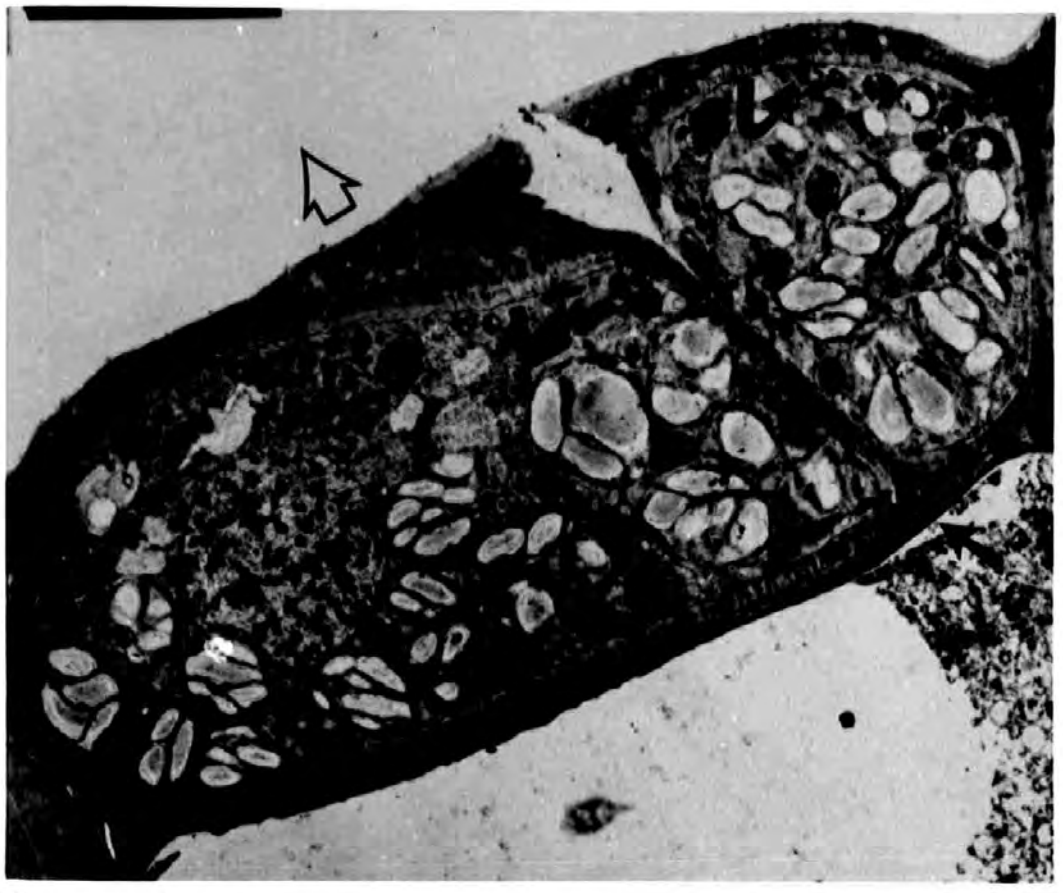
Plate 4.5.

Ultrastructure of mature Polypodium vulgare guard cells, IV.

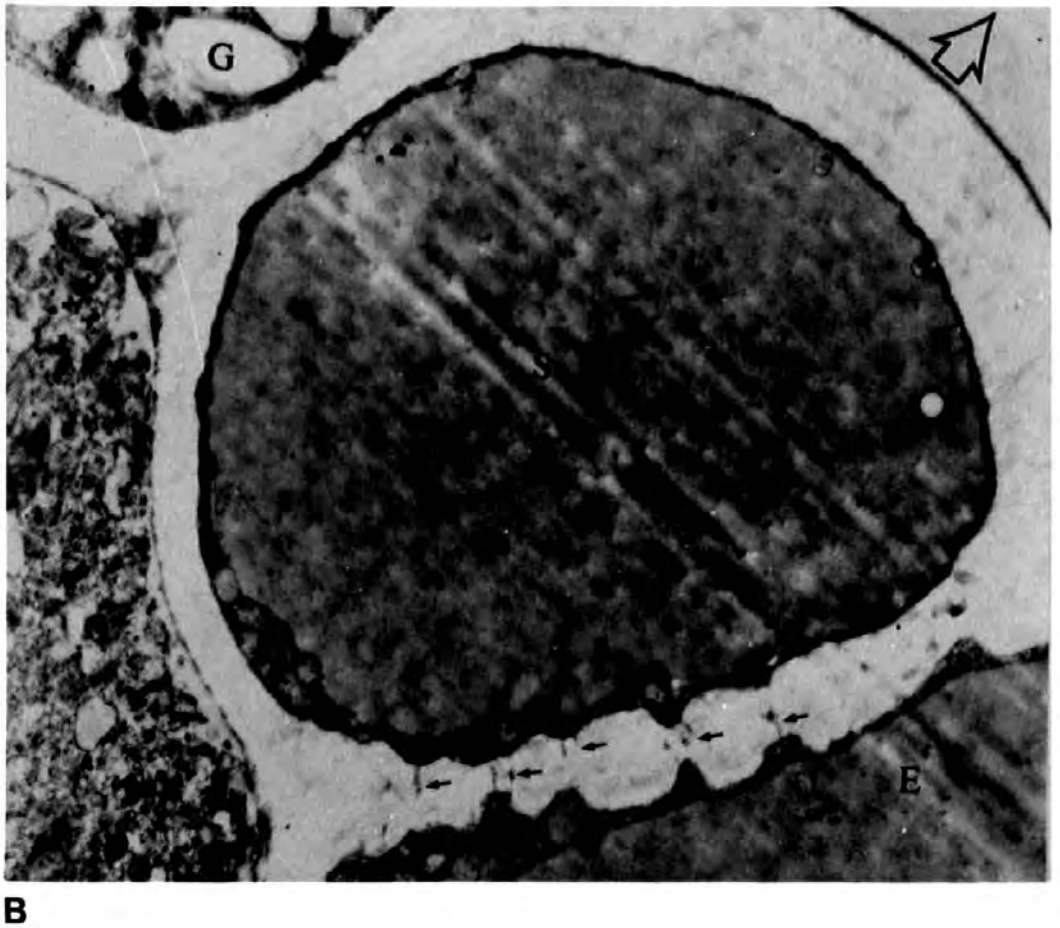
Transmission electron micrographs of tissue fixed in glutaraldehyde and osmic acid; post-stained with uranyl acetate and lead citrate. The hollow arrows indicate abaxial orientation.

A. Oblique vertical section, x 4 500. The section, besides illustrating the densely packed protoplast typical of the species, shows certain anomalies in the lower periclinal guard cell walls (straight arrow). The stoma is clearly subtended by a cell wall element which arises from the lower periclinal wall of the left hand guard cell, passes under that of the right hand guard cell and merges insensibly into the lower periclinal wall of the adjacent epidermal cell. The bent arrows indicate electron dense organelles which are believed to be spherosomes.

B. Vertical section, x 8 600, incorporating a subsidiary cell (S), a guard cell (G), and an epidermal cell (E). Plasmodesmata (small arrows) and associated pit fields are clearly shown in the anticlinal cell wall between the subsidiary cell and the epidermal cell. The cytoplasm associated with the pit fields is extremely rich in endoplasmic reticular elements. No plasmodesmata were observed in the walls of any mature guard cells examined. The densely staining bodies in the guard cell are probably spherosomes.



A



B

Plate 4.6.

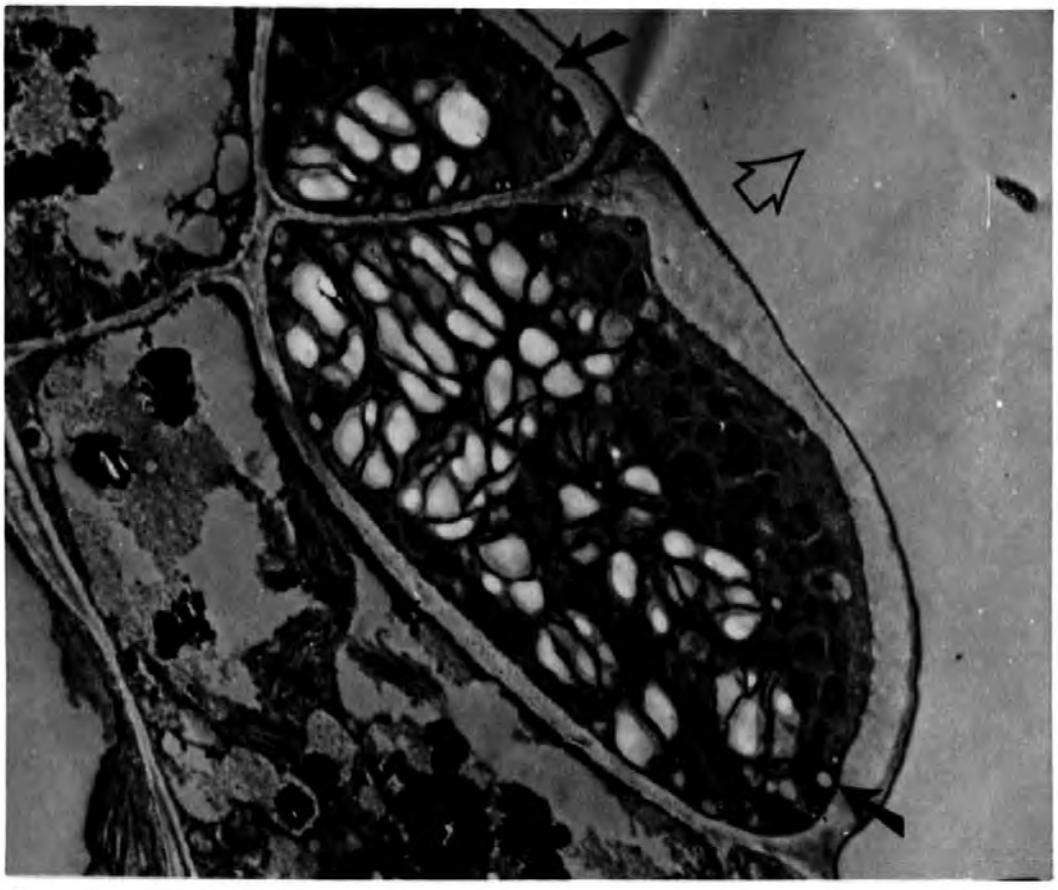
Ultrastructure of mature Polypodium vulgare guard cells, V.

Transmission electron micrographs of oblique transverse vertical sections fixed in glutaraldehyde and osmic acid; post-stained with uranyl acetate and lead citrate. The hollow arrows indicate abaxial orientation.

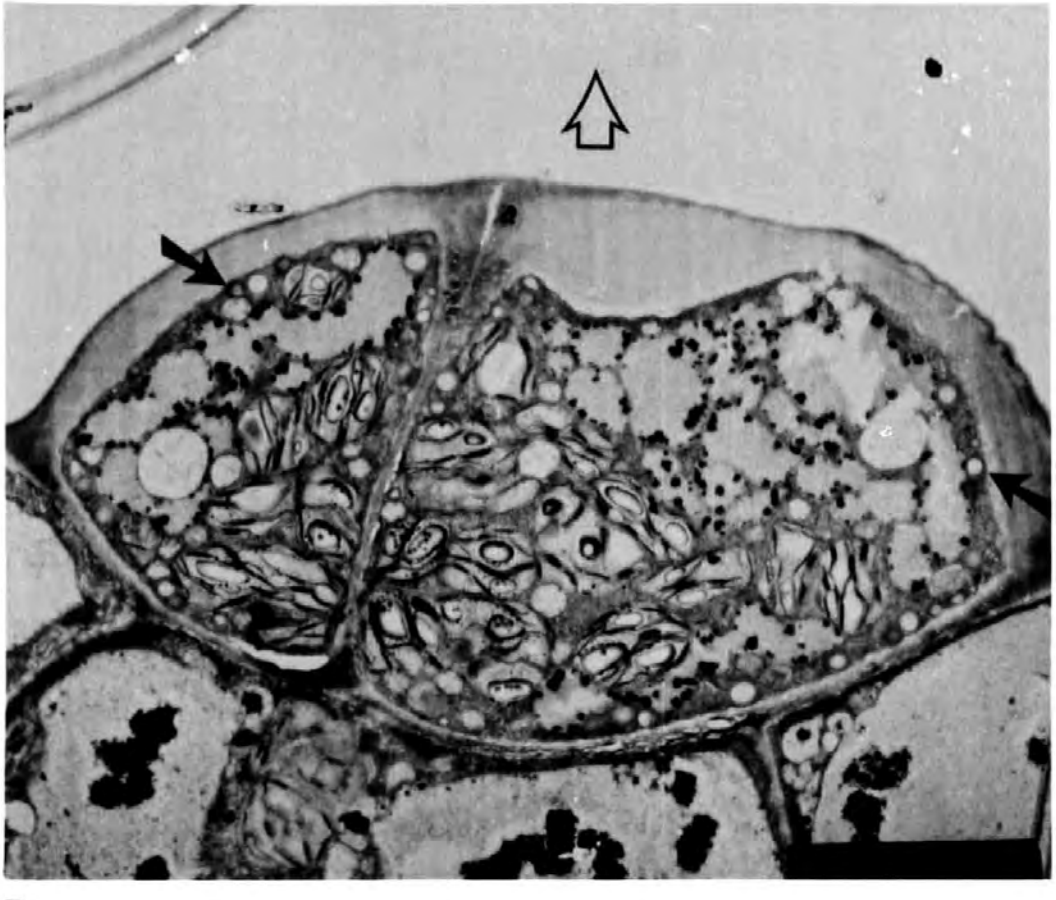
A. x 5 400.

B. x 5 300.

The two plates are directly comparable, both in plane of section and cellular contents. The vacuolar elements of the two sections are very different in nature, those in A being smaller and electron dense, whilst those in B are larger, electron transparent and contain electron dense particles around their periphery. Unidentified bodies (solid arrows), with electron translucent cores and electron dense cortices, are associated with the periphery of the guard cell protoplast in both micrographs.



A



B

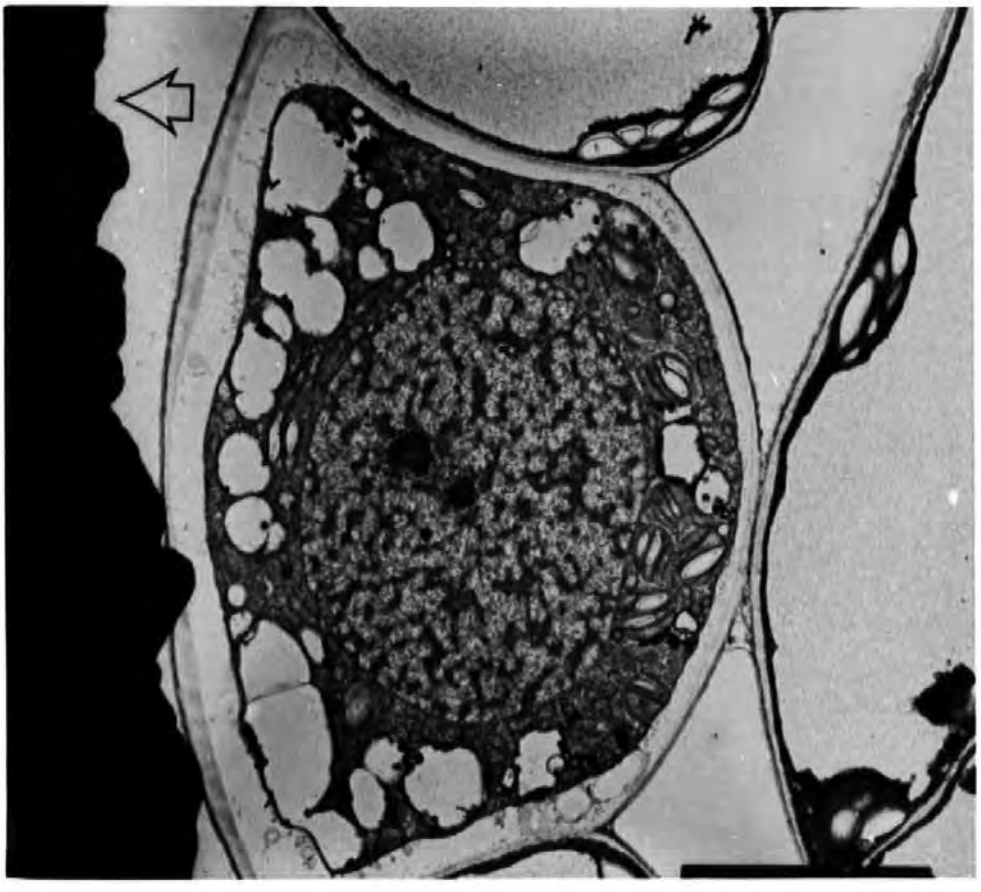
Plate 4.7.

Ultrastructure of developing guard cells of Polypodium vulgare, I.

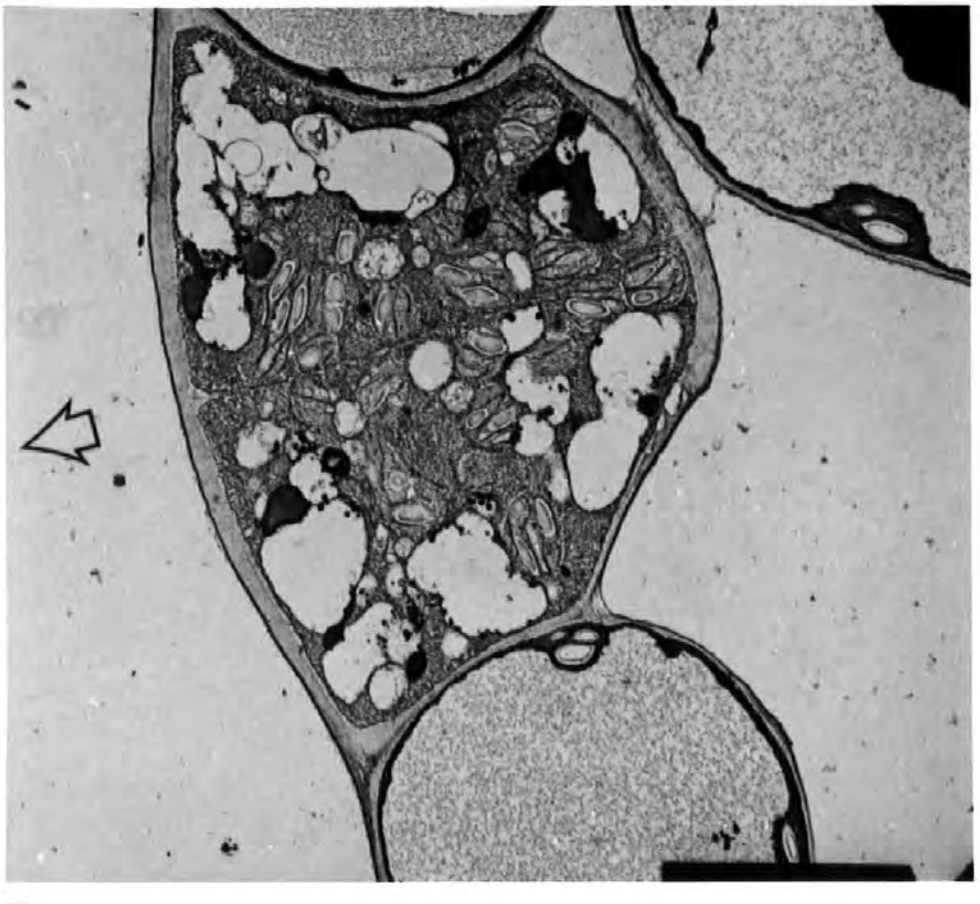
Transmission electron micrographs of transverse vertical sections fixed in glutaraldehyde and osmic acid; no post-staining. The hollow arrow indicates abaxial orientation.

A. Guard-cell mother-cell, x 5 400. The section indicates that chloroplast development is well advanced even at this early ontogenetic stage and that starch inclusions are present in the guard-cell mother-cell, epidermal and mesophyll chloroplasts. The vacuolar element of the guard-cell mother-cell is suprisingly large when considering that the cell is still meristematically active.

B. Young guard cell complex, x 5 300. Similar ultrastructural details to those seen in A are evident in this section. In addition, formation of the common anticlinal wall of the complex has commenced and is represented by the presence of elongated membranous vesicles in the plane of the phragmoplast. The protoplasts of the two cells are still connected through the breaks in the developing cell wall. There is evidence of the initiation of differential cell wall thickening, to form the protective ridges of the stoma, at the junction of the common anticlinal cell wall and the outer periclinal cell wall. There are some highly osmiophilic elements in the vacuoles which are suprisingly well developed.



A



B

Plate 4.8.

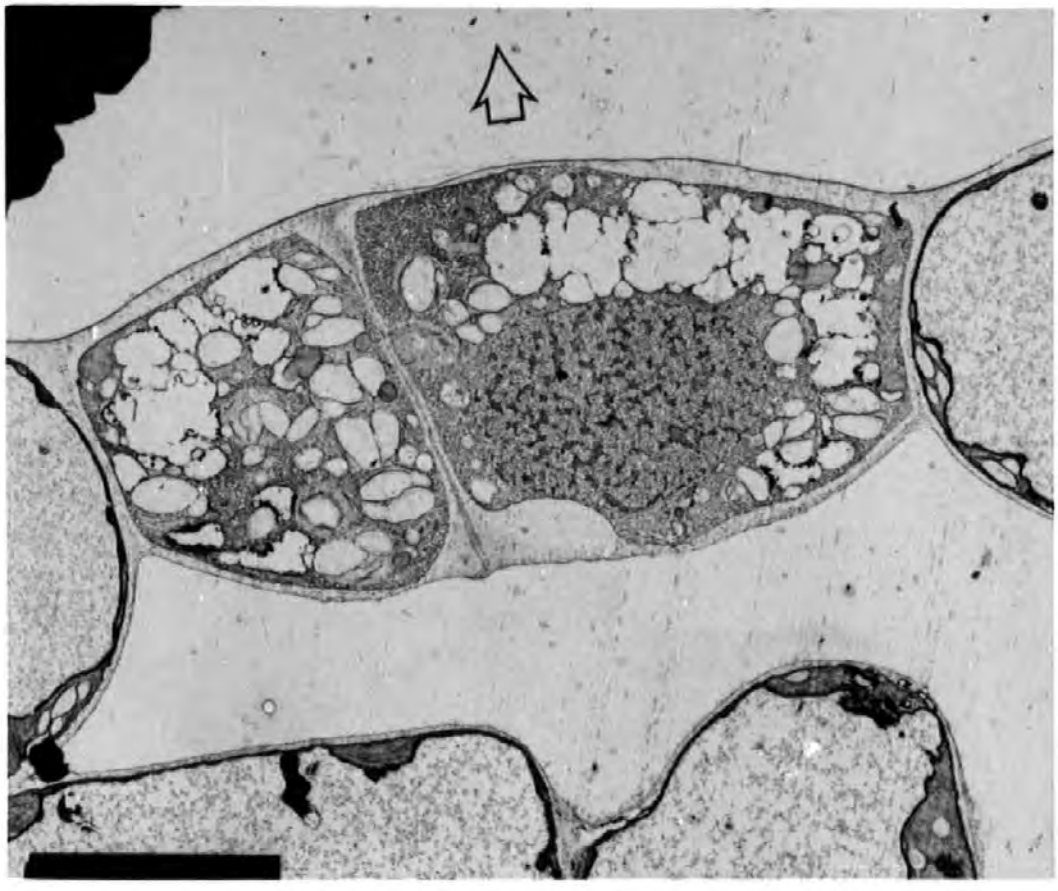
Ultrastructure of developing guard cells of Polypodium vulgare, II.

Transmission electrom micrographs of tissues fixed in glutaraldehyde and osmic acid; no post-staining. The hollow arrows indicate abaxial orientation.

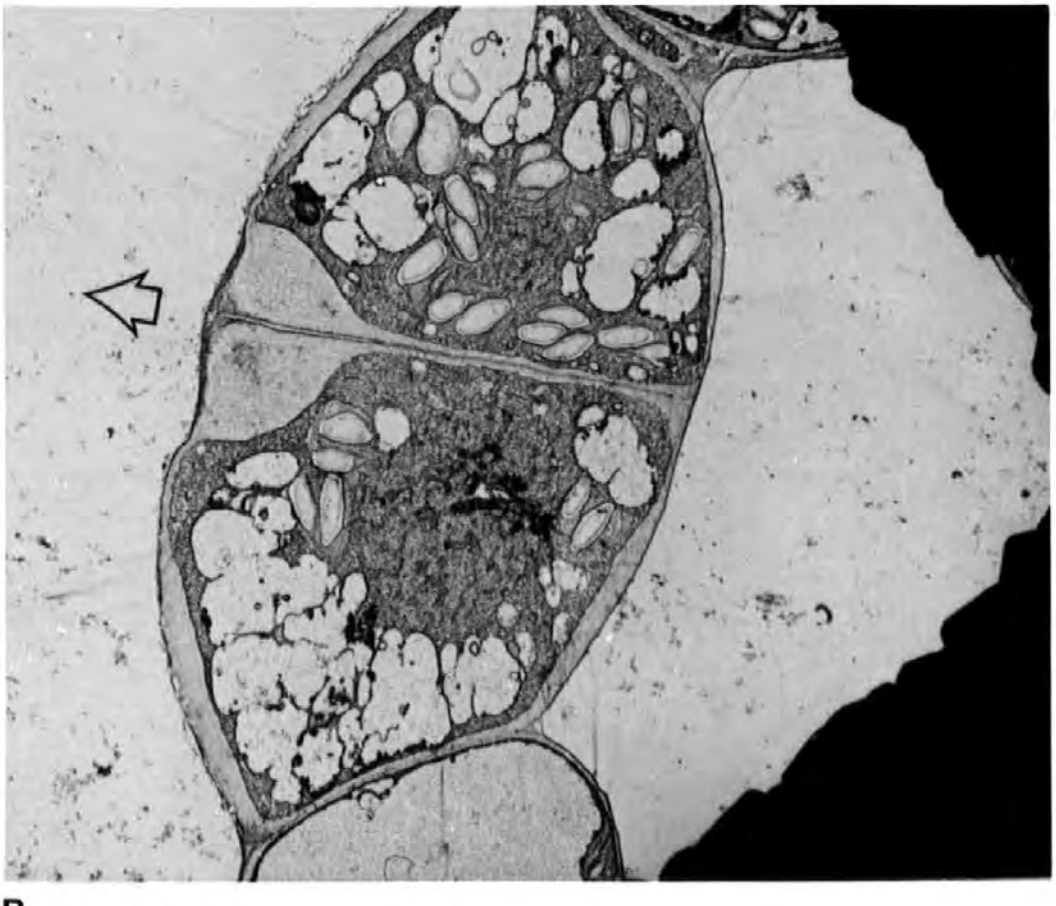
A. Oblique transverse vertical section, x 3 700.

B. Oblique transverse vertical section, x 4 400.

The sections show the development of the differentially thickened ridges which protect the stoma. Both sections are oblique so that development is only shown at one end of the anticlinal wall. The cell wall depositions are laid down on the inner face of the cell walls and considerably distort the lumina of the guard cells. Two distinctive features of these developing ridges are that the outer surface of the periclinal walls immediately adjacent to the ridges become sunken in relation to the rest of this wall and that the adjacent areas of the periclinal walls remain exceptionally thin in comparison to regions further away from the ridges.



A



B

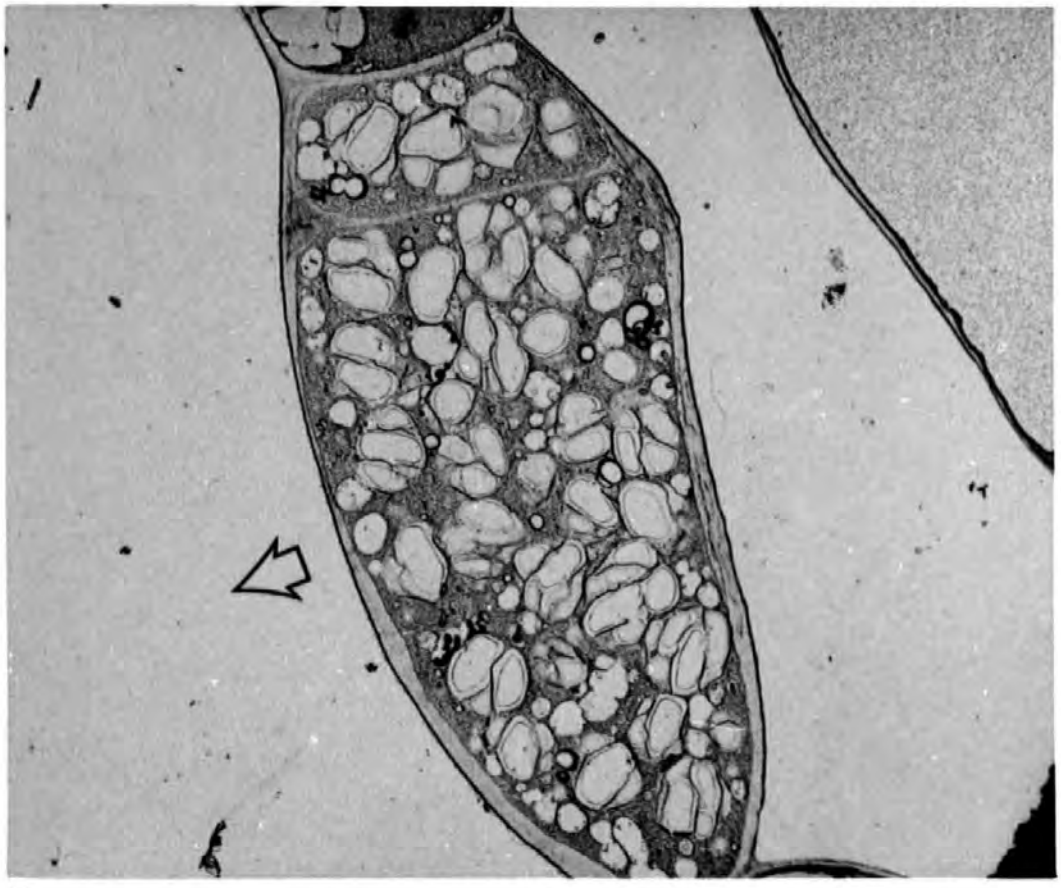
Plate 4.9.

Ultrastructure of developing guard cells of Polypodium vulgare, III.

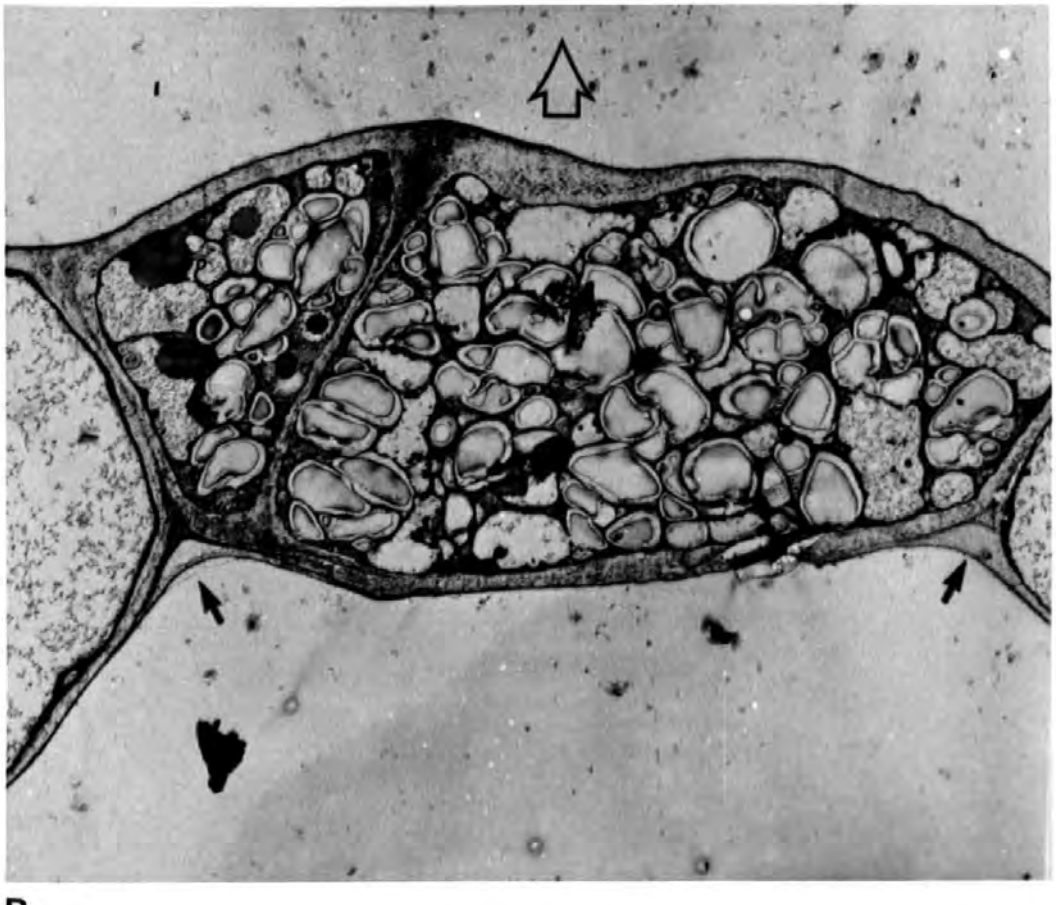
Transmission electron micrographs of obliquely longitudinal vertical sections fixed in glutaraldehyde and osmic acid; no post-staining. The hollow arrows indicate abaxial orientation.

A. x 3 700. The section illustrates the rich starch content of the chloroplasts, and the developing common anticlinal walls of the guard cell complex.

B. x 4 300. Similar ultrastructural features are illustrated in this section as in A. Additionally, this section shows interesting development associated with the lower periclinal cell walls. The solid arrows indicate structures which could be endocuticular trabeculae. Examination of the lumen of the structure on the right hand side shows that its matrix is both identical to and contiguous with that of the lower periclinal cell walls of the adjacent subsidiary cell.



A



B

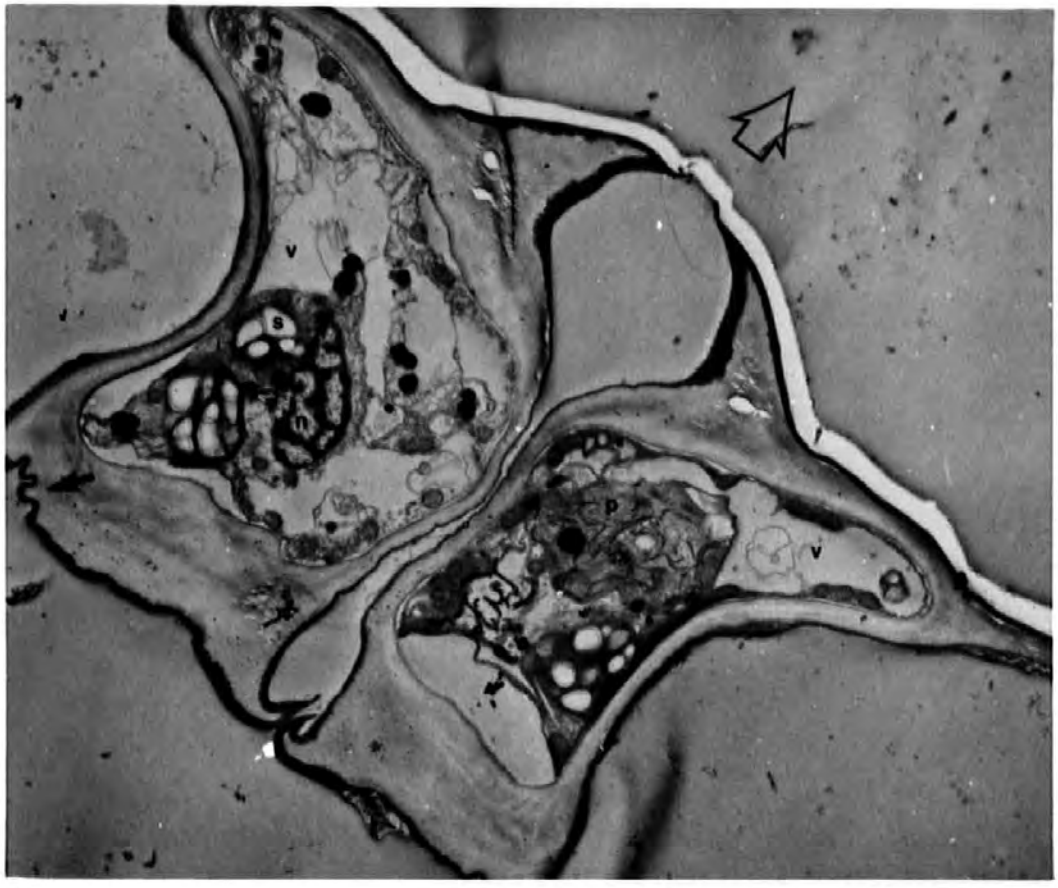
Plate 4.10.

Ultrastructure of mature guard cells of Tradescantia pallidus.

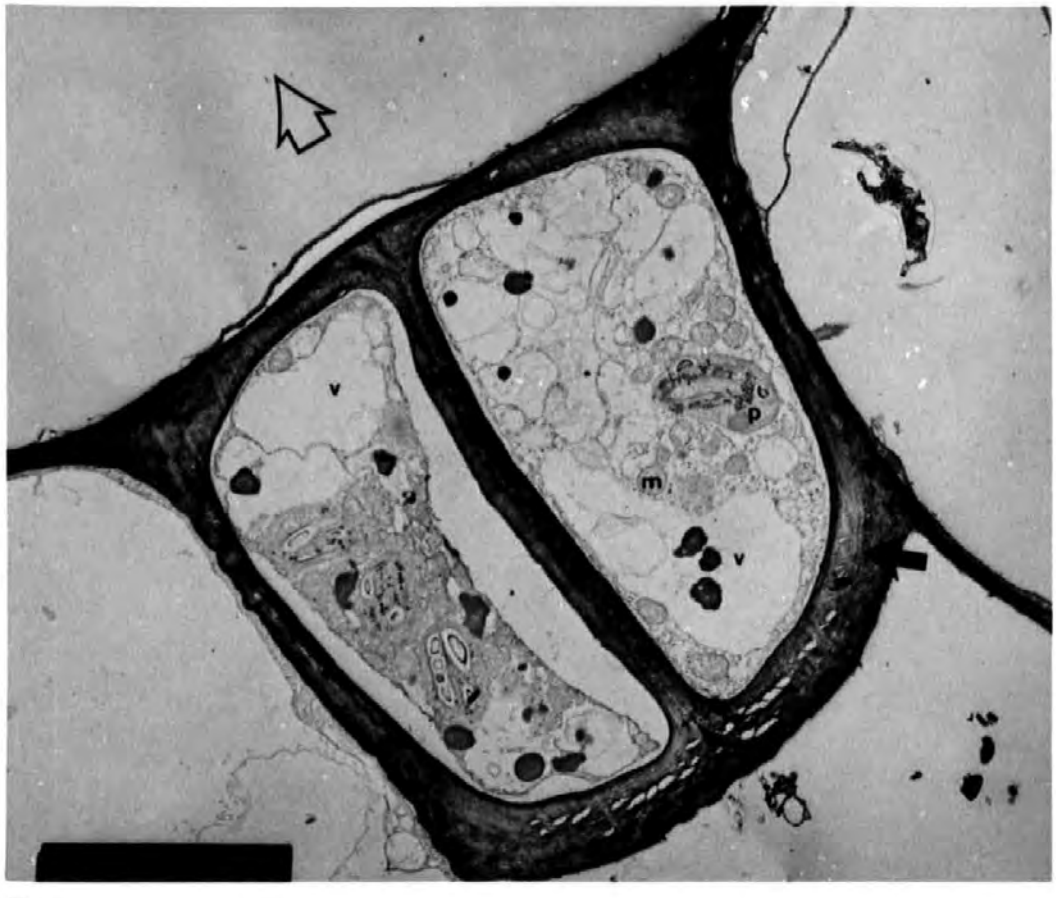
Transmission electron micrographs of vertical transverse sections fixed in glutaraldehyde and osmic acid; post-stained with uranyl acetate and lead citrate.

A. Section through the stoma, x 5 500. This section shows the differentially thickened nature of the lower periclinal cell wall which exhibits characteristic crenulations (arrowed) on its outer face adjacent to the lateral subsidiary cells, and the massively developed thickened ridges protecting the stoma. The cuticle is well developed and stains darkly. The protoplast shows typical ultrastructural organisation with a reticulate chromatin network in the nucleus (n), many starch inclusions (s) in the chloroplasts (p), and vacuoles (v) with membranous and osmiophilic inclusions.

B. Section through the polar regions of the guard cell complex, x 6 500. The ultrastructural organisation is similar to that in A, but shows in addition many mitochondria (m) and the chloroplasts (p) contain numerous osmiophilic bodies. The lower periclinal cell wall is not so thickened in the polar regions but exhibits a bi-lamellar appearance (arrowed) with the outer region being fairly distinct from the inner region. The outer region appears to be more closely associated with the lower periclinal walls of the adjacent subsidiary cell than with the outer anticlinal walls of the guard cells.



A



B

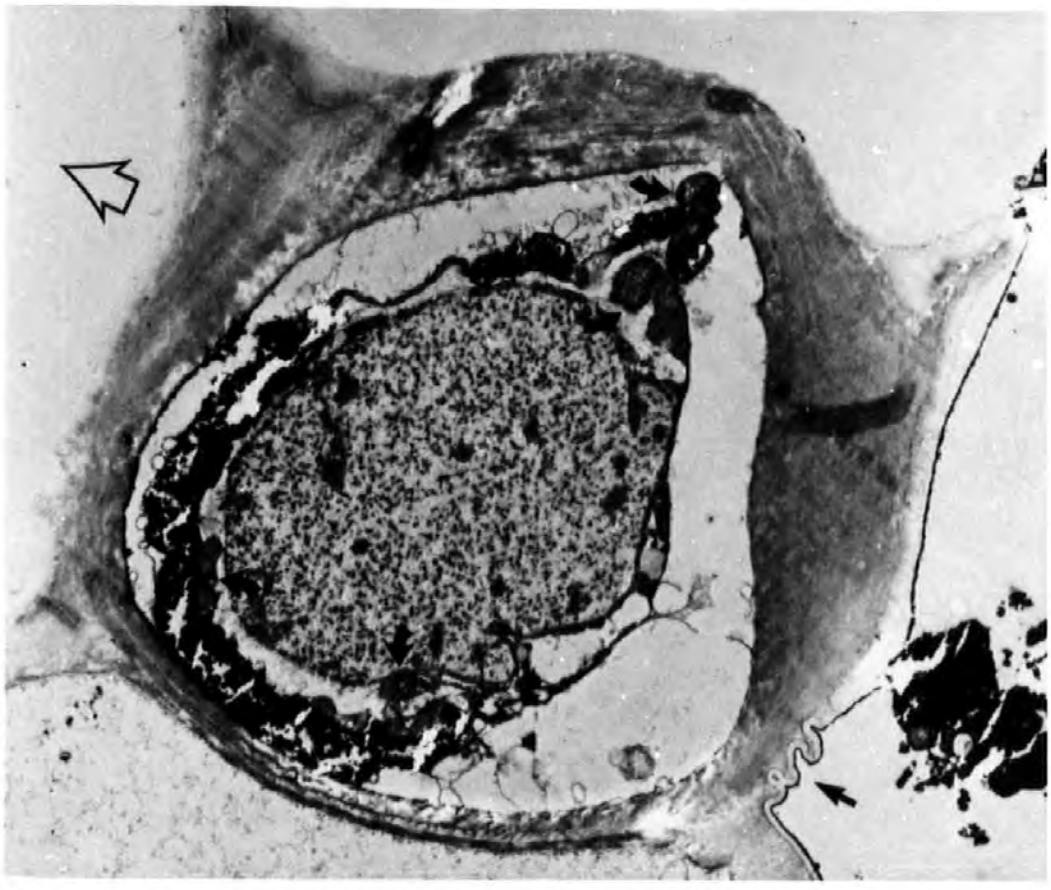
Plate 4.11.

Macallum stain accumulation in Commelina communis, I.

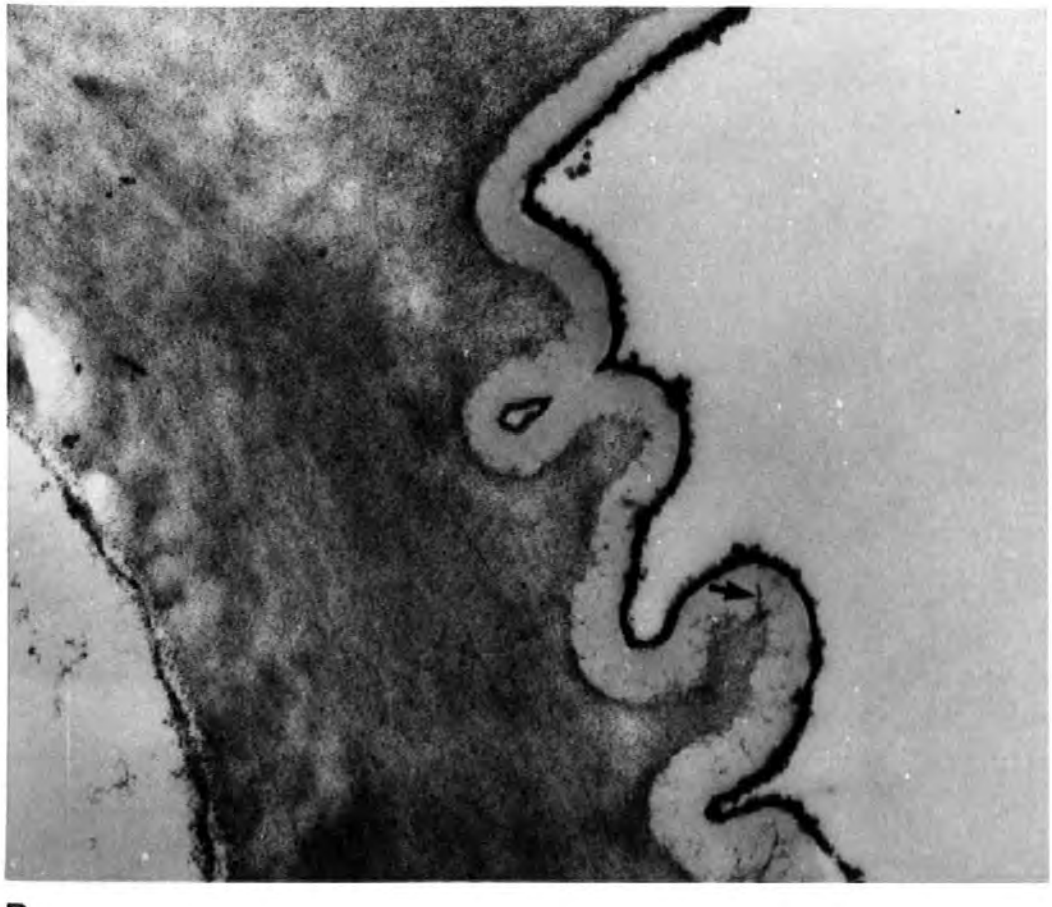
Transmission electron micrographs of transverse vertical sections pretreated with 7.5% (w/v) modified Macallums solution, fixed in glutaraldehyde and osmic acid; no post-staining.

A. Single guard cell, x 7 750; the hollow arrow indicates abaxial orientation. The cobalt stain has become adsorbed into the cytoplasm which has pulled away from the cell wall in places. There is no indication of precipitation within the vacuoles or onto organelles such as chloroplasts (arrowed). Similarly no definite precipitations can be observed within the cell walls although a layer of cobalt has become adsorbed onto the endocuticle of the lower periclinal cell walls which, as in Tradescantia pallidus, are plicated in the region immediately adjacent to the lateral subsidiary cell.

B. Magnification of the plicated region of the lower periclinal guard cell wall, x 47 300. The electron translucent region to the outside of the cell wall is the endocuticle which is covered to its outside by a layer of cobalt. The endocuticle is infiltrated by fibrous elements of the cell wall (arrowed).



A



B

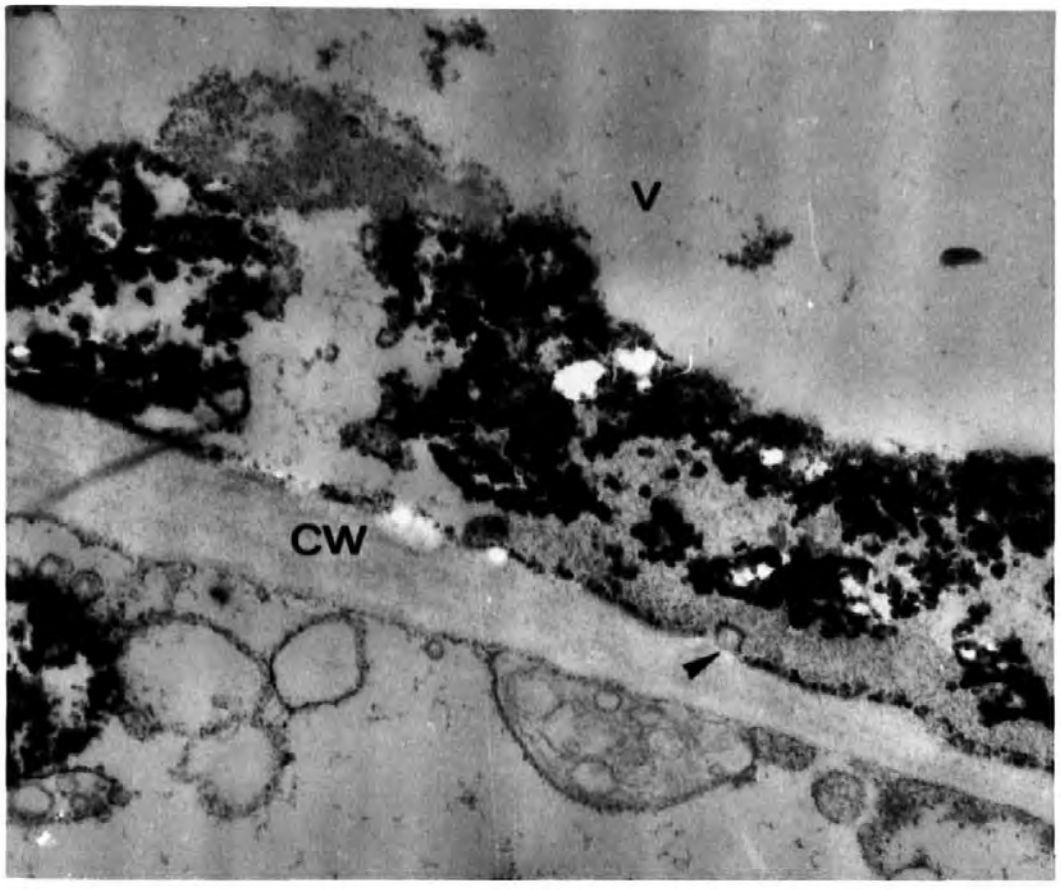
Plate 4.12.

Macallum stain accumulation in Commelina communis, II.

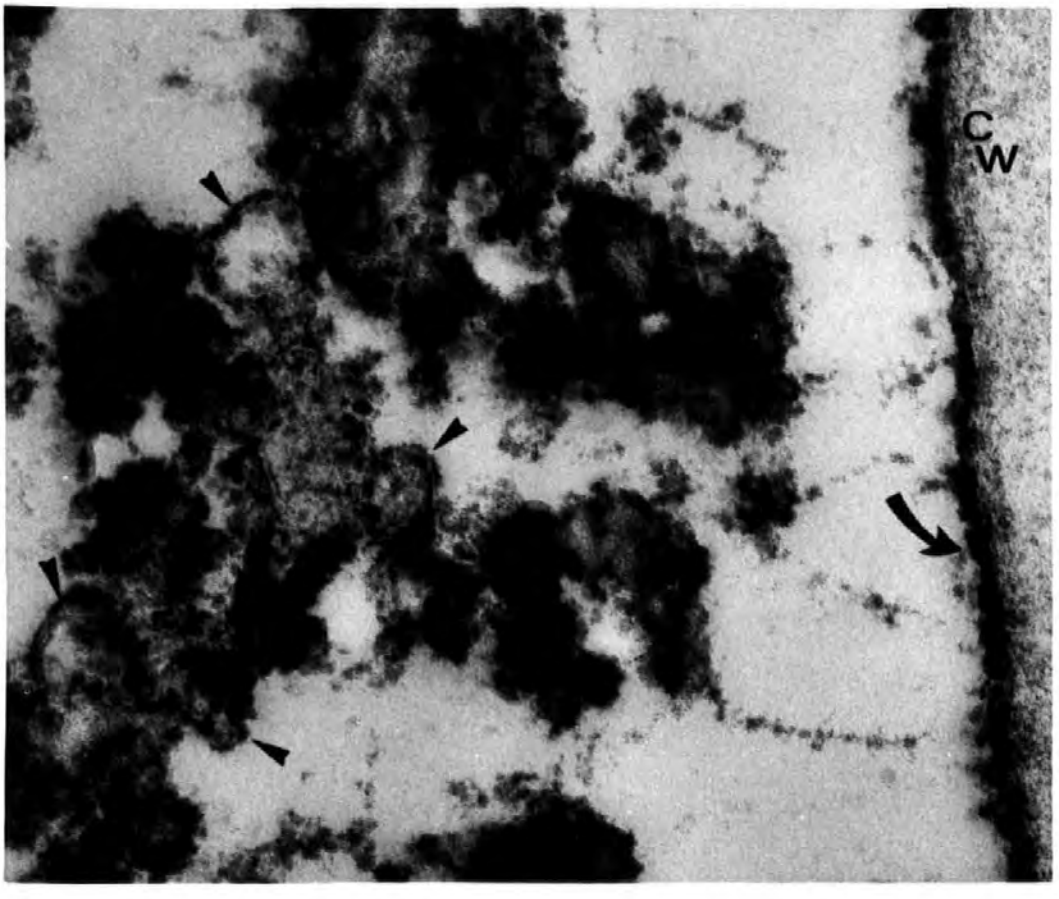
Transmission electron micrographs of vertical sections pretreated with 7,5% (w/v) modified Macallum solution, fixed in glutaraldehyde and osmic acid; no post-staining.

A. x 24 000. The micrograph includes portions of two epidermal cells with an intervening cell wall (CW). The cobalt is precipitated into localised areas of the cytoplasm, and does not appear to be present within the vacuole (V). The trace of cobalt immediately adjacent to the cell wall is presumed to be associated with the plasmalemma which, in other sections, is particularly rich in cobalt-rich pinocytotic-like vesicles of which a poor example is present in this micrograph (arrowed).

B. x 60 850. A high power micrograph of the cobalt deposits. There is a heavy deposit (curved arrow) associated with what is presumed to be the plasmalemma adjacent to the cell wall (CW). The cobalt-rich cytoplasmic mass is characterised by vesicular structures, which, in less densely precipitated areas, can be seen to be formed of unit membrane-like structures (small arrows).



A



B

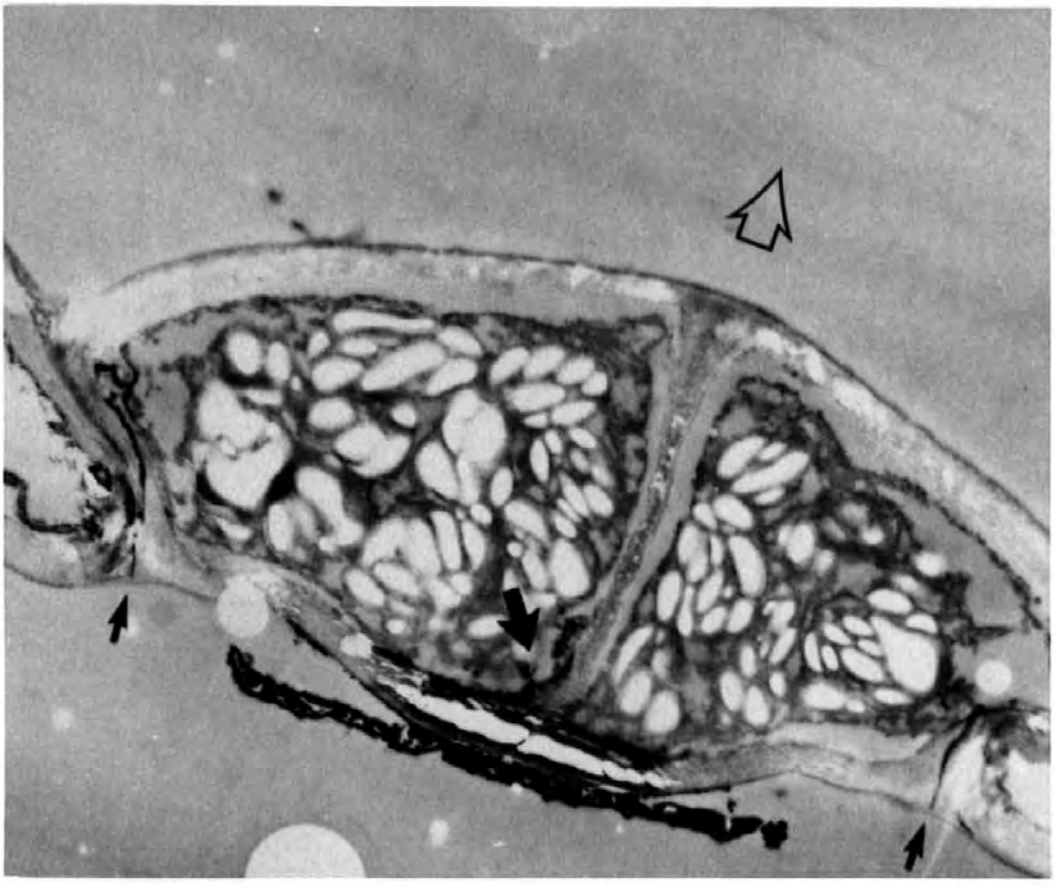
Plate 4.13.

Macallum stain accumulation in Polypodium vulgare, I.

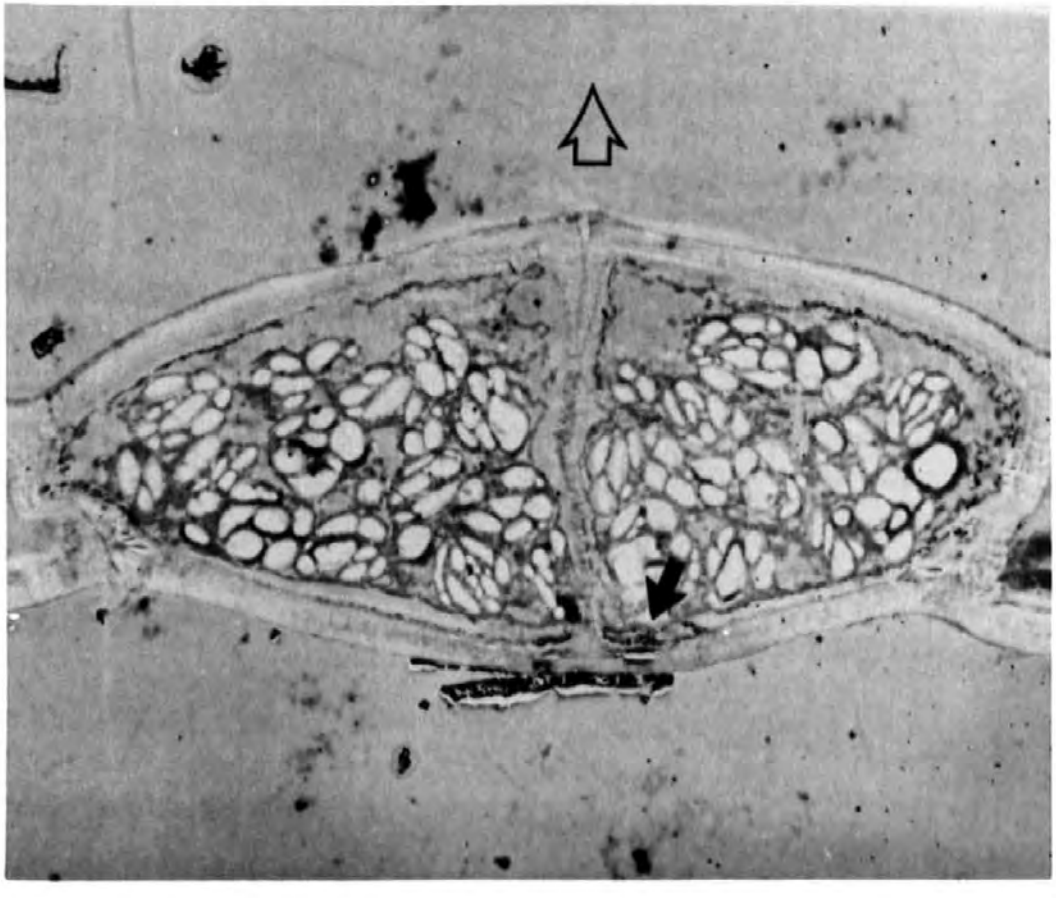
Transmission electron micrographs of vertical transverse sections through the polar regions of guard cell complexes which have been pretreated with 7,5% (w/v) modified Macallum solution, and fixed in ethanol; no post-staining. The hollow arrows indicate abaxial orientation. The stain can be seen to be accumulated in an extracellular body immediately below the common anticlinal guard cell walls. There is little evidence of cobalt deposits elsewhere in the sections except in the cell wall and edge of the protoplast immediately above the bodies (Heavy arrows). A characteristic of many sections such as these illustrated here is the presence of cobalt traces in the lower halves of the outer anticlinal guard cell walls (small solid arrows in A).

A. x 4 600

B. x 3 600



A



B

Plate 4.14.

Macallum stain accumulation in Polypodium vulgare, II.

Transmission electron micrograph of a vertical transverse section through the lower half of a guard cell complex. The tissue was pretreated with 7,5% (w/v) modified Macallum solution, fixed in ethanol, but not post-stained. The pale spots on the micrograph result from water contamination of the formvar coating.

Cobalt precipitated onto a body below the common anticlinal cell walls of the complex in the polar region, x 6 600. The body appears to be bilobed with a 'break' occurring immediately below the common anticlinal guard cell walls (solid arrow). The walls of the body appear to be contiguous with the outer cell wall elements (curved hollow arrow).

Plate 4.15.

Macallum stain accumulation in Polypodium vulgare, III.

Transmission electron micrograph of a vertical transverse section through a single guard cell, x 8 150. The tissue was pretreated with 7,5% (w/v) modified Macallum stain, fixed in ethanol, and not post-stained. There is very little evidence of cobalt within the cell walls except, perhaps, at the juncture of the outer anticlinal guard cell wall and the lower periclinal walls. There is a trace of cobalt around the periphery of the protoplast associated with what is believed to be the plasmalemma. The most striking feature of the section is that the cobalt is adsorbed onto the starch grains (S) of the chloroplasts (C).

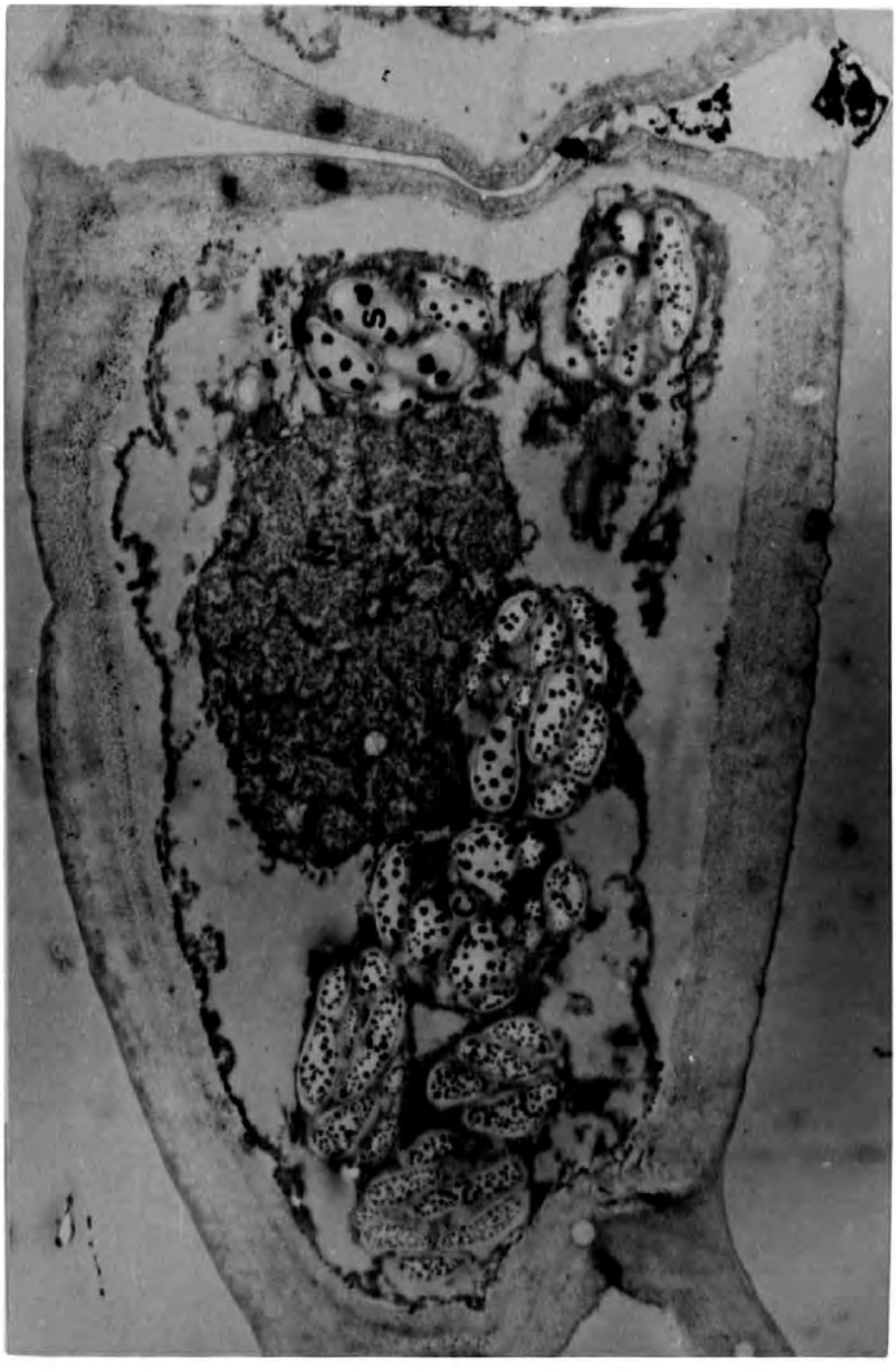

S

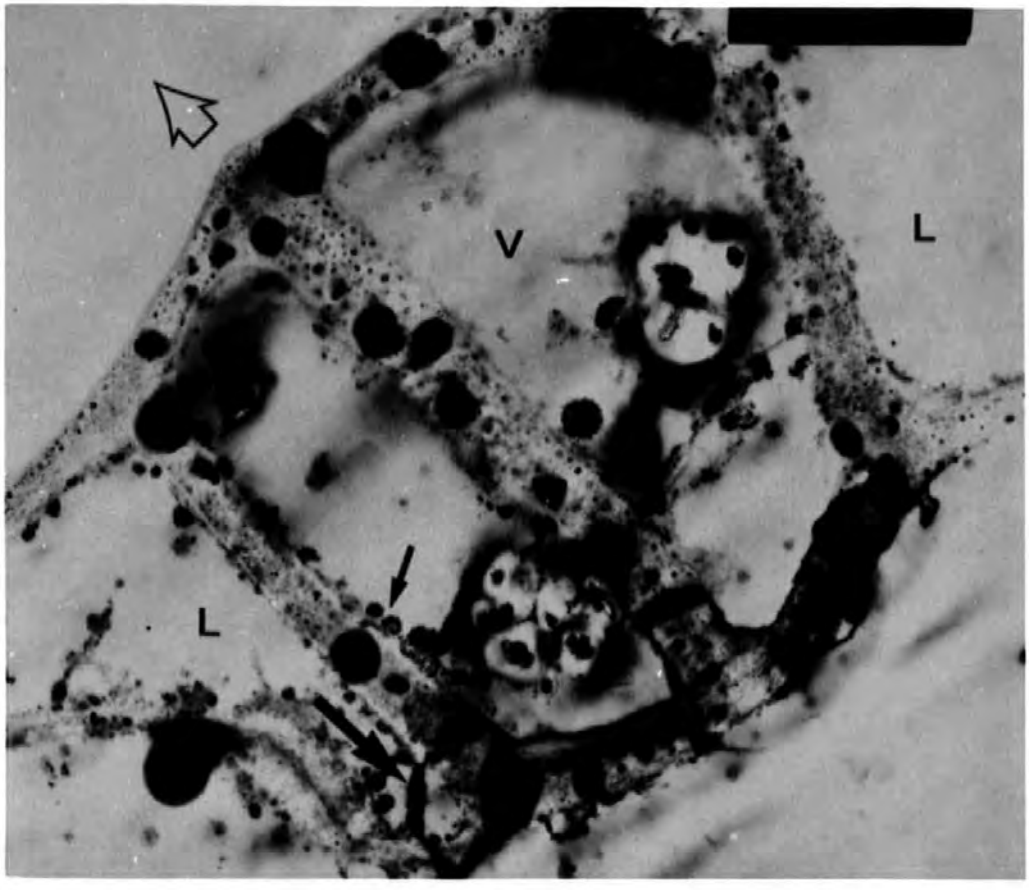
Plate 4.16.

Macallum stain accumulation in Commelina communis, III.

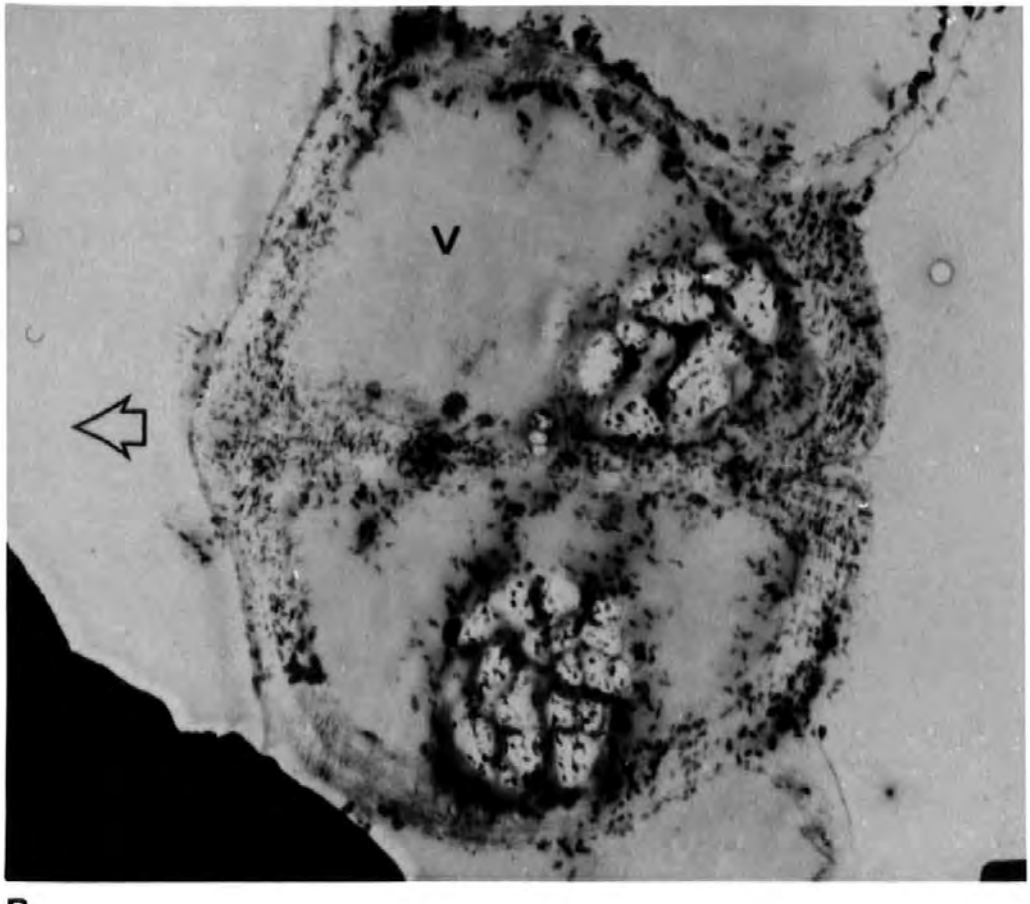
Transmission electron micrographs of vertical transverse sections through guard cell complexes. The tissues were pretreated with 7,5% (w/v) modified Macallum stain, fixed in ethanol, and not post-stained.

A. Section through the polar region, x 8 250. The most notable feature of the section is the 'membrane' delimited cobalt deposit centred on the lower periclinal walls of the complex, but also including the lower parts of the common anticlinal guard cell walls, a part of the adjacent lateral subsidiary cell periclinal wall, and the corner of the subsidiary cell protoplast (large arrow). Elsewhere the cobalt is largely contained within the cell walls and in the cytoplasm of the guard cells; the vacuoles (V) are largely free of deposits. There is evidence of cobalt-rich pinocytotic vesicles forming on the guard cell plasmalemma (small arrow).

B. Section adjacent to the stoma, x 8 000. As in A, the cobalt is restricted to the guard cell walls and protoplast. In this section the cobalt precipitation is in the form of acicular crystals within the cell walls, and the crystals are orientated in a regular manner.



A



B

Plate 4.17.

Macallum stain accumulation in Commelina communis, IV.

Transmission electron micrograph of a vertical transverse section through the edge of the stoma, x 6 600. The tissue was pretreated with 7,5% (w/v) modified Macallum stain, fixed in ethanol, and not post-stained. Cobalt precipitation is primarily located within the anticlinal walls of the complex. Traces of cobalt can also be seen in the middle lamellar region of the lower regions of the outer anticlinal walls of the complex (straight arrow). The cuticle in this section is more electron dense than the cell walls and examination of the endocuticle in the region of the straight arrow reveals the presence of a thin precipitate of cobalt between the endocuticle and the cell wall. The cobalt distribution within the guard cell protoplast is rather sparse but pinocytotic-like vesicles can be seen to be associated with the protoplast immediately adjacent to the anticlinal walls (curved arrow).

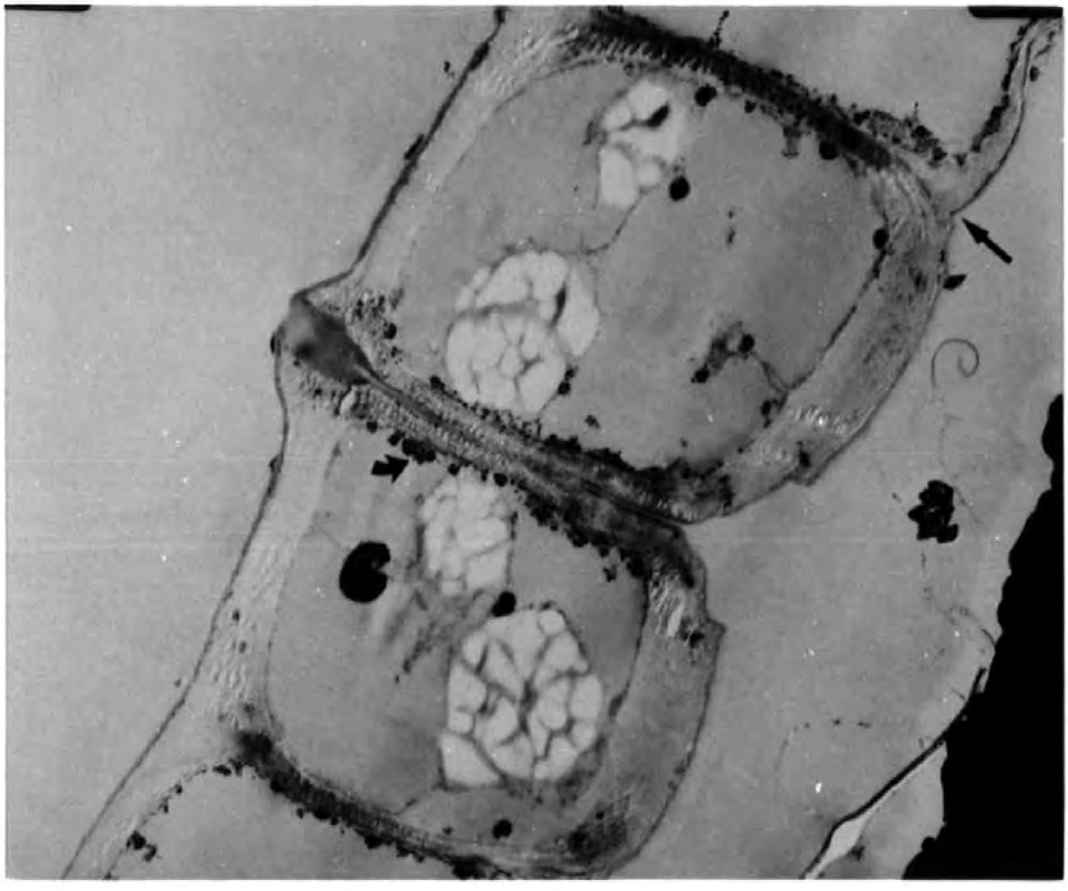


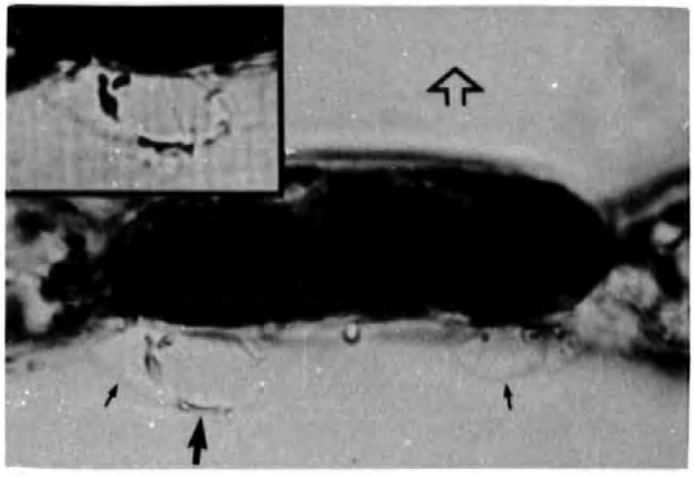
Plate 4.18.

Polar structures in Polypodium vulgare.

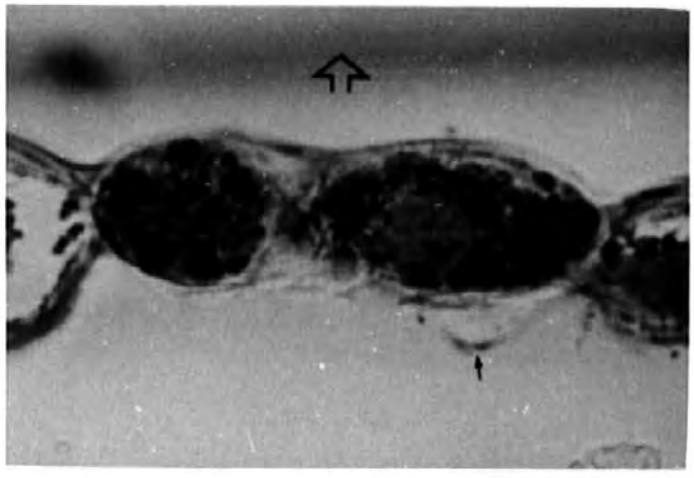
Light micrographs of thick resin-embedded sections fixed in glutaraldehyde and osmic acid, post-stained with methylene blue. Longitudinal vertical sections through guard cells, x 1 200. The hollow arrows indicate abaxial orientation.

A. The small arrows indicate what are believed to be substomatal sacs, whilst the large solid arrow indicates a structure which is thought to be the binding site. The inset is of the substomatal sac containing the binding site which can be seen to be filled with resin (evidenced by the knife marks).

B. Another section with what is believed to be a substomatal sac at one pole of the guard cell (arrowed).



A



B

Plate 4.19.

Macallum stain accumulation in Polypodium vulgare, IV.

Light micrograph of a thick resin-embedded section pretreated with 7,5% (w/v) modified Macallum stain, fixed in ethanol, and post-stained with methylene blue. Transverse vertical section, x 1 100. The hollow arrow indicates abaxial orientation. This section is through the pole of a complex and shows a heavy cobalt deposit immediately beneath the lower periclinal walls.

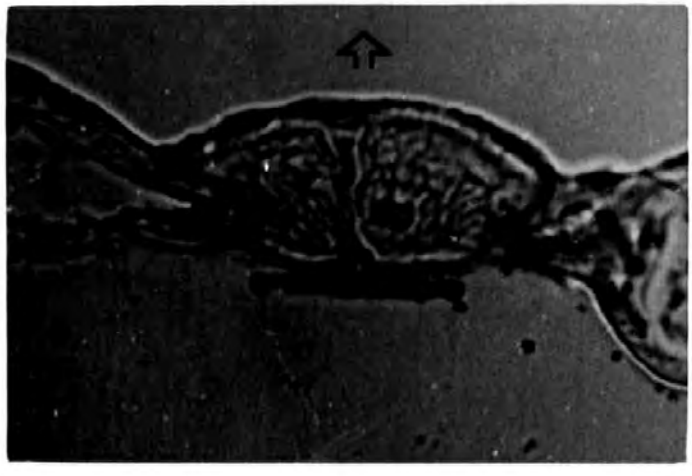


Plate 4.20.

Macallum stain accumulation in Polypodium vulgare, V.

Light micrographs of thick resin-embedded tissue pretreated with 7,5% (w/v) modified Macallum solution, fixed in ethanol, and post-stained with methylene blue. Vertical transverse sections, 1 100. The hollow arrows indicate abaxial orientation.

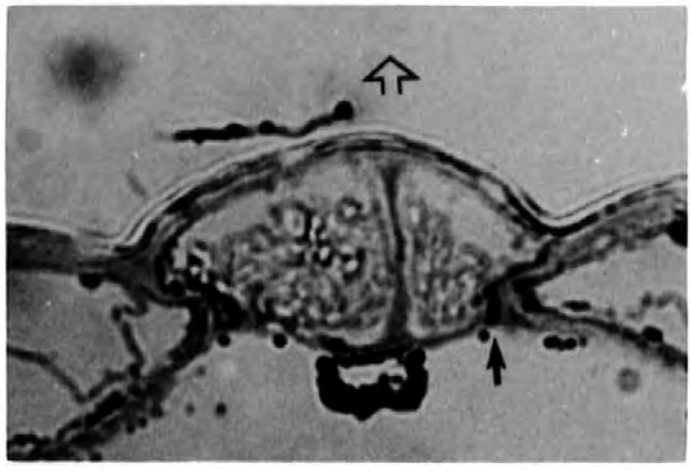
The three sections illustrated here are from the same complex but are not consecutive.

A. Through the extreme pole of the complex.

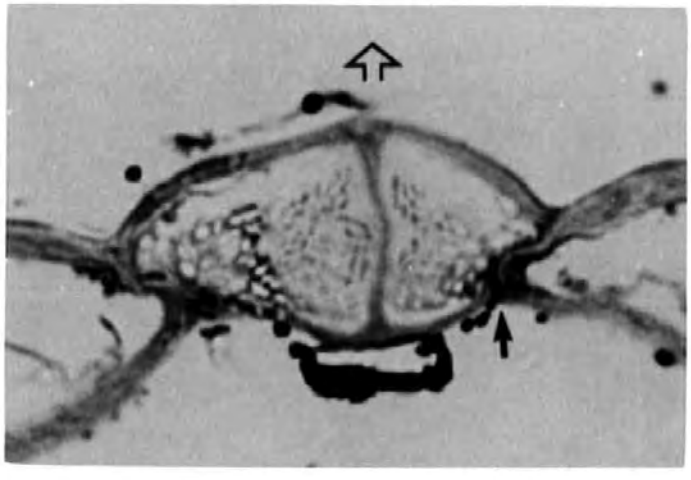
B. Through a region closer to the stoma than A.

C. Through the centre of the polar stain localisation.

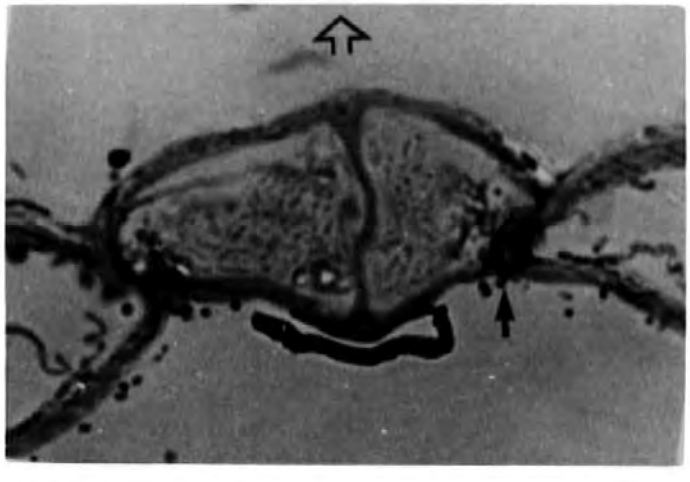
The substomatal body is represented as a hollow structure attached to the lower periclinal wall of the complex. The small solid arrow in each micrograph indicates a cobalt trace in the outer anticlinal guard cell wall.



A



B



C

Plate 4.21.

Macallum stain accumulation in Tradescantia pallidus, I

Light micrographs of resin-embedded thick sections pretreated with 7,5% (w/v) modified Macallum stain, fixed in ethanol, and post-stained with safranin. Transverse vertical sections, x 1 500. The hollow arrows indicate abaxial orientation.

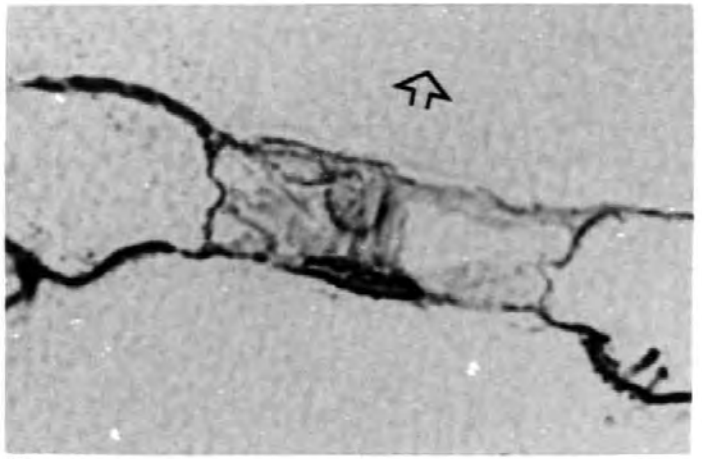
The sections are not from the same guard cell complex.

A. Section through the extreme pole of the guard cell complex and includes only parts of the anticlinal polar guard cell walls. The cobalt is precipitated into a sac-like structure which is located at the interface of the guard cells and the polar subsidiary cell.

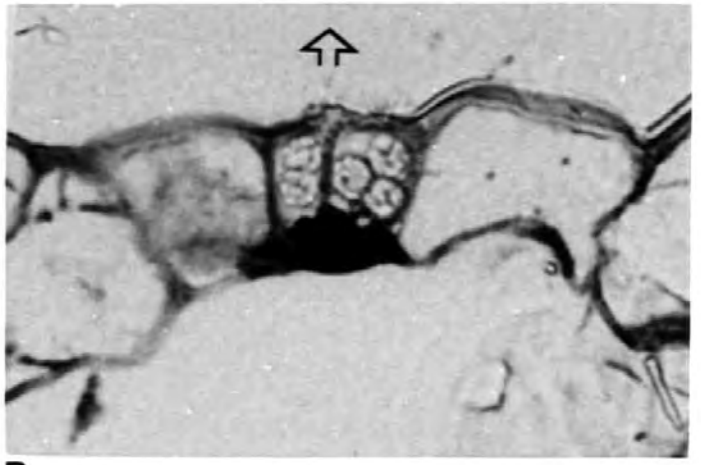
B. Section through the central polar region. The cobalt is precipitated in a 'structure' which encloses the lower walls of the guard cells, parts of the guard cell protoplast, and part of one of the lateral subsidiary cell protoplasts.

C. This section is rather closer to the stoma than section B. The cobalt precipitate is not quite so extensive as in B.

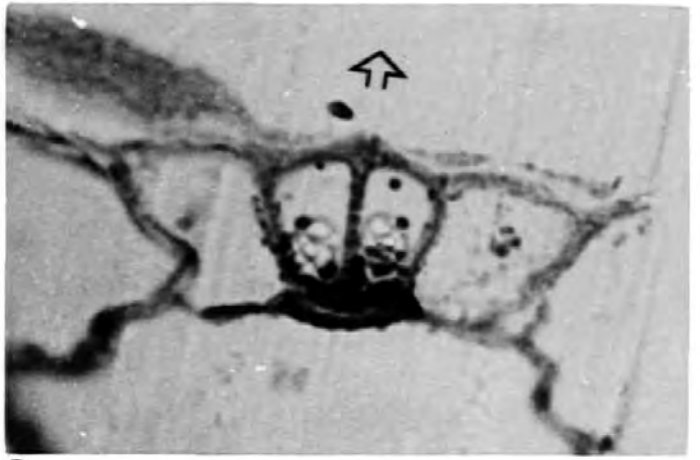
D. Section through the stoma. The sac-like structures illustrated in A, B, and C are lacking in this section. The cobalt deposits appear to be associated with what could be peripheral elements of vacuoles.



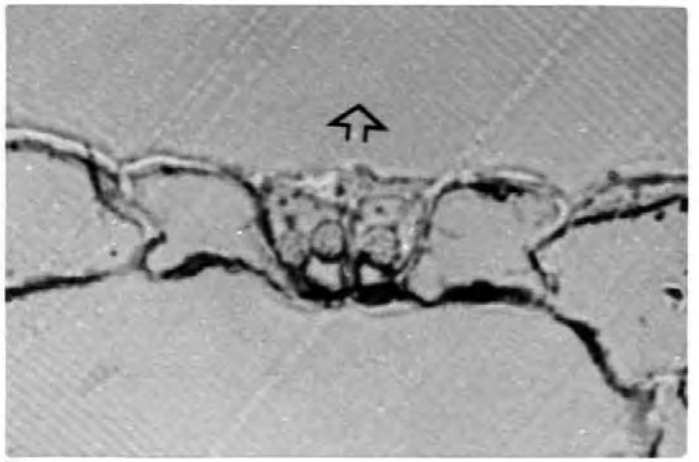
A



B



C



D

Plate 4.22.

Macallum stain accumulation in Tradescantia pallidus, II.

Light micrograph of a thick resin-embedded section pretreated with 7,5% (w/v) modified Macallum stain, fixed in ethanol, and post-stained with safranin. Transverse vertical section, x 2 750.

The section passes through the polar region of the guard cell complex and the cobalt precipitate can be seen to contained within a structure below the lower periclinal cell walls.

CHAPTER 5

SCANNING ELECTRON MICROSCOPE STUDIES

INTRODUCTION

The light and transmission electron microscope studies provided very strong evidence for the presence of polar substomatal structures in certain plants. It had been anticipated that the transmission electron microscopy would have confirmed and elucidated the extracellular binding sites in fresh tissue. However, the binding sites are either completely electron translucent in thin section, or become denatured during preparative procedures. The only way that they could be examined under the transmission electron microscope was after the tissue had been treated with the Macallum stain (1905) prior to a very abbreviated fixation procedure. The results were unsatisfactory as precipitated cobalt occluded structural details of the binding sites, although they did confirm their extracellular location in Polypodium vulgare.

The problems encountered during light and transmission electron microscopy led to a scanning electron microscope investigation being undertaken using fresh material mounted on a cryostage after freezing in liquid nitrogen. The problems encountered and techniques attempted in this relatively new field of biological investigation are detailed in Chapter 2.

OBSERVATIONS IN *Polypodium vulgare*

Epidermis

The external features of the epidermis were examined in a piece of whole leaf which was affixed to the stub, abaxial surface uppermost. The abaxial surface is composed of interlocking sinuous-margined epidermal cells in which the guard cell complexes are arranged in bands (Plate 5.1A). A full description of epidermal cell organisation is given in Chapter 9. The leaf surface is sparsely covered with bi-celled, club-shaped trichomes which arise from the interstices of three or four adjacent epidermal cells. The individual guard cells are barely distinguishable from their pairs due to the cuticle almost totally occluding their common anticlinal cell wall sutures. The cuticle is continuous over the outer surface of the epidermis and there appears to be no wax 'bloom' in this species.

The inner face of the epidermis was examined in epidermal peels affixed to the stub with the side adjacent to the mesophyll uppermost. The cell organisation on the inner face is identical to that of the outer face except, of course, the trichomes are absent. The cuticle is more obvious on the inner face and forms a continuous sheet which covers the cell sutures (Plate 5.1B). The guard cell complex has a very different appearance on this face and is characterised by endocuticular processes traversing the peripheral sutures of the complex between the guard cell and the immediately adjacent epidermal and subsidiary cells. The endocuticle completely occludes the common anticlinal guard cell wall sutures and has a distinctly pouched appearance in the polar regions below these cell walls. The pouches,

in Plate 5.1B, are clearly plicated at one pole suggesting that they are capable of distension.

Mesophyll

The mesophyll was examined by removing the abaxial epidermis from a piece of whole leaf and mounting the remaining tissue on a stub. There is no recognisible palisade or spongy mesophyll tissue in this species, instead it is replaced by an aerenchymatous-like mesophyll tissue (Plate 5.2) of reticulate appearance. The mesophyll tissue is covered by a membranous-like cuticle and the surface of the tissue adjacent to the epidermis is characterised by peg-like protrusions by which the two tissues are connected. The strength of this connection is variable since some epidermes are easier to strip off pinnae than others. In the tissue illustrated, the epidermis was easily stripped off and it appears from the micrograph that the connection between the two tissues is very weak. No sign of attachment was observed on the inner face of the epidermis.

Polar structures of guard cell complex

The epidermis illustrated in Plate 5.1B is clearly under water stress as evidenced by the sunken nature of the lower periclinal epidermal cell walls and the loose folded nature of the endocuticle. The following observations were made on epidermal tissue which was prepared as rapidly as possible so as to minimise water loss.

A comparison of Plate 5.1B and 5.3A clearly indicates that the turgor of the tissue has been maintained in Plate 5.3A. The polar pouches observed in Plate 5.1B are evident in Plate 5.3A as very distinct

substomatal endocuticular sacs. The possibility that the sacs could result from the guard cells having dilated polar regions, as in graminaceous guard cells, is rejected from the evidence in Plate 5.3A. At one pole of the complex illustrated, the common anticlinal guard cell wall suture is clearly visible beneath the substomatal sac which supports the hypothesis that the structures are extracellular.

Material which had been fixed in 70% ethanol prior to examination on the cryostage appeared very dehydrated with the endocuticle collapsed onto the underlying tissues (Plate 5.3B). The guard cell complexes in this tissue are characterised by the presence of discoid bodies, of ca. 11 μm diameter, lying between the lower periclinal cell walls and the endocuticle.

OBSERVATIONS IN THE COMMELINACEAE

Epidermis and mesophyll of Commelina communis

Plate 5.4A illustrates a piece of whole leaf tissue from which the abaxial epidermis has been removed. The substomatal chamber is shown to be formed by a gap in the palisade mesophyll tissue due to the mesophyll cells being directly attached to all the epidermal cells except those of the stomatal complex. The mesophyll tissue, in this species, is formed by a monolayer of palisade cells and a monolayer of spongy mesophyll cells. The substomatal chambers of the abaxial surface are formed similarly to those of the adaxial surface with breaks occuring in the spongy mesophyll below the stomatal complexes. The mesophyll in Plate 5.4A appears to be covered with a cuticle, and cuticular strands can be seen extending between adjacent mesophyll

cells and between the mesophyll and epidermal cells. The inner face of the adaxial stomatal complex, illustrated in Plate 5.4A, differs from that of Polypodium vulgare in that there are no endocuticular trabeculae around the periphery of the complex and there are no distinct polar substomatal sacs although the polar regions are slightly bulbous.

Plate 5.4B is of the inner surface of an abaxial stomatal complex. As in the case of the adaxial complexes, there are no endocuticular trabeculae or substomatal sacs. The endocuticle seems to form only a loose covering over the guard cell complex with many longitudinal folds running the length of the guard cells. These folds could accomodate both the cell wall distention which may occur during stomatal opening, and the hypothetical binding sites. An interesting feature of this micrograph is that the endocuticle of the stomatal complex exhibits a specific orientation of rugose striations. The polar and inner lateral subsidiary cell endocuticles show a radial orientation whilst that of the outer lateral subsidiary cell is longitudinal in respect of the leaf axis. The guard cell endocuticle exhibits no such orientation. It is thought that these rugose striations are not a characteristic of the endocuticle itself but probably reflect underlying features of the cell walls.

Epidermis and mesophyll of Tradescantia pallidus

The external abaxial epidermal surface in this species is covered with a dense waxy bloom (Plate 5.5) which does not appear to be present on the adaxial surface (Plate 5.6B). Plate 5.6B is of a longitudinal section through leaf segments and shows a pair of stomatal complexes subtended by substomatal cavities formed by gaps in the underlying mesophyll tissue. There is no palisade mesophyll in this

species.

Polar structures of the guard cell complex in Tradescantia pallidus

The inner face of the abaxial guard cell complex in fresh tissue (Plate 5.6A) does not show any distinct substomatal sacs, as found in Polypodium vulgare, but its polar regions are bulbous and the endocuticle in these regions is highly plicated. The internal face of the stomatal complexes in epidermal strips which have been floated on distilled water for 30 minutes prior to examination on the cryostage appear different to the fresh ones, as illustrated in 5.6A. The guard cells seem fully turgid (Plate 5.7A) and have weakly bi-lobed, yet distinct swellings at the poles of the guard cell complex with a single strand of up to 2,5 µm thickness running from the polar extremity of each lobe to the polar subsidiary cell.

Sometimes freshly stripped epidermal tissue examined on the cryostage was covered with extensive ice deposits which showed an unusual distribution over the stomatal complex (Plate 5.7B). The ice deposits are particularly heavy and localised over the poles of the guard cell complex and epidermal cells, whilst the rest of the guard cells and the subsidiary cells are relatively free of ice deposits.

The structures underlying the swollen poles of the guard cell complex were sucessfully demonstrated in whole leaf material which had been cut longitudinally in the vertical plane immediately prior to freezing in liquid nitrogen. Plate 5.8A is of such a section and shows a guard cell complex which has been cut down its centre so that the guard cells have been cleaved, rather than cut apart. The binding site, as hypothesised in Chapter 3, is clearly visible as a

discrete subtetrahedral body lying in the intercellular space between the guard and polar subsidary cell. The body is ca. 12,5 μm deep by 12,5 μm long (from Plate 5.8) by ca. 8,0 μm wide (from Plate 5.7A), and whilst one of its faces is adpressed onto the subsidiary cell wall, there appear to be no direct connections between the body and either the guard or subsidiary cells. Plate 5.9 is of a similar section before and after ca. 5 minutes exposure to the electron beam at 8 KeV. In Plate 5.9A, which is of the tissue as it appeared under the scanning electron microscope immediately after freezing, ice crystals can be seen in the substomatal chamber and a membrane-covered structure (curved arrow) is associated with the pole of the guard cell which has been sectioned. The membrane covering this polar structure and the ice crystals disappeared under the heat generated by the electron beam (Plate 5.9B) so that the polar body was exposed as a substantial rugose structure ca. 16 μm deep by 13 μm wide lying in the intercellular space between the pole of the guard cell and the polar and lateral subsidiary cells.

Sections through the lower periclinal guard cell walls in the polar regions exhibit an interesting internal organisation (Plate 5.10) in that they possess intramural laminate interruptions as predicted in Chapter 3. These interruptions give rise to laminate cavities within these cell walls.

DISCUSSION

Polypodium vulgare

This scanning electron microscope survey confirms the

presence of an extensive endocuticle and its modification around the periphery of the guard cell complex into trabeculae traversing the outer anticlinal cell wall sutures of the complex. Furthermore, the presence of distinct substomatal sacs were confirmed by these studies. Failure to obtain good vertical sections through the polar structures prevented direct observations of the ion-adsorbent bodies lying within the substomatal sacs although their presence was confirmed in material in which the endocuticle had been collapsed onto the underlying tissues.

The present study has not revealed any new information about the polar structures which was not previously known or predicted. It is not possible, therefore, to elaborate further on the model of the polar ion-adsorbent bodies in this species as proposed in Chapter 4.

Commelinaceae

The scanning electron microscope studies confirmed the presence of an endocuticle in the Commelinaceae. The regularly orientated striations observed in the endocuticle in Plate 5.4B are thought to reflect the orientation of the underlying cell wall micellae which were first reported in stomatal complexes by Ziegenspeck (1937/9). The micellar striations are not visible in the guard cells of Plate 5.4B and this is probably because the stoma is closed and the endocuticle, consequently, is in a relatively 'loose' condition with numerous longitudinal folds and not stretched over the guard cells. If they are manifestations of the micellae of the cell walls, it is interesting to note that those of the polar subsidiary cell are orientated at right angles to those illustrated in the related plant, Rhoeo discolor (Ziegenspeck, 1937/9; Fig. 8, p 303). The radial orientation of the micellae in the inner lateral subsidiary cells

may indicate that the turgor movements in these cells is in a longitudinal direction during stomatal opening rather than in a radial direction as is suggested by microscopic observations.

The external manifestation of the substomatal structures on the inner face of the guard cell complex is not as pronounced as in P. vulgare. Indeed, only a slight polar swelling was observed in fresh material. However, the polar structures appear to become swollen when strips are floated on water (Plate 5.7A) so that sac-like structures do appear at the poles. The explanation for this poor external manifestation of the polar structures in T. pallidus, and probably other members of the Commelinaceae, is due to the difference in the position of the binding site when compared with P. vulgare. In P. vulgare it lies below the lower periclinal guard cell walls in the polar region, whilst in T. pallidus it lies in the intercellular space between the pole of the guard cell and the adjacent subsidiary cells.

A revised model of the polar structures in T. pallidus is presented in Fig. 5.1 to accomodate the findings of the scanning electron microscope studies. Its basic difference to that proposed in Chapter 3 is that the binding sites have been re-located in the intercellular space at the pole of the guard cell complex.

The deposition of ice crystals in freshly-stripped epidermes, as illustrated in Plate 5.7B, is of considerable interest. When strips are removed from Tradescantia spp., cell sap exudes from the exposed surfaces. Scanning electron microscopy suggests that the sap must come from the mesophyll since the epidermal cells do not usually appear to be damaged. The majority of the ice deposition which covers the epidermal strip is restricted to the epidermal cells (as opposed

to those of the stomatal complex) and may well represent frozen cell sap. This explanation may not apply to the polar ice deposits in the guard cell complex since the complex is not in contact with the mesophyll (Plate 5.6B). These polar ice deposits may, therefore, arise from the guard cells themselves and could represent liquid which has been forced out at these sites as the strip is bent back on itself during removal. Such an interpretation infers that either the poles of the guard cells are highly hydrated or else that this region of the complex is particularly permeable.

Fig. 5.1.

Polar structures in Tradescantia pallidus.

This is a diagrammatic representation of the polar structures as determined by the scanning electron microscope studies and is based on Plates 5.8, 5.9, and 5.10.

The sectioned cell walls are single-stippled; the binding sites are double-stippled.

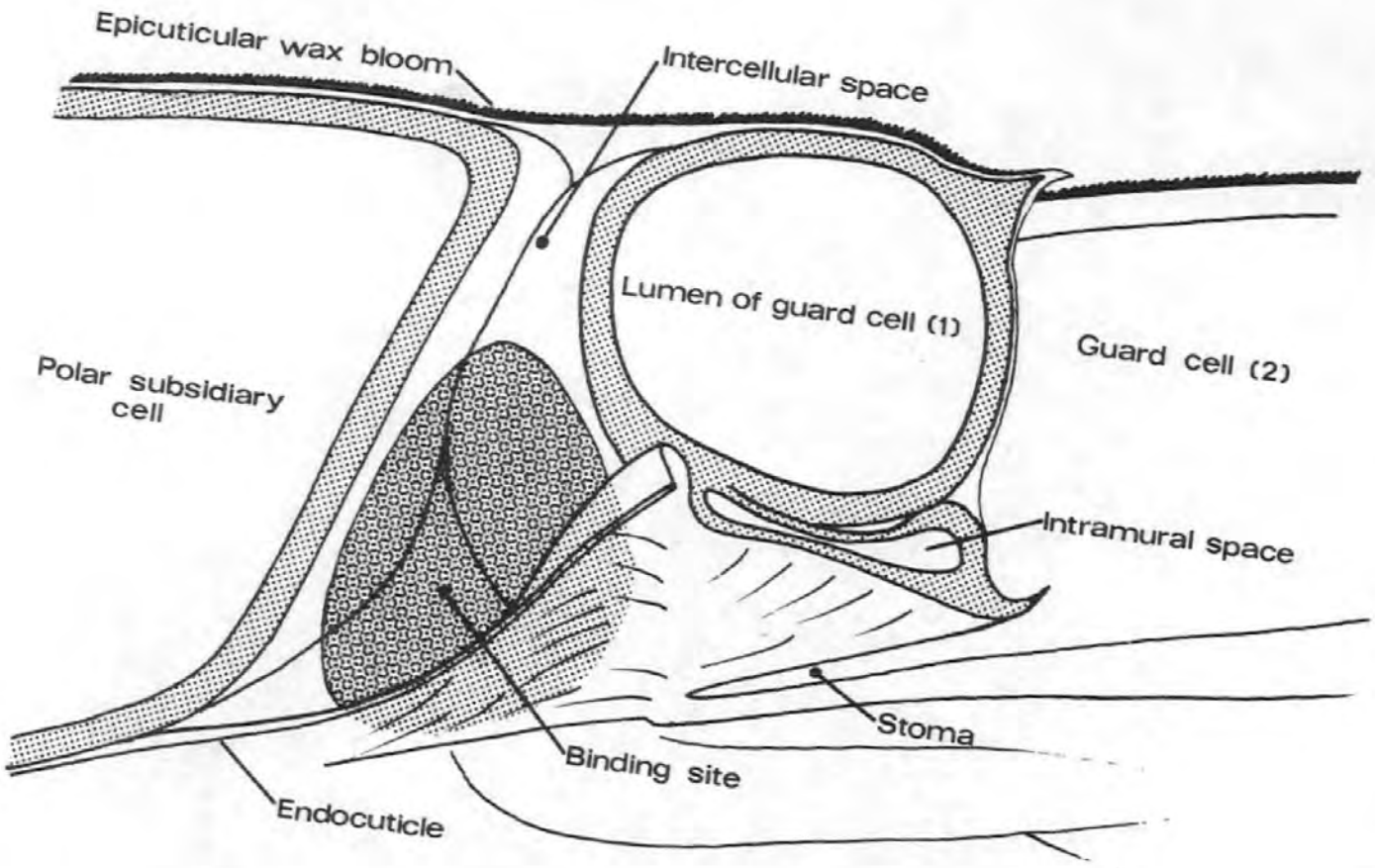
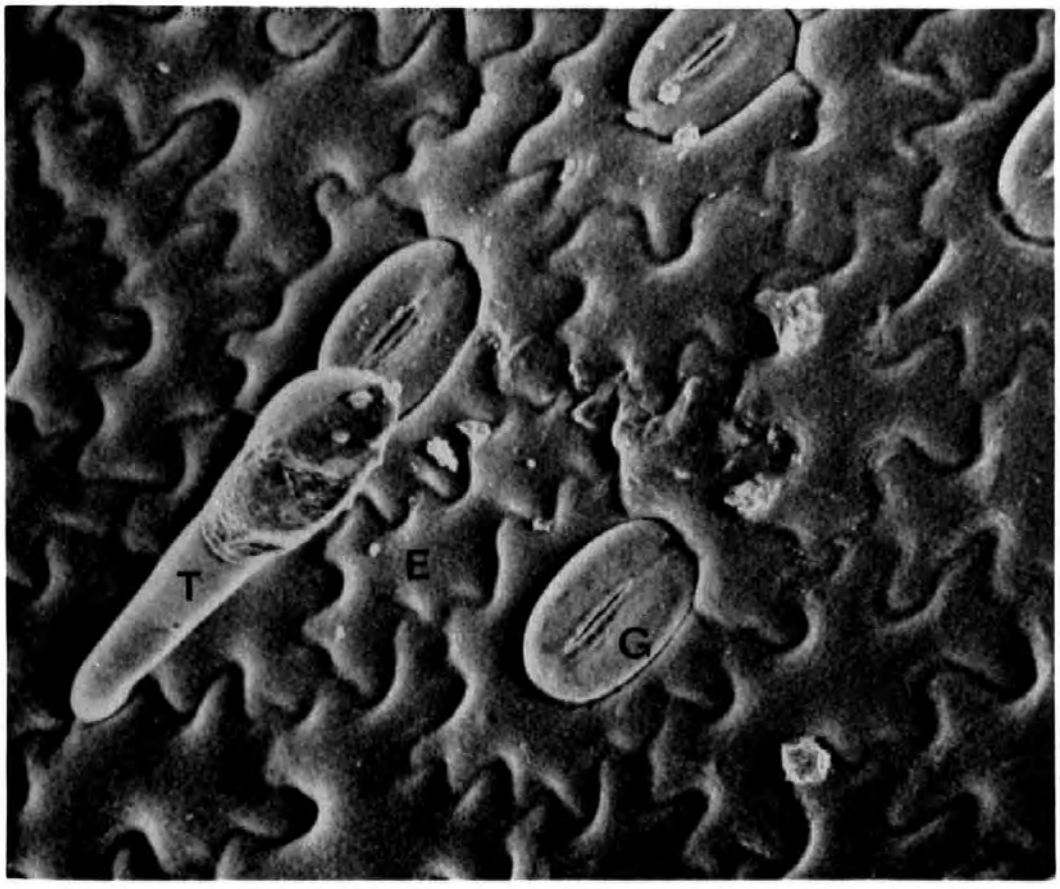

Epicuticular wax bloom
Intercellular space
Lumen of guard cell (1)
Guard cell (2)
Polar subsidiary cell
Intramural space
Stoma
Binding site
Endocuticle

Plate 5.1.

Stomata in Polypodium vulgare, I.

A. 10 KeV scanning electron micrograph of the abaxial surface of the pinna. The guard cells (G) can be seen to be associated with a horseshoe-shaped subsidiary cell. The epidermal cells (E) are characterised in many ferns by their sinuous anticlinal cell wall sutures. The epidermis is sparsely covered with bicellular trichomes (T). (x 450)

B. 10 KeV scanning electron micrograph of the internal face of a guard cell complex, x 2 000. There is no indication of the common anticlinal guard cell wall suture, the whole complex being covered with an endocuticle. A distinctive feature of the guard cell complex (G) is the presence of a plicated sac-like structure (P) at either pole. Endocuticular trabeculae can be seen traversing the outer anticlinal walls of the complex onto the subsidiary cell (S) and epidermal cells.



A

B

Plate 5.2.

Mesophyll of Polypodium vulgare.

10 KeV scanning electron micrograph of mesophyll tissue, x 1 030. The mesophyll is of an aerenchymatous nature and appears to be connected to the epidermis in life by the peg-like processes illustrated in this micrograph.

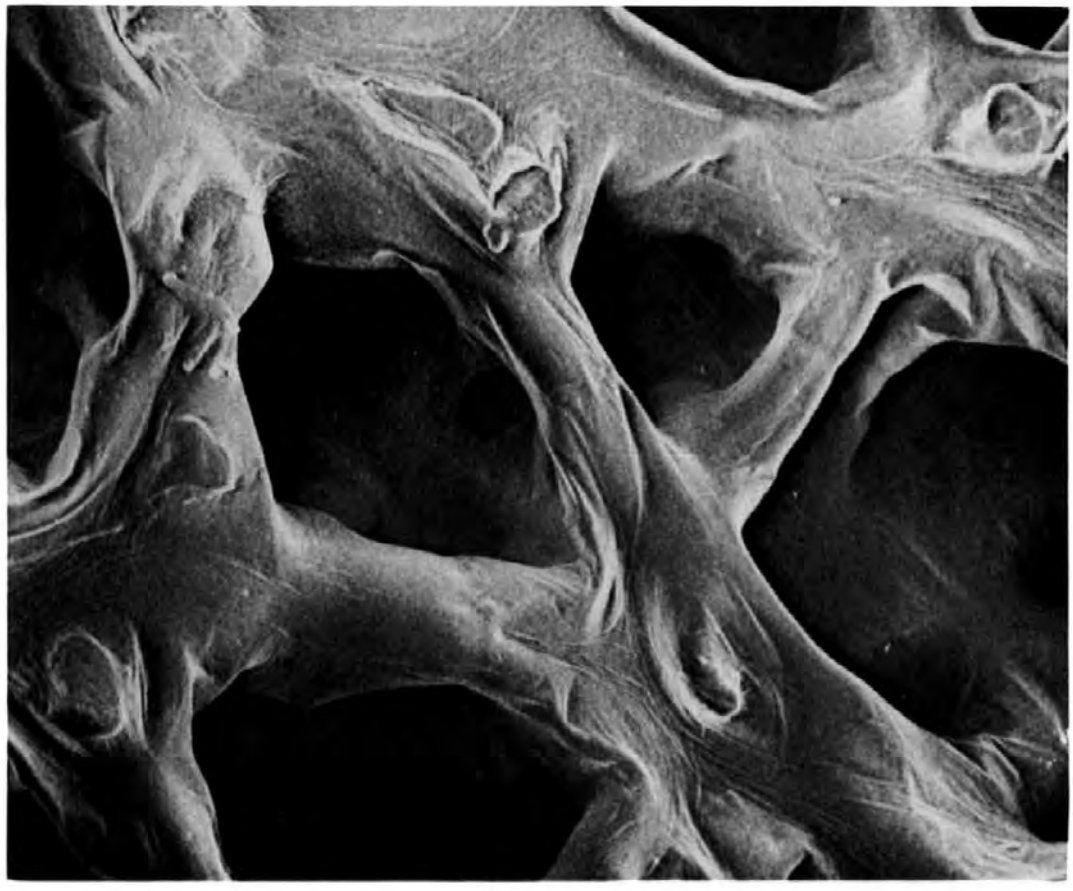
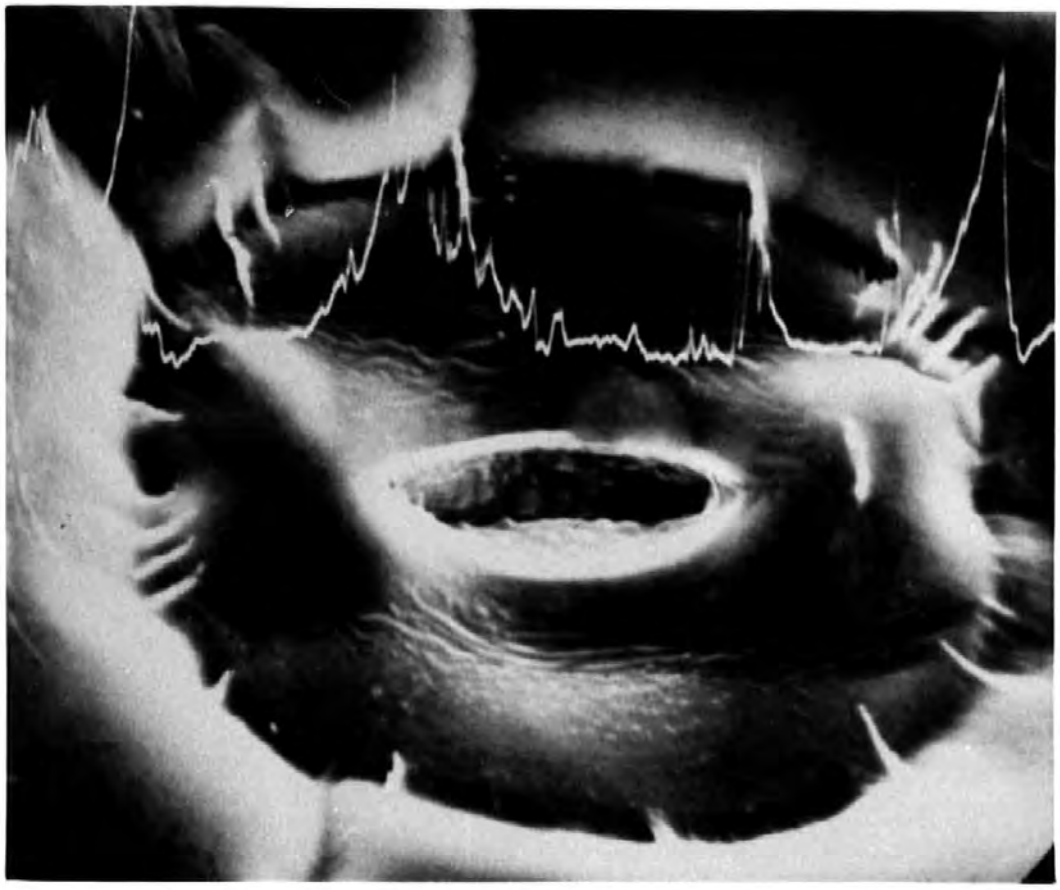


Plate 5.3.

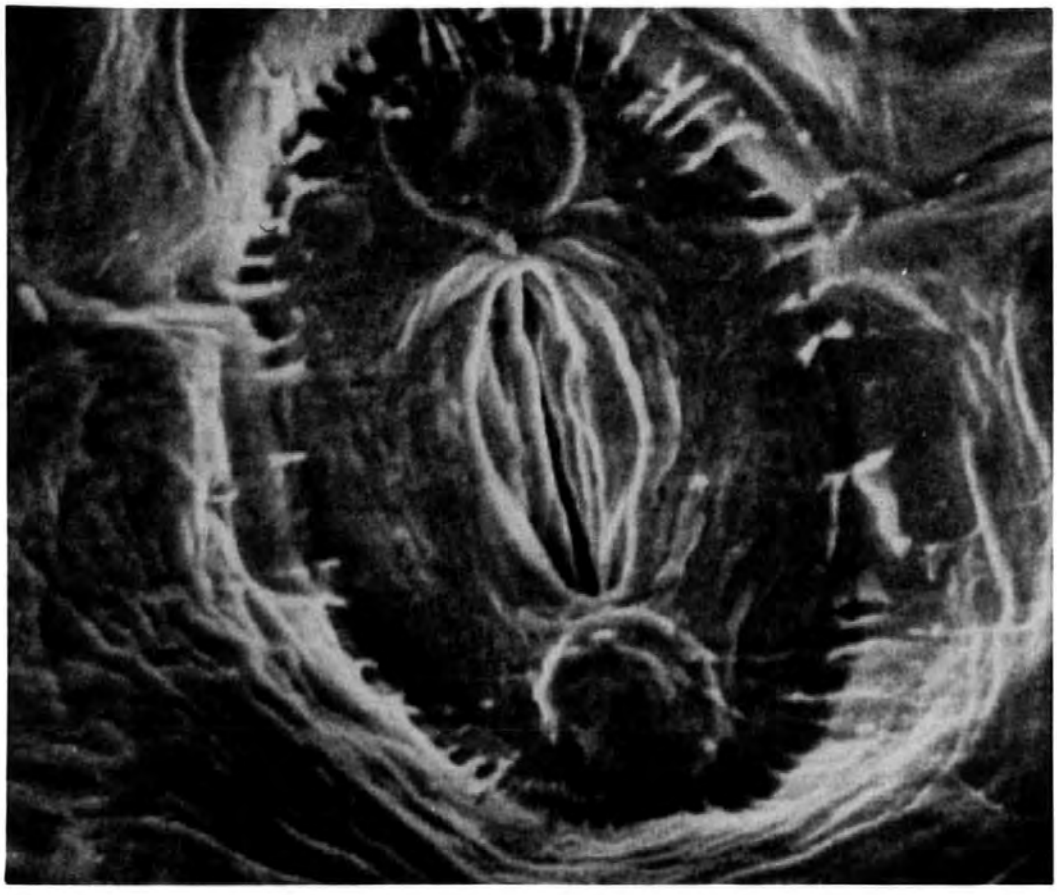
Stomata in Polypodium vulgare, II.

A. 12 KeV scanning electron micrograph of the stomatal complex viewed from the side adjacent to the mesophyll. Distinct substomatal sacs can be seen at the poles of the complex - the secondary signal distribution along the axis of the pore gives an idea of the surface topography of the specimen. Endocuticular trabeculae cen be seen extending between the guard cell complex and the adjacent epidermal tissue. The common anticlinal guard cell wall suture can just be seen at the right hand pole of the complex. (x 2 200)

B. 9 KeV scanning electron micrograph of a stomatal complex fixed in 70% ethanol viewed from the side adjacent to the mesophyll, x 2 270. The endocuticle can be seen to be collapsed onto distinct structures at the poles of the guard cell complex.



A



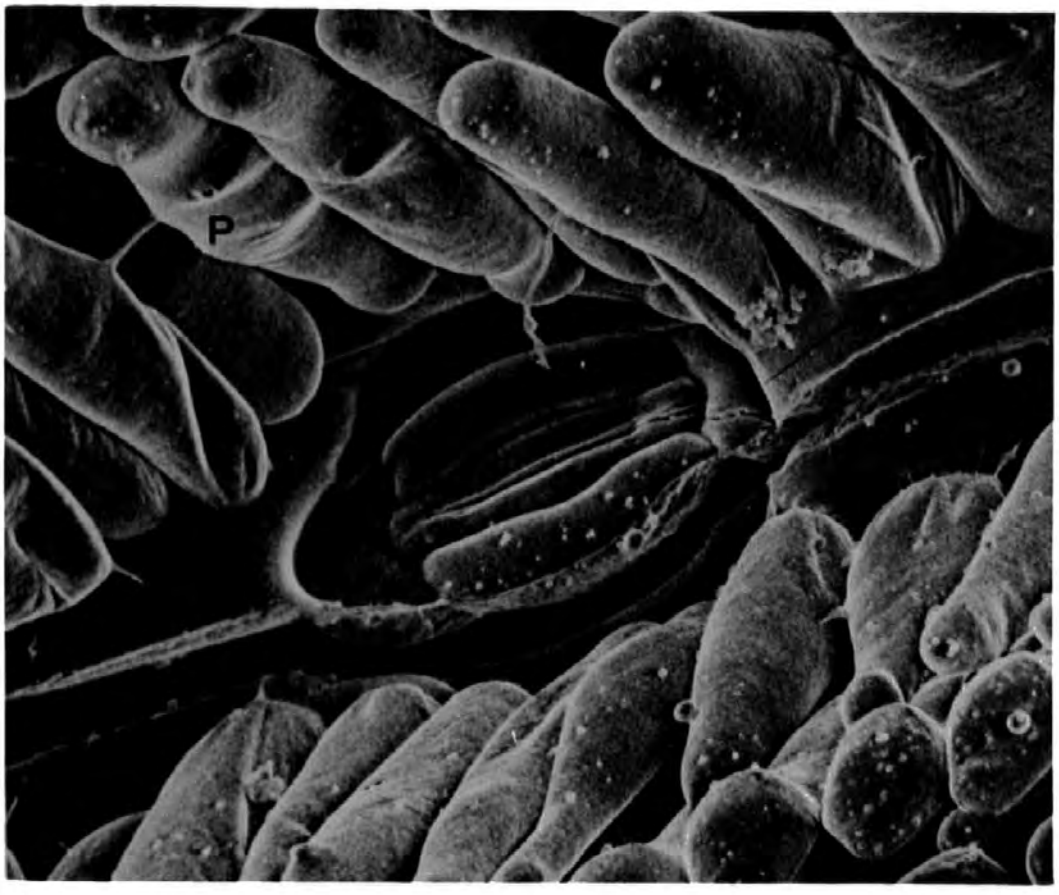
B

Plate 5.4.

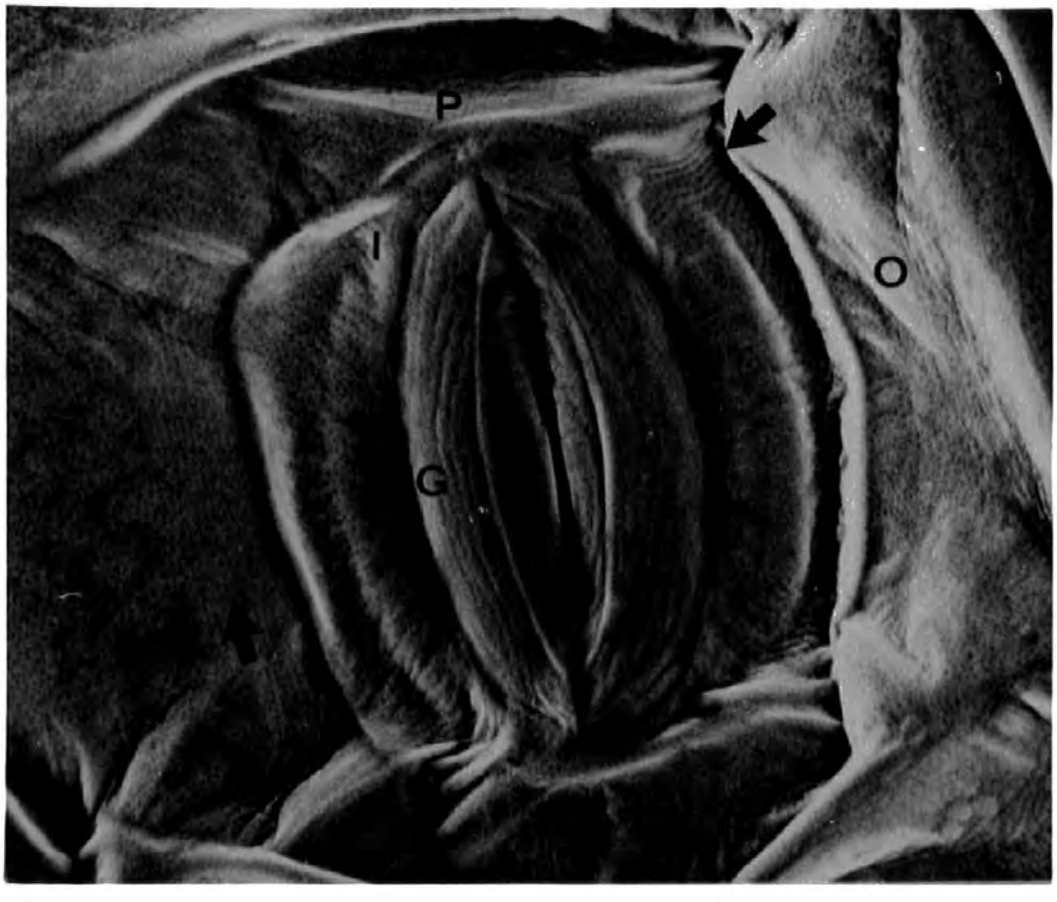
Stomata in Commelina communis.

A. 8 KeV scanning electron micrograph of an adaxial stomatal complex viewed through the palisade mesophyll (P), x 970. The palisade mesophyll cells seem to be connected to each other and to the epidermal cells by what are presumed to be endocuticular strands. The substomatal chamber is clearly illustrated in this micrograph.

B. 10 KeV scanning electron micrograph of an abaxial stomatal complex, x 2 100. The endocuticular covering of the guard cells (G) appears to be very loose and plicated. The endocuticle of the subsidiary cells bears rugose striations. Those of the polar subsidiary cells (P) and the inner lateral subsidiary cells (I) are orientated radially in respect of the stoma, whilst those of the outer lateral subsidiary cells (O) are orientated parallel to the stoma. The arrows indicate the striations and their orientation in the lateral subsidiary cells.



A



B

Plate 5.5.

Stomata in Tradescantia pallidus, 1.

A 10 KeV scanning electron micrograph of a stomatal complex, x 2 000. This micrograph illustrates the dense waxy bloom which covers the abaxial surface of the leaf in this species. The outer anticlinal guard cell wall suture is concealed beneath this bloom, but its position is indicated by the arrows. S = lateral subsidiary cell.

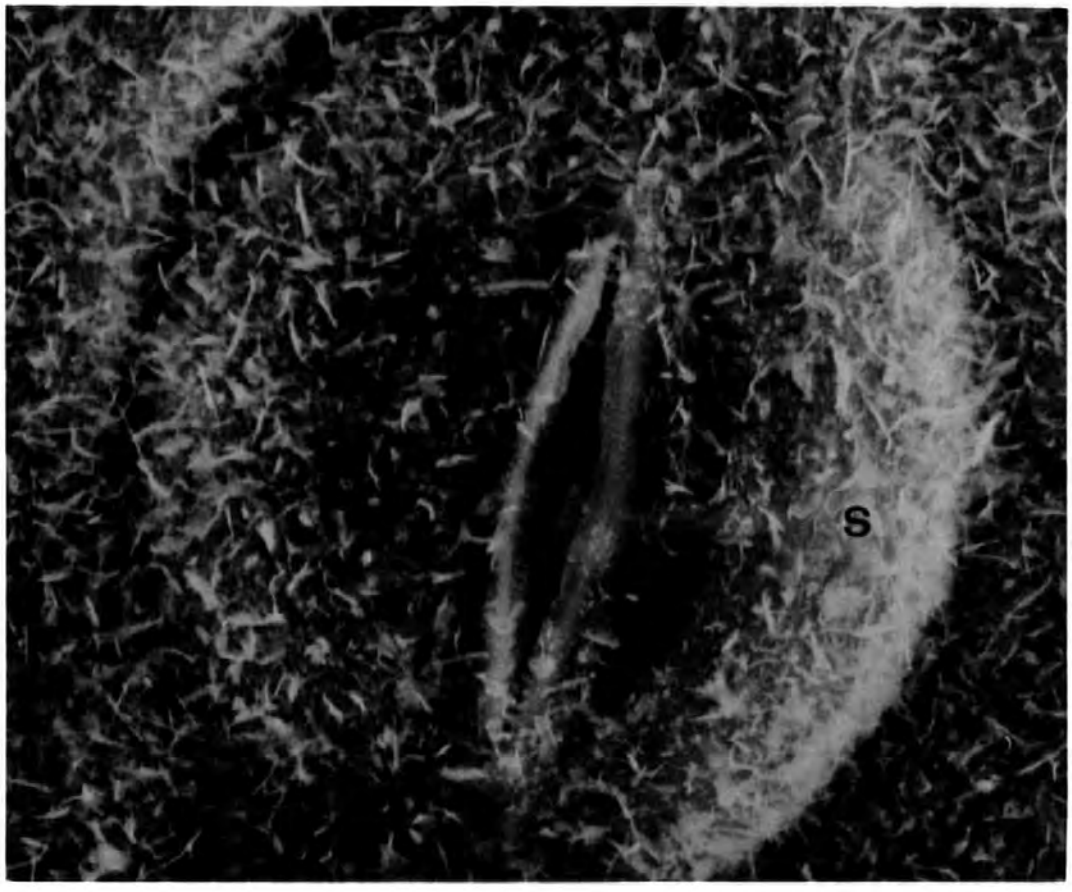

S

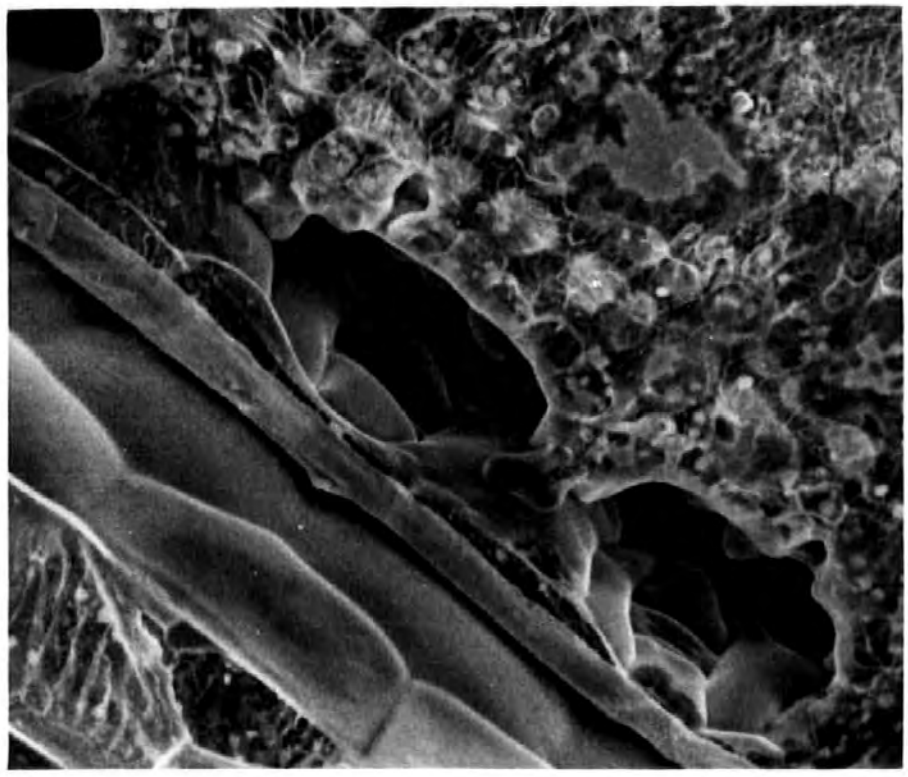
Plate 5.6.

Stomata in Tradescantia pallidus, II.

A. 10 KeV scanning electron micrograph of a guard cell complex viewed obliquely from the side adjacent to the mesophyll, x 2 700. The poles of the complex are slightly bulbous and the endocuticle in these regions is considerably plicated.

B. 10 KeV scanning electron micrograph of a section through a leaf, x 280. This micrograph clearly illustrates the substomatal chambers.

A



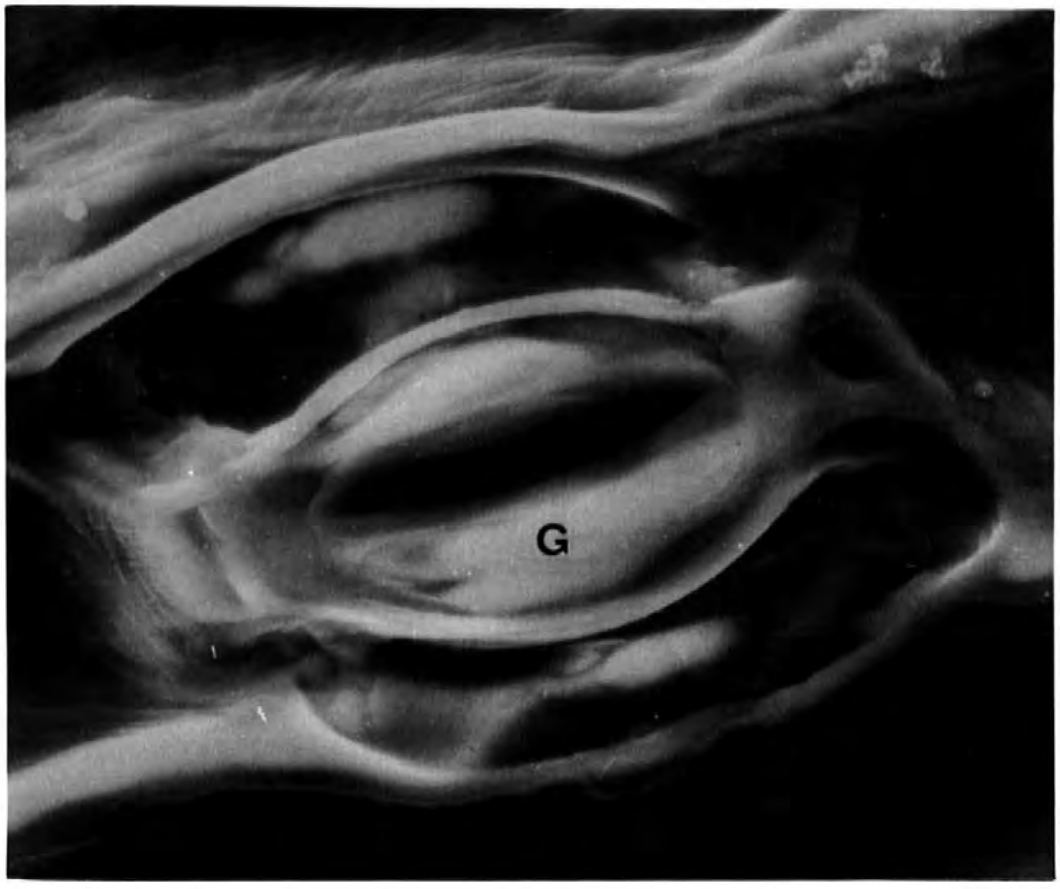
B

Plate 5.7.

Stomata in Tradescantia pallidus, III.

A. 10 KeV scanning electron micrograph of a guard cell complex viewed from the side adjacent to the mesophyll, x 1 750. This tissue had been floated on water prior to examination and clearly shows polar substomatal swellings (arrowed) at both ends of the guard cell complex (G). Strands extend from these polar swellings onto the polar subsidiary cells

B. 8 KeV scanning electron micrograph of a stomatal complex viewed from the side adjacent to the mesophyll, x 1 100. The specimen is badly iced over, but it is interesting to note that the polar regions of the guard cells are the only area of the stomatal complex which are iced over.



A

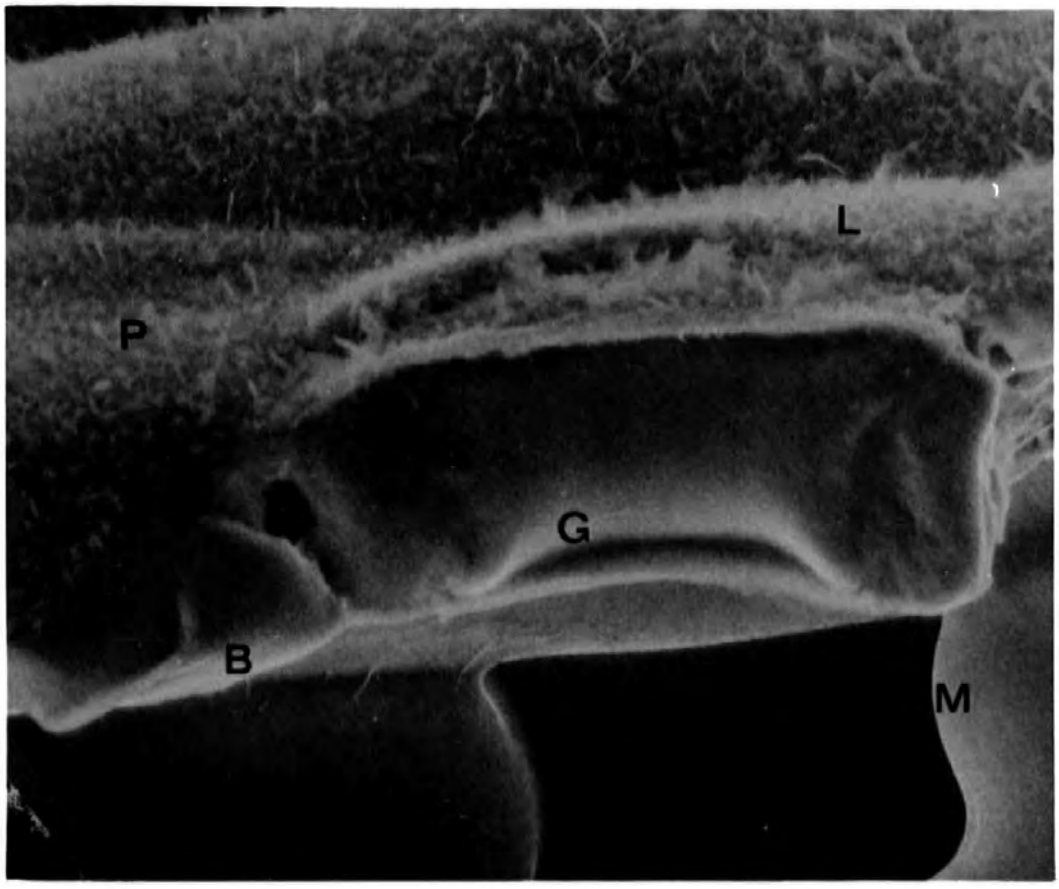
B

Plate 5.8.

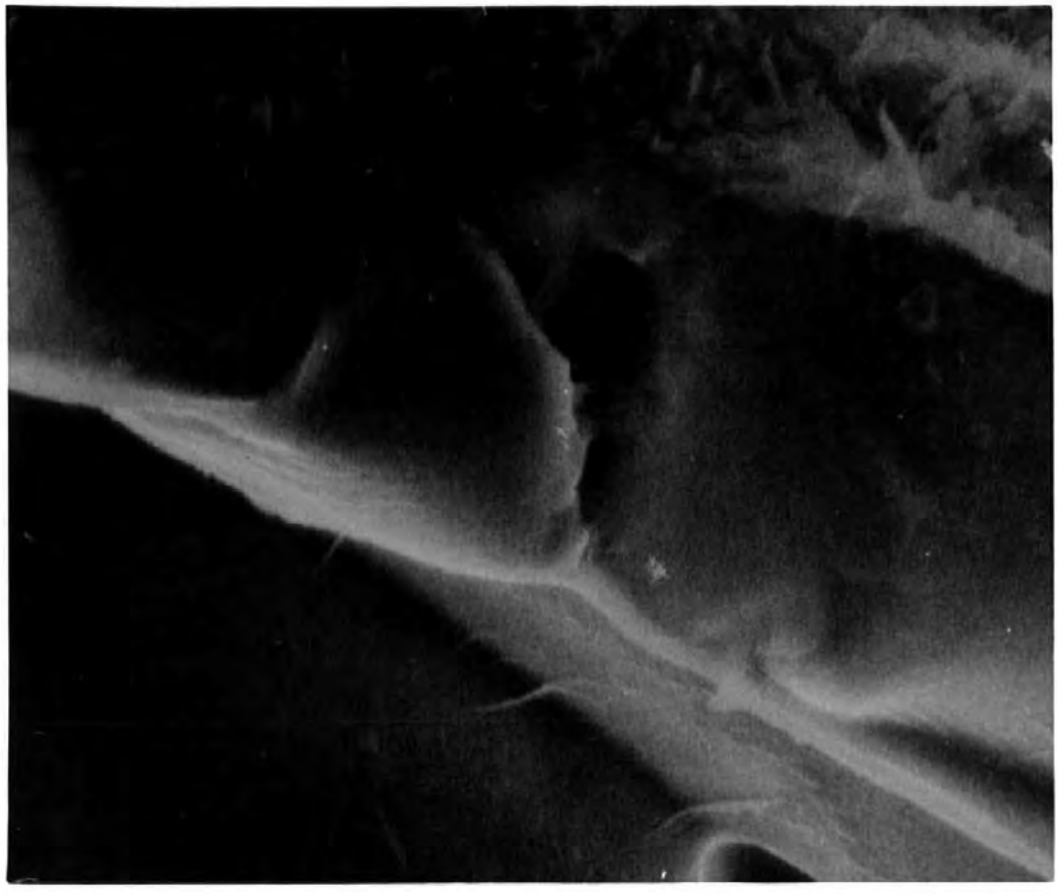
Substomatal ion-adsorbent bodies in Tradescantia pallidus, I.

A. 10 KeV scanning electron micrograph of a bisected stomatal complex viewed from a lateral aspect, x 2 000. During sectioning the scalpel blade passed exactly down the centre of the guard cell complex so as to leave the guard cell (G) intact. The polar subsidiary cell (P), likewise, has not been damaged. Other tissue visible in the micrograph is the mesophyll (M) and a lateral subsidiary cell (L). An ion-adsorbent body (B) is clearly visible wedged between the pole of the guard cell and the adjacent polar subsidiary cell.

B. An enlargement of the ion-adsorbent body illustrated in A, x 4 200. Strands can be seen extending between the body and the polar subsidiary cell and also between the body and the guard cell wall.



A



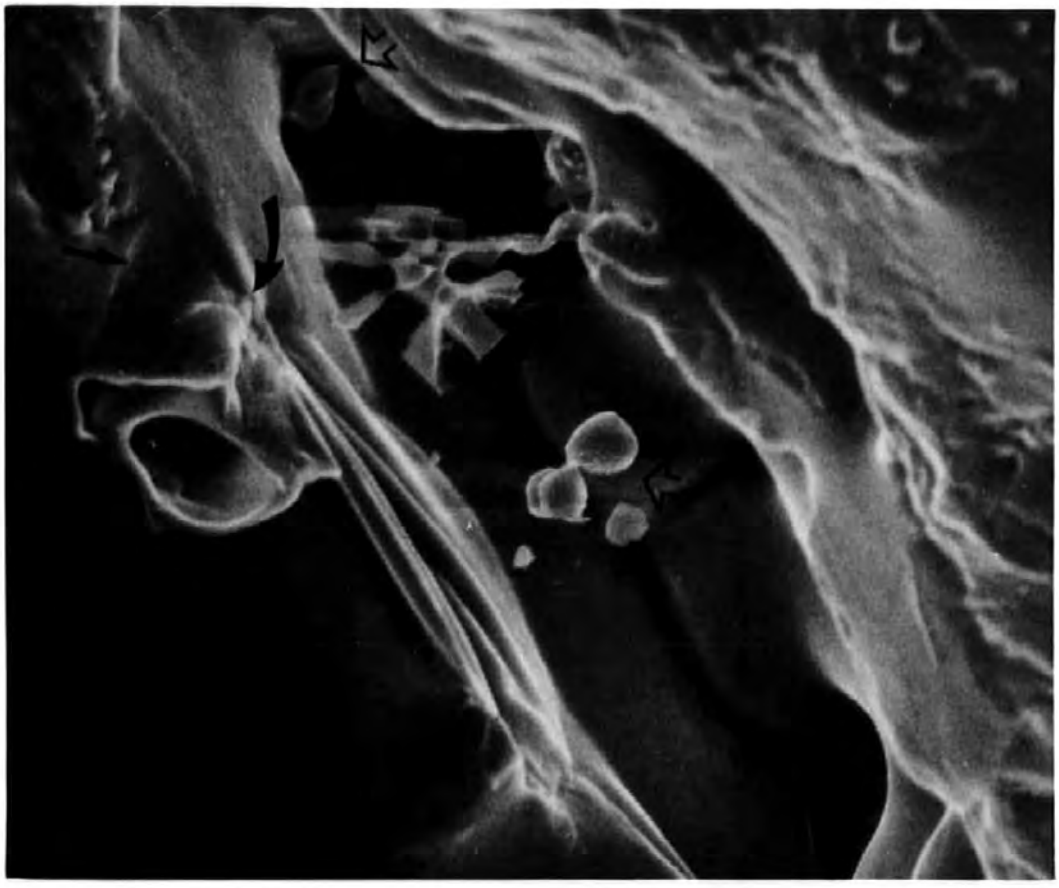
B

Plate 5.9.

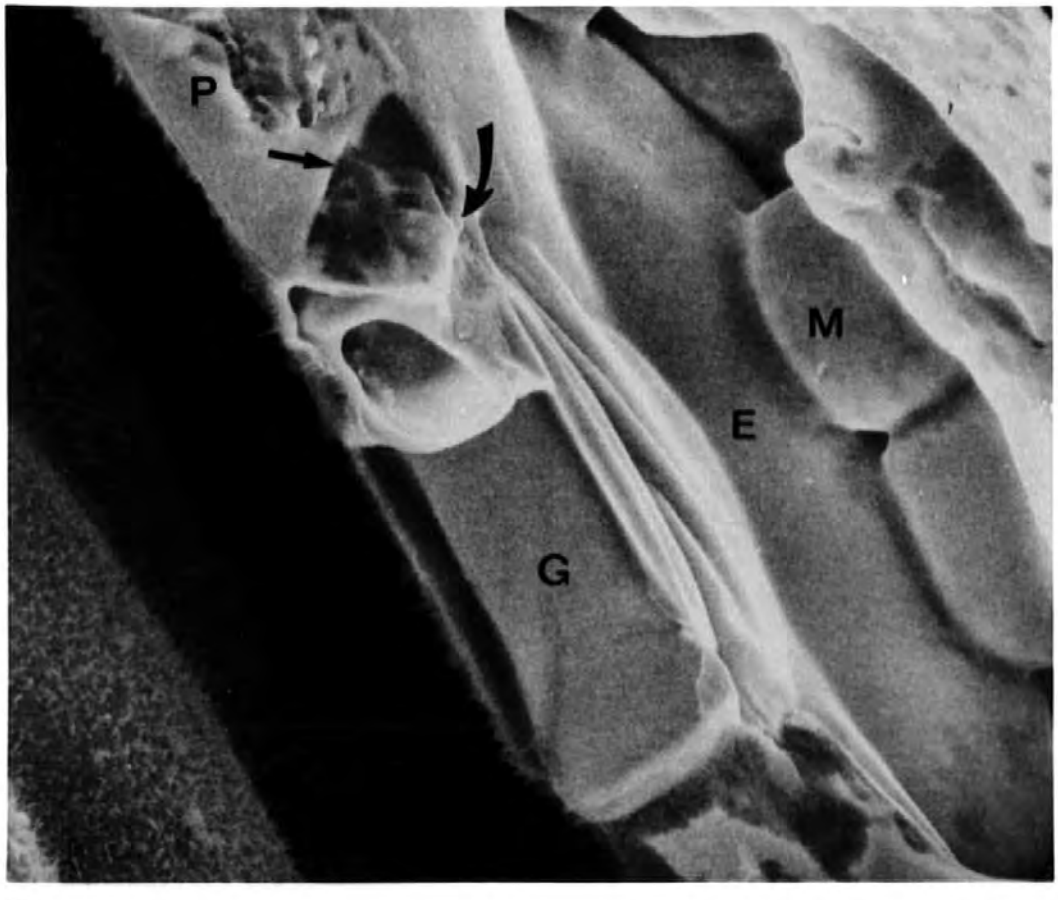
Substomatal ion-adsorbent sites in Tradescantia pallidus, II.

A. 10 KeV scanning electron micrograph of a bisected stomatal complex viewed from a lateral aspect, x 1 600. There are several ice crystals lodged in the substomatal chamber (hollow arrows). The curved solid arrow indicates the ion-adsorbent body lying behind what appears to be a membrane between the pole of the guard cell and the polar subsidiary cell wall (straight solid arrow).

B. This is exactly the same tissue as it appeared after being left in the electron beam for a few minutes. Note how the ice crystals have disappeared, as has the membrane covering the ion-adsorbent body. E = epidermal cell, M = mesophyll cell, P = polar subsidiary cell, and G = guard cell.



A

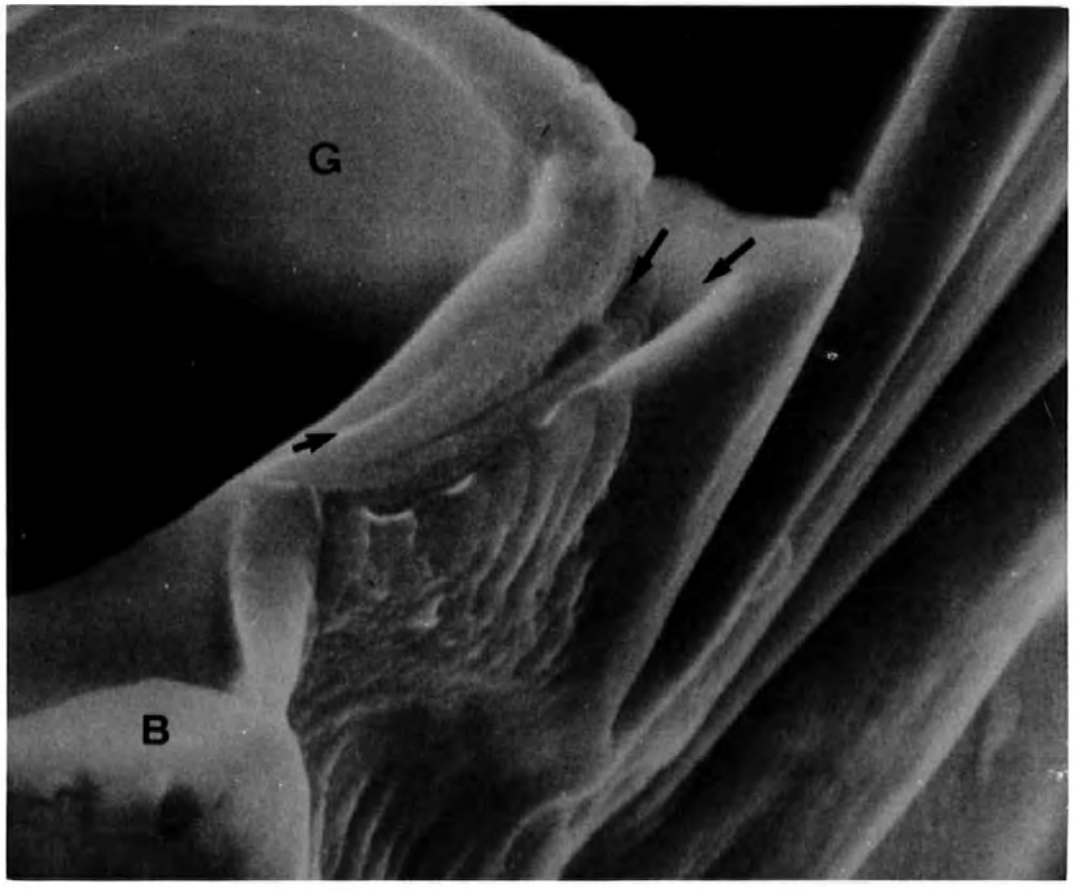


B

Plate 5.10.

Lower periclinal guard cell wall in Tradescantia pallidus.

10 KeV scanning electron micrograph of a section through the lower periclinal guard cell wall, x 10 200. Lamellar gaps occur in the thickened lower periclinal walls as indicated by the arrows. G = guard cell lumen; B = ion-adsorbent body.


G
B

CHAPTER 6

FURTHER ASPECTS OF SUBSTOMATAL STRUCTURES

BINDING SITES

In both the plant species studied in detail, the binding sites are located in an extracellular position adjacent to the poles of the guard cell complex. The scanning electron micrographs of the bodies in Tradescantia pallidus (Plate 5.8A) indicate that they are solid structures in fresh tissue and, whilst direct observation has not been possible in Polypodium vulgare, circumstantial evidence (Plate 5.3B) indicates that they too are solid. The electron microscope results eliminate the possibility that the cobalt localisations observed in tissues treated with the Macallum stain are artefacts, such as Liesegang bands which have been reported in tissues treated with this histochemical stain (Lloyd, 1925). Since the binding sites are discrete bodies which are capable of adsorbing a wide variety ions from both the transpiration stream and external solutions (vide Chapter 11), it is proposed to refer to these structures as ion-adsorbent bodies.

Although the ion-adsorbent bodies appear solid in external aspect (Plate 5.8A), the light microscopical examination of resin-embedded sections of P. vulgare shows that the bodies are ~~are~~ bag-like (Plates 4.18A, and 4.20) with 30 - 40 nm thick walls surrounding the hollow lumen. The ultrastructural nature of these walls is not

known at present. The hollow character of the ion-adsorbent bodies and the great variability in the size and form of the substomatal structures suggest that they are capable of inflation. It would also seem that the degree of inflation may be related to the state of hydration of the tissue. This is suggested by the small size and plicated nature of the substomatal sacs in P. vulgare (Plate 5.1B), which is clearly under water stress, when compared with the dilated sacs present in Plate 5.3A, in which the tissue is turgid. Similar evidence has been found in T. pallidus in which the polar regions of freshly-stripped material are only slightly swollen (Plate 5.6A), whilst these regions in material which has been floated on water for thirty minutes, after stripping, are distinctly swollen (Plate 5.7A).

An unresolved feature of the ion-adsorbent sites is whether one single, one semi-divided, or two separate bodies are present at each pole of the guard cell complex. The evidence produced from the microscopical studies is confusing. In P. vulgare, the light microscopy favours a paired structure as does transmission electron microscopy, whereas scanning electron microscopy and light microscopy of thick resin-embedded sections favours the concept of a single polar ion-adsorbent body. All the evidence indicates that a pair of bodies are associated with each pole of the guard cell complex in T. pallidus and that the two halves are in weak contact, mesially.

The presence of an endocuticle covering the internal surface of the epidermis and mesophyll has been confirmed by scanning electron microscopy which indicates that it also covers the ion-adsorbent bodies as substomatal sacs. The substomatal sacs are believed to be formed by the ion-adsorbent bodies becoming inflated against the endocuticle.

Whilst the endocuticle is clearly visible in several of the transmission electron micrographs of guard cell complexes as either an electron dense outer element of the cell wall or else as an electron sub-transparent region in the same position (Plate 4.11), no indication of endocuticular substomatal sacs was found. Thus, it must be assumed that the substomatal sacs either are not extensions of the endocuticle or else they are composed of endocuticular material which becomes denatured during the preparative procedures.

Present knowledge of cuticle structure is far from complete (Martin & Juniper, 1970) but, in this discussion, their model of a conventional mesophytic cuticle will be adopted (Fig. 6.1A). The cell wall (sensu stricta) is delimited, on its outer surface, by a layer of more or less pure pectin which is contiguous with the pectinaceous middle lamellae of the anticlinal walls. Outside the pectin layer there is a cutinised layer in which cellulose and cutin are laid down in a pectinaceous matrix. On the outer surface of the cutinised layer is a cuticularised layer of rather uncertain composition. Martin & Juniper (1970) state that this layer is constructed of more or less pure cutin, whilst Crisp (1965) considers it to be lipoidal and to represent the cuticle proper. On cell faces exposed to the atmosphere, there is very often a layer of waxy secretions over the external face of the cuticularised layer, but in considering the endocuticle here, this layer is irrelevent.

Plate 4.14 indicates that, in P. vulgare, the walls of the ion-adsorbent bodies are continuous with the outer elements of the lower periclinal walls of the guard cell complex. This outer element is stained more darkly than than the rest of the cell wall which

indicates structural differences as would be expected of the endocuticle. It is, therefore, suggested that the walls of the ion-adsorbent bodies are composed of the pectin and, perhaps, the cutinised layers of the endocuticle, and that the endocuticular substomatal sacs are formed by the lipoidal layer (Fig. 6.1B). This is compatible with the failure to demonstrate the substomatal sacs using transmission electron microscopy since the lipoidal element would be denatured by the pure ethanol and propylene oxide, or acetone, during the preparative procedures, or even the epoxy-resin. The collapsed endocuticular substomatal sacs illustrated in Plate 5.3B presumably retained their integrity because the material was fixed in only 70% ethanol which may be insufficiently strong to denature its lipoidal nature.

It is also possible that the endocuticular trabeculae, observed in light microscopy studies (Plate 3.3), are not visible under the transmission electron microscope for the reasons discussed above. The scanning electron micrographs (Plates 5.1B and 5.3) confirm their presence and it is, therefore, unlikely that they are artefacts. Their association with the guard cell complexes in both P. vulgare and Tradescantia spp. is particularly interesting since the guard cells do not appear to be connected to surrounding epidermal or subsidiary cells by plasmodesmata. The trabecular system could be an important apoplastic pathway between the guard and adjacent subsidiary or epidermal cells and could act as preferential ion channels between these cells as evidenced in Plate 3.3A.

INTRAMURAL ION PATHWAYS

The transmission electron micrographs (Plates 4.13, and 4.17), and the light micrographs (Plate 4.20) show that cobalt, from the Macallum stain (Macallum, 1905), is deposited in the form of a trace in the lower parts of the outer anticlinal walls of the guard cell complex. An enlargement of one of these traces in P. vulgare (Plate 6.1A) shows that the trace begins beneath the endocuticle, passes upwards through the middle lamellar region of the common guard/subsidiary cell wall, and then crosses the guard cell wall obliquely to enter the lumen of this cell. Although no structural modifications of the cell wall are observable in these regions, other observations suggest that preferential pathways exist in this region. When epidermal tissue is heated excessively in the electron beam of the scanning electron microscope, the first sign of damage is the appearance of endocuticular 'bubbles' over the outer anticlinal walls of the guard cell complex (Plate 6.1B). When epidermal strips are treated with the basic Macallum stain (Macallum, 1905), vesicular localisations of cobalt have been observed to collect around the periphery of the guard cell complex (Plate 6.1C). This may result from guard cell sap being 'forced' rapidly out of the cells as a result of the high concentration of the cobaltinitrite solution employed in the basic formulation of the stain (vide Chapter 2). Air can also bubble out of these anticlinal sutures when whole leaf segments are mounted in water under a coverslip and exposed to a strong light (Plate 6.1D).

Martin & Juniper (1970) have produced pictorial evidence

of ectodesmata in their Fig. 4.6 which bears a striking similarity to Plate 6.1A, especially since neither structure penetrates the cuticle. In order to determine whether the preferential pathways in the anticlinal walls could be ectodesmata, some material was subjected to to fixation in Gilson's solution as described by Schnepf (1959). The resultant demonstrations of ectodesmata were rather disappointing and only very small areas of Tradescantia pallidus exhibited the classical distribution pattern described and illustrated by Schonherr & Bukovac (1970), Sievers (1959), Schnepf (1959), Franke (1969), and others. No distinctive ectodesmata were found in Polypodium vulgare. The distribution of ectodesmata in T. pallidus is illustrated in Plate 6.2 where the structures occur primarily above the anticlinal sutures of the epidermal cells and particularly above the anticlinal sutures of the outer walls of the guard cells on the inner face of the epidermis; the distribution on the outer face of the epidermis is random and no evidence was obtained to suggest they are associated with the anticlinal cell walls on this face. These findings are not in agreement with those of other workers (op. cit.) who find that ectodemata occur primarily on the outer face of the epidermis. However, the present findings on the structures in T. pallidus are consistent with the the evidence of preferential ion channnels in the lower regions of the outer anticlinal guard cell walls and it is possible that the cobalt traces found in Plate 6.1A could be ectodesmata. This aspect of stomatal organisation clearly requires further study. If ectodesmata are particularly associated with the inner suface of the epidermis, as is suggested here, their role in apoplastic movements of water and solutes between the lumen of the guard cells and the subcuticular regions of the stomatal complex (i.e. the transpiration stream, Meidner, 1975)

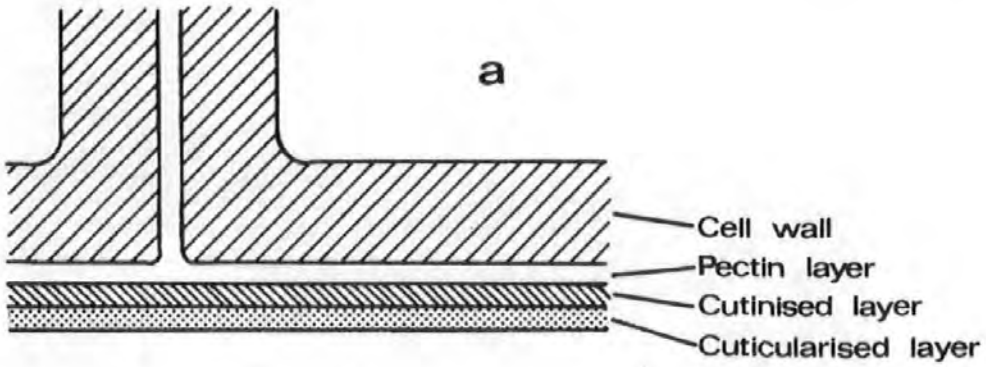
may be of great importance.

Fig. 6.1.

Hypothetical composition of the ion-adsorbent body walls.

a. The composition of a hypothetical cuticle (redrawn after Martin & Juniper, 1970).

b. Hypothetical composition of the ion-adsorbent body walls. cw = cell wall, p = pectin layer, cn = cutinised layer, and ct = cuticularised layer.


a
Cell wall
Pectin layer
Cutinised layer
Cuticularised layer

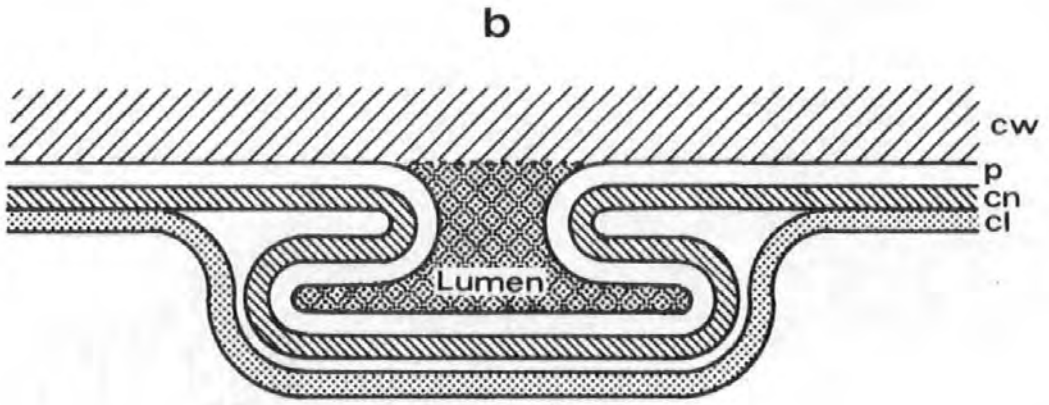

b
cw
p
cn
cl
Lumen

Plate 6.1.

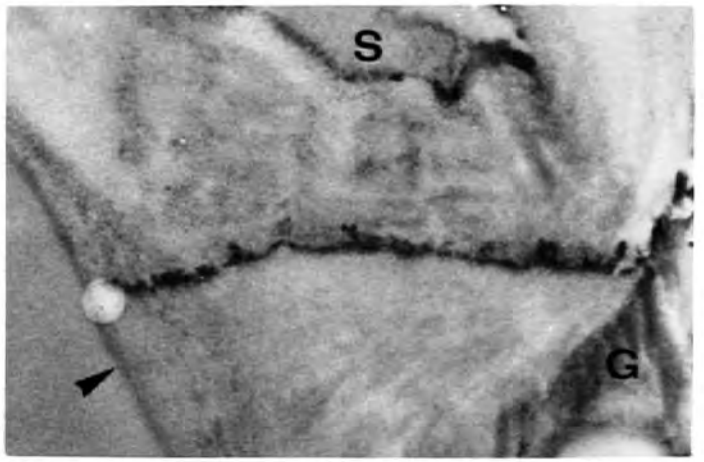
Intramural pathways in Polypodium vulgare.

A. Transmission electron micrograph, x 21 000. The micrograph illustrates a cobalt trace through the lower regions of the outer anticlinal guard cell walls similar to those illustrated in Plate 4.13A. The tissue was pretreated with modified Macallum's stain (7,5% w/v), and fixed in ethanol; no post-staining. The cobalt trace can be seen to extend from beneath the endocuticle (arrowed), through the common outer anticlinal cell wall between the guard cell (G) and adjacent subsidiary cell (S) to the guard cell protoplast.

B. Scanning electron micrograph of the stomatal complex, x 1 000. The tissue has been exposed to the electron beam for too long which has resulted in the tissue becoming heated up and characteristic blisters appearing, particularly over the outer anticlinal cell wall sutures on the side adjacent to the mesophyll.

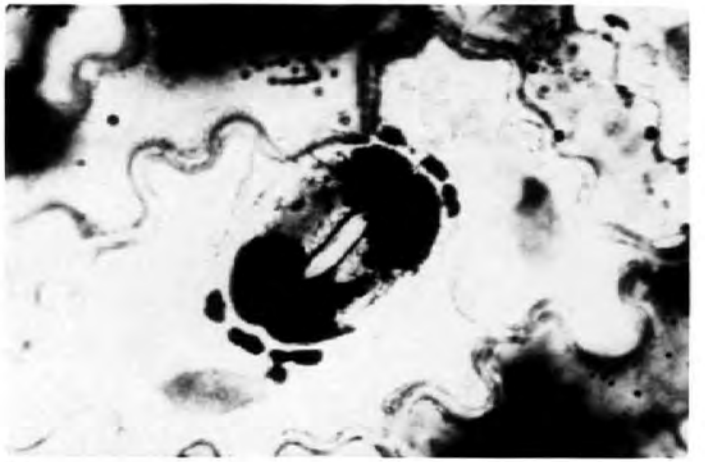
C. Light micrograph of stomatal complex stained with the basic Macallum formulation, x 525. Besides showing the usual stain localisations, this micrograph also shows beads of stain associated with the outer anticlinal cell walls of the guard cell complex. Careful focussing indicates that the beads are in a very superficial location on the inner surface of the epidermis.

D. Light micrograph of fresh tissue, x 325. The whole leaf segment has been illuminated for about one minute prior to the photograph having been taken and shows bubbles of gas (arrowed) emanating from the outer anticlinal walls of the guard cell complex.



A

B



C

D

Plate 6.2.

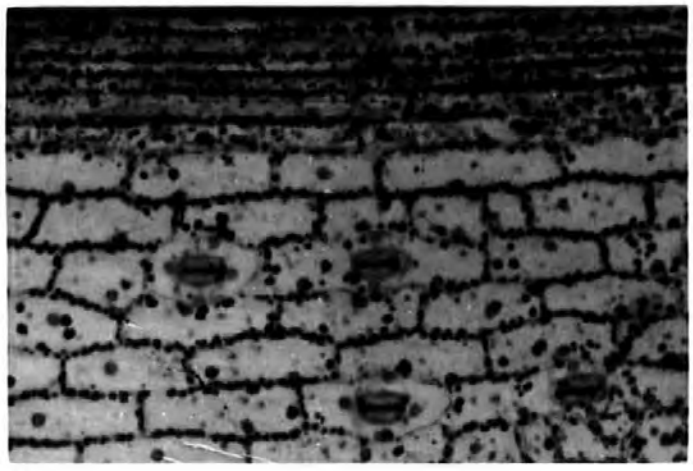
Ectodesmata in isolated epidermal tissue of Tradescantia pallidus.

Light micrographs of epidermal tissue treated with Gilson's solution and viewed from the side adjacent to the mesophyll.

A. Lower power micrograph, x 110. The mercury precipitates can be seen to be closely associated with the anticlinal cell wall sutures and are particularly abundant in the suprafascicular epidermal tissue.

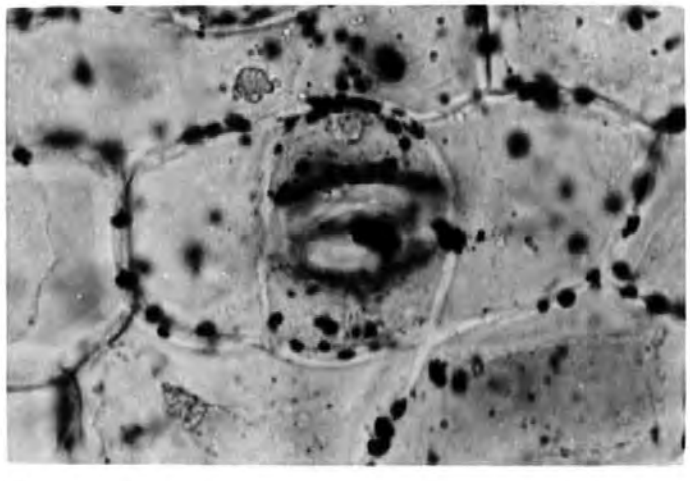
B. Stomatal complex, x 425. The micrograph is focussed on the dense aggregations of mercury precipitates on the inner face of the guard cell complex below the outer anticlinal guard cell walls.

C. This is an identical micrograph to B, but is focussed on the surface of the epidermal tissue adjacent to the mesophyll.



A

B



C

CHAPTER 7

CHEMICAL NATURE OF THE ION-ADSORBENT BODIES

INTRODUCTION

Circumstantial evidence was offered in Chapter 6 to suggest that the ion-adsorbent bodies might be constructed of cuticularised outer cell wall elements of the lower periclinal walls at the poles of the guard cell complex. The present study is a histochemical examination of the polar regions of guard cells for characteristic elements of the pectin and cuticularised layers in this region.

OBSERVATIONS

Cuticularised element of the endocuticle

This element is considered to be highly lipoidal (Crisp, 1965) and was tested for with the Nile Blue procedure (Cain, 1947), and Sudan IV and Sudan Black B (Baker, 1947; Gomori, 1952) on epidermal strips of Polypodium vulgare, Commelina communis, and Tradescantia spp. In no case was distinct staining observed in epidermal peels viewed with their endocuticular surfaces uppermost, or when strips of P. vulgare were sectioned by hand. It was concluded that the lipoidal content of the endocuticle in these mesophyte species is too thin to stain sufficiently for microscopical examination.

Having been unsuccessful in demonstrating the lipoidal nature of the cuticularised layer of the endocuticle histochemically, it was decided to isolate it by acid digestion as described in Chapter 2. Just before complete digestion of the underlying cells is achieved, the endocuticle of P. vulgare can be removed and be seen to retain weak 'imprints' of the underlying cells (Plate 7.1A). At this stage, the 'imprints' of the substomatal structures can often be distinguished at the poles of the guard cell complexes. The 'imprints' of both the anticlinal cell wall sutures and the polar structures eventually disappear on complete digestion of the non-lipoidal constituents of the epidermis to leave a flat lipid layer punctuated only by slits left by the stomata. The completely isolated lipid layer of the endocuticle in C. communis is also featureless except for the stomatal slits. Observations of the endocuticular surface during acid digestion in this species indicate that the lipid element is very weakly attached to the outer anticlinal cell wall sutures of the guard cell complex and quickly becomes freed (Plate 7.1B). No 'imprints' of the substomatal structures are seen in the partially isolated endocuticle, although the endocuticle in this region is particularly wrinkled (Plate 7.1B).

Pectin element of the cell wall / endocuticle

The polar regions of guard cell complexes in fresh epidermal strips of P. vulgare stain a pale purple-pink with toluidine blue (Plate 7.2A & B), as detailed in Chapter 2. The subsidiary and epidermal cells abutting onto the stomatal complex do not take up the stain immediately, whilst other epidermal cells stain blue, with the exception of the suprafascicular tissue which stains purple. After

about ten minutes immersion in the stain, the epidermal cells abutting onto the guard cells become blue, whilst after 20 to 30 minutes, the staining at the poles of the guard cells becomes masked as the stain suffuses into the body of the guard cells. If the epidermal strip of P. vulgare is pretreated with a ten second immersion in ethanol, the component cells of the epidermis produce a very interesting staining pattern. The poles of the guard cells stain blue-black, the epidermal and subsidiary cells abutting onto the guard cell complex stain pink, the epidermal cells stain blue, and the suprafascicular epidermal cells stain either very deep blue or violet.

Polychromatic effects were not observed in the epidermal tissue of Tradescantia pallidus when stained with toluidine blue. All the epidermal cells, except those of the stomatal complex, stain blue, whilst the subsidiary cells or those parts of them adjacent to the guard cells remain unstained. Usually the poles of the guard cells only take up the stain very slightly as in Plate 7.2C, but occasionally the poles may stain very distinctly (Plate 7.2D). In the majority of of guard cell complexes which do not exhibit strong polar staining, the common and polar anticlinal walls of the guard cell complex take up the stain very strongly (Plate 7.2C). After about five minutes immersion in the stain, the subsidiary and guard cells become suffused with the dye.

In order to assess the involvement of pectin in the ion-adsorbent structures, epidermal strips of T. pallidus were incubated in pectinase as detailed in Chapter 2. After varying periods of time, epidermal strips were removed and treated with 2% (w/v) sodium cobaltinitrite in the modified Macallum technique (Chapter 2) to

determine the effect of the enzyme on the binding properties of the ion-adsorbent bodies. The results are illustrated in Plate 7.3. The enzyme was observed to have little effect on the ion-adsorbent bodies during the first 2.5 hours of the treatment, although there was an unusually strong reaction to the Macallum stain in the stomatal complex (Plate 7.3A). After 6 hours treatment, the ion-adsorbent bodies appeared to be largely denatured and their polar positions only weakly visible (Plate 7.3C). All signs of the bodies had disappeared after 12 hours pectinase treatment (Plate 7.3D).

DISCUSSION

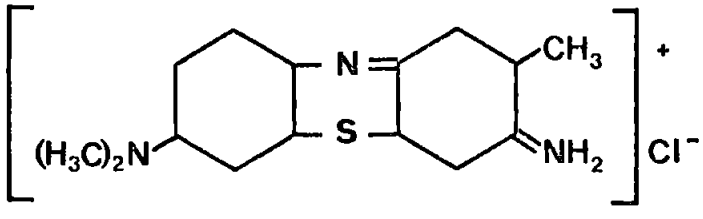
Cuticularised element of the endocuticle

The present findings tend to confirm the position of the cuticularised element of the endocuticle as proposed in Chapter 6. It appears that although this membrane is not directly involved in the ion-adsorbent bodies, it contributes to the endocuticular substomatal sacs which cover the bodies.

Pectin element of the cell wall / endocuticle

Toluidine blue is a basic thiazine dye (Fig. 7.1) with a positively charged dye ion. Generally, it will stain any tissue

Fig. 7.1. The structure of toluidine blue.



element which is acidic in reaction by ionic bonding. It is histochemically metachromatic under certain conditions which means that on staining a substrate, the absorption spectrum of the substrate-dye complex may differ sufficiently from that of the original dye colour (orthochromatic state) to give a marked contrast in colour (metachromatic state). The shift of the absorption maximum of a metachromatic dye is always towards shorter wavelengths. The work of Michaelis & Granick (1945) suggests that the metachromatic effects result from polymer formation of the dye at the substrate face. In the case of toluidine blue, it is thought that the monomeric form of the dye produces the orthochrome state, the dimer produces a purple metachromatic colouration, and the trimer produces the pink fully metachromatic state (Fig. 7.2). Polymerisation is favoured by a

Fig. 7.2. Metachromasia.

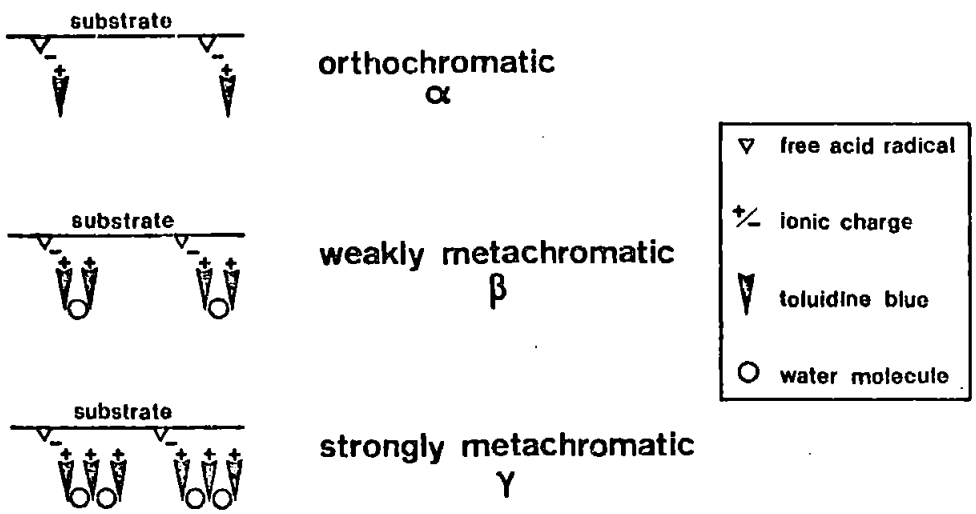
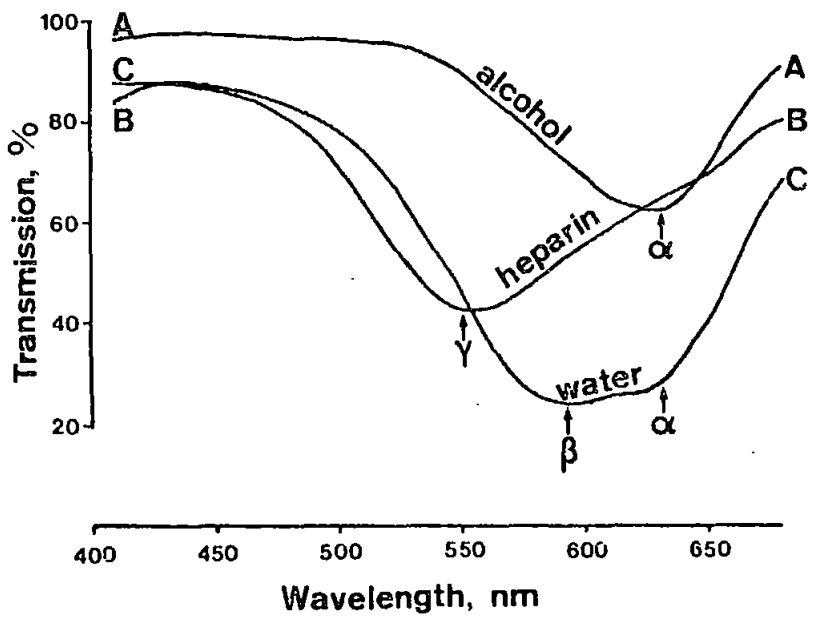


Diagram illustrating the dependance of metachromasia on polymerisation of dye molecules and the requirement of a certain minimum substrate density of negative charges. (Based on Pearse, 1968).

sufficiently high dye concentration and a sufficient degree of hydration since the molecules of the dye are held together as polymers by water molecules and van der Waal's forces (Pearse, 1968). Baker (1958) has shown that toluidine blue has a transmission spectrum with three troughs (Fig. 7.3). The blue orthochromatic trough (α) is situated

Fig. 7.3. Transmission spectrum of toluidine blue.

(redrawn from Baker, 1958)



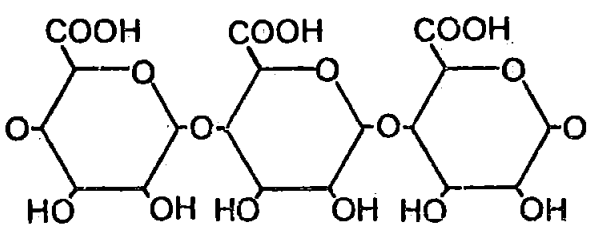
Graph showing the transmission of light through a 1 cm thick solution of toluidine blue.

A. 0,0002% toluidine blue in 80% alcohol.
B. 0,001% toluidine blue in distilled water.
C. 0,001% toluidine blue in distilled water + 2 drops heparin solution (5 000 i.u. ml^{-2}) per 3.4 ml of solution.

at ca. 640 nm , the purple metachromatic trough (β), which results from the simultaneous presence of α and γ forms, is at 590 - 600 nm, and the

pink metachromatic trough (γ) at 550 nm (520 nm, according to Pearse, 1968). Metachromasia can take place on substrates in which a certain minimum density of negative charges exists. The distance between free acid radicals in polygalacturonic acid (Fig. 7.4), the prime constituent of pectin, is 0,5 nm which is sufficient to produce weak metachromasia (Pearse, 1968).

Fig. 7.4. The structure of polygalacturonic acid.



The weak purple-pink metachromasia observed at the poles of P. vulgare guard cells when stained with toluidine blue suggests that the metachromatic state lies between the β and γ states which would have a light transmission trough between 550 and 600 nm (Fig. 7.3). This observed metachromasia is of the right order if the reacting substrate is of a pectic nature since the absorption maxima for the production of metachromasia in pectinate is 560 nm (Sylven, in Pearse, 1968).

There is only one published report (Drawert, 1942) of structures which can be interpretated as substomatal ion-adsorbent sites. This paper on the ontogeny and morphology of the stomatal complex in Tradescantia virginica (sic.) includes a report on the distribution of toluidine blue in isolated epidermes. The present

findings largely agree with those of Drawert (1942) except that the present study does not confirm that the polar subsidiary cells of T. pallidus or T. x andersoniana differ markedly from the lateral subsidiary cells in that the dye is taken up preferentially by the former cells. Plate 7.2C & D show that the dye is not taken up immediately by any of the subsidiary cells. Furthermore, he suggests that there is a difference between the uptake of the dye by the lateral subsidiary cells in the light and in the dark. This has not been confirmed in this investigation. It was noted by Drawert (1942) and illustrated in Plate 7.2D that some subsidiary cells took up the dye in regions distal to the complex and that a clearly defined dye front was formed within the subsidiary cell. No explanation can be offered for this phenomenon although it may be an artefact similar to the Leisgang bands reported on by Lloyd (1925) in his critique of the Macallum stain (Macallum, 1905).

The absence of metachromatic staining in T. pallidus and the excessively polychromatic appearance of P. vulgare pretreated with ethanol are probably both due to available ion-binding sites. The number of free acid radicals is dependant on the ambient pH of the toluidine blue solution used. A higher pH will maintain a higher state of hydration in the tissue and induce greater ionisation of the carboxyl groups on the substrate, both of which are conducive to metachromasia. The alcohol is thought to enhance the reaction by improving the penetration of the dye by the partial dissolution of the lipoidal endocuticle and may also remove any polar lipids which may be adsorbed onto the bodies.

The explanation given by Drawert (1942) for the polar uptake

of toluidine blue is interesting. He states that polar staining is restricted to the outer membrane, but it is not clear whether he is referring to the endocuticle or the periclinal cell wall. He correctly points out that the density of the dye in the tissue is proportional to the isoelectric point of the substrate and makes the point that the isoelectric point of cell surfaces increases with age so that the older the cell wall, the deeper the toluidine blue staining. He concludes that the polar staining of the guard cells is a result of premature ageing of the cell surface. Whilst accepting that this may be a plausible explanation which should be investigated further, the metachromasia observed in P. vulgare guard cells would seem to suggest that these areas of the guard cells are particularly rich in pectin.

This suggestion is confirmed by the enzymatic degradation of the ion-binding sites by pectinase treatment (Plate 7.3). The failure of enzyme-incubated epidermal tissue to adsorb the cobalt stain is not thought to be a case of auto-degradation brought about by the death of the tissue since the ion-adsorbent sites of epidermal strips killed in boiling in water for ten minutes and those stripped off dead leaves (Chapter 3) retain the ability to adsorb the cobalt cations.

The densely-staining anticlinal walls at the poles of the guard cell complex when stained with toluidine blue (Plate 7.3C) are very similar to the anomalies observed in these walls by Raju et al. (1975). As mentioned in the discussion of Chapter 3, no evidence has been found during this study to support their contention that these regions of the guard cell walls are differentially thickened. The anticlinal walls of the guard cells in these regions have a greater perpendicular depth than other anticlinal walls of the guard cell complex, which are curved when viewed with a microscope, so that any

stain which lodges in these polar cell walls will appear denser than elsewhere in the guard cell walls since it will be compounded by the depth of the wall.

Other stains were used to determine the involvement of pectin in the ion-adsorbent bodies. Weak polar fluorescence was obtained with fluorescent periodic acid-Schiff's reaction (de Tomasi, 1936), but no distinct polar staining was observed with the standard periodic acid-Schiff's stain (Hotchkiss, 1948; McManus, 1948). Similarly no positive reactions were obtained with the more specific stains for pectin, ruthenium red (Johansen, 1940) and hydroxylamine-ferric chloride (Reeve, 1959), but this may may have been due to insufficient substrate being present since, ruthenium red, at least requires "fairly high concentrations" of pectin (Jensen, 1962).

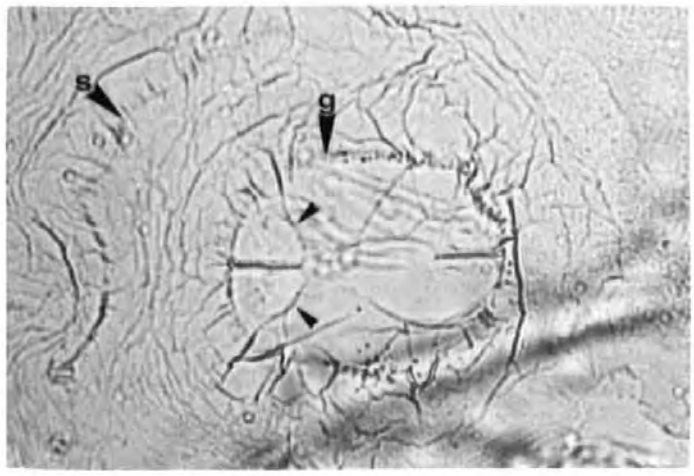
Plate 7.1.

Quasi-isolated endocuticles.

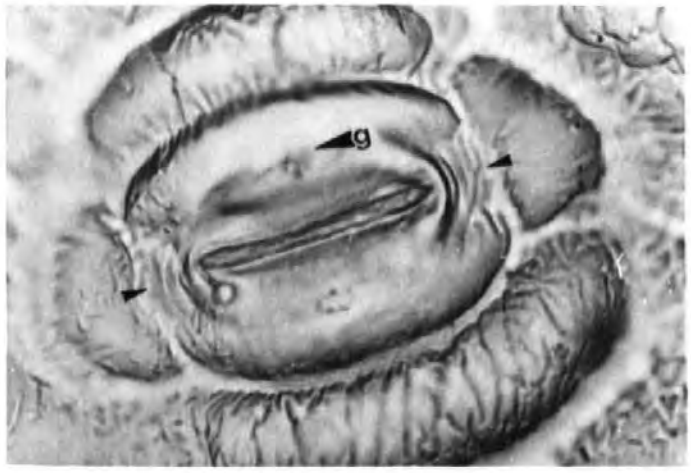
Light micrographs of acid-digested cuticular membranes, unstained.

A. Stomatal complex of Polypodium vulgare, x 550. Weak imprints of the anticlinal cell wall sutures between the guard cell complex and the subsidiary cell (g), and between the subsidiary and adjacent epidermal cell (s) are visible. The guard cell complex retains an impression of the underlying ion adsorbent bodies (small arrows) which disappear on complete digestion of the non-lipoidal elements of the cell wall / endocuticle.

B. Stomatal complex of Commelina communis, x 850. This micrograph is of an early stage during the acid digestion and shows the weak connection between the endocuticle and the outer anticlinal guard cell wall sutures which separates very early during the treatment (g). No direct signs of the underlying ion-adsorbent bodies are apparent in this species although the endocuticle in this region is particularly wrinkled (small arrows).



A



B

Plate 7.2.

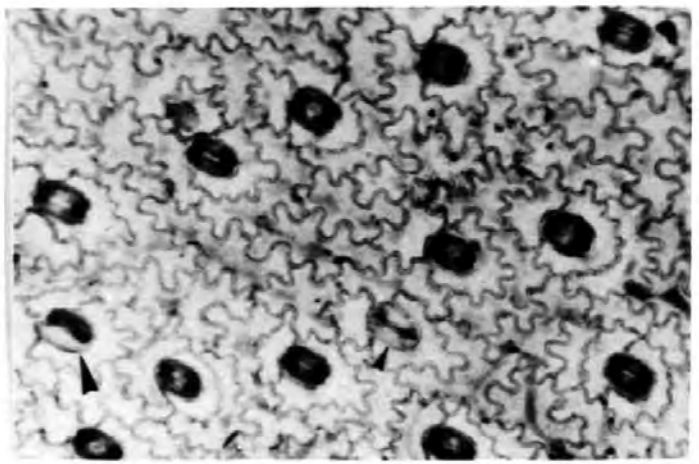
Toluidine blue staining of isolated epidermes.

A. Light micrograph of Polypodium vulgare, x 100. The poles of the guard cells stain pale purple-pink, the cells immediately adjacent to the guard cell complex are unstained, and the rest of the epidermal tissue stains blue. The small arrow indicates a dead complex in which the poles are stained, whilst the larger arrow indicates a complex in which one of the guard cells is dead and the other living.

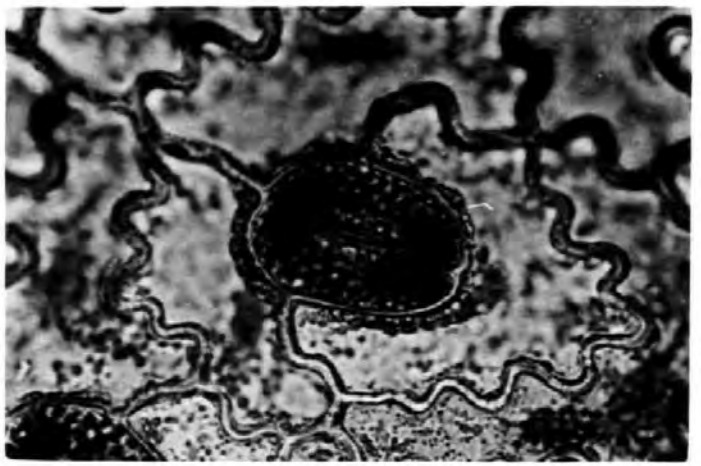
B. Light micrograph of a single guard cell complex in Polypodium vulgare, x 450.

C. Light micrograph of stomatal complex of Tradescantia pallidus, x 430. The stain has been taken up by the epidermal cells, but not by the subsidiary cells. There is only a very weak polar accumulation of the dye in the guard cell complex although the polar anticlinal cell walls appear to have adsorbed it preferentially (arrowed, but see text).

D. Light micrograph of stomatal complex of Tradescantia pallidus showing a very strong polar reaction, x 750. A dye front is clearly visible in the polar subsidiary cells (arrowed).



A



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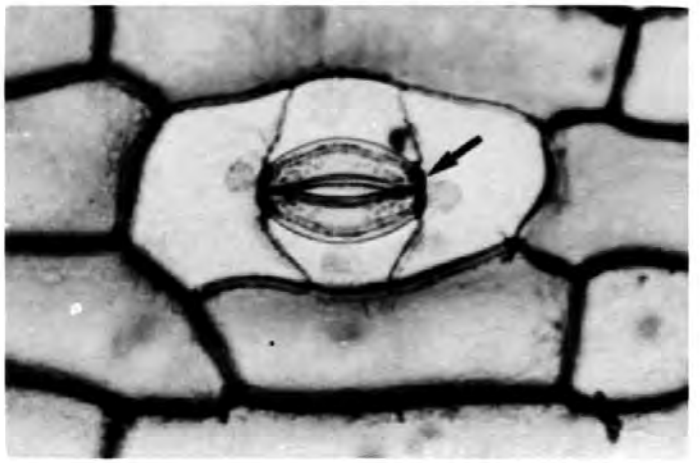

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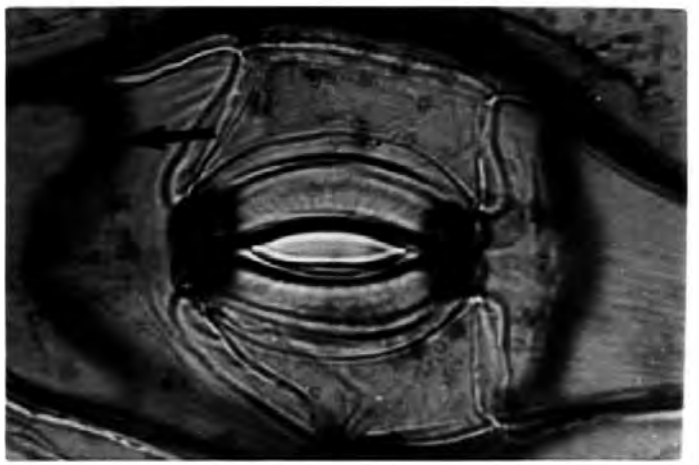


D

Plate 7.3.

Pectinase treatment of isolated epidermes.

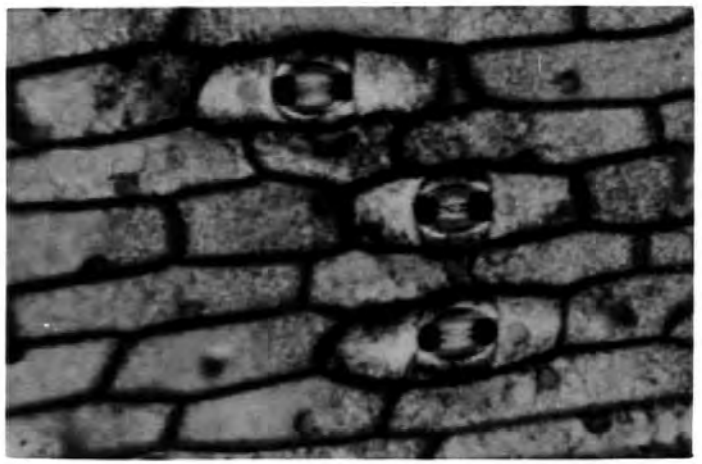
Light micrographs of epidermal strips from Tradescantia pallidus which were incubated in pectinase for varying periods before being treated with the modified Macallum's reagent (2% w/v).

A. Control tissue, incubated in distilled water for 2.5 h, x 200.

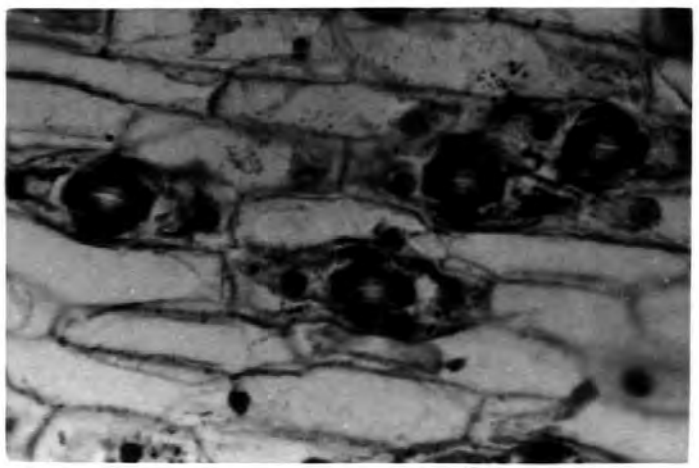
B. Enzyme-treated tissue after 2.5 h, x 200. Note how the entire stomatal complex reacts very positively to the stain when compared with the surrounding epidermal tissue, or the stomatal complexes in the control (A, above).

C. Enzyme-treated tissue after 6 h, x 200. Note the very weak polar reactions in the guard cells.

D. Enzyme-treated stomatal complex after 12 h, x 900. No distinct polar localisations were observable although a very positive Macallum stain-reaction was present over much of the tissue.



A



B

C

D

CHAPTER 8

DISTRIBUTION OF SUBSTOMATAL ION-ADSORBENT STRUCTURES IN THE PLANT KINGDOM

INTRODUCTION

Since substomatal ion-adsorbent sites are described and reported on here for the first time, there are obviously no reports in the literature regarding their distribution in the plant kingdom. It is clearly advantageous to know their distribution since, once known, correlations can perhaps be found between the presence of the structures and some particular ecological requirement, phylogenetic or taxonomic assemblage, anatomical organisation, physiological function, or some other criterion. To achieve such a correlation it would be necessary to carry out a systematic and comprehensive survey which is obviously outside the scope of this study. However, a preliminary, non-systematic, survey was carried out on a wide variety of plant types using the modified Macallum technique (7,5% w/v) described in Chapter 2.

OBSERVATIONS

Bryophyta

No indication of substomatal ion-adsorbent sites was found in sporophytic tissue of Polytrichum, Mnium, and Funaria spp.. It was

noticeable that peels from these mosses adsorbed such large quantities of cobalt from the stain as to make observations on the stomatal complex extremely difficult.

Pteridophyta

An examination of representative members of the Psilopsida (Psilotum nudum), Lycopsida (Lycopodium, Huperzia, and Selaginella spp.) and Sphenopsida (Equisetum spp.) indicate that the ion-adsorbent bodies are absent from these rather primitive pteridophytes, although it is possible that they may occur in a rudimentary form in Equisetum spp..

Within the eusporangiate Filicopsida, the substomatal structures appear to be absent in the Ophioglossales (Botrychium and Ophioglossum spp.), but are particularly well developed in the Marattiales (Marattia and Angiopteris spp.), (Plate 8.1). The structures are almost ubiquitous in the leptosporangiate Filicales, but absent in the Marsileaceae (Marsillea and Pillularia spp.) and Salvineaceae (Salvinia natans). The ion-adsorbent bodies in the Filicales look similar to those described for Polypodium vulgare. Representative examples are illustrated in Plates 8.2 and 8.3. A large number of genera and species have been examined, and the structures have only been found lacking in Pellaea sagittata and Ceratopteris thalictroides

Gymnospermae

The ion-adsorbent sites have only been looked for in the more primitive types such as Cycas revoluta, Bowenia sp. indet., and Ginko biloba and found to be absent from these representatives although

the lower periclinal guard cell walls appear to be differentially thickened/cuticularised in the polar regions.

Angiospermae

Despite extensive searches being made in this group, ion-adsorbent sites have only been positively identified in the Commelinaceae and the Liliaceae. The structures are generally well-developed in the Commelinaceae and have been found in all the genera and species of this family which have been examined. Representative examples are illustrated in Plates 8.4, 8.5, and 8.6. The structures vary greatly in size between species, even within a single genus, (Plate 8.6), and are very insignificant in Callisia fragrans (Plate 8.5C) and Cyanotis kewensis (Plate 8.6C), indeed many of the stomatal complexes in these two species appear to lack the structures completely. The ion-adsorbent sites found in Chlorophytum elatum appear to be atypical of the Liliaceae since all the other genera and species examined show no sign of the structure (Plate 8.7A).

There is a strong possibility that the genus Maranta (Marantaceae) possess a type of ion-adsorbent body quite unlike those described for Polypodium vulgare or Tradescantia pallidus. In this genus the structure is represented by a string of bodies lying immediately below the outer anticlinal walls of the guard cell complex, adjacent to the lateral subsidiary cells (Plate 8.7C). Scanning electron microscopy has confirmed the presence of these structures in M. lubberstana (Plate 8.7D).

Ion-adsorbent bodies do not appear to be present in the wide range of dicotyledons examined, although what appear to be

extensions of the neighbouring cells onto the inner face of the guard cell complex in Dianthus x plumarius (Caryophyllaceae) may represent yet another type of ion-adsorbent structure present in certain plants (Plate 8.7B). The substomatal structures in D. x plumarius have not been investigated in any detail.

DISCUSSION

No firm conclusions can be drawn from the fragmentary survey carried out, except to surmise that ion-adsorbent structures are fairly ubiquitous amongst the Filicales and the monocotyledon family, Commelinaceae. The physical differences already noted between the bodies in Polypodium vulgare, which is considered to be a typical member of the Filicales, and Tradescantia pallidus, which is considered to be typical of the Commelinaceae, may indicate that a variety of morphological types of bodies could be scattered throughout diverse plant groups, as is exemplified by the structures found in Maranta spp. and, possibly, in Dianthus x plumarius.

The distribution of the structures within the plant kingdom seems to be extremely fragmented and, at present, cannot be roughly correlated with any particular ecological requirement or phylogenetic assemblage. The distribution within the Pteridophyta is centred within the filicopsid ferns but excludes the phylogenetically-primitive Ophioglossales and the phylogenetically-advanced aquatic and semi-aquatic members of the Marsileaceae and Salvineaceae. The filicopsid ferns which appear to lack the ion-adsorbent structures have a common feature in that their fronds are particularly soft and appear to be only weakly cutinised - the Ophioglossales, Marsileaceae,

Salvineaceae, Ceratopteris, and Pellaea. This observation may lead one to consider that the structures are particularly associated with ferns which are exposed to a harsher environment and which have, presumably, developed a thicker cuticle. This does not appear to be substantiated amongst our native ferns, since the bodies are found in both Blechnum spicant, a species which thrives in exposed upland conditions, and Dryopteris spp., which are typical shaded woodland ferns. This is corroborated bythe fact that the structures in Polypodium vulgare appear to be identical in samples collected from sun-baked walls and in those collected from epiphytic stands in shaded woodlands.

Similarly there appears to be no relationship between the presence of the ion-adsorbent bodies and a particular stomatal type. The ion-adsorbent bodies do not appear to be dependant on the presence of subsidiary cells since, although the majority of plants which exhibit the structures possess subsidiary cells (i.e. all the ferns and the Commelinaceae), the structures are also present in Chlorophytum and, possibly, Dianthus, which lack subsidiary cells. The stomata of Commelina communis are generally considered to be of a typical mesophyte-type, whereas the stomata of ferns are structurally very different (vide Chapter 4), and yet both have ion-adsorbent bodies.

If ion-adsorbent structures function in the stomatal mechanism, a fundamental question is raised; i.e. what structure or system replaces them in plants which apparently lack them? This is unclear at present but it is perhaps significant that, in recent years, a large body of physiological research into the mechanisms behind stomatal movements has been carried out on Commelina spp., which are

now known to possess ion-adsorbent structures. In this respect, Commelina cannot be regarded as typical. However, the stomata of other plant species such as Pelargonium, Vicia, Phaseolus, and Zea (all of which have been examined and shown to lack substomatal ion-adsorbent bodies), basically respond in a similar way to Commelina without the 'advantage' of these structures.

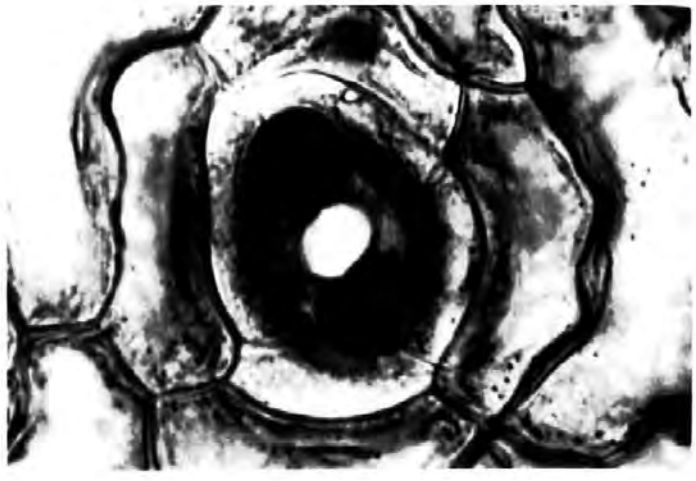
Plate 8.1.

Substomatal ion-adsorbent bodies; Ferns I.

Light micrographs of epidermal tissue treated with the modified Macallum' stain (7,5% w/v), x 750.

A. *Marratia* sp. indet.

B. *Angiopteris evecta*.



A

B

Plate 8.2.

Substomatal ion-adsorbent bodies; Ferns II.

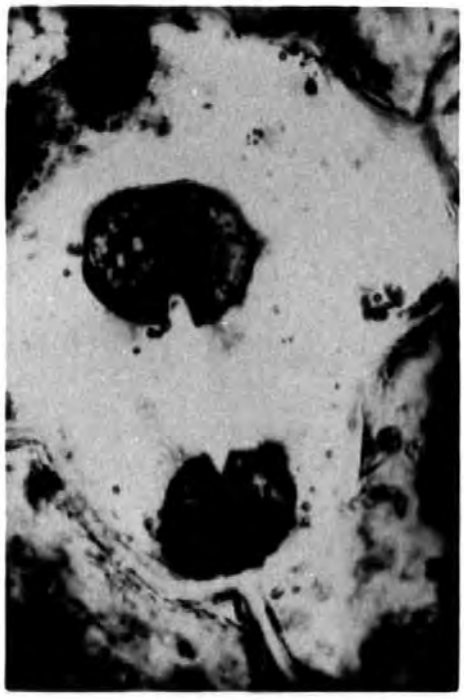
Light micrographs of epidermal tissue treated with the modified Macallum stain (7,5% w/v), x 950.

A. Polypodium aureum.

B. Platycerium bifurcatum.

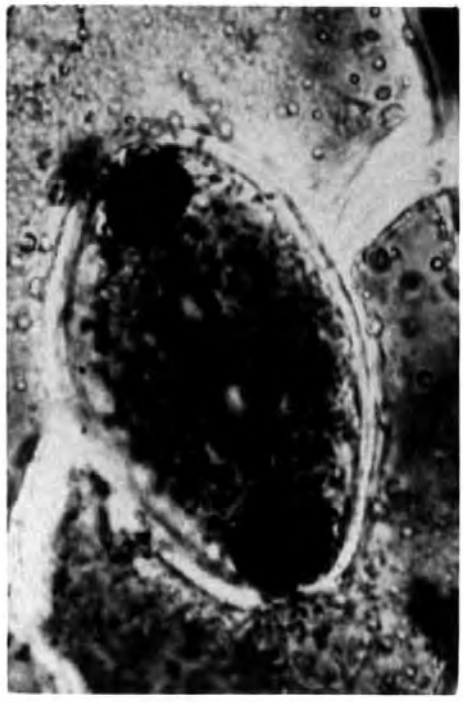
C. Polystichum acrostichiodes.

D. Lygodium scandens.



A

B



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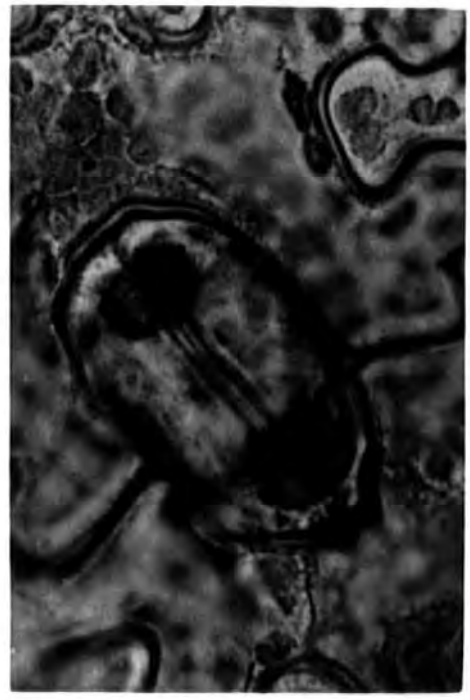


D

Plate 8.3.

Substomatal ion-adsorbent bodies; Ferns III.

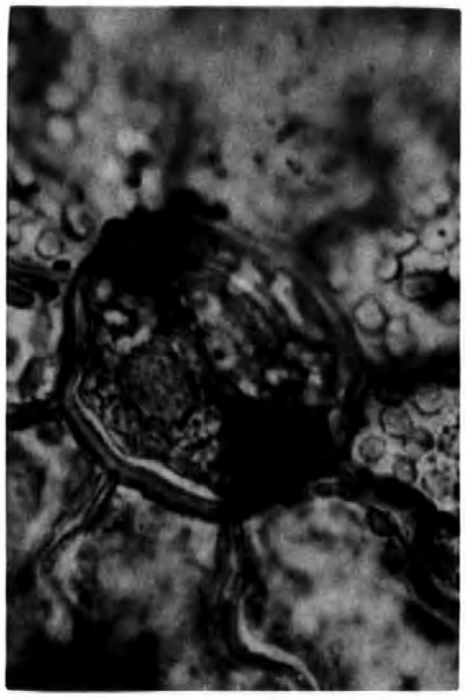
Light micrographs of epidermal tissue treated with the modified Macallum's stain (7,5% w/v), x 750.

A. Osmunda regalis.

B. Asplenium adiantum-nigrum.

C. Dryopteris pseudo-mas.

D. Polystichum aculeatum.



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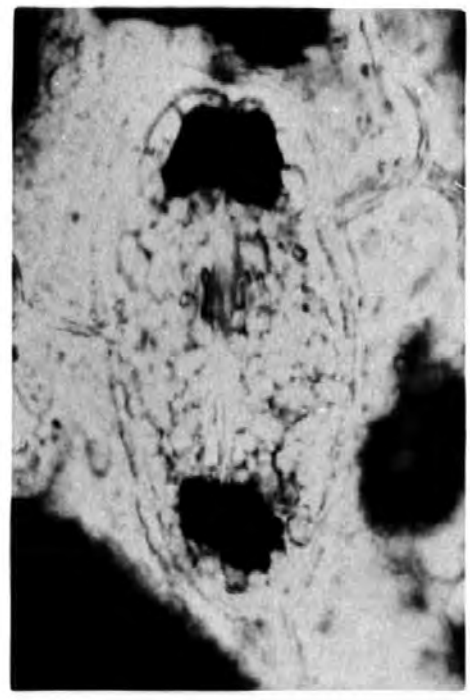
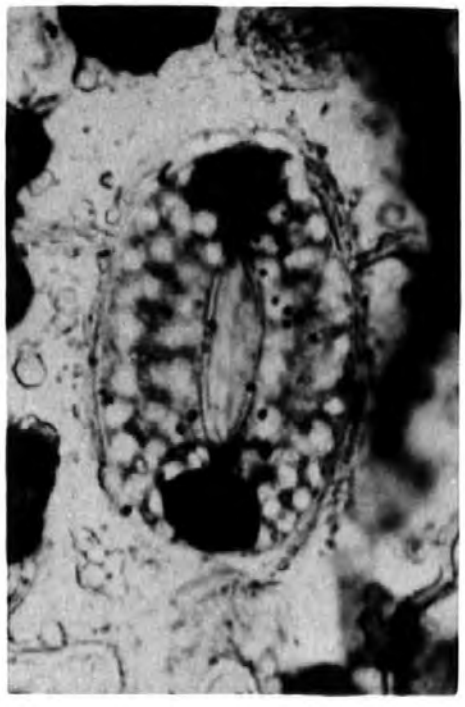


B



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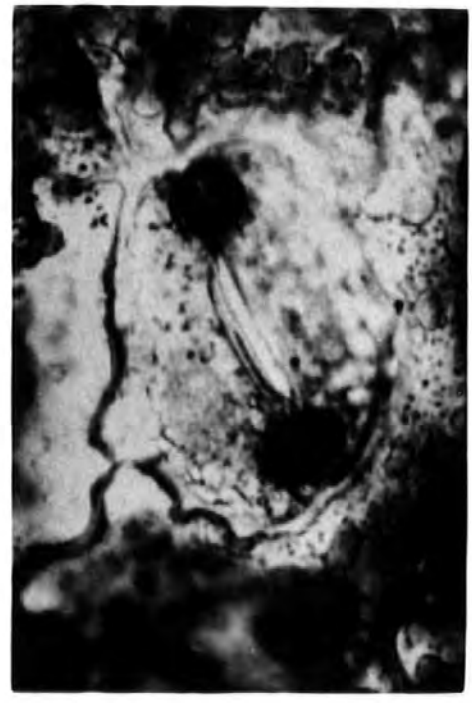


D

Plate 8.4.

Substomatal ion-adsorbent bodies: Commelinaceae I.

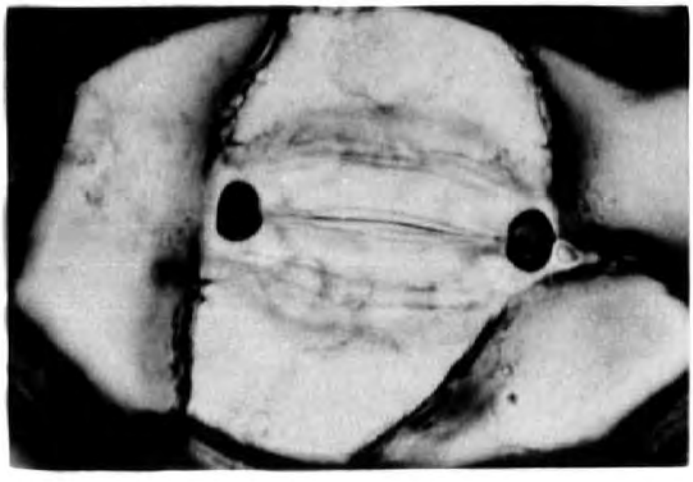
Light micrographs of epidermal tissue treated with the modified Macallum stain (7,5% w/v), x 1 000.

A. Tradescantia blossfeldiana.

B. Coleotrype natalensis

C. Setcreasea purpurea.

A



B

C

Plate 8.5.

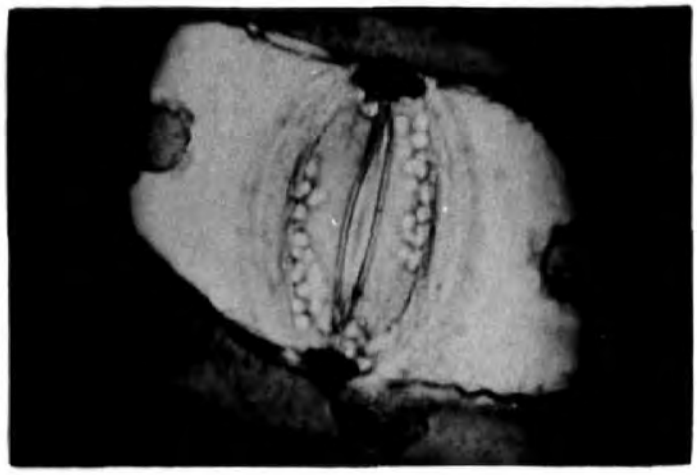
Substomatal ion-adsorbent bodies; Commelinaceae II.

Light micrographs of epidermal tissue treated with the modified Macallum's stain (7,5% w/v), x 1 000.

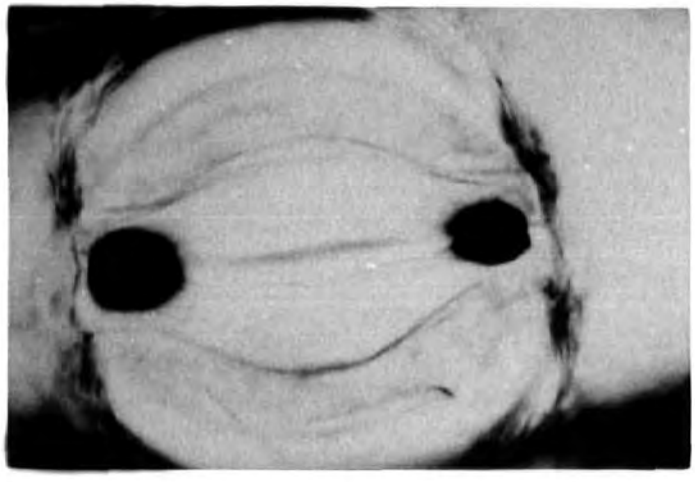
A. Palisota barteri.

B. Rhoeo discolor.

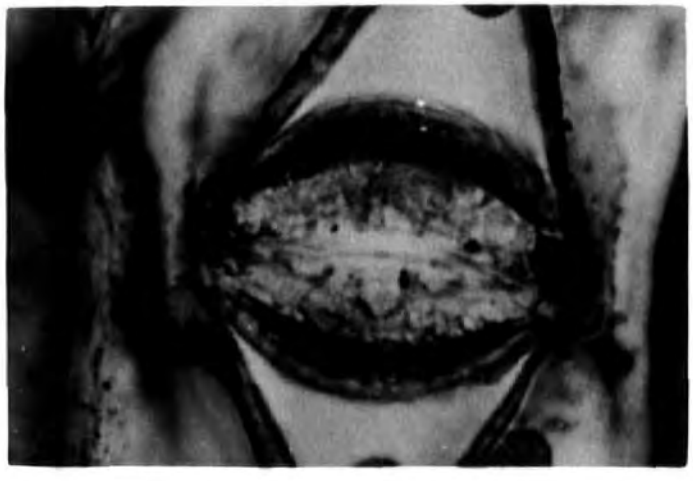
C. Callisia fragrans.



A



B



C

Plate 8.6.

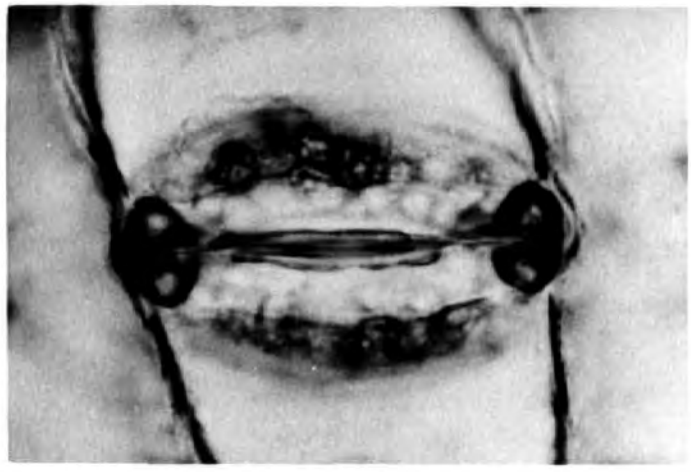
Substomatal ion-adsorbent bodies; Commelinaceae III.

Light micrographs of epidermal tissue treated with the modified Macallum's stain (7,5% w/v), x 1 000.

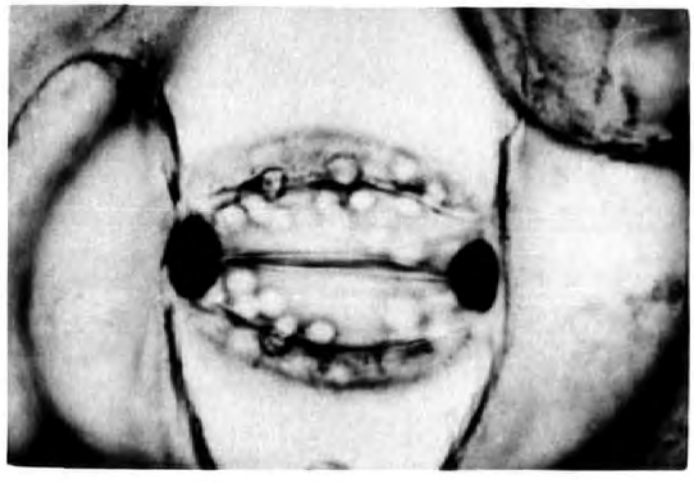
A. Cyanotis mollucana.

B. Cyanotis pillosa.

C. Cyanotis kewensis.



A



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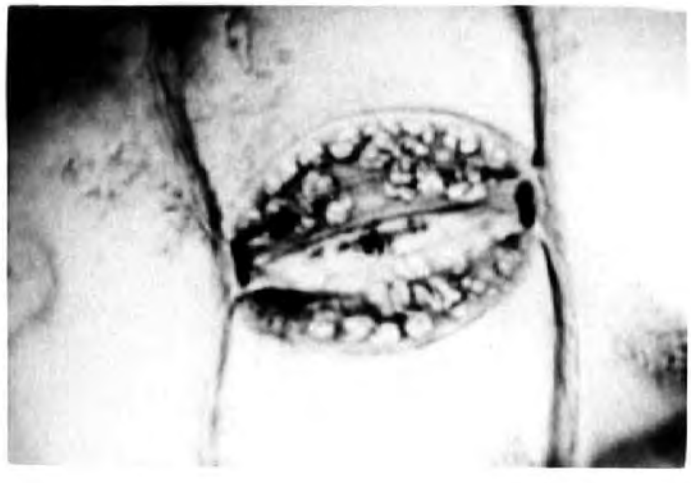


C

Plate 8.7.

Substomatal ion-adsorbent bodies; Miscellaneous.

A - C are light micrographs of epidermal tissue treated with the modified Macallum's stain (7,5% w/v). D is a scanning electron micrograph.

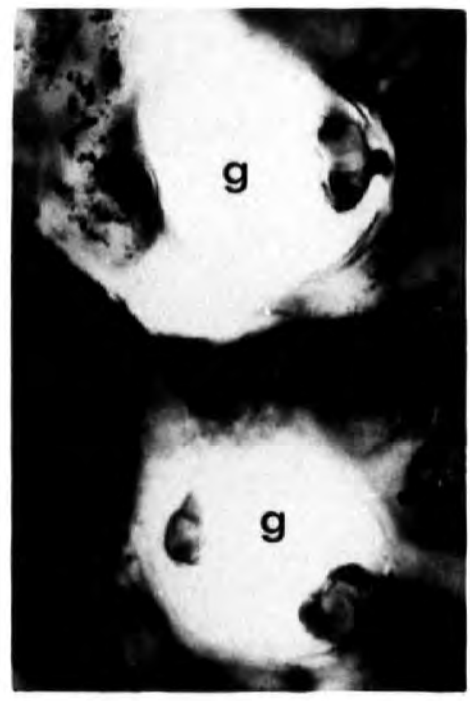
A. Chlorophytum elatum, x 950

B. Dianthus x plumarius, x 750. This is a composite micrograph of two non-adjacent guard cell complexes (g).

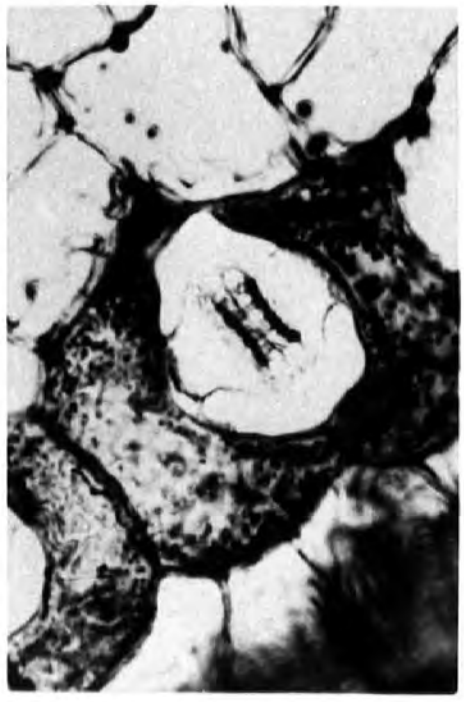
C. Maranta lubberstana, x 650

D. Maranta lubberstana, x 3 000. The bodies (arrowed) can be seen adjacent to the guard cells (g) and appear to be highly hydrated as evidenced by surface charging. s = subsidiary cell.

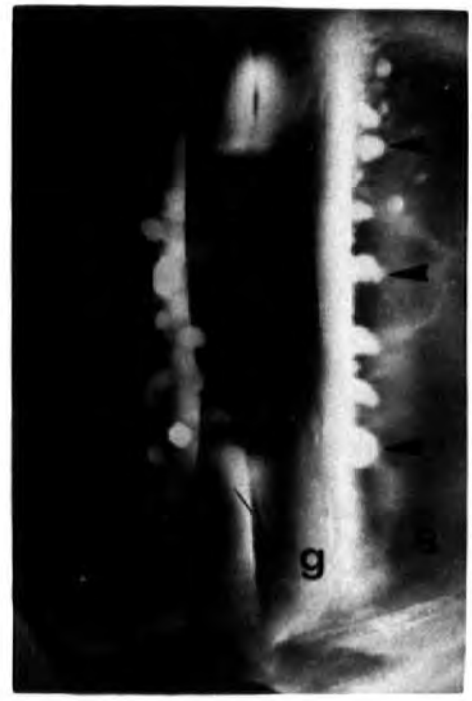
A



B



C



D

CHAPTER 9

MORPHOLOGY AND ONTOGENY OF THE STOMATAL COMPLEX

INTRODUCTION

In order to study the morphogenesis of substomatal ion-adsorbent sites, it is necessary to examine immature epidermes and relate observed development of the sites with ontogenetic stages of the developing stomata. Although there is a considerable body of literature on the ontogeny of stomata and their morphological forms (vide Fryns-Claessens & Van Cotthem, 1973), certain inconsistencies are apparent in the descriptions of the various types of cells which contribute to the mature stomatal complex, and also in the interpretation of the ontogenetic roles played by these cells. Consequently, the ontogeny of the stomata in Tradescantia pallidus and Polypodium vulgare were examined in an attempt to resolve current anomalies in the accepted interpretation of developmental events leading up to stomatal formation.

A mature stomatal complex is made up of a pair of guard cells surrounded by subsidiary and/or neighbouring cells. Subsidiary cells are related ontogenetically to their associated guard cells and are derived de novo from either the stomatal meristemoid (defined in the following paragraph) or protodermal cells adjacent to the stomatal meristemoid and which, at maturity, are generally morphologically distinct from other epidermal cells. Neighbouring cells are protodermal cells which lie immediately adjacent to a

stomatal meristemoid and which mature in this position without undergoing any cell division or differentiation so that, at maturity, they are indistinguishable from other epidermal cells except for their contact with the guard cell complex.

During stomatal development, certain protodermal cells, cut off from the leaf meristem, undergo an asymmetrical division. The smaller product of this cell division, which is cut off distal to the leaf meristem, becomes the stomatal meristemoid and ultimately gives rise to a pair of guard cells. The stomatal meristemoid is known as the guard-cell mother-cell when the meristemoid undergoes only a single symmetrical division to form the guard cells. When the stomatal meristemoid undergoes more than a single division, the first division(s) is of an asymmetrical nature and gives rise to a subsidiary cell(s), whilst the final division of the meristemoid, which now is technically the guard-cell mother-cell, is of a symmetrical type and gives rise to the guard cells. The subsidiary cells derived from the stomatal meristemoid are referred to as mesogene subsidiaries. However, subsidiary cells can also be derived from protodermal cells adjacent to the stomatal meristemoid which assume meristematic activity to cut off perigene subsidiary cells. It is proposed to refer to these meristematic protodermal cells adjacent to the stomatal meristemoid as subsidiary meristemoids.

Modern ontogenetic terminology is based on that of Florin (1931, 1933) and Pant (1965). Florin (1931) distinguished two types of stomatal development in gymnosperms; a haplocheilic type in which the stomatal meristemoid acts as a guard-cell mother-cell and forms the guard cells only, and a syndetocheilic type in which the stomatal meristemoid gives rise to one or more subsidiary cells before forming

the guard cell complex. He later (Florin, 1933) proposed that the two ontogenetic types should be referred to as perigenous and mesogenous types, respectively. Pant (1965) adopted this latter terminology and added a third category, mesoperigenous, to cover those stomatal types which contained both mesogenous and perigenous elements. His definitions of the three basic ontogenetic types have been elaborated on and re-defined by Fryns-Claessens and Van Cotthem (1973) as follows:

Perigenous. "The stomatal initial or meristemoid acts as a guard-cell mother-cell. It divides only once to produce the two guard cells directly. All surrounding cells (generally neighbouring, but sometimes subsidiaries) are derived from normal epidermal cells."

Mesogenous. "The meristemoid divides by a series of divisions into a guard-cell mother-cell and either a number of mesogenous subsidiaries or a ring-like mesogenous cell surrounding the guard-cell mother-cell completely."

Mesoperigenous. "The meristemoid divides into two unequal cells of which the smaller forms the guard-cell mother-cell directly or divides a second time into two cells of which one is the guard-cell mother-cell. The larger daughter cell of the first division or those of both divisions become mesogenous neighbouring or subsidiary cell. The guard cells are formed after two or three divisions of the meristemoid. The surrounding cells are of dual origin: one or more are mesogenous, the other(s) perigenous."

Tradescantia pallidus

The leaves of this species are amphistomatous and the stomata are of a phaneropore type with their longitudinal axes lying parallel to the longitudinal axis of the leaf. The guard cells of the abaxial epidermis are between 41 and 72 µm long ($\bar{x}$ = 51,6) by 10 to 24 µm wide ($\bar{x}$ = 13,8) and are arranged in linear files throughout the interfascicular epidermis (Plate 9.1B). Those of the adaxial surface are between 47 and 69 µm long ($\bar{x}$ = 55,0) by 9 to 15 µm wide ($\bar{x}$ = 12,9) and are restricted to between one and four longitudinal rows on the suprafascicular epidermis (Plate 9.1A). There are, on average, 34,2 stomata mm^{-2} on the total abaxial epidermis with an average stomatal density of 44,6 mm^{-2} on the interfascicular tissue. The density of stomata on the adaxial surface averages 6,8 mm^{-2}. The stomatal index (Salisbury, 1928) of the total abaxial epidermis averages 8,9 whilst that of the total adaxial epidermis averages 3,6. The average stomatal ratio between adaxial and abaxial leaf surfaces is 1:5.

The basic morphological form of the stomatal complex is of a pair of guard cells surrounded by four subsidiary cells which are morphologically distinct from other epidermal cells (Plate 9.2A). The subsidiary cells are clearly paired. A pair of lateral subsidiary cells lie adjacent to the outer anticlinal walls of the guard cell complex, and a pair of polar subsidiary cells abut onto the poles of the complex. The polar subsidiary cells quite frequently fail to develop in this species so that stomatal complexes containing only a pair of lateral subsidiary cells are not uncommon (Plate 9.2B).

The stomatal pattern on the abaxial epidermis is usually of a more constant nature than that of the adaxial epidermis. A wide variety of aberrations in the stomatal pattern have been observed which generally fall into one of three categories:

a/ Failure of certain subsidiary cells to develop,

b/ Development of supernumerary subsidiary cells, and

c/ Two (seldom more) guard cell complexes developing in such a position that they become directly attached to to each other, generally at the poles.

Most of the aberrations noted and illustrated by Drawert (1942) in T. viginica (sic.) have also been observed in T. pallidus, a few examples of which are illustrated in Plate 9.3. Since these are thought to represent aberrations, as opposed to variations, they will not be considered further here.

Polypodium vulgare

The pinnae of this species are hypostomatous and the stomata are of a phaneropore type with their longitudinal axes lying parallel to the secondary vascular tissue of the pinnae. The guard cells are between 45 and 73 μm long ($\bar{x}$ = 64,9) by 20 to 25 μm wide ($\bar{x}$ = 22,0) and are scattered randomly, but evenly, throughout the interfascicular epidermis (Plate 9.4A).

The basic morphological form of the stomatal complex consists of a horseshoe-shaped subsidiary cell circumscribing the pole of the guard cell proximal to the main vein of the pinna, to enclose between one-half and three-quarters of the guard cell complex (Plate 9.4B). Exceptionally, this subsidiary cell may be represented by a pair of subsidiary cells (Plate 9.4B). Of equal abundance are stomatal

complexes with a pair of horseshoe-shaped subsidiary cells at the same pole of the complex, so arranged that only the inner one is in contact with the guard cells whilst the outer one partially surrounds the inner one (Plate 9.4C). Very occasionally there may be three polar subsidiary cells (Plate 9.4D). A very small percentage of stomatal complexes (<5%) suggest that subsidiary cells of a different nature are present at the opposite pole of the guard cell complex to the normal subsidiary cells described above. They are manifest as the result of a cell division in the epidermal cell abutting onto this pole of the complex causing a straight-sided subsidiary cell to be formed against the complex (Plate 9.5B). A variant of this type also includes a neighbouring cell in the complex as well as the two types of subsidiary cell already described (Plate 9.5A).

DEVELOPMENT OF THE STOMATAL COMPLEX

Tradescantia pallidus

Isodiametric protodermal cells are cut off in linear files from the intercalary meristem located at the base of the leaf. Some of these protodermal cells subsequently assume meristematic activity and divide asymmetrically in a vertical plane at right angles to the leaf axis (Plate 9.6). The smaller distal product of this division becomes the stomatal meristemoid which is compressed baso-distally initially. The nucleus of a protodermal cell immediately laterad to the stomatal meristemoid migrates towards the meristemoid before undergoing an oblique asymmetrical division in a plane sub-parallel to the leaf axis, the smaller product of which becomes associated with

the stomatal meristemoid and eventually develops into a lateral subsidiary cell (Plate 9.7A). The protodermal cell on the opposite side of the meristemoid undergoes a similar division to give rise to the other lateral subsidiary cell. Shortly afterwards, the protodermal cells immediately distal and immediately proximal to the stomatal meristemoid undergo asymmetrical cell divisions in a vertical plane at right angles to the leaf axis and give rise to the polar subsidiary cells (Plate 9.7B). During the development of the subsidiary cells, the stomatal meristemoid is quiescent but elongates slightly prior to dividing symmetrically in a vertical plane parallel to the long axis of the leaf to form the pair of guard cells (Plate 9.7C).

This sequence of developmental events is not pursued in any rigid order, although it generally follows the progression outlined above. In no case was it observed that the guard cells were formed before the differentiation of the lateral subsidiary cells. Stomatal differentiation is strictly gradate and proceeds acropetally so that all the stomata in a single transverse transect across the epidermis are at a similar stage of development. Exceptionally, individual stomatal meristemoids may remain latent until adjacent stomatal development is complete before differentiating (Plate 9.8A).

After the the guard-cell mother-cell divides to form the guard cells, the latter gradually become more rounded in cross-section and elongate. As they elongate, they bow out laterally and eventually their common anticlinal cell walls separate mesially to form the stoma. The maturation of the guard cells is accompanied by progressive vacuolation during which the chloroplasts assume a parietal position (Plate 9.9). During epidermal maturation, the epidermal cells elongate whilst the lateral subsidiary cells become rather flattened

in a vertical longitudinal plane to accomodate the enlargement of the guard cells.

The ontogeny of stomata in T. pallidus is summarised diagramatically in Fig. 9.1. All stages have been observed microscopically. Since the stomatal meristemoid undergoes only a single cell division to form the guard cells, it is correct to refer to the meristemoid as the guard-cell mother-cell immediately on its inception from the protoderm.

Polypodium vulgare

Protodermal cells are cut off from the marginal meristem of circinately-folded pinnae in this species. Certain protodermal cells divide asymmetrically in an oblique plane at right angles to the secondary vascular tissue of the pinna to cut off a distal subspherical smaller product which becomes the stomatal meristemoid. The meristemoid subsequently divides once or twice in an oblique plane at right angles to the secondary vascular tissue of the pinna to cut off one or two horseshoe-shaped subsidiary cells on the side distal to the leaf margin (Plate 9.10A & B). The divisions are such that the smaller product (stomatal meristemoid/guard-cell mother-cell) is partially subtended by the larger product (subsidiary cell). Immediately following the formation of the subsidiary cells, the stomatal meristemoid undergoes a symmetrical division in a vertical plane parallel to the secondary vascular tissue of the pinna to give rise to the pair of guard cells (Plates 9.10C & 9.11). Occasionally, protodermal cells abutting onto the pole of the meristemoid, proximal to the leaf margin, become meristematic after the formation of the subsidiary cell(s) outlined above. In these cases, the protodermal

cell divides in a vertical plane subparallel to the margin of the meristemoid so that a resultant subsidiary cell becomes interposed between the neighbouring epidermal cell (its mother-cell) and the guard cells (Plate 9.12B).

Stomatal differentiation proceeds basipetally from regions immediately behind the marginal meristem of the pinna in a non-gradate fashion so that, whilst the majority of stomatal meristemoids commence development immediately on inception from a protodermal cell, a considerable proportion remain latent until adjacent ones have fully differentiated, but not completely matured, before commencing development (Plate 9.11A).

As the stomatal complex matures, it expands so that the subsidiary cell(s) and/or neighbouring cell(s) in contact with the guard cells pull back from their original relative position(s), where they partially subtend the guard cells, to lie adjacent to the poles of the guard cell complex. At the same time, the characteristic sinuosities of the anticlinal walls of the subsidiary cells (but not those walls abutting onto the guard cell complex) and the epidermal cells develop, and the stoma appears between the common anticlinal cell walls of the guard cells.

These sequences of events are summarised diagramatically in Figs. 9.2 & 9.3. All the stages illustrated have been observed with the exception of 9.2b, where the stomatal meristemoid is cut off from a protodermal cell. The stomatal meristemoid is readily distinguished from surrounding protodermal cells by its marginally smaller size, its more heavily-staining nucleus, and denser cytoplasm. The stomatal meristemoid is involved in either two or three (exceptionally four) divisions depending on whether it gives

rise to one, two (or three) polar subsidiary cells. The meristemoid, therefore, does not become a guard-cell mother-cell until after it has cut off its subsidiary cell(s) prior to its final division which results in the formation of a pair of guard cells.

DEVELOPMENT OF SUBSTOMATAL CHAMBERS

Tradescantia pallidus

In this species, it appears that a break in the mesophyll forms naturally as a result of developmental events in the epidermis. Protodermal cells become associated with protomesophyll cells lying immediately below and, as the cells of the stomatal complex develop, they become interposed into existing protodermal tissue which is thus displaced along with the underlying mesophyll tissue to form a natural gap in the mature mesophyll (Fig. 9.4). This natural break is illustrated in Plate 5.6A.

Polypodium vulgare

The mode of development of substomatal chambers in the mesophyll in P. vulgare is rather debateable. The mesophyll tissue of mature fronds is aerenchymatous with lateral processes from adjacent cells leaving large air passages between the cells of the tissue (Plate 5.2). This structure could well preclude the necessity for special breaks to form in the mesophyll immediately below the stomata (Fig. 9.4). However, examination of very young epidermal strips from circinately-folded pinnae show that a small proportion of the developing stomata have, what appear to be, small atrophying mesophyll

cells loosely attached to the inner face of the stomata. This feature was also noted in a number of other fern species and is particularly prevalent in the related fern, Phyllitis scolopendrium, (Plate 9.13). In this species, it appears probable that mesophyll cells immediately below young stomata atrophy under the influence of the developing stomata so that naturally-induced breaks occur in the substomatal position of the mature mesophyll (Fig. 9.4).

DISCUSSION

Morphology of stomatal complexes

The stomatal complex of Tradescantia pallidus does not appear to have been described previously although it is very similar to that of its congeners which have been documented by Strasburger (1867), Campbell (1881), Benecke (1892), Drawert (1942), and Tomlinson (1969). The basic morphological form, as illustrated in Plate 9.2A, is typical of the genus and is tetracytic (Metcalfe, 1961). The abundance of complexes with only lateral subsidiary cells (Plate 9.2B) is significant so that the dicytic* form must be regarded as a variant rather than an aberration (a condition found in the related genera Cuthbertia and Triceratella, Tomlinson, 1966). All other stomatal patterns observed in this species are considered

* This self-explanatory form is used here for the first time since the only reference to this morphological form in the literature is that of Prat (1960) who described it as 'stomates quadricellulaires'. Compound terminology, which includes ontogenetic expressions, refer to this type of stomata as 'biperigenous' (Pant, 1965) and 'diperigenous' (Van Cotthem in Fryns-Claessens & Van Cotthem, 1973).

to be aberrant and, therefore, not worthy of nomenclatural identity.

The morphological forms found in the stomatal complex of Polypodium vulgare have been described by Reuter (1942) and later workers. The two basic forms, as illustrated in Plates 9.4B and 9.4C, have been described as eupolocytic and copolocytic (Van Cotthem, 1970) depending on whether they have one or two polar subsidiary cells, respectively. The condition where there are three polar subsidiary cells (Plate 9.4D) has not been recorded before in the literature and, if a special terminology is required, it is proposed to term them polypolocytic.

The new stomatal types observed in P. vulgare (Plate 9.5) raise a nomenclatural problem since there are no terms available to cover the morphological types produced by the mesoperigenous developmental pathways described. It seems advisable to retain the polocytic expression, which is the common morphological type found in this species, and to couple it to the anisocytic type to give the compound morphological expression, aniso-polocytic. It is hoped that this morphological expression adequately portrays the unequalness of the two types of subsidiary cells whilst emphasising their polarity. There is little point in complicating the expression with the prefixes, eu-, co-, or poly-, to denote the number of mesogene subsidiary cells present.

Ontogeny of stomatal complexes

During the present study it became clear that subsidiary and neighbouring cells are treated synonymously by Pant (1965) and subsequent workers in their interpretations of perigenous and mesoperigenous stomatal types. However, his definition of mesogenous

stomata is such that the cells surrounding the guard cells must be derived from the stomatal meristemoid and therefore, whilst including subsidiary cells, neighbouring cells are mutually excluded. This failure to differentiate between the respective roles played by subsidiary and neighbouring cells in the perigenous and mesoperigenous modes of development results in a biassed classification. As a consequence, it is impossible to identify which types of cells are present in perigenous and mesoperigenous stomatal complexes from the terminology alone, and it becomes necessary to refer back to original descriptions in the literature or else resort to the microscope and developing epidermes.

Ontogenetic studies of the stomatal complex are concerned with the development of the component cells, as present in mature complexes, from protodermal origins. The most important cells, therefore, are those which are derived de novo from meristematic protodermal cells (i.e. meristemoids), and not those derived directly from the leaf meristem. These are the guard and subsidiary cells. Neighbouring cells do not fit into this category since they are no more than undifferentiated protodermal cells and direct products of the leaf meristem which, by accident, happen to be in contact with the guard-cell mother-cell and which eventually mature in juxtaposition to the guard cell complex. Neighbouring cells are clearly not directly involved in stomatal development but do contribute to the morphology of the mature stomatal complex. It appears unreasonable to afford them the same status as subsidiary cells when discussing developmental events. This basic difference between subsidiary and neighbouring cells unfortunately does not seem to have been considered relevant in standard ontogenetic nomenclature. This, in itself, is rather

suprising since the etymology of the expressions used bears strong connotations of cell inception (the base genous is derived from the Greek for 'offspring'). In its current useage, both neighbouring and subsidiary cells are included in the perigene and mesoperigene conditions whilst only subsidiary cells are included in the mesogene condition.

The anomalous situation which exists at present needs to be rationalised so that the terminology is more meaningful and summarises ontogenetic events more accurately. This can be quite easily accomplished by re-defining and restricting the basic ontogenetic classes to include only those events pertaining to the development of the subsidiary cells.

Proposed new ontogenetic classification of stomatal types

The proposed restricted definitions of the basic ontogenetic categories are as follows:

Mesogenous. Stomatal complexes in which the guard cells are only in contact with one or more subsidiary cells derived from the stomatal meristemoid.

Perigenous. Stomatal complexes in which the guard cells are only in contact with one or more subsidiary cells derived from one or more subsidiary meristemoids.

Mesoperigenous. Stomatal complexes in which the guard cells are only in contact with subsidiary cells which are derived from both the stomatal meristemoid and the subsidiary meristemoid(s).

To these basic classes, it is necessary to add a fourth one to accomodate those stomatal complexes which are devoid of subsidiary

cells (= aperigenous type of Fryns-Claessens & Van Cotthem, 1973). It is proposed to refer to such complexes as agenous (from the Greek for 'without' + 'offspring'). It is defined as:

Agenous. Stomatal complexes in which the guard cells are only in contact with neighbouring cells.

The basic ontogenetic categories, as defined above, clearly indicate the origin of the subsidiary cells involved but fail to identify the presence of neighbouring cells which also may be in contact with the guard cells of the mature complex, except in the case of the agenous group. To remedy this, it is proposed to attach the Greek prefixes, eu- (= whole), and hemi- (= half) to the three basic ontogenetic categories which are associated with subsidiary cells. The prefixes will be indicative of whether the guard cell complex is completely surrounded by subsidiary cells or whether it is surrounded by a mixture of subsidiary and neighbouring cells, respectively.

The result of the present proposals is the erection of seven new ontogenetic classes to replace the three of Pant (1965). The two classifications are compared with each other in Table 9.1. The different developmental pathways involved in the formation of the various ontogenetic classes proposed in the new classification and that of Pant (op. cit.) are represented diagramatically in Figs. 9.5 and 9.6.

The proposed new ontogenetic categories are defined as follows:

Agenous. Guard cells completely surrounded and contacted by undifferentiated neighbouring cells. (Fig. 9.7a).

Hemiperigenous. Guard cells partly surrounded and contacted by

Table 9.1.

Comparison of the proposed new ontogenetic classification with that of Pant (1965).

Pant's terminology	Proposed new terminology
Perigenous	Agenous Hemiperigenous Euperigenous
Mesoperigenous	Hemimesoperigenous Eumesoperigenous Hemimesogenous
Mesogenous	Eumesogenous

subsidiary cell(s) derived from subsidiary meristemoid(s) and partly by undifferentiated neighbouring cell(s). (Fig. 9.7b)

Euperigenous. Guard cells completely surrounded and contacted by subsidiary cell(s) derived from subsidiary meristemoid(s). (Fig. 9.7c)

Hemimesogenous. Guard cells partly surrounded and contacted by subsidiary cell(s) derived from the stomatal meristemoid, and partly by undifferentiated neighbouring cell(s). (Fig. 9.7d)

Eumesogenous. Guard cells completely surrounded and contacted by subsidiary cell(s) derived from the stomatal meristemoid. (Fig. 9.7e).

Hemimesoperigenous. Guard cells partly surrounded and contacted by subsidiary cell(s) derived from the stomatal meristemoid, partly by subsidiary cell(s) derived from subsidiary meristemoid(s), and partly by undifferentiated neighbouring cells. (Fig. 9.7f).

Eumesoperigenous. Guard cells partly surrounded and contacted by subsidiary cell(s) derived from the stomatal meristemoid, and partly by subsidiary cell(s) derived from subsidiary meristemoid(s). (Fig. 9.7g).

The only category of Pant (1965) which is not modified in the new classification is his mesogene class which now becomes the eumesogenous class. The inclusion of both perigene subsidiary cells and neighbouring cells in his perigenous and mesoperigenous classes results in the fragmentation of these classes in the new classification. Accordingly, both of Pant's categories have had their perigene elements (sensu lato) divided into three new classes, one of which contains only perigene subsidiary cells (sensu stricta), the second of which contains both perigene subsidiary cells and neighbouring cells (perigenous, sensu lato in partem) and the third of which contains only neighbouring cells (perigenous, sensu lato in partem).

The new classification has been incorporated into that of Fryns-Claessens & Van Cotthem (1973), which uses a compound terminology containing expressions of both morphological form and ontogenetic type, in Table 9.2. Besides modifications to the ontogenetic element of the terminology, the only other changes involve the partitioning of their tetraperigenous class into two new categories (tetra-euperigenous and tetra-hemiperigenous), and the erection of two new classes (aniso-polo-hemimesoperigenous and aniso-polo-eumesoperigenous). The latter two classes are not

Table 9.2.

Modified classification of stomatal types.

(after Fryns-Claessens and Van Cotthem, 1973)

Original terminology	Modified terminology.
Aperigenous	Agenous
Monoperigenous Diperigenous Tetraperigenous	Mono-hemiperigenous Di-hemiperigenous Tetra-hemiperigenous
Hexaperigenous Polyperigenous	Tetra-euperigenous Hexa-euperigenous Poly-euperigenous
Anomo-mesoperigenous Dia-mesoperigenous Hemi-para-mesoperigenous Eupolo-mesoperigenous Copolo-mesoperigenous Aniso-mesoperigenous Stauro-mesoperigenous Para-mesoperigenous	Anomo-hemimesogenous Dia-hemimesogenous Hemi-para-hemimesogenous Eupolo-hemimesogenous Copolo-hemimesogenous Aniso-hemimesogenous Stauro-hemimesogenous Para-hemimesogenous
Cyclo-mesoperigenous	Cyclo-eumesoperigenous Aniso-polo-eumesoperigenous
	Aniso-polo-hemimesoperigenous
Desmo-mesogenous Euperi-mesogenous Coperi-mesogenous Duploperi-mesogenous Dia-mesogenous Para-mesogenous Cyclo-mesogenous Allelo-mesogenous Aniso-mesogenous Helico-mesogenous Tetra-mesogenous	Desmo-eumesogenous Euperi-eumesogenous Coperi-eumesogenous Duploperi-eumesogenous Dia-eumesogenous Para-eumesogenous Cyclo-eumesogenous Allelo-eumesogenous Aniso-eumesogenous Helico-eumesogenous Tetra-eumesogenous

represented in their classification; indeed the hemimesoperigenous ontogenetic type does not appear to have been reported on in the literature before. Both types came to light during the ontogenetic studies in Polypodium vulgare (vide infra).

Ontogenetic types in Tradescantia & Polypodium

Two basic morphological types of stomatal complexes have been identified in Tradescantia pallidus; tetracytic and dicytic (vide supra, and Plate 9.2). From the ontogenetic studies in this species, it is clear that the subsidiary cells arise from subsidiary meristemoids and not the stomatal meristemoid (Fig. 9.1). In the tetracytic type, the guard cell complex is completely surrounded and contacted by the subsidiary cells and, therefore, it can be classed as an euperigenous ontogenetic type or, to give it its full compound terminology (Table 9.2), tetra-euperigenous. In the dicytic type, neighbouring cells abut onto the poles of the guard cell complex which is also bordered laterally by subsidiary cells. They are, therefore, of a hemiperigenous type or, to give them their full terminology, di-hemiperigenous.

Polypodium vulgare also has two basic morphological types of stomatal complex; eupolocytic and copolocytic (vide supra, and Plate 9.4B & C). The horseshoe-shaped subsidiary cell(s) in these types are mesogenous since they arise from the stomatal meristemoid (Fig. 9.2). The guard cell complexes of these types are also contacted by undifferentiated neighbouring cells, which makes them hemimesogenous ontogenetic types or, to give them their full compound terminology (Table 9.2), eupolo-, or copolo-hemimesogenous types. The existence of a polypolo-hemimesogenous type (Plate 9.4D) is reported on here for

the first time.

In addition to the basic ontogenetic types of stomatal complex in P. vulgare, identified in the preceding paragraph, two completely new morphological types (Plate 9.5, and Fig. 9.3) have been found in this species which arise from different ontogenetic pathways. Besides the usual mesogenous subsidiary cells, they also have perigenous subsidiary cells at the opposite pole of the complex. In one case, the guard cell complex is contacted by both types of subsidiary cell and a neighbouring cell (Plate 9.5A) derived by a hemimesoperigenous ontogenetic pathway. They are, therefore, aniso-polo-hemimesoperigenous types (Table 9.2). In the other case, the guard cell complex is contacted by both mesogene and perigene subsidiary cell elements only (Plate 9.5B), and are thus of an eumesoperigenous type or, aniso-polo-eumesoperigenous, to give them their full compound terminology (Table 9.2).

Relevance of the new ontogenetic classification

The importance of this new ontogenetic classification is to remove an inherent bias in the existing terminology which fails to differentiate between the ontogenetic significance of perigene subsidiary cells and the purely structural significance of neighbouring cells. Payne (1970) recognised a basic difference between these two types of cells but did not pursue the matter further. This observation by Payne was not followed up by Fryns-Claessens and Van Cotthem (1973) who, whilst accepting the position of perigene subsidiary cells (op. cit., p.77), failed to recognise their ontogenetic significance and continued to group them together with neighbouring cells in their revised classification. It is, perhaps, this unequal

weighting in the classification which prompted Paliwal (1969) to devise a special nomenclature for monocotyledon stomatal development which, he claims, is warranted by virtue of their specialised type of development. Although he fails to specify which aspect of monocotyledon development is of a specialised type, there are good grounds for supporting his contentions.

The vast majority of dicotyledons with subsidiary cells fall into Pant's mesogenous and mesoperigenous categories. This effectively means that all dicotyledons with both subsidiary and neighbouring cells in contact with the guard cell complex are mesoperigenous (except for the very few species classified as cyclo-mesoperigenous by Fryns-Claessens and Van Cotthem, 1973, = cyclo-eumesoperigenous in present classification), and all those whose guard cells are completely surrounded by subsidiary cells are mesogenous. Thus in the dicotyledons a distinction, albeit inadvertent, is drawn between subsidiary and neighbouring cells. The majority of monocotyledons, however, fall into Pant's perigenous class where no such distinction can be drawn. Paliwal (op. cit.) appears to have recognised this inconsistency but then complicates the issue by proposing that perigene subsidiary cells are not ontogenetically related to the guard cells but do have a special structural relationship with them. Whilst recognising that there is a distinction between perigene and mesogene subsidiary cells, the former do have more than just a structural affinity to the guard cells and must be afforded at least an indirect ontogenetic relationship. To relegate perigene subsidiary cells to a specialised structural relationship is tantamount to recognising these cells as little more than neighbouring cells, which is clearly unacceptable.

It is worth noting that Fryns-Claessens and Van Cotthem (1973) were possibly correct in asserting in their description of perigenous stomata (quoted in the introduction to this chapter) that the surrounding cells were generally neighbouring cells rather than subsidiary cells since they included the new agenous group in their perigenous class. However with the removal of agenous types from the perigenous groups in the present classification, the surrounding cells are usually subsidiary cells and only rarely neighbouring cells. The new agenous class contains all stomatal complexes which are not associated with subsidiary cells. Fryns-Claessens and Van Cotthem (op. cit.) referred to such stomata as 'aperigenous' which, strictly speaking, is indicative of these complexes lacking perigene subsidiary cells, but it would be just as correct to refer to them as 'amesogenous' in that they also lack mesogene subsidiary cells. Since these stomata lack both types of subsidiary cell, and since the basic terms must relate to the mode of development of subsidiary cells, it is considered that the proposed term, agenous, is preferable. No reason can be found why the agenous class should be retained in the perigenous group.

Unresolved problems

As Baranova (1975) points out, "it is desirable to avoid changes in accepted nomenclature until the classification of stomata is completed". The classification proposed here is based on accepted terminologies but lacks the ambiguities of previous schemes in that it clearly differentiates between both types of subsidiary cells and neighbouring cells. It is believed that the new classification is completely explicit and accurately portrays the involvement of the

component cells found in developing and mature stomatal complexes.

The new scheme has proved viable for a wide range of stomatal types and plant species, and the only problem encountered has been the exact definition of the term 'subsidiary cell'. A classical definition of subsidiary cells is given by Esau (1965, p. 138) as "two or more . . . cells adjacent to the guard cells (which) appear to be associated functionally with them and (which) are morphologically distinct from the other epidermal cells". As Tomlinson (1974) points out, the functional aspect is largely an assumed one. Most modern workers, however, emphasise the point in their definitions that subsidiary cells bear some developmental relationship to guard cells (Pant, 1965; Payne, 1970). Ignoring the functional characteristics of subsidiary cells, it was anticipated that the definition of subsidiary cells, as given in the glossary, would adequately cover these morphologically-distinct and developmentally-related cells in all types of stomatal complexes. Unfortunately this does not appear to be so. Commelina spp. have a very distinct stomatal complex with two pairs of lateral subsidiary cells and a pair of polar subsidiary cells which, judging from the physiological studies carried out on this genus (Penny & Bowling, 1974, etc.), are functionally implicated in the stomatal mechanism. However, Tomlinson (1966) indicates that the outer lateral subsidiary cells are not true subsidiary cells according to the definition in the main glossary. Usually when a subsidiary meristemoid (and a stomatal meristemoid, for that matter) divides to form a subsidiary cell, only one of the products becomes a subsidiary cell whilst the other reverts to the role of an epidermal cell (or guard-cell mother-cell, or stomatal meristemoid). In the case of Commelina, both products of the lateral subsidiary

meristemoids become subsidiary cells. Whether this genus is exceptional in this respect is not known, although the closely related genus Geogenanthus, which is also hexacyclic, has true outer lateral subsidiary cells (Tomlinson, 1966). Besides complicating the formulation of an accurate definition for subsidiary cells, it also raises another important question. If both products of meristemoids are to be referred to as subsidiary cells, many species will then have 'subsidiary cells' which are morphologically indistinguishable from ordinary epidermal cells. More seriously, this interpretation would invalidate present ontogenetic classifications. As Fryns-Claessens and Van Cotthem (1973) point out, in connection with another argument, the larger product of the protodermal cell which gives rise to the stomatal meristemoid would technically become a mesogene subsidiary cell. Such an interpretation would mean that all stomatal complexes would have a mesogene element which, in the case of euperigenous types, would be situated outside the perigene subsidiary cells encircling the stomata and would be indistinguishable from other epidermal cells. There could be no agenous, euperigenous, or hemiperigenous classes.

It is, perhaps, better to accept the definition of subsidiary cells, as given in the glossary, but it is essential to remember that there are exceptions. In the present state of knowledge, it seems advisable to exclude functional criteria from any definition, since it may well be that not all subsidiary cells have a functional significance, whilst some epidermal neighbouring cells may well have in the agenous category, at least.

Fig. 9.1.

Ontogeny of the stomatal complex in Tradescantia pallidus.

A diagrammatic representation of the ontogenetic sequences involved in the formation of the euperigenous stomatal complex. The stomatal meristemoid, guard-cell mother-cell, and guard cells are cross-hatched, whilst the perigene subsidiary cells are single-hatched. Mitotic figures indicate cell division.

a. Protodermal tissue.

b. Protodermal cell assuming meristematic activity and cutting off the stomatal meristemoid.

c. Protodermal cell immediately laterad to the stomatal meristemoid assuming meristematic activity (i.e. becoming a subsidiary meristemoid), and cutting off a lateral subsidiary cell.

d. Protodermal cells immediately distal and basal to the stomatal meristemoid assuming meristematic activity (i.e. becoming subsidiary meristemoid), and cutting off polar subsidiary cells.

e. Guard-cell mother-cell dividing to form guard cells.

f. Immature stomatal complex prior to the formation of the stoma.

g. Mature euperigenous stomatal complex.

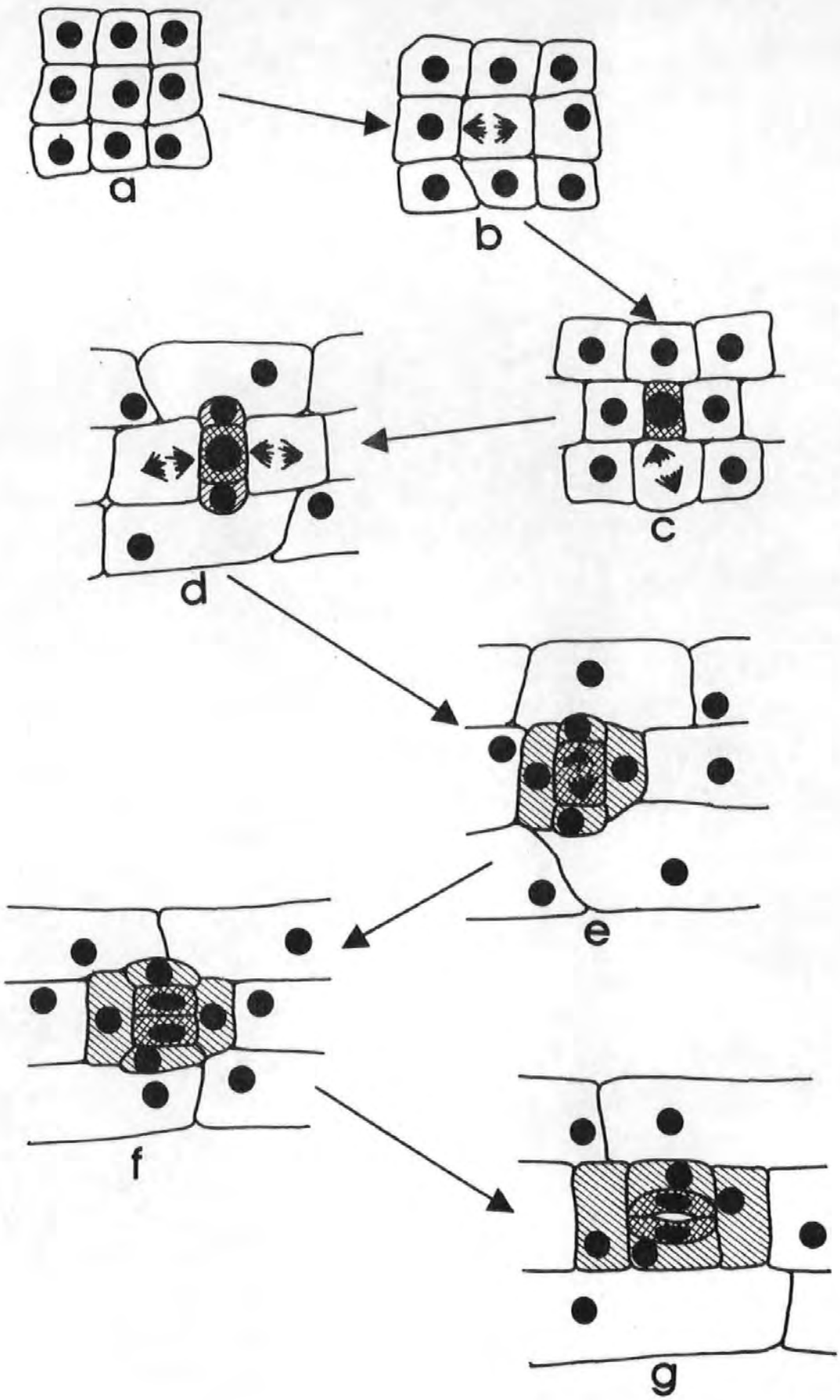

a
b
c
d
e
f
g

Fig. 9.2.

Ontogeny of the stomatal complex in Polypodium vulgare, I.

A diagrammatic representation of the ontogenetic sequences involved in the formation of hemimesogenous stomatal complexes. The stomatal meristemoid, guard-cell mother-cell and guard cells are cross-hatched, whilst the mesogenous subsidiary cells are single-hatched. Mitotic figures indicate cell division.

a. Protodermal tissue

b. Protodermal cell assuming meristematic activity and cutting off the stomatal meristemoid.

c. Stomatal meristemoid dividing to form the mesogene subsidiary cell.

d. Second division of the stomatal meristemoid to form a second mesogene subsidiary cell.

e. Guard-cell mother-cell dividing to form the guard cells in copolocytic complex.

e' Guard cell mother-cell dividing to form the guard cells in eupolocytic complex.

f. Immature copolocytic stomatal complex prior to formation of the stoma.

f' Immature eupolocytic stomatal complex prior to formation of the stoma.

g. Mature copolocytic hemimesogenous stomatal complex.

g' Mature eupolocytic hemimesogenous stomatal complex.

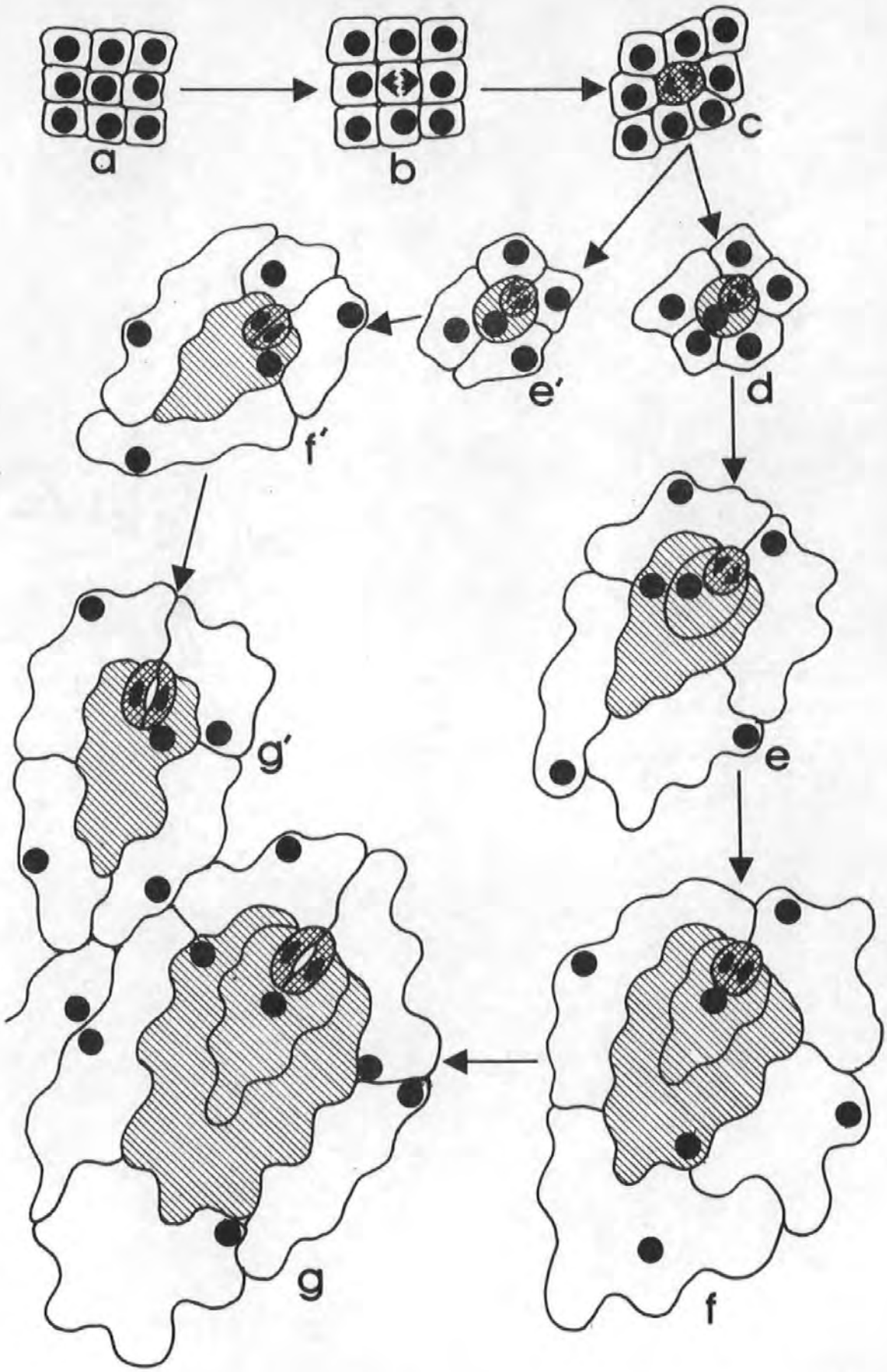

a
b
c
d
e'
f'
e
f
g'
g

Fig. 9.3.

Ontogeny of the stomatal complex in Polypodium vulgare, II.

Diagrammatic representation of the sequences involved in the ontogeny of hemimesoperigenous (left hand side), and eumesoperigenous (right hand side) stomatal complexes. The stomatal and subsidiary meristemoids are stippled, the mesogenous subsidiary cells cross-hatched, and the perigenous subsidiary cells single-hatched. Mitotic figures indicate cell division.

a. Stomatal meristemoid dividing to form a polar mesogenous subsidiary cell.

b. Neighbouring protodermal cell assuming meristematic activity as a subsidiary cell to form a perigene subsidiary cell. Guard-cell mother-cell dividing to form guard cells.

c. Immature hemimesoperigenous (left hand side) and eumesoperigenous (right hand side) complexes prior to formation of the stomata.

d. Mature hemimesoperigenous (left hand side) and eumesoperigenous (right hand side) stomatal complexes.

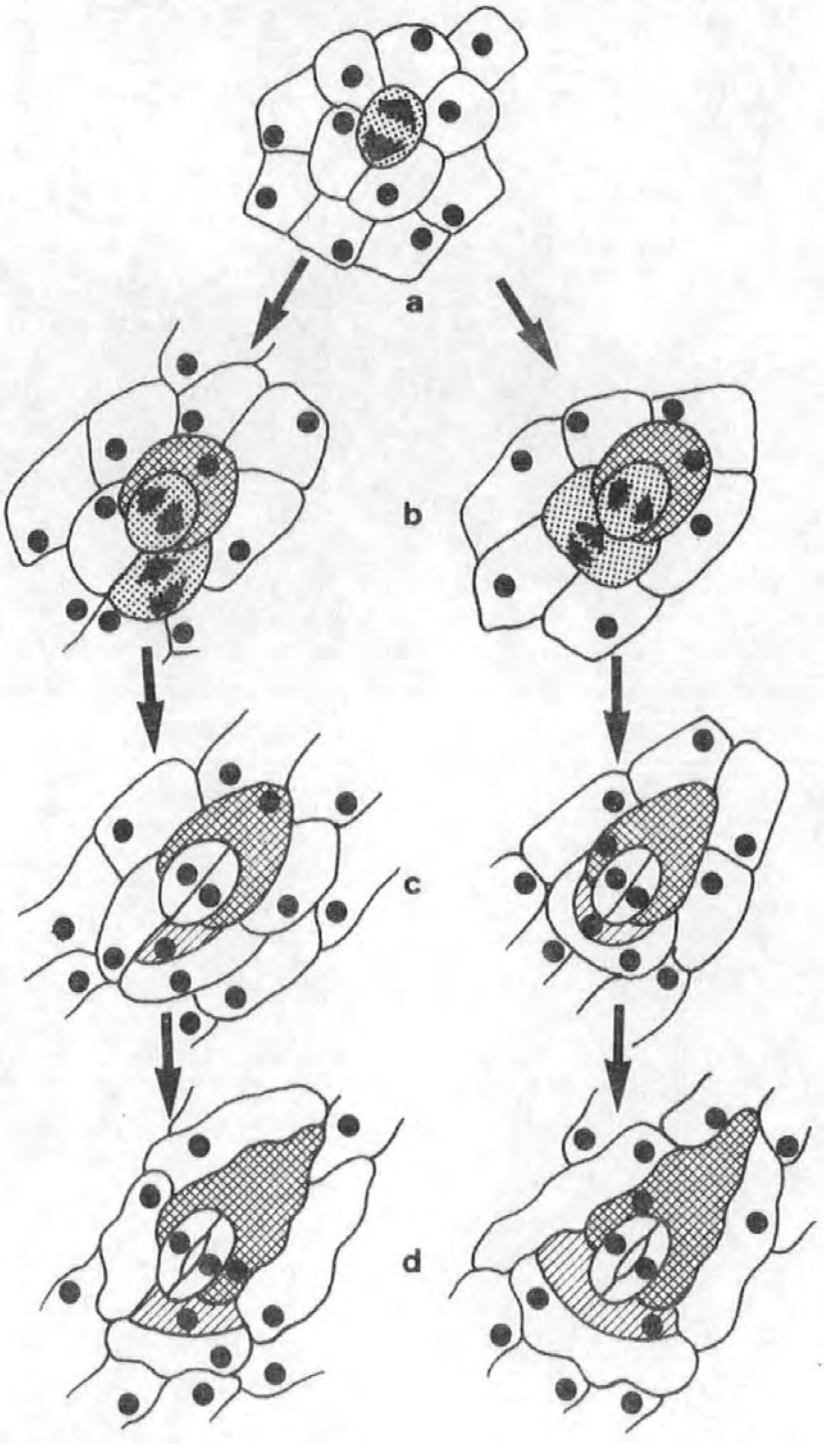

a
b
c
d

Fig. 9.4.

Development of substomatal chambers.

Diagrammatic sequences of substomatal chamber development in Polypodium vulgare (top), Phyllitis scolopendrium (centre), and Tradescantia pallidus (bottom). Guard-cell mother-cell and guard cells are shaded solid, subsidiary cells stippled, protodermal/epidermal cells unshaded, and protomesophyll/mesophyll cells cross-hatched.

1. Protodermal tissue.

2. Mesogenous subsidiary cell development (P. vulgare, and Ph. scolopendrium only). Protomesophyll cell below stomatal complex of Ph. scolopendrium starting to atrophy. P. vulgare protomesophyll becoming extended and aerenchymatous as the epidermis expands.

3. Perigenous subsidiary cell development (T. pallidus only) indicating how the underlying mesophyll is separated below the stomatal complex as the subsidiary cells become interposed into the epidermal tissue. Abortion of mesophyll cell attached to the underside of the stomatal complex in Ph. scolopendrium almost complete.

4. Mature tissues showing the resultant substomatal chambers.

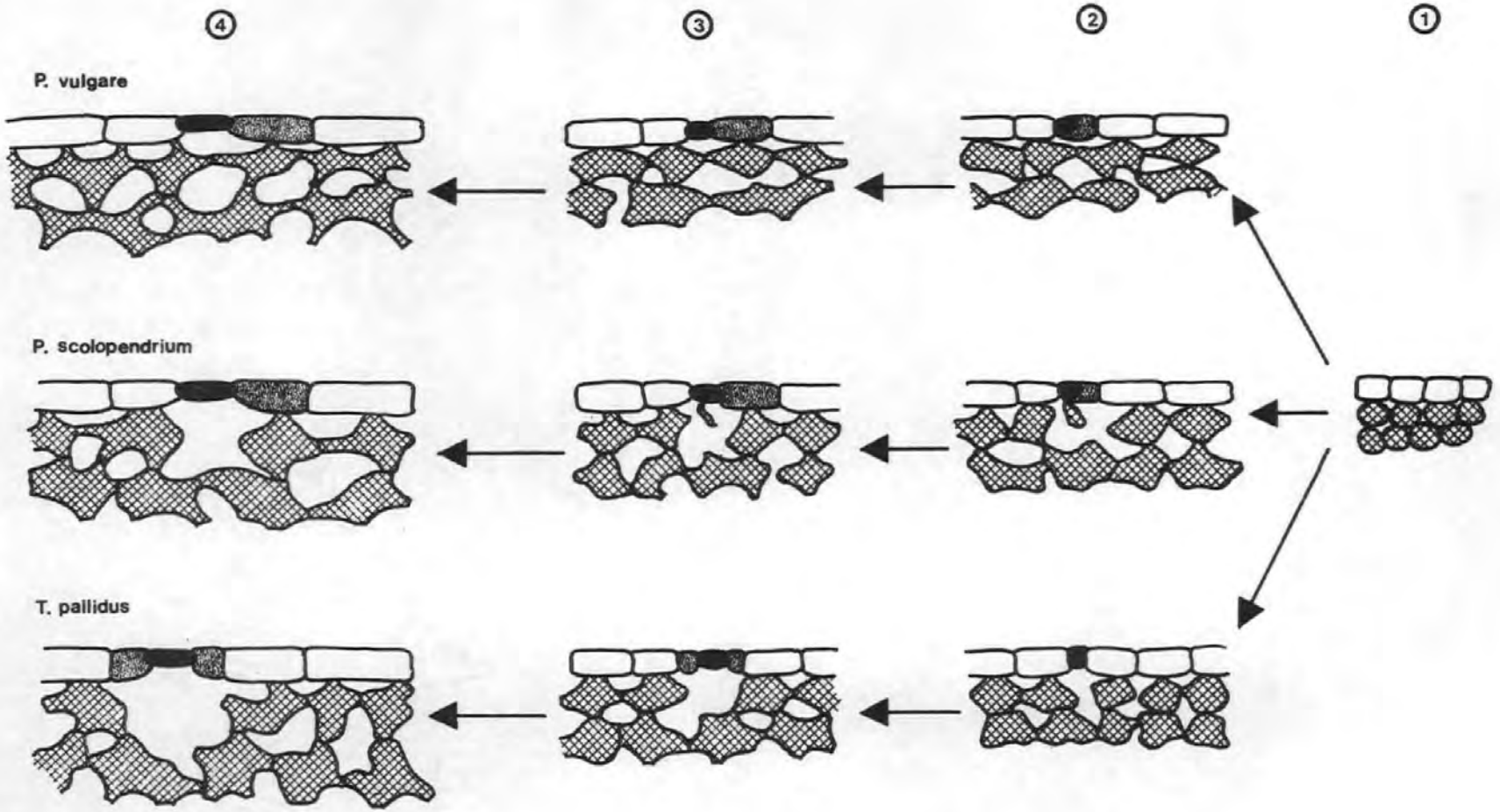
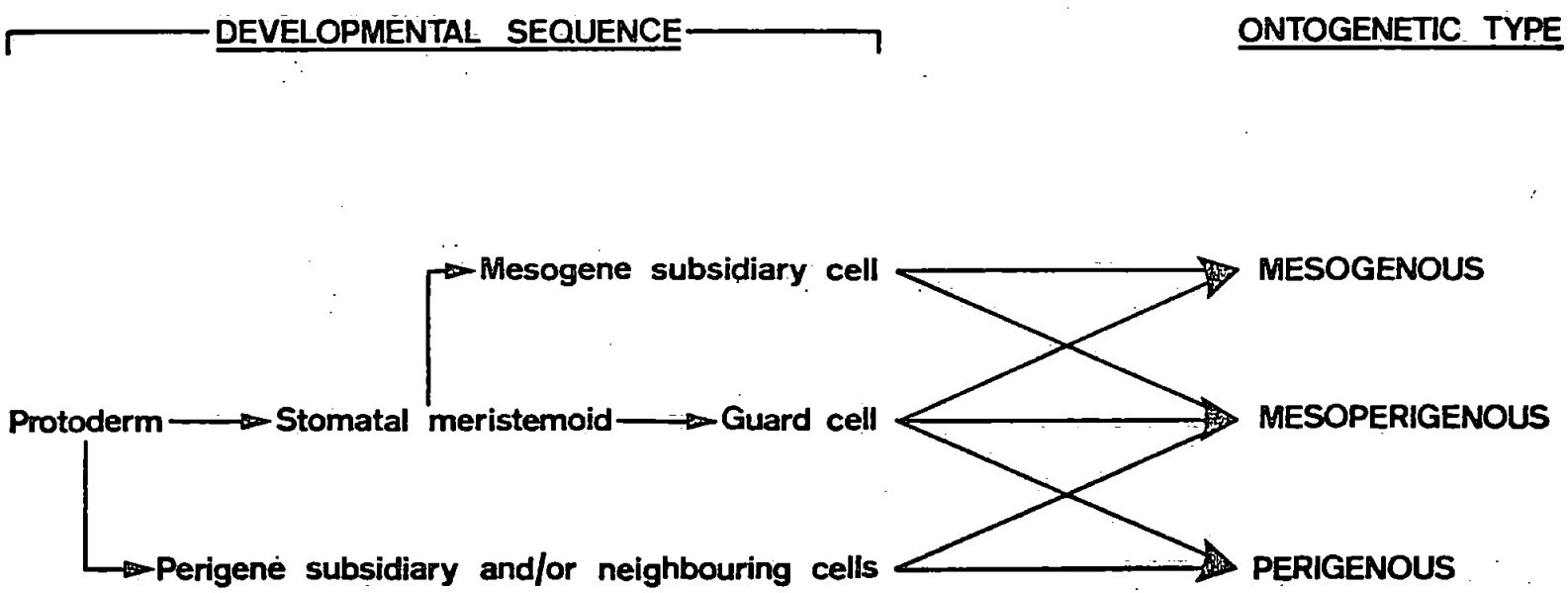

④
③
②
①
P. vulgare
P. scolopendrium
T. pallidus

Fig. 9.5.

Ontogenetic derivation of cells of stomatal complex, I.

Derivation of the basic ontogenetic types of stomatal complex, according to Pant (1965).

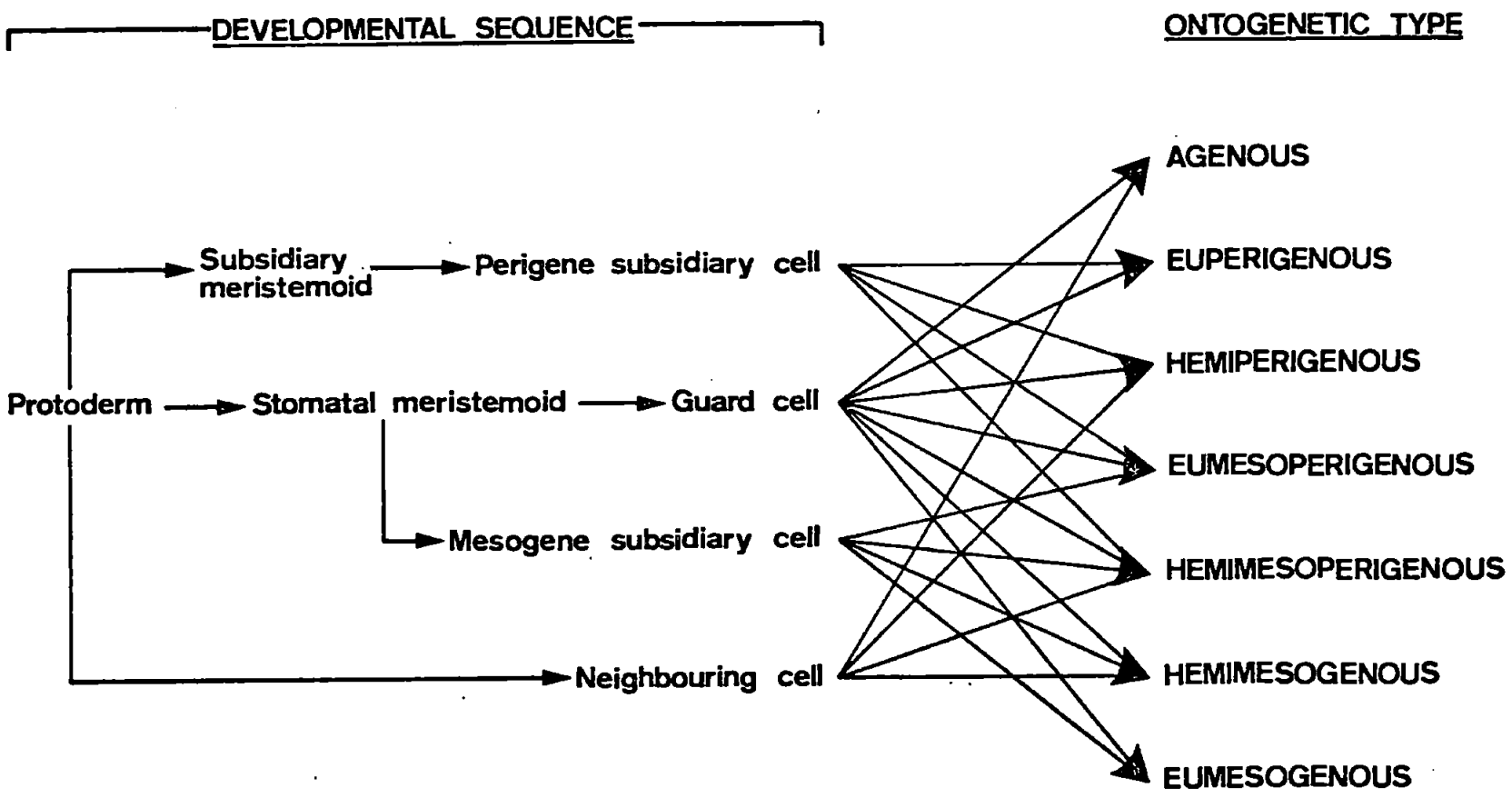


ONTOGENETIC PATHWAYS OF PANT (1965)

Fig. 9.6.

Ontogenetic derivation of cells of stomatal complex, II.

Derivation of the basic ontogenetic types of stomatal complex, according to the proposed new classification.



PROPOSED ONTOGENETIC PATHWAYS

Fig. 9.7.

Ontogenetic derivation of cells of stomatal complex, III.

Derivation of the basic ontogenetic types of stomatal complex, according to the proposed new classification. Neighbouring cells are stippled, mesogenous subsidiary cells cross-hatched, and perigenous subsidiary cells single-hatched. The arrows associated with the individual subsidiary cells indicate their source and direction of derivation.

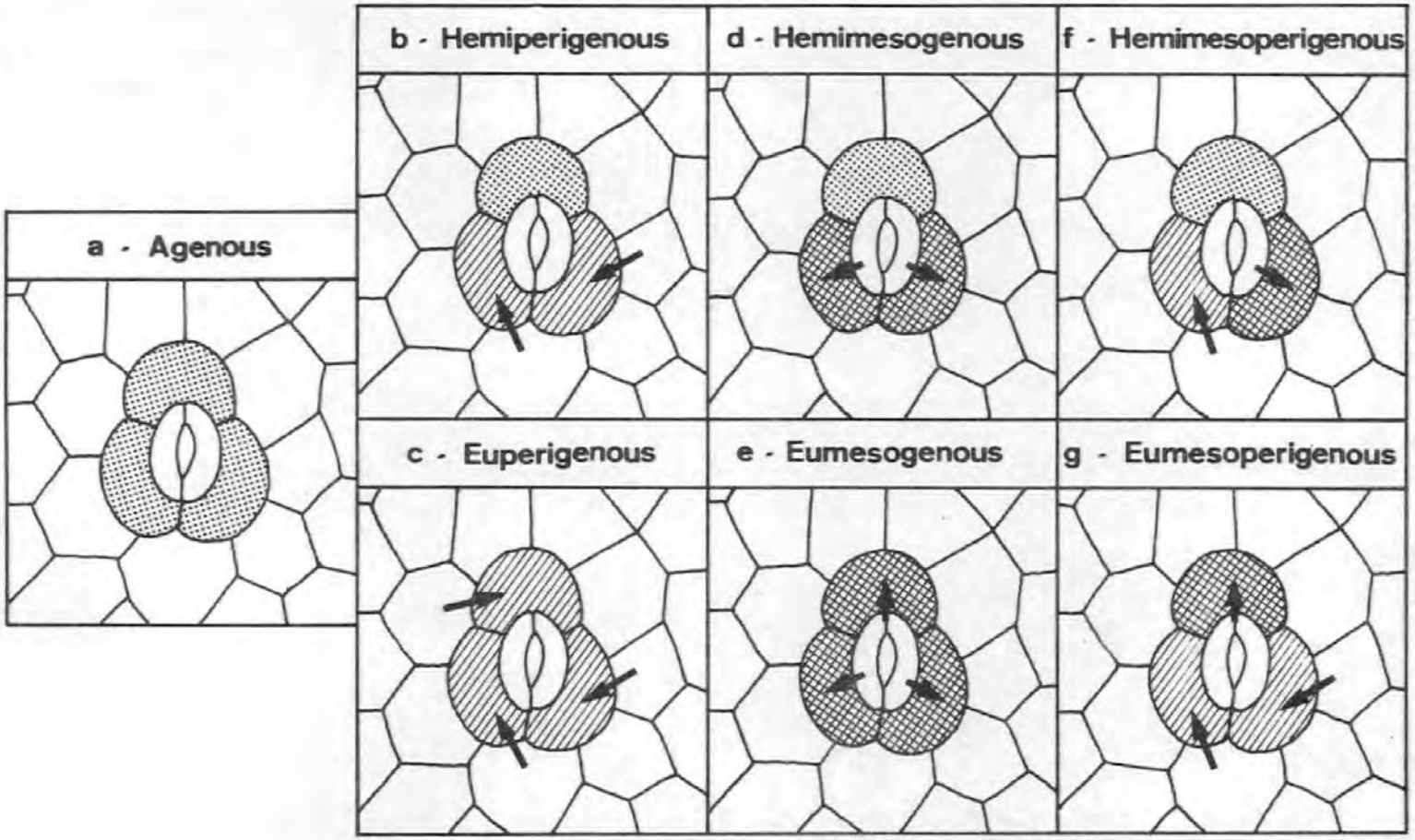

a - Agenous
b - Hemiperigenous
c - Euperigenous
d - Hemimesogenous
e - Eumesogenous
f - Hemimesoperigenous
g - Eumesoperigenous

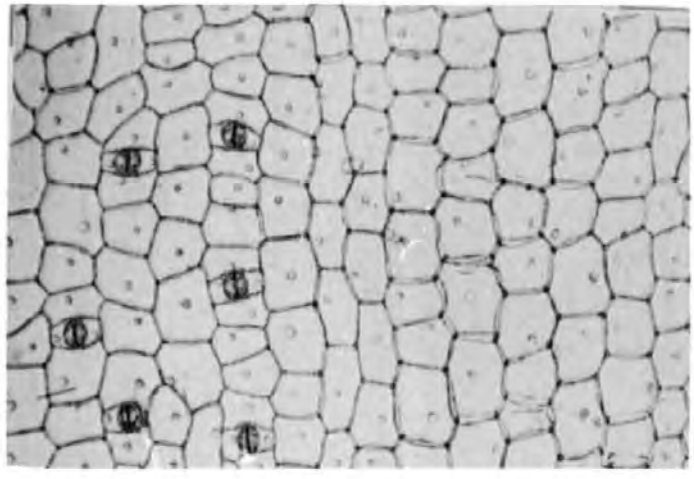
Plate 9.1.

Epidermes of Tradescantia pallidus.

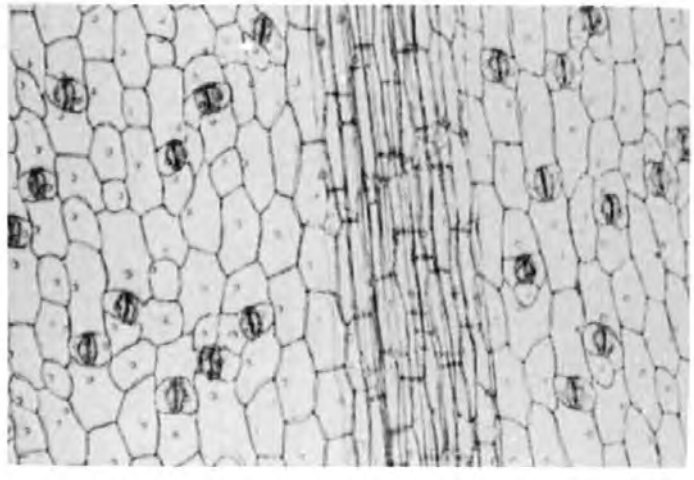
Light micrograph of unstained tissue, x 70.

A. Adaxial epidermis. Note how the stomata are restricted to the suprafascicular tissue.

B. Abaxial epidermis. Note how the stomata are restricted to the interfascicular region.



A



B

Plate 9.2.

Morphological forms of the stomatal complex in Tradescantia pallidus, I.

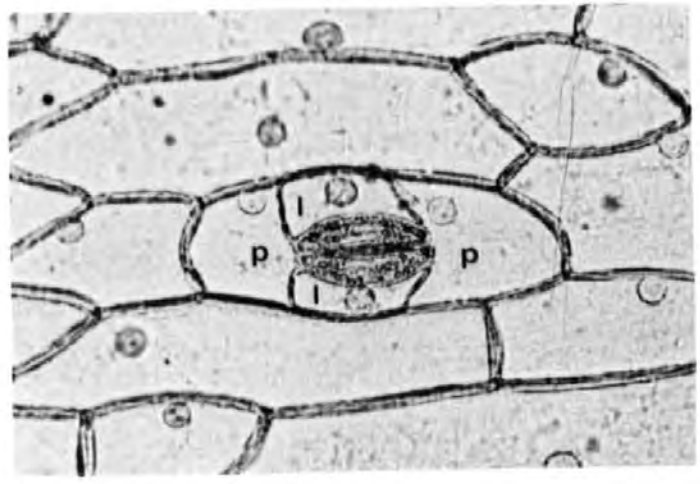
Light micrographs of unstained tissue, x 350.

p = polar subsidiary cell, l = lateral subsidiary cell, and

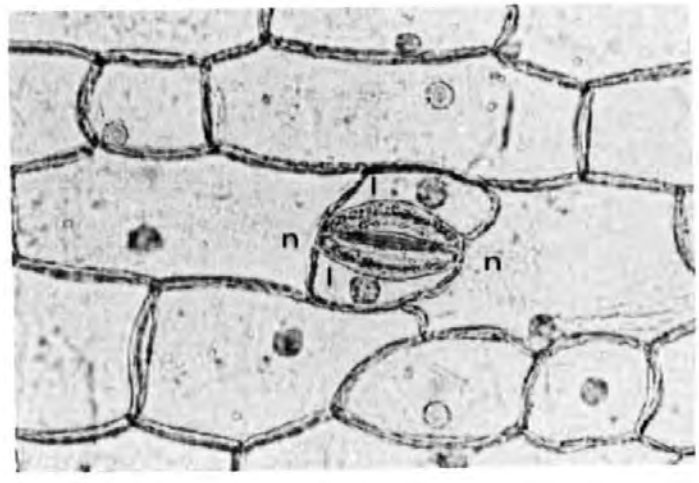
n = neighbouring cell.

A. Normal tetracytic form.

B. Normal dicytic form resulting from the failure of the polar subsidiary cells to develop.



A



B

Plate 9.3.

Morphological forms of the stomatal complex in Tradescantia pallidus, II.

Light micrographs of unstained tissue, x 350

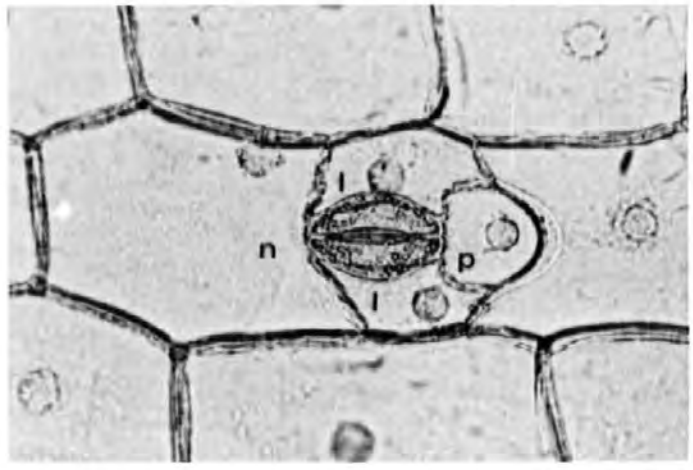
p = polar subsidiary cell, l = lateral subsidiary cell, and

n = neighbouring cell.

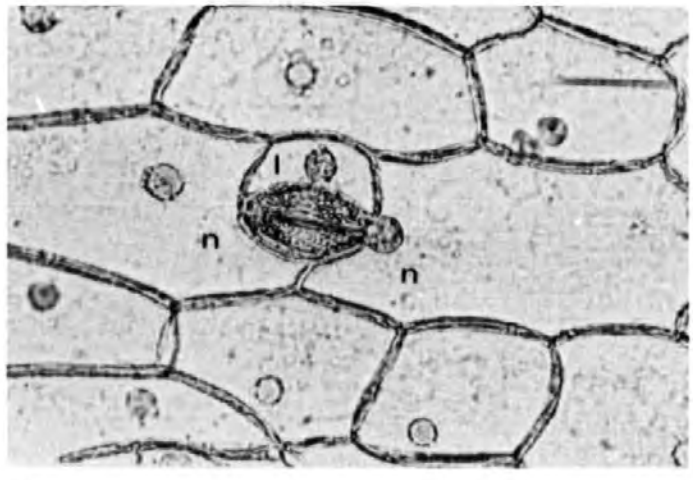
A. Aberrant tricytic form in which one of the polar subsidiary cells has failed to develop.

B. Aberrant monocytic form in which only one subsidiary cell (lateral) has developed. The nucleus of the right hand side neighbouring cell is particularly interesting in this example since it appears to be associated with the polar regions of the guard cells in some way. During this investigation, it was not uncommon to find nuclei of protodermal cells, in Tradescantia spp., being shared by adjacent cells, presumably via some fistula in the cell wall.

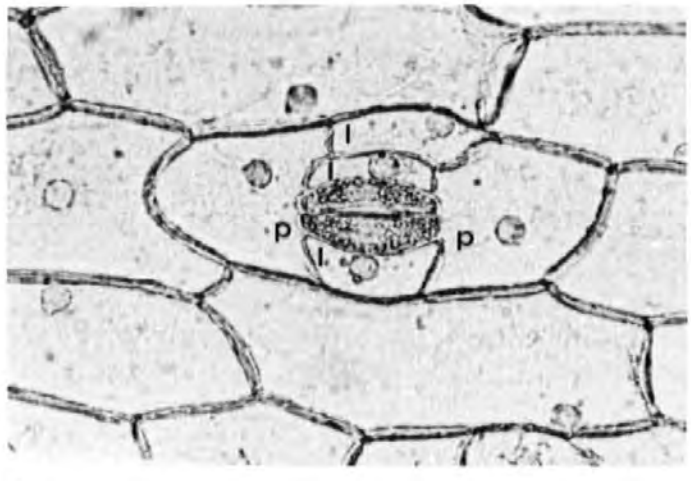
C. Aberrant stomatal complex with supernumerary subsidiary cells; a pair of lateral subsidiary cells are present on one side of the complex.



A



B



C

Plate 9.4.

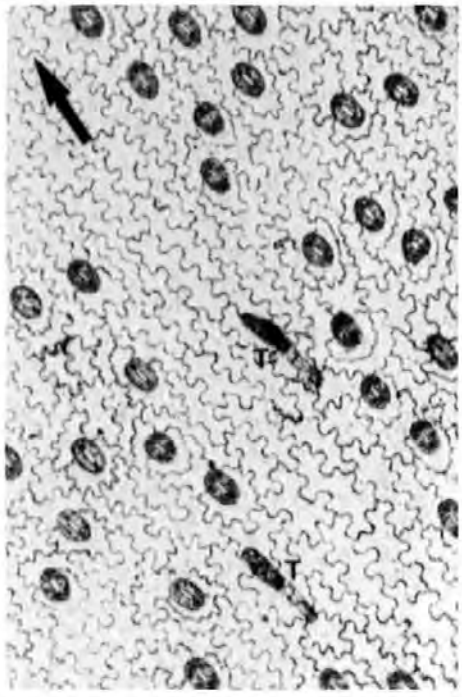
Morphology of the epidermis and stomatal complex in Polypodium vulgare.

A. Light micrograph of unstained abaxial epidermis, x 75. The arrow indicates the orientation of the tissue in respect of the leaf margin. The stomata are randomly distributed throughout the epidermis but not on the suprafascicular tissue. Bicelled trichomes (T) are scattered over the abaxial epidermis.

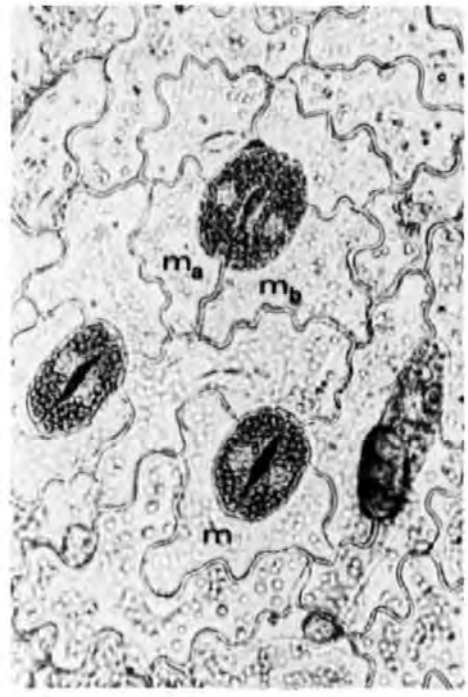
B. Light micrograph of unstained eupolocytic stomatal complex, x 250. The lower stomatal complex is typical with a single horseshoe-shaped mesogene subsidiary cell (m). The upper stomatal complex is of the same morphological type except that the mesogene subsidiary cell has divided into two (m_a and m_b) after it had been cut off from the stomatal meristemoid.

C. Light micrograph of unstained copolocytic stomatal complex, x 350. The complex illustrated has two mesogene horseshoe-shaped subsidiary cells. The outer one (m_1) was cut off from the stomatal meristemoid before the inner one (m_2).

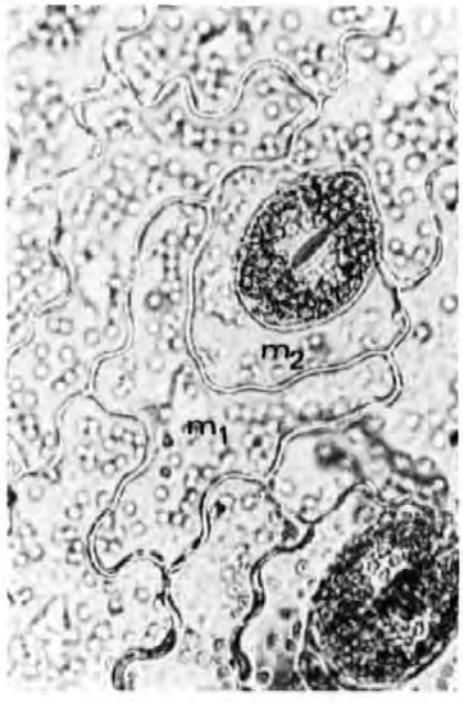
D. Light micrograph of unstained polypolocytic stomatal complex, x 325. A previously unrecorded type which has three horseshoe-shaped mesogene subsidiary cells. The outermost one (m_1) was cut off from the stomatal meristemoid first, followed by the middle one (m_2), whilst the inner one (m_3) was cut off last.



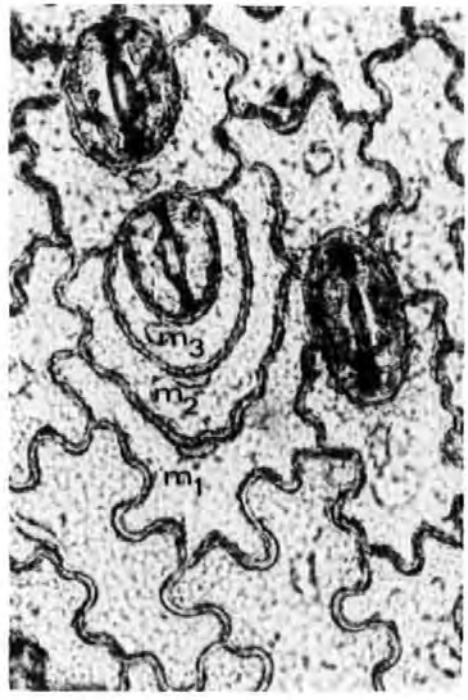
A



B



C



D

Plate 9.5.

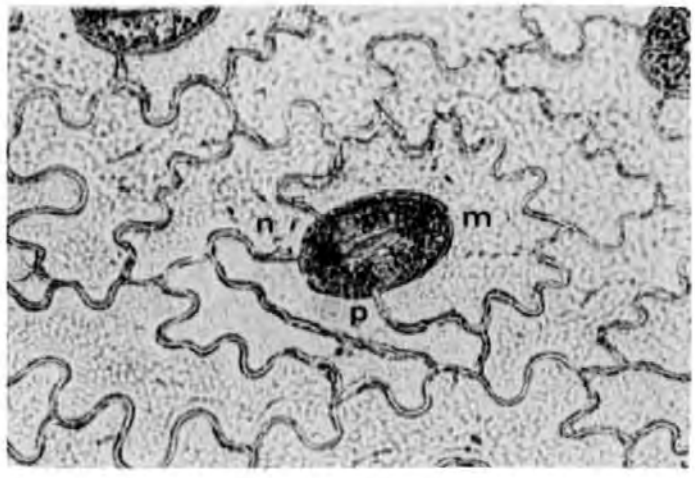
New stomatal types found in Polypodium vulgare.

Light micrographs of unstained aniso-polocytic stomatal complexes, x 325.

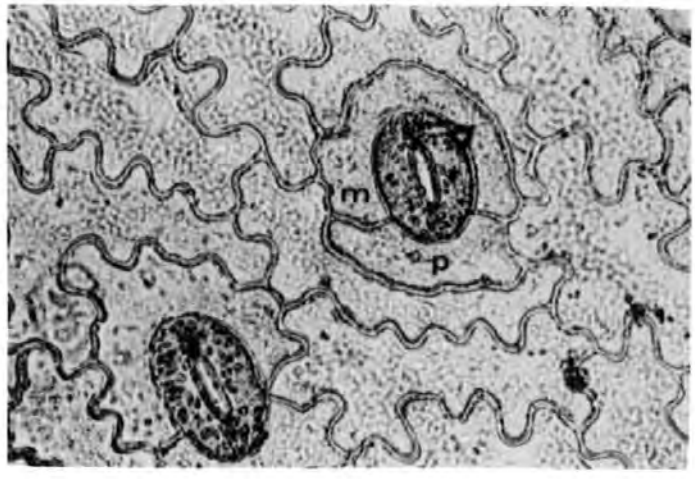
m = mesogene subsidiary cell, p = perigene subsidiary cell, and n = neighbouring cell.

A. A hemimesoperigenous type in which the guard cell complex is in contact with a mesogene subsidiary cell, a perigene subsidiary cell and a neighbouring cell.

B. An eumesoperigenous type in which the guard cell is surrounded by a mesogene and a perigene subsidiary cell.



A



B

Plate 9.6.

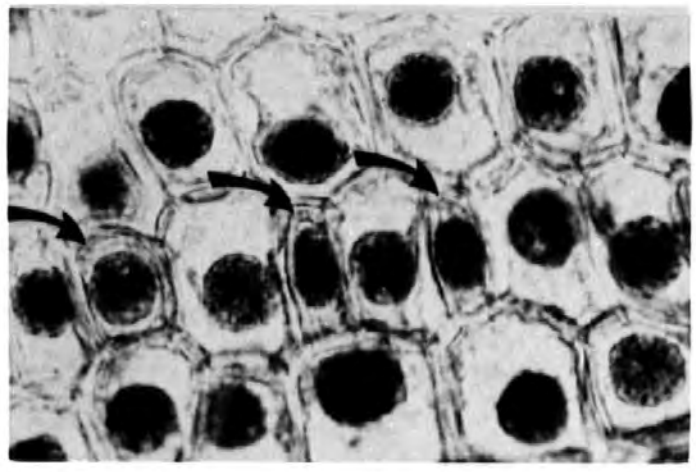
Stomatal ontogeny in Tradescantia pallidus, I.

Light micrographs of acetic orcein-stained epidermes, x 650.

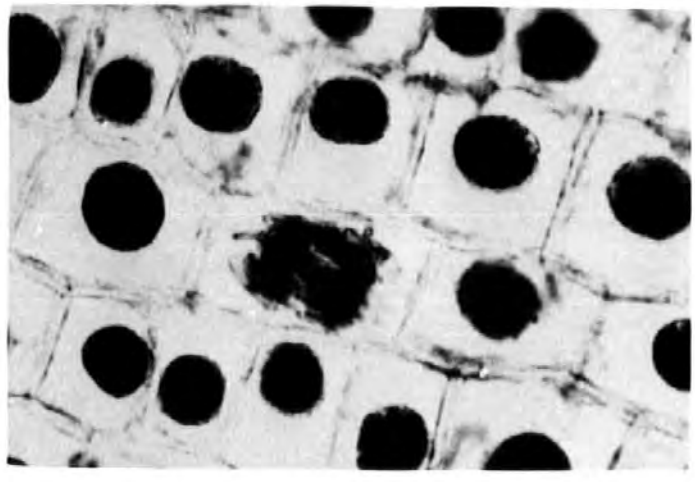
A. Protodermal tissue with newly-formed stomatal meristemoids (arrowed).

B. Protodermal tissue showing a protodermal cell in anaphase during stomatal meristemoid formation. Note the migration of the nucleus in the protodermal cell immedately laterad to the mitotic figure as it becomes meristematically active prior to dividing to form a lateral subsidiary cell.

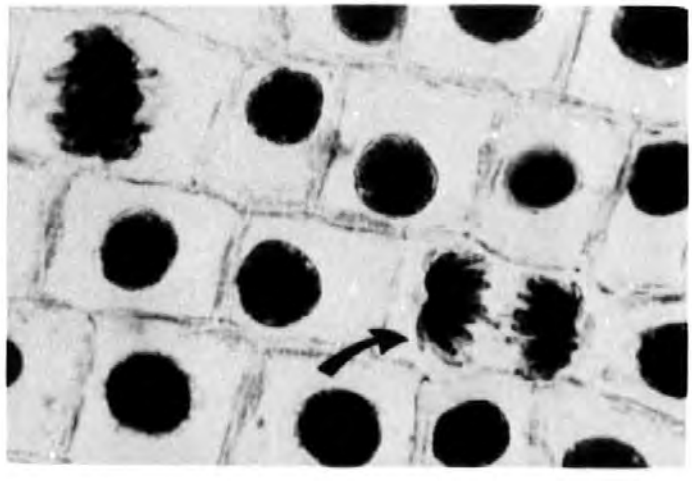
C. Protodermal tissue illustrating the asymmetrical nature of the cell division which gives rise to the stomatal meristemoid. The stomatal meristemoid develops from the smaller, distal product (arrowed element of the late anaphase mitotic figure)



A



B



C

Plate 9.7.

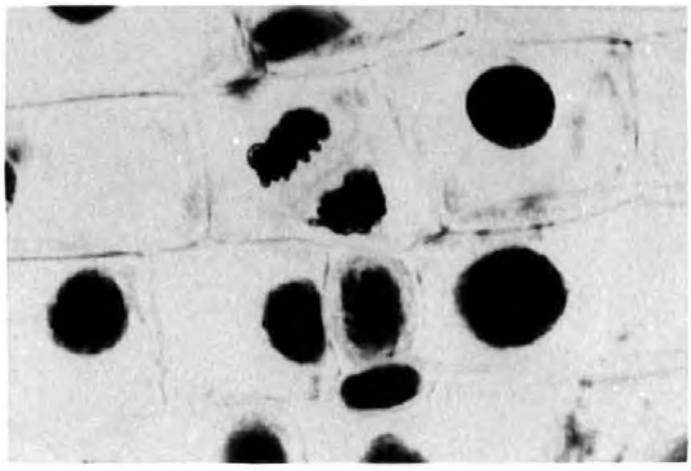
Stomatal ontogeny in Tradescantia pallidus, II.

Light micrographs of acetic orcein-stained epidermes, x 650.

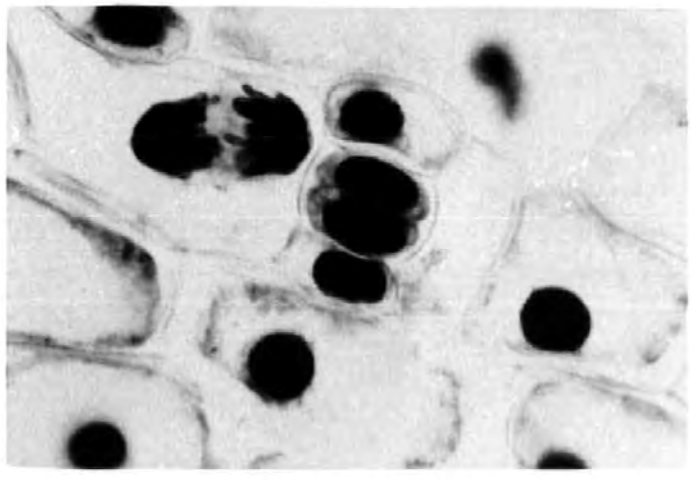
A. Lateral subsidiary cell formation. The lower lateral subsidiary cell is already formed and the new cell wall nearing completion. The upper lateral subsidiary cell is in the process of formation as the subsidiary meristemoid divides. The mitotic figure is in telophase and the equatorial plate is clearly visible.

B. Polar subsidiary cell formation. The right hand polar subsidiary cell is already formed, although its nucleus has been lost. The other polar subsidiary cell is in the process of formation and is at a late anaphase stage.

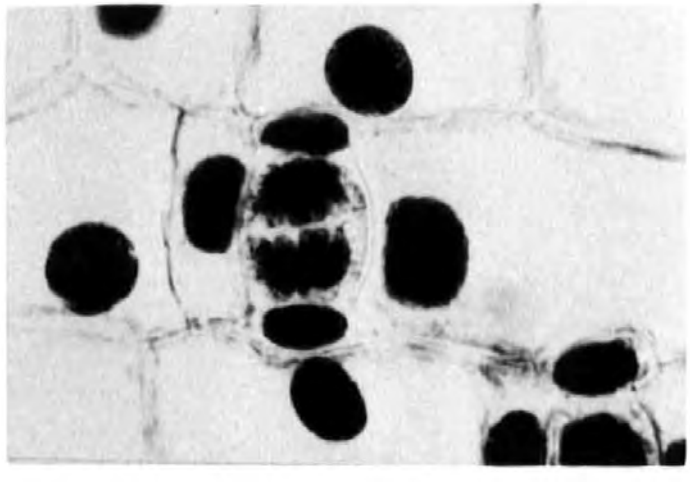
C. Guard cell formation. The young complex has three subsidiary cell developed already and the guard-cell mother-cell is in the process of dividing to form the pair of guard cells.



A



B



C

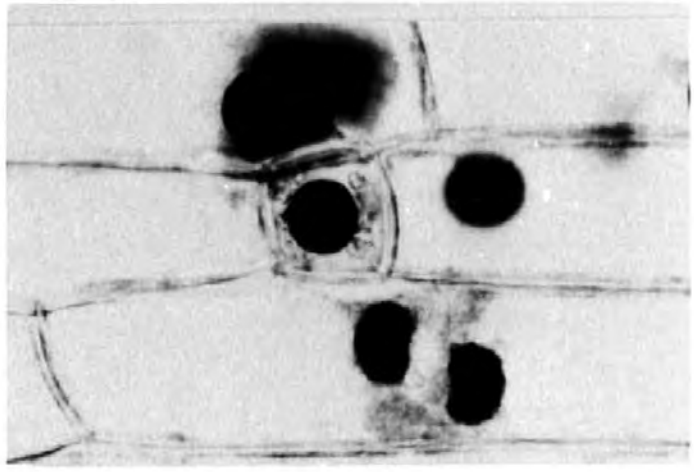
Plate 9.8.

Stomatal ontogeny in Tradescantia pallidus, III.

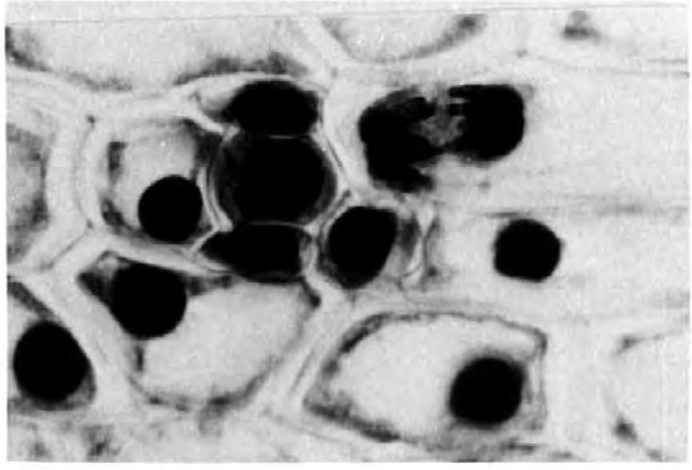
Light micrographs of acetic orcein-stained epidermes, x 650.

A. Delayed stomatal development in submature epidermis. The lateral subsidiary cells are in the process of being formed.

B. Aberrant stomatal development. The complex is developing supernumerary subsidiary cells. There are two polar subsidiary cells on the right hand side; one being in late anaphase.



A



B

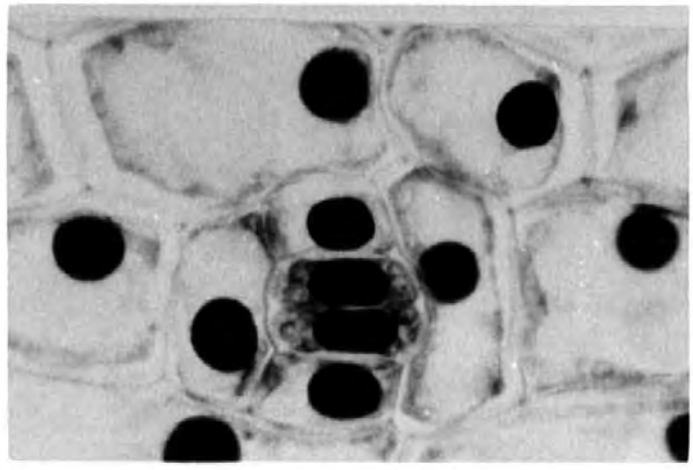
Plate 9.9.

Stomatal ontogeny in Tradescantia pallidus, IV.

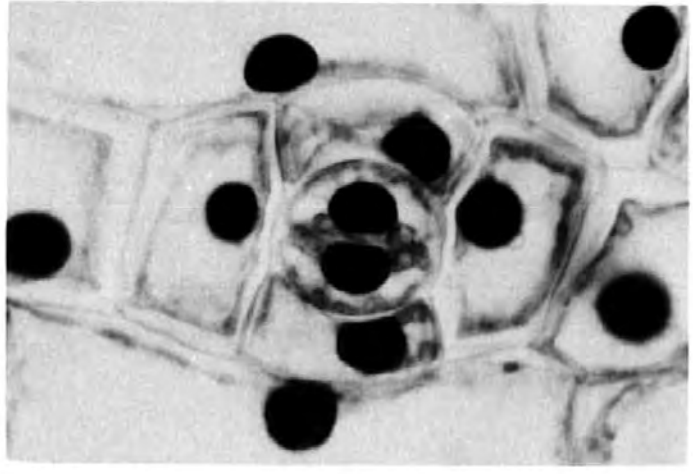
Light micrographs of acetic orcein-stained epidermes, x 650.

A. Stomatal complex just after the division of the guard-cell mother-cell to produce the guard cells.

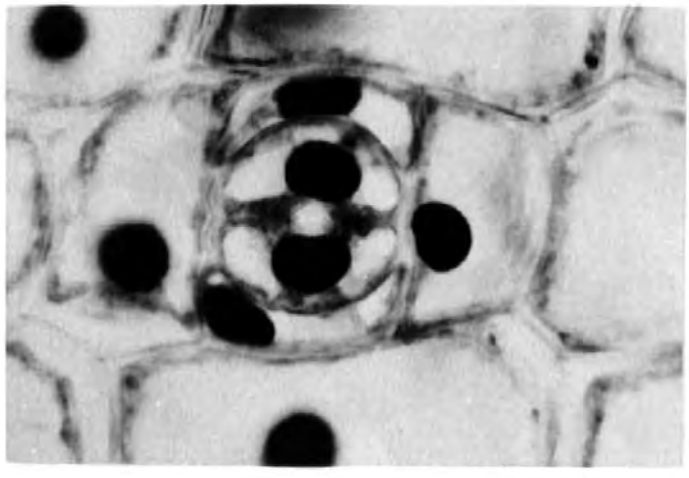
B, & C. Stages in the maturation of the guard cell complex. In B, the guard cell volume is increasing as vacuolation starts to take place and the guard cells begin to bow out into the lateral subsidiary cells. In C, vacuolation is well advanced and stoma formation has been initiated. The lateral subsidiary cells can be seen to be distorted by the enlarging guard cell complex.



A



B



C

Plate 9.10.

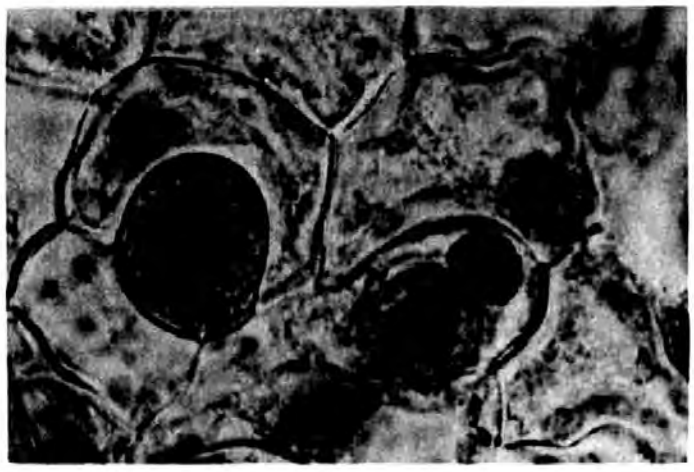
Stomatal ontogeny in Polypodium vulgare, I.

Light micrographs of acetic orcein-stained epidermes, x 1 150 (oil immersion).

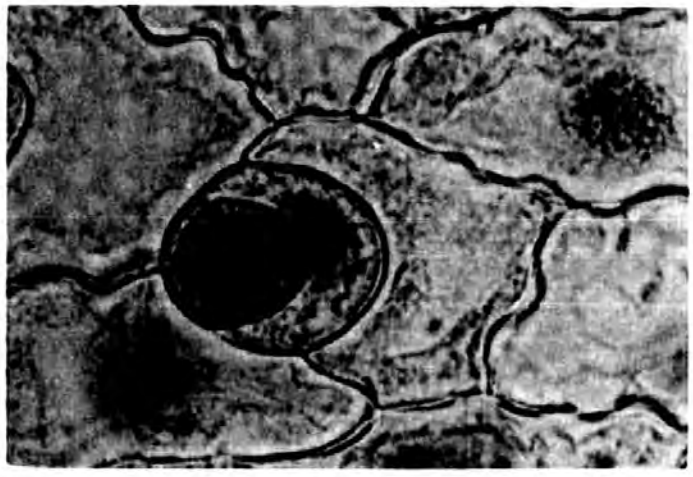
A. Young developing complexes after the stomatal meristemoid has cut off a single mesogene subsidiary cell.

B. Young developing complex after the stomatal meristemoid has cut off two mesogene subsidiary cells.

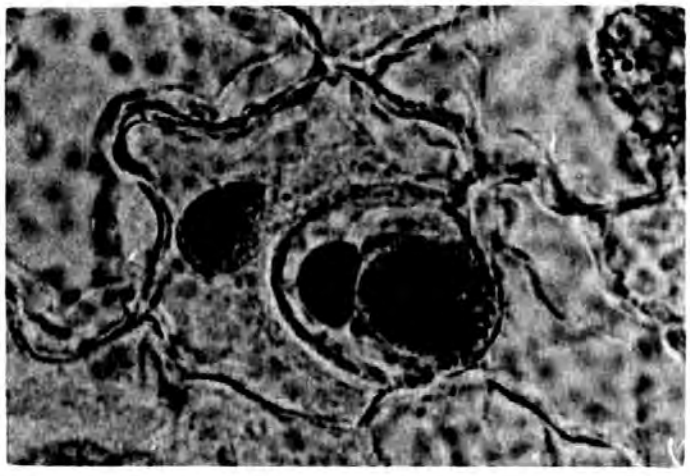
C. Young eupolocytic stomatal complex with the guard-cell mother-cell dividing to form the guard cells.



A



B



C

Plate 9.11.

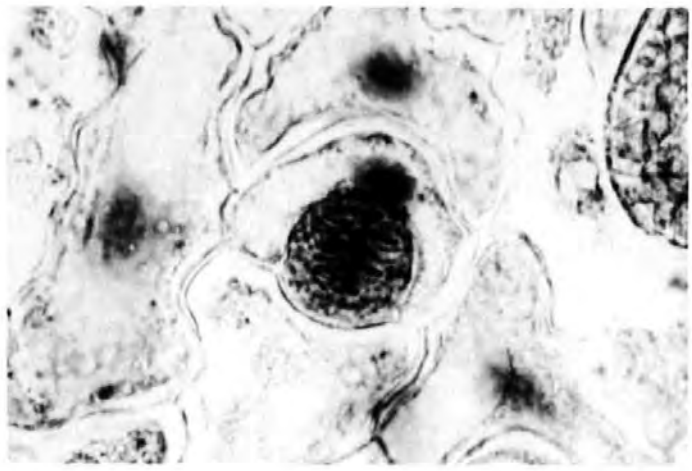
Stomatal ontogeny in Polypodium vulgare, II.

Light micrographs of guard cell development in acetic orcein-stained epidermes, x 1 000. (oil immersion)

A. Guard-cell mother-cell in metaphase.

B. Guard-cell mother-cell in telophase with the equatorial plate clearly visible.

C. Young guard cell complex with the common anticlinal walls just beginning to form.



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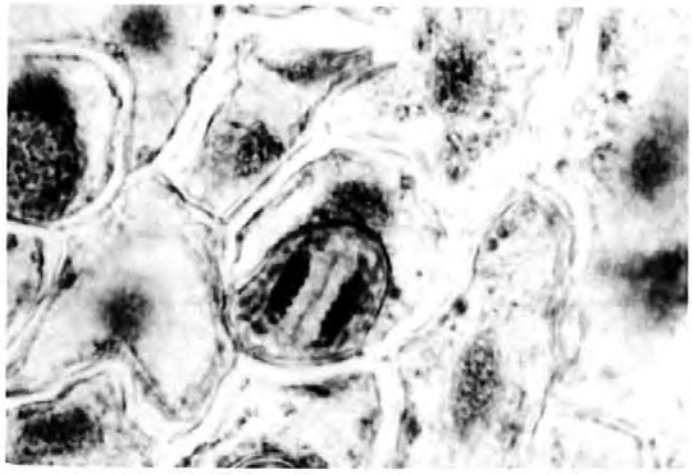

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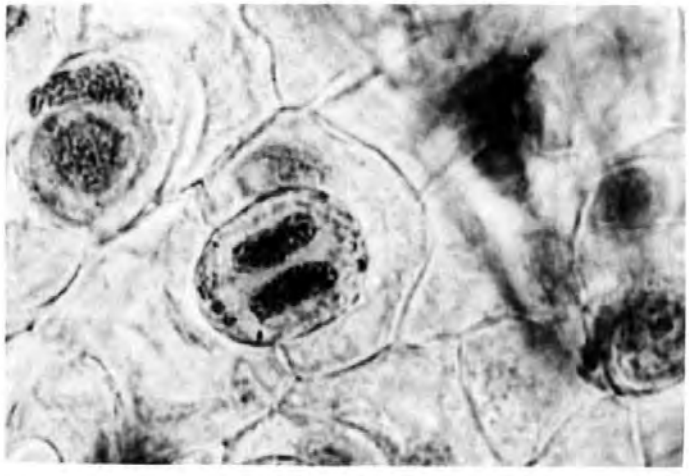


C

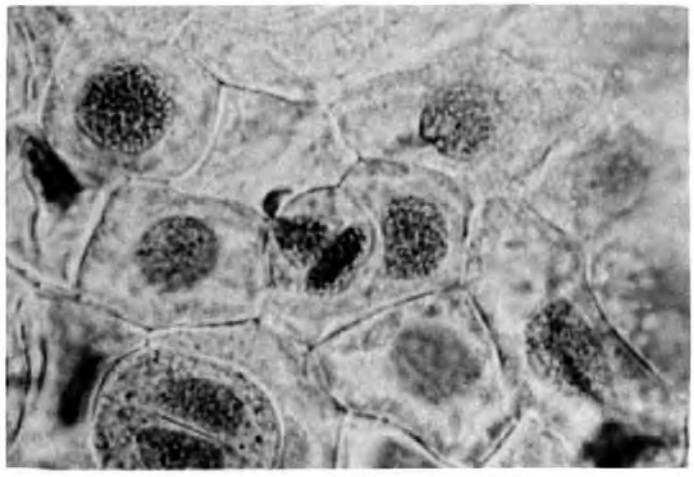
Plate 9.12.

Stomatal ontogeny in Polypodium vulgare, III.

Subsidiary cell formation. Light micrographs of acetic orcein-stained epidermes, x 900. (oil immersion).

A. Stomatal meristemoid dividing to form a mesogene subsidiary cell.

B. Subsidiary meristemoid dividing to form a perigene subsidiary cell.



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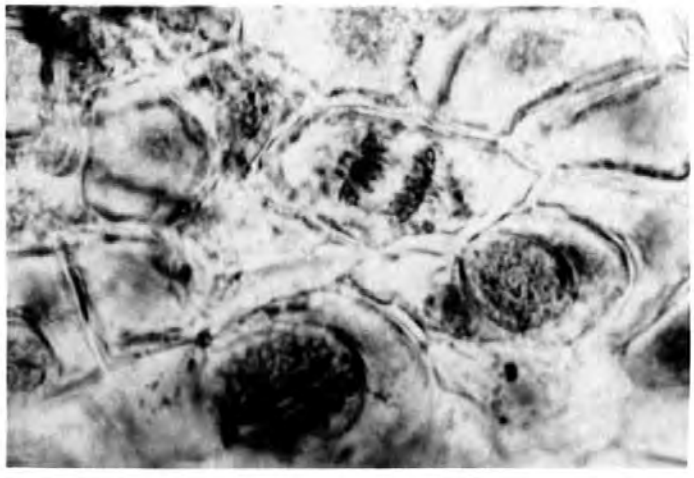


B

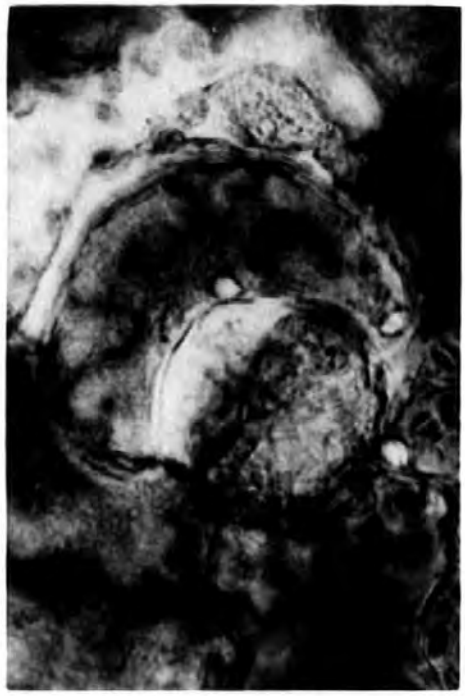
Plate 9.13.

Substomatal chamber formation in Phyllitis scolopendrium.

Light micrographs of unstained epidermes, x 1 000. The left hand micrographs are focussed on the face of the developing guard cell complex, whilst those on the right hand side are focussed on the body of the atrophying protomesophyll cell.

A. Very young stomatal complex before the guard-cell mother-cell has divided showing the aborting protomesophyll cell associated with the inner face of the stomatal complex. The rugosities of the protomesophyll cell are largely caused by plastid inclusions.

B. A more mature complex with its associated atrophying protomesophyll cell.



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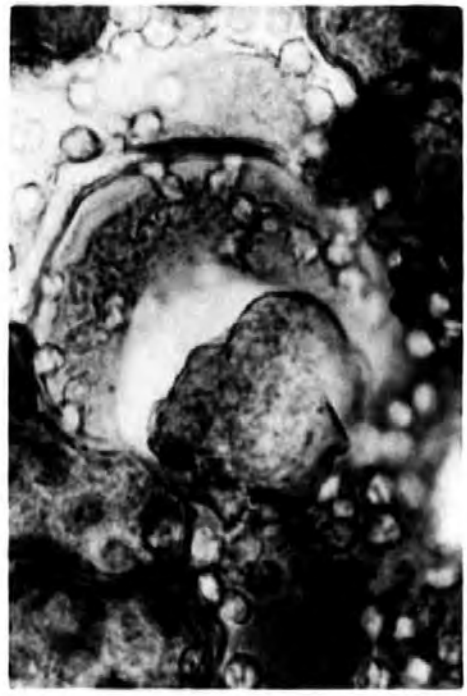

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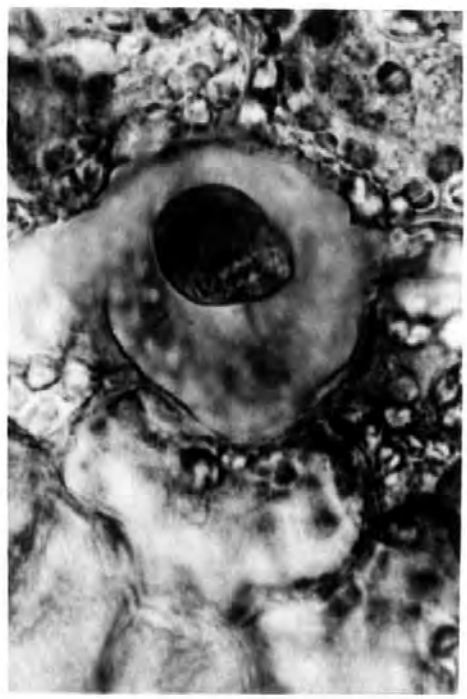


D

CHAPTER 10

THE MORPHOGENESIS OF SUBSTOMATAL STRUCTURES

INTRODUCTION

Immature epidermes of Polypodium vulgare and Tradescantia spp. were stained with 7,5% (w/v) cobaltinitrite solution, as detailed in Chapter 2, in order to determine the morphogenetic sequences which lead to the mature substomatal structures. The histochemical findings were corroborated by by scanning electron microscopy carried out on fresh tissue of P. vulgare.

OBSERVATIONS IN Polypodium vulgare

Modified Macallum's technique

Shortly after the stomatal meristemoid cuts off its (last) subsidiary cell, a heavy cobalt precipitate is usually observed at that pole of the guard-cell mother-cell adjacent to the (inner) mesogenous subsidiary cell (Plate 10.1.1A). Evidence of the early formation of endocuticular trabeculae is already apparent at this early ontogenetic stage in many developing complexes (Plate 10.1.1A). Immediately following this polarisation of the cobalt precipitation, a transverse cleavage develops across the guard-cell mother-cell

(Plate 10.1.2A) which is at right angles to the eventual plane of division of the guard-cell mother-cell which occurs during guard cell formation. At this stage a superficial disc-like structure is often visible at the pole of the guard-cell mother-cell adjacent to the mesogene subsidiary cell (Plate 10.1.2A). This stage is followed by one in which the cobalt precipitations become polarised at both ends of the guard-cell mother-cell and the transverse cleavage commences to pull apart (Plate 10.1.3A). The tear occurs in a very superficial position on the inner face of the guard-cell mother-cell. The torn edges of the cleavage start to draw back towards their respective poles (Plate 10.2.1A) before the first indications of the guard-cell mother-cell dividing to form the guard cell complex occur. By the time this cell division takes place, the remnants of the cleavage are usually established in the polar positions and seem to delimit the extent of the cobalt precipitation (Plates 10.2.2A, and 10.2.3A). By the time the stoma appears, there is little evidence of this cleavage remaining, and its place is taken taken by the polar cobalt precipitations which are similar to, but not as dense as, those found in mature complexes (Plate 10.3.1A).

Whilst the above account describes the normal sequence of events, young complexes have been observed in which the guard-cell mother-cell commences division before the two halves of the cleavage pull apart towards their respective poles (Plate 10.3.2A).

Scanning electron microscopy

Immediately after the (last) subsidiary cell has been cut off from the stomatal meristemoid, a disc-like structure of ca. 6 μm diameter, can be observed at that pole of the guard-cell mother-cell

adjacent to the (inner) mesogene subsidiary cell (Plate 10.1.1B). This structure, whilst superficial in nature, clearly lies beneath what is believed to be the endocuticle. The transverse cleavage observed with the histochemical stain (Plate 10.1.2A) is clearly visible in Plate 10.1.2B as a groove across the inner face of the guard-cell mother-cell between the lateral extremities of the (inner) mesogene subsidiary cell. In this micrograph, a structure is apparent at the mesogene subsidiary cell pole of the gurad-cell mother-cell below the endocuticle. The tearing apart of the transverse cleavage of the guard-cell mother-cell appears to involve the polarisation of superficial elements of the guard-cell mother-cell wall immediately below the endocuticle (Plate 10.1.3B). This polarisation of the cell wall elements gives rise to very distinct polar swellings in the guard-cell mother-cell (Plate 10.2.1B) which, on the division of the underlying guard-cell mother-cell , are transiently connected by a longitudinal ridge (Plate 10.2.2B). This ridge is undoubtably related to the formation of the thickened lips which protect the throat of the stoma in the mature guard cell complex. It presumably involves the integration of the endocuticle in this region into the thickened cell wall ridges of the developing guard cells (Plate 10.2.3B). Eventually the stoma appears and the endocuticle covering the ridges is torn apart (Plate 10.3.1B). In such submature guard cell complexes, the endocuticular substomatal sacs are not as well developed as those of mature tissues. What is believed to be a precociously developed guard-cell mother-cell is illustrated in Plate 10.3.2B, in which the mother-cell has commenced cell division before the polarisation of the superficial cell wall elements has been completed.

OBSERVATIONS IN Tradescantia spp.

The morphogenesis of substomatal structures has not been as intensively studied in this genus as in Polypodium vulgare. No preferential cobalt precipitation, associated with the stomatal complex, occurs in this genus until after the guard-cell mother-cell divides to form the guard cell complex. At this stage, cobalt is precipitated around the entire periphery of the young guard cell complex so as to completely occlude the lateral subsidiary cells (Plate 10.4A). As the complex matures, the cobalt precipitation over the lateral subsidiary cells migrates towards the poles (Plate 10.4B) until it eventually approaches the form of polar localisation found in the mature complex (Plate 10.4C). Interestingly, whilst the guard cells are still submature, and have not attained their final size, the polar precipitations of cobalt become very dense and subcircular in shape which, relatively speaking, are much more extensive than those found in mature complexes (Plate 10.4D).

OBSERVATIONS IN Lygodium scandens

This fern (Schizaceae) shows a morphogenetic sequence which is intermediate between that found in Polypodium and that of Tradescantia. In this species, the guard-cell mother-cell is completely occluded by a dense precipitate of cobalt (Plate 10.5A, right hand side). As the guard-cell mother-cell divides and the anticlinal walls are formed, the cobalt precipitate migrates centrifugally away from the region below

the future stoma (Plate 10.5A, left hand side). As the stoma develops, the remains of the now peripherally situated precipitate migrate to the poles of the complex (Plate 10.5B). An adult guard cell complex is illustrated in Plate 8.2D.

DISCUSSION

The changes observed in the morphological features on the undersurface of the developing guard cell complex with the scanning electron microscope relate to the formation of the substomatal sacs, whilst the histochemical stain relates more directly to the underlying ion-adsorbent sites. It is believed, however, that the size and shape of the endocuticular sacs may reflect the developmental/physiological state of the ion-adsorbent sites.

The transverse cleavage observed in P. vulgare is clearly caused by a rift occurring in cell wall elements underneath the endocuticle in this species, but does not directly involve the cuticular membrane. Transmission electron microscopy observations (Chapter 4) implicate the outer layers of the lower periclinal guard cell walls in the structure of the ion-adsorbent body, whilst the histochemical and enzymatic techniques employed in Chapter 7 support the hypothesis, first proposed in Chapter 6, that a major constituent of the body is pectin derived from the interface of the cell wall and the endocuticle. It would seem feasible, therefore, that the rift in the outer region of the cell wall could be symptomatic of pectin migrating towards the poles of the developing complex as it becomes incorporated into the ion-adsorbent bodies. This migration could be induced by the pectin layer

in the polar regions becoming distended into a sac-like form which will result in a strain being set up in the pectin layer of the young guard cell wall which will eventually lead to its tearing apart.

The morphogenesis of the ion-adsorbent bodies in Tradescantia spp. has not been clearly elucidated in the present investigation and clearly differs considerably from that in P. vulgare, but this is probably to be expected in view of the different location of the bodies in Tradescantia. Being situated in the intercellular space at the poles of the guard cell complex, it is unlikely that scanning electron microscopy will contribute additional information about morphogenesis in this genus.

In addition to obvious differences in the location of the ion-adsorbent bodies, the morphogenesis of the structures commences immediately after the stomatal meristemoid becomes the guard-cell mother-cell in P. vulgare whereas it apparently only commences after the guard-cell mother-cell in Tradescantia has divided to form the guard cell complex. Lygodium is intermediate. The entire ontogeny of the stomatal complex occurs in the light in P. vulgare whereas, in Tradescantia spp, it proceeds in the relative darkness within the basal sheaths of older leaves. Since the morphogenesis of the substomatal structures in Tradescantia appears to take place in the region where the emergent leaf starts to turn green, it was thought that the structures might not develop until they are exposed to light.

To test this hypothesis, a specimen of Tradescantia pallidus was defoliated and the root stock, in its pot, was sealed in an aluminium foil bag and kept in a light-tight darkened cupboard. After eight weeks, the aluminium foil was removed and epidermal strips from the etiolated leaves treated with the modified Macallum stain

(vide Chapter 2). The stomatal complexes from this material were similar to those from normal leaves except that their guard cells lacked chloroplasts. The ion-adsorbent bodies in the etiolated tissue appeared to be of normal size but the cobalt precipitates were very weak in comparison with those from normal tissues (Plate 10.6). The presence of light, therefore, does not appear critical in the development of the ion-adsorbent bodies but may affect its functional capacity.

These aspects of the substomatal structures in P. vulgare suggest that, whilst they are formed at an early stage of stomatal ontogeny, they only become functional at a later stage after the guard cell complex matures. This is exemplified when the rather poor cobalt precipitations found in early ontogenetic stages studied are compared with the localisations found in mature complexes (c.f. Plate 10.3.1A with 10.3.3A). In the immature, but differentiated guard cells (Plate 10.3.1A), the cobalt precipitation, although rather weak, is discretely delimited and the associated substomatal sacs are less pronounced than those observed in ontogenetically younger complexes. In mature guard cell complexes, the cobalt precipitate is much heavier and more discretely delimited (Plate 10.3.3A) whilst the substomatal sacs are correspondingly more pronounced (Plate 10.3.3B).

The Macallum stain indicates that potassium may accumulate in the guard cells before they become functional but it should be remembered that the modified Macallum stain used in the foregoing studies was designed to demonstrate ion-adsorbent sites rather than potassium localisations (vide Chapter 2 and the concluding remarks of Chapter 11). However, a similar conclusion was reached by

Dayanandan & Kaufman (1975) who detected the accumulation of potassium in immature complexes of eight species with the Macallum stain.

An interesting aspect of this study has been the confirmation of the inequality of the ion-adsorbent sites within a single guard cell complex. In P. vulgare, the ion-adsorbent sites at the pole of the guard cell complex adjacent to the mesogene subsidiary cell are usually better developed than those at the opposite pole. Initially, this difference was thought to be connected with the asymmetric position of the subsidiary cell in relation to the guard cell complex. However, a similar inequality of the ion-adsorbent sites occurs in Tradescantia spp. where the subsidiary cells are symmetrically arranged around the guard cell complex. This inequality is now thought to be related to ontogenetic events since the larger ion-adsorbent sites are formed at the ontogenetically-distal ends of the guard cell complex although the stomatal meristemoid arises from a lateral meristem in P. vulgare and from a basal intercalary meristem in Tradescantia. This may be significant since in the mature stomata of P. vulgare, the dominant ion-adsorbent site is located proximal to the epidermal transpiration stream, whilst in Tradescantia it is located distally in respect of the transpiration stream. The opposite relative positions of the dominant ion-adsorbent sites may indicate that the inequality observed results from developmental pressures rather than any functional criteria such as the availability of water and ions.

Plate 10.1.

The morphogenesis of substomatal structures in Polypodium vulgare, I.

The vertical series, A, are stained with 7,5% (w/v) modified Macallum stain. The vertical series, B, are scanning electron micrographs viewed from the side adjacent to the mesophyll. All the micrographs are x 2 160. Laterally adjacent micrographs are of developmentally-identical complexes and are directly comparable.

1. Guard-cell mother-cell immediately after the (last) mesogene subsidiary cell has been cut off. Note the cobalt-rich trabeculae in A, and how the cobalt precipitation in A corresponds to a disc-like structure under the endocuticle in B.

2. Guard-cell mother-cell illustrating the transverse cleavage which, in B, can be seen to occur beneath the endocuticle.

3. Guard-cell mother-cell illustrating the transverse cleavage developing into a distinct split. There is a polarisation of cobalt precipitate in A, which is typical of this and subsequent morphogenetic stages.

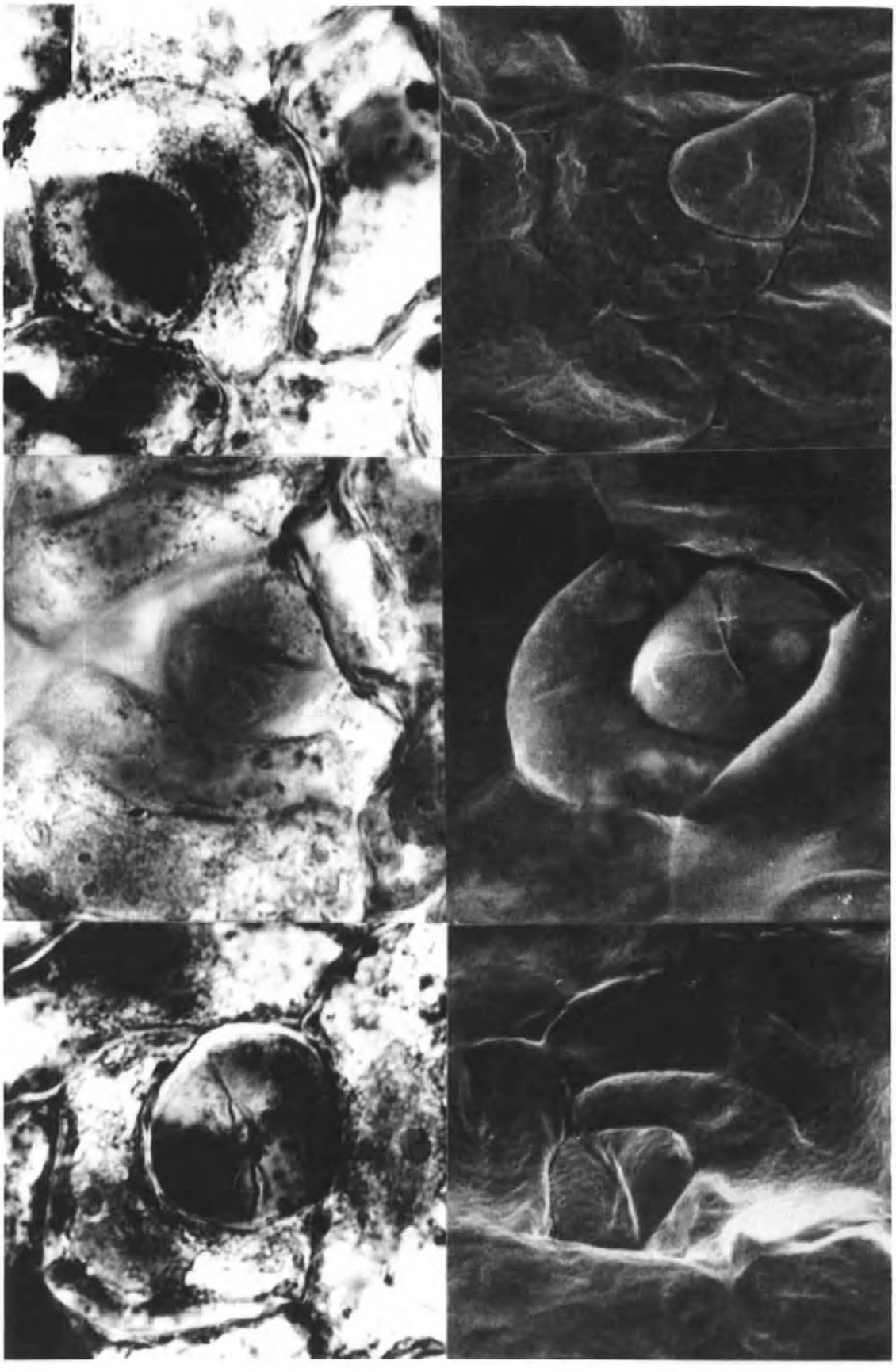

1
2
3
A
B

Plate 10.2.

The morphogenesis of substomatal structures in Polypodium vulgare, II.

The vertical series, A, are stained with 7,5% (w/v) modified Macallum reagent. The vertical series, B, are scanning electron micrographs viewed from the side adjacent to the mesophyll. All the micrographs are x 2 160. Laterally adjacent micrographs are of developmentally-identical complexes and are directly comparable.

1. Guard-cell mother-cell as the cleavage develops into a distinct polarisation which results in the formation of very distinct polar substomatal swellings in B.

2. A very early stage after the guard-cell mother-cell has divided to form the guard cell pair. The ridge adjoining the polar substomatal swellings in B, is believed to result from the incorporation of the endocuticle into the thickened ridges of the underlying cell walls.

3. A later stage of guard cell development just prior to the formation of the stoma.

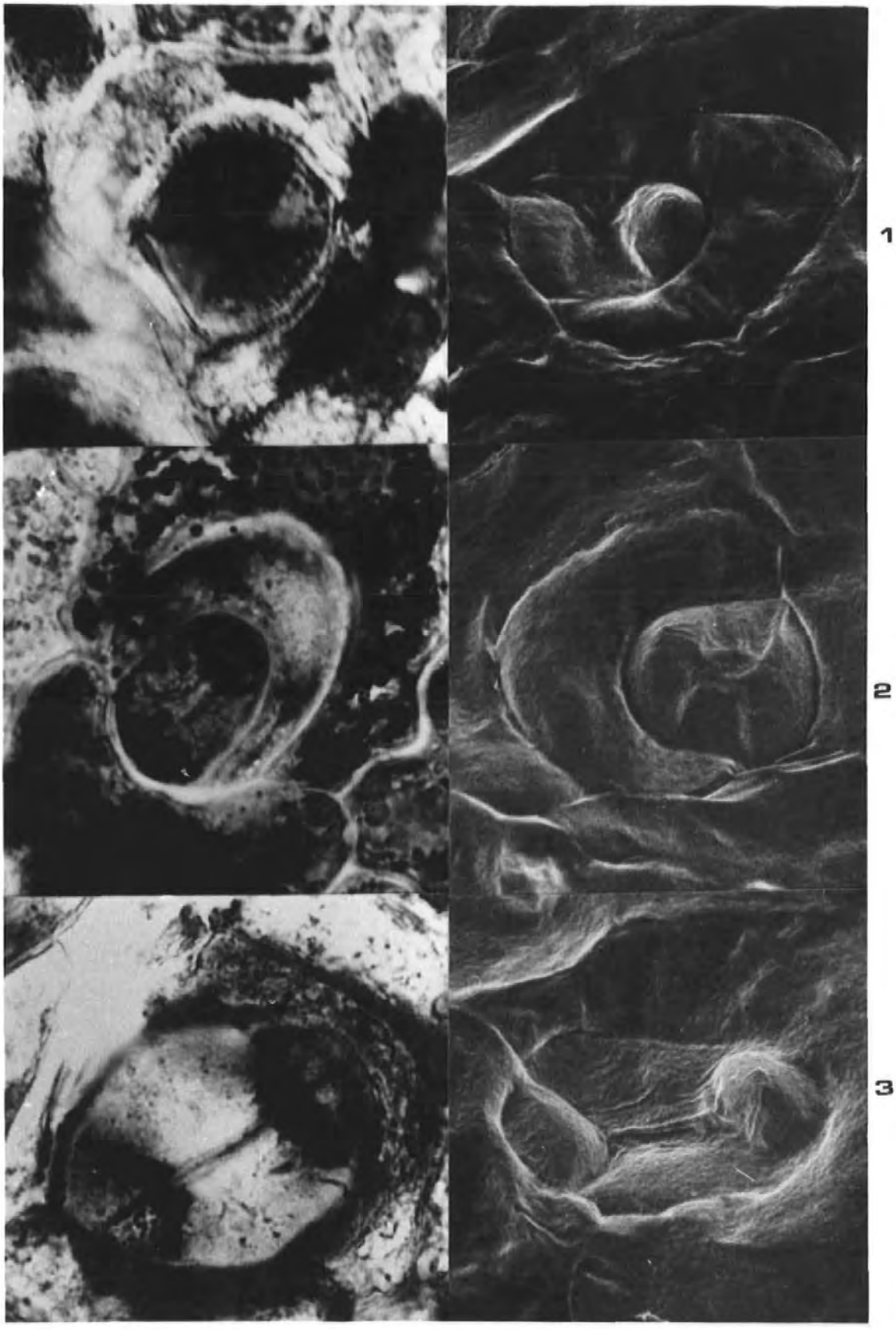

1
2
3
A
B

Plate 10.3.

The morphogenesis of substomatal structures in Polypodium vulgare, III.

The vertical series, A, are stained with 7,5% modified Macallum reagent. The vertical series, B, are scanning electron micrographs viewed from the side adjacent to the mesophyll. Laterally adjacent micrographs are of developmentally-identical complexes which are directly comparable.

1. Submature guard cell complex, x 2 160. The stoma has just formed at this stage. Although there is a distinct polar localisation of cobalt precipitate, it is not as intense as that found in mature stomata. Similarly, the substomatal endocuticular sacs are not very well developed at this stage.

2. Precocious development, x 2 160. The guard-cell mother-cells illustrated here have started to divide to form the guard cell pair before the cleavage has developed into a distinct polarisation.

3. Mature guard cell complexes, x 1 000.

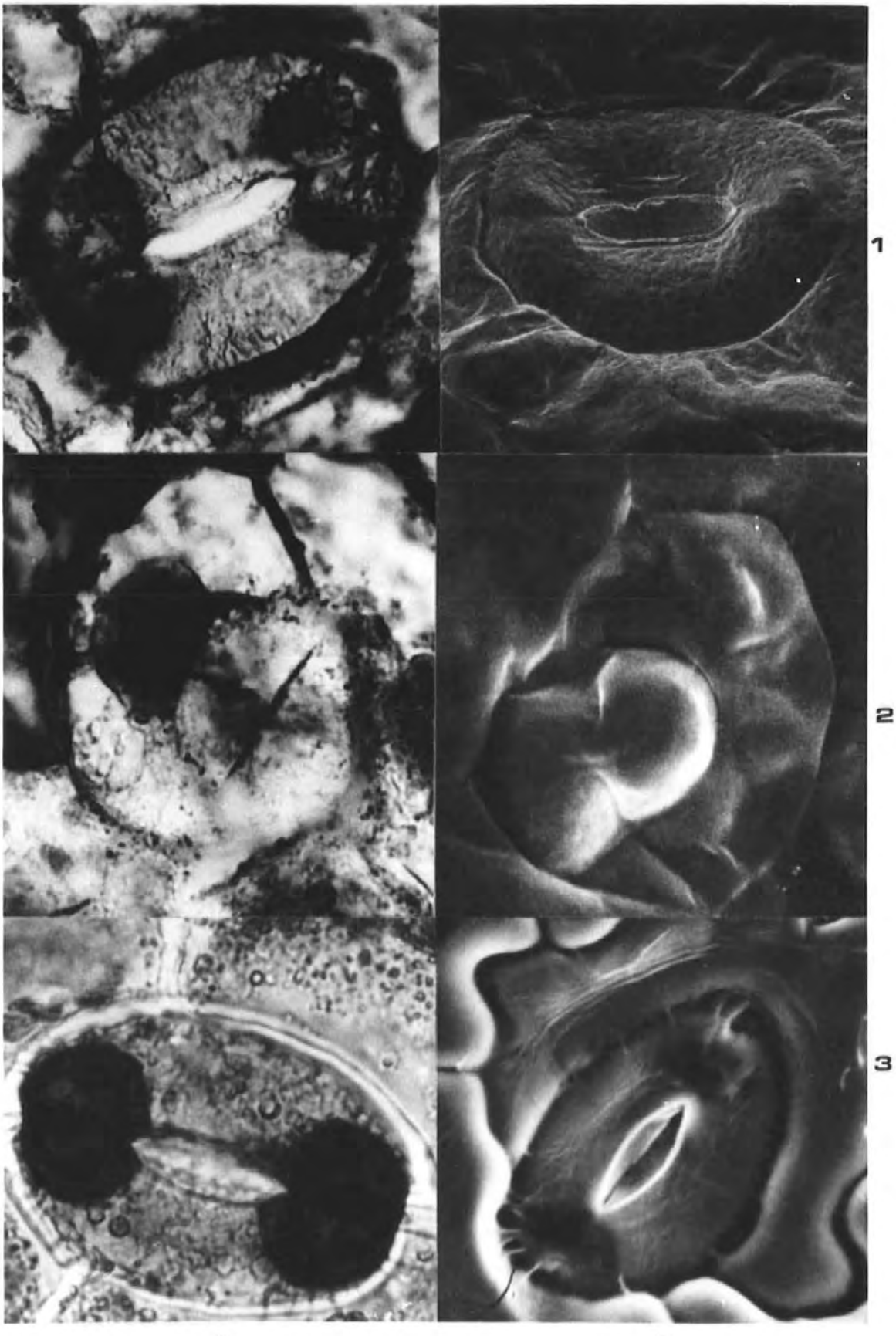

1
2
3
A
B

Plate 10.4.

The morphogenesis of substomatal structures in Tradescantia pallidus.

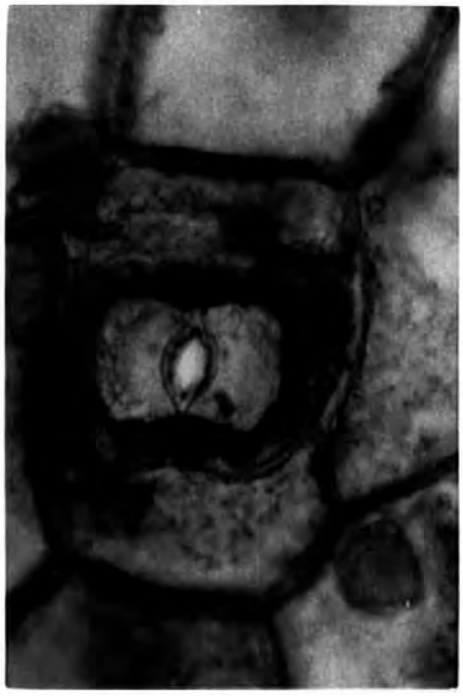
Light micrographs of stomatal complexes stained with 7,5% (w/v) modified Macallum reagent, x 1 100.

A. Cobalt precipitation restricted to the periphery of the young guard cell complex and the lateral subsidiary cells.

B. The cobalt precipitation becoming localised in the polar regions of the young complex, and the precipitation becoming less extensive and heavy over the lateral subsidiary cells.

C. The cobalt precipitation almost completely restricted to the polar regions of the young guard cell complex.

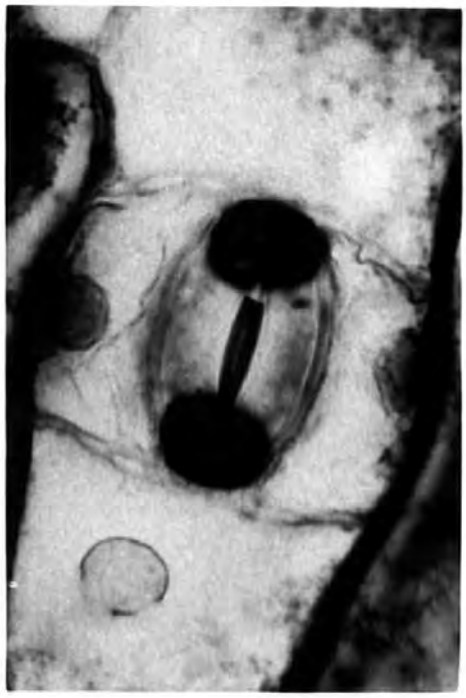
D. Submature guard cell complex showing complete localisation of the cobalt to polar precipitations which are particularly heavy and extensive when compared with those found in mature complexes.



A

B

C



D

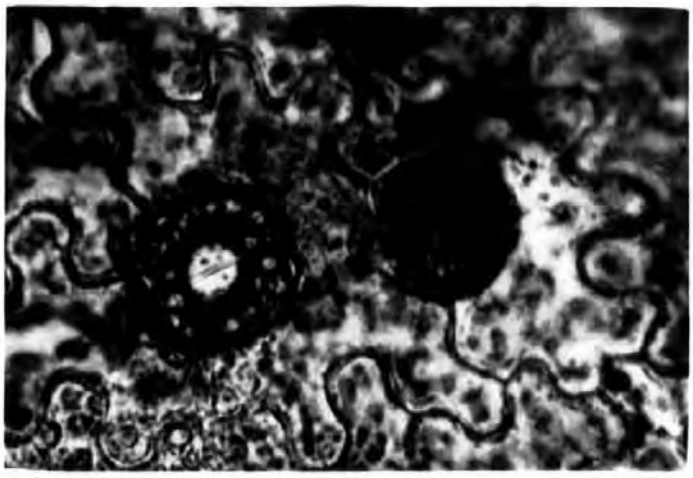
Plate 10.5.

Morphogenesis of substomatal structures in Lygodium scandens.

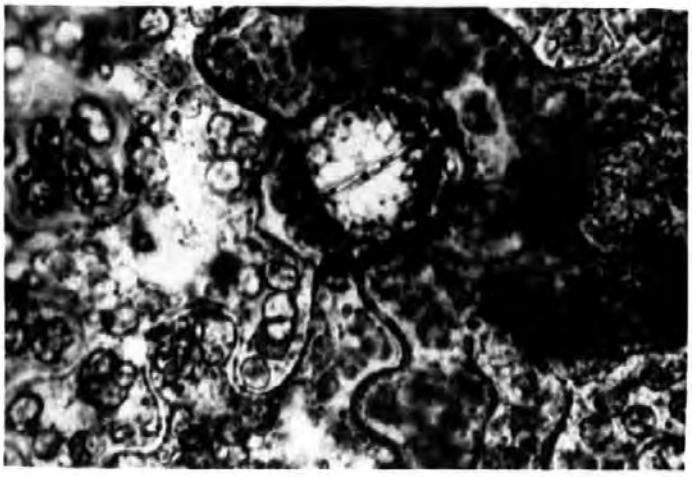
Light micrographs of tissue stained with 7,5% (w/v) modified Macallum reagent, x 800.

A. Micrograph illustrating two early stages in the morphogenesis of the structure. The complex on the left hand side is of either a guard-cell mother-cell or a very young guard cell complex. The cobalt has precipitated onto the complex so densely as to preclude positive identification of the ontogenetic stage involved. The complex on the left hand side is of a young guard cell complex and shows the cobalt precipitation migrating centrifugally away from the eventual site of the stoma.

B. A more advanced ontogenetic stage to the two illustrated in A. The cobalt precipitate is becoming localised at the poles of the young guard cell complex.



A



B

Plate 10.6.

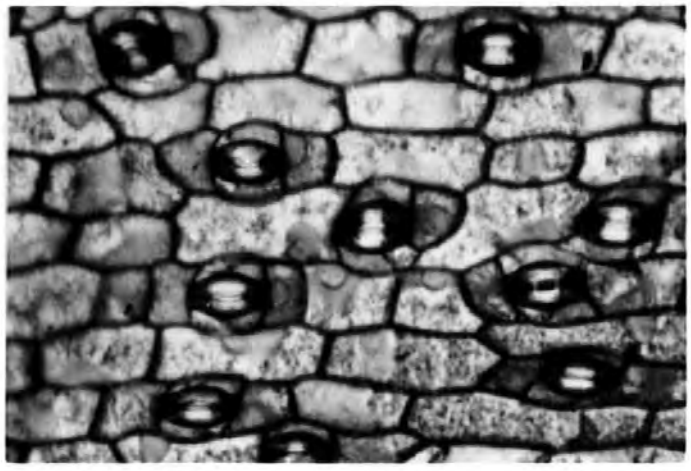
The morphogenesis of substomatal structures in etiolated leaves of Tradescantia pallidus.

Light micrographs of tissue stained with 7,5% modified Macallum reagent.

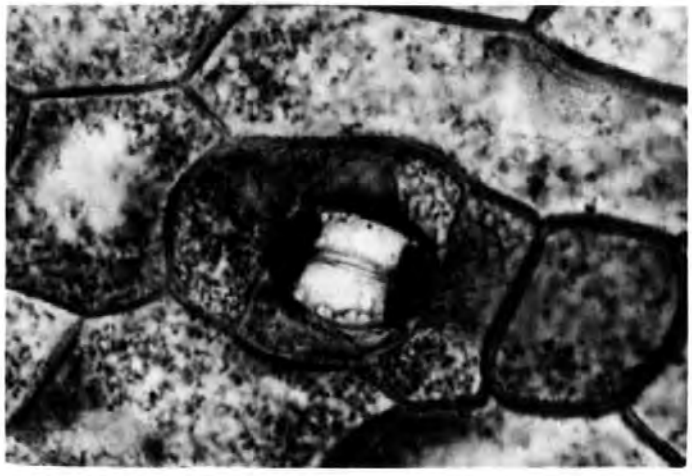
A. Low power view of tissue, x 260, showing the polar localisations of the cobalt in the guard cell complexes.

B. Guard cell complex, x 525. The size and shape of the polar localisations are similar to those found in ordinary epidermal tissue but the intensity of precipitation is considerably reduced.

C. Guard cell complex, x 525. Besides illustrating supernumerary lateral subsidiary cells (two on each side) and the absence of polar subsidiary cells, this complex shows very extensive polar localisations of the cobalt precipitate.



A



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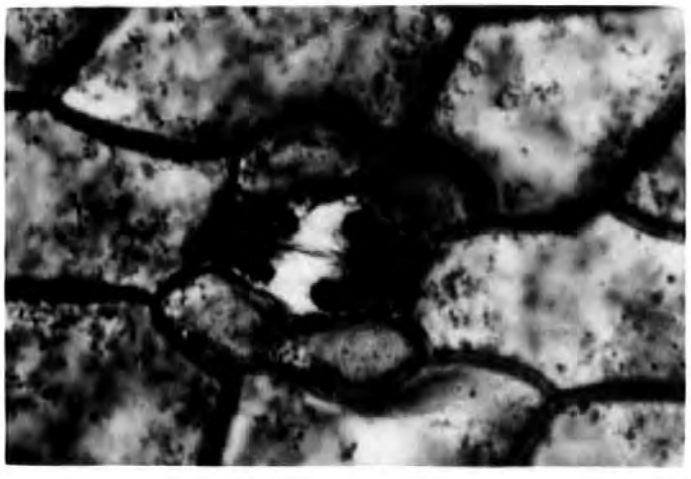


C

CHAPTER 11

FUNCTIONAL AND BEHAVIOURAL ASPECTS OF STOMATA

INTRODUCTION

A considerable volume of physiological data contributing to current understanding of the stomatal mechanism have been obtained from the study of stomatal behaviour in epidermal tissue floated on ionic buffers (reviewed by Thomas, 1975). It was considered that this technique offered the possibility of investigating the role, if any, of the substomatal ion-adsorbent bodies in stomatal movements. Some of the experiments in this section are of a preliminary nature, carried out prior to the emergence of the main theme of the investigation, whilst others are essentially investigations into the basic methodology employed in in vitro experimentation using isolated epidermal tissue.

STOMATAL BEHAVIOUR IN ISOLATED EPIDERMES

Experimental

Experiment 11.1. pH effects on stomatal opening in isolated epidermal tissue of Commelina communis. Results are summarised in Fig. 11.1a, and statistical analyses are presented in Appendix 4.1.

Experiment 11.2. The effect of darkness and buffer concentration on stomatal opening in isolated epidermal tissue of Commelina communis. Results are summarised in Fig. 11.1b, and statistical analyses are presented in Appendix 4.2.

Experiment 11.3. pH effect on stomatal opening in isolated epidermal tissue of Polypodium vulgare. Results are summarised in Fig. 11.1c & d, and statistical analyses are presented in Appendix 4.3 and 4.4.

Experiment 11.4. The effect of light on stomatal opening in isolated epidermal tissue of Commelina communis. Results are summarised in Fig. 11.2, and statistical analyses are presented in Appendix 4.5.

Discussion

The foregoing experiments provided basic information about the behaviour of stomata in isolated epidermal strips of Commelina communis when incubated on citrate buffer. Suitable pH treatment appears to kill the majority of epidermal and subsidiary cells which eliminates any osmotic constraints they may have on the guard cell complex. This effective isolation of the guard cell complex means that any stomatal movements in epidermal strips of this species must be independant of active physiological involvement by the subsidiary cells. This must be considered as an experimental artefact since the subsidiary cells of this species have been strongly implicated in stomatal movements by Willmer & Pallas (1974), and Penny & Bowling (1974, & 1976). The present study indicates that stomatal opening in epidermal strips of this species is ion-stimulated and largely independant of

light below ca. 100 J m^{-2} s^{-1}. Although ion-stimulated opening largely results from potassium uptake from the buffer (Willmer & Mansfield, 1969a, 1970, and Squire & Mansfield, 1972), it is also possible that metabolism of the citrate ions may contribute to the opening process. Citrate, like malate, may be taken up and metabolised by guard cells (Dittrich & Raschke, 1977). For these reasons, it is considered that ion-stimulated stomatal opening in isolated epidermal strips is not achieved by the same mechanism(s) as in life, a fact which should be borne in mind when evaluating data obtained by this technique.

Since the undersurface of the epidermis is in direct contact with the buffer medium, the buffer will affect the osmotic relations of the guard cells because the osmotic potential of the guard cells varies with changes in stomatal aperture (Meidner & Mansfield, 1968). Stomata are extremely sensitive to carbon dioxide concentrations (Meidner & Mansfield, 1968) and the carbon dioxide gradient between the guard cells and the substomatal chamber probably is important in stomatal responses. In the epidermal strip technique, the substomatal chamber is saturated by the buffer medium. Thus the normal moderating effect of mesophyll photosynthesis on stomatal responses will no longer exist and the stomata will only respond to the levels of carbon dioxide dissolved in the buffer and the ambient atmosphere.

The behaviour of isolated epidermal strips of Polypodium vulgare, incubated on ionic buffers, differs markedly from that of C. communis. Mansfield & Willmer (1969) reported similar subdued responses in the closely related fern, Phyllitis scolopendrium, a species whose guard cell potassium levels fails to exhibit consistent correlation with stomatal aperture (Mansfield et al, 1973). In addition, it has only been possible to demonstrate very limited

stomatal movements in intact P. vulgare pinnae (silicon rubber impression technique), although Lange et al (1971) elicited stomatal responses to certain environmental conditions imposed on isolated epidermal strips of this species.

It is possible that the semi-encompassing nature of the horseshoe-shaped subsidiary cells in P. vulgare exert a structural effect on the operation of the guard cells. Under conditions where the turgor of the subsidiary cells exceeds that of the guard cells, any opening movements of the guard cells could be restricted. The potential structural advantage of the subsidiary cells could also be compounded by a turgor advantage derived from the metabolism of subsidiary cell chloroplasts; organelles which are normally lacking in mesophyte subsidiary cells.

EFFECT OF CERTAIN PHENOLICS ON STOMATAL BEHAVIOUR

Experimental

Experiment 11.5. The effects of chlorogenic acid on stomatal opening in light-incubated isolated epidermal tissue of Commelina communis. Results are summarised in Fig. 11.3a, and statistical analyses are presented in Appendix 4.6.

Experiment 11.6. The effects of chlorogenic acid on stomatal opening in dark-incubated isolated epidermal tissue of Commelina communis. Results are summarised in Fig. 11.3b, and statistical analyses are presented in Appendix 4.7.

Experiment 11.7. The effects of chlorogenic acid on

open stomata in light-incubated isolated epidermal tissue of Commelina communis. Results are summarised in Fig. 11.3c, and statistical analyses are presented in Appendix 4.8.

Experiment 11.8. The effects of ferulic acid on stomatal opening in light-incubated isolated epidermal tissue of Commelina communis. Results are summarised in Fig. 11.4a, statistical analyses are presented in Appendix 4.9.

Experiment 11.9. The effects of ferulic acid on stomatal opening in dark-incubated isolated epidermal tissue of Commelina communis. Results are summarised in Fig. 11.4b, and statistical analyses are presented in Appendix 4.10.

Experiment 11.10. The effects of ferulic acid on open stomata in light-incubated isolated epidermal tissue of Commelina communis. Results are summarised in Fig. 11.4c, and statistical analyses are presented in Appendix 4.11.

The foregoing experiments with chlorogenic and ferulic acids were all repeated on epidermal strips of Polypodium vulgare. The results were largely inconclusive in that the responses obtained were very variable and of small magnitude. However, a single experiment (Expt. 11.11, below) did provide significant results.

Experiment 11.11. The effects of ferulic acid on stomatal opening in light-incubated isolated epidermal tissue of Polypodium vulgare. Results are summarised in Fig. 11.5, and statistical analyses are presented in Appendix 4.12.

Discussion

As mentioned in the introduction to this chapter, the physiological studies reported on here were carried out prior to the emergence of the main theme of the investigation (i.e. substomatal ion-adsorbent bodies). Consequently they represent basic experimentation which requires further ratification before any major conclusions can be drawn about the mode of action of the phenolics.

Chlorogenic acid has been shown to be inhibitory to stomatal opening in tobacco leaf discs (Zelitch, 1967), in whole tobacco and sunflower seedlings treated with the acid via a nutrient solution (Einhellig & Kuan, 1971), and in isolated epidermal strips of Commelina communis (Ogunkanmi et al., 1973). Ferulic acid has been reported on as stimulating the transpiration rate of deracinated Phaseolus vulgaris in potometer experiments (Michniewicz & Rozej, 1974).

The present study confirms that chlorogenic acid will inhibit stomatal opening in the light but will only slightly do so in the dark. It appears to have no effect on open stomata and does not induce their closure in the light. The inhibitory action of chlorogenic acid on stomatal opening would, therefore, appear to be light dependant. It has already been established that stomatal opening on citrate buffer is induced primarily by potassium uptake by the guard cells which implies that the inhibition of opening observed in the light results from chlorogenic acid blocking this process. The question arises, therefore, why chlorogenic acid does not inhibit this process in the dark because ion-stimulated opening does not appear to be light dependant (vide Expt 11.2). Dayanandan & Kaufman (1975), however, have demonstrated potassium uptake by Crassula argentea guard cells

during night opening which is typical of such plants which possess Crassulacean acid metabolism. This apparent failure to block ion uptake in the dark deserves further investigation since it implies that two ion-uptake mechanisms may be involved in the guard cells of isolated epidermal strips.

The present findings that chlorogenic acid does not induce stomatal closure of open stomata in epidermal strips are not in agreement with those of Ogunkanmi et al (1973) who indicate very significant closure at 10^{-3}M. The position needs some clarification, however, since Ogunkanmi et al (op. cit.) only imply that they used already opened stomata and they do not specify whether their control values represent stomatal apertures at the beginning or the end of the incubation period.

Polypodium vulgare epidermal strips appear to be completely unaffected by chlorogenic acid in either a water or a buffer incubation medium, and are not considered here.

Stomatal opening in isolated epidermal strips of Commelina communis is inhibited by ferulic acid in both the light and the dark, but stomatal closure is not induced in open stomata in the light. The results of the chlorogenic acid investigations imply that two separate mechanisms exist for ion uptake by guard cell. If this is the case, ferulic acid appears to be able to block both processess. In contrast, ferulic acid stimulates stomatal opening in epidermal strips of Polypodium vulgare when in aqueous solution but not in citrate buffer. This suggests that ferulic acid, in this case, is not stimulating ion uptake by the guard cells but is either modifying the metabolism of the guard cells themselves or, in view of the suspected

involvement of subsidiary cells in this species, it may affect the metabolism, or even viability of these cells.

Once again, the present findings are not in agreement with those in the literature (Michniewicz & Rozej, 1974) but may not be directly comparable since epidermal tissue was used in the present investigation whilst they used deracinated plants. P. vulgare appears to behave similarly to their Phaseolus vulgaris although they, apparently, began their experiments with stomata in the open state. The present findings in C. communis contradict those of Michniewicz & Rozej (op. cit.) and indicate that, in isolated epidermes of this species, ferulic acid inhibits the opening process.

These observations indicate that the study of the action of phenolics on the stomatal mechanism may provide useful information about the mechanism(s) of stomatal movements both in vivo and in vitro. More importantly, they reveal basic differences between the responses of stomata under in vivo and in vitro conditions. For this reason alone, further investigations into the use of isolated epidermal tissues in in vitro investigations should be carried out.

The difficulties in interpreting this data are further highlighted by the fact that no differences in potassium content could be detected using the basic Macallum reagent (Macallum, 1905).

ALTERNATIVE TECHNIQUES FOR MONITORING STOMATAL BEHAVIOUR IN VITRO

As a result of the problems encountered with the epidermal strip technique, alternative techniques were introduced to study

stomatal behaviour *in vitro*. Whole leaf discs and semi-leaf discs were incubated on distilled water. The semi-leaf discs were prepared by removing the adaxial epidermis and floating the remaining leaf tissue on the exposed mesophyll tissue. The discs were maintained above the water surface by air trapped between the mesophyll cells. The epidermal cell layer was never in direct contact with the incubating medium and, consequently, was not able to directly take up exogenous materials.

Early results of such experimentation with *Commelina communis* are summarised in Fig. 11.6. Positive stomatal responses were obtained with lower epidermal semi-leaf discs which, in the presence of normal air, opened to 7 μm. This value is less than the 11 μm commonly achieved with isolated epidermal strips floated on citrate buffer (Fig. 11.2) but is large enough for accurate measurement. One great advantage of using both semi-leaf, and whole leaf discs is their ability to open and close in response to consecutive light and dark treatments. They also respond to imposed carbon dioxide regimes. The 7 μm stomatal opening achieved with semi-leaf discs in the light is independant of exogenous ions and, therefore, compares very favourably with the 2 - 3 μm non ion-stimulated opening exhibited by isolated epidermes floated on citrate buffer under the same light regime (Fig. 11.2). The stomatal behaviour of both semi-leaf, and whole leaf discs, unlike that of epidermal strips, is very similar to that found in nature as monitored by viscous flow porometry (*vide infra*).

Whilst semi-leaf discs may prove a useful addition to *in vitro* techniques in certain physiological studies where the only parameter being monitored is stomatal aperture, they are not suitable for studies involving observations of the substomatal ion-adsorbent

bodies. This is because it is impossible to separate the epidermis from the underlying mesophyll cells without damage. Whilst it is possible to remove the epidermis from whole leaf discs, it is difficult and not always sucessful. Consequently a variation of the whole leaf disc technique was developed specifically for Tradescantia spp., but which could be attempted with any plant species whose leaves are sufficiently large (vide Expt. 11.12). The stomata, whilst not opening as widely as those in epidermal strips or semi-leaf discs, open comparably to those of leaf discs and are easily measurable. After the treatment period, the abaxial epidermis can be easily stripped off in order to observe the ion-adsorbent bodies.

STOMATAL MOVEMENTS AND ION-ADSORBENT BODIES

Effect of abscisic acid

2 cm lengths were excised from Tradescantia pallidus leaves one hour prior to the commencement of the photoperiod. The lengths were placed, on end (vide Plate 2.3), in 1 cm^3 aliquots of 10^{-11}, 10^{-9}, 10^{-7}, 10^{-5}, and 10^{-3} M abscisic acid dissolved in distilled water and incubated at 137 J m^{-2} s^{-1}. After 4 h, 30 stomatal apertures were measured from each treatment and an abaxial epidermal strip removed. The epidermal strip was treated with the modified Macallum stain (vide Chapter 2) and sample fields photographed under the microscope. A control, incubated in distilled water, was employed. The experiment was replicated on three separate occasions.

Stomatal aperture progressively decreased with increasing

concentration of abscisic acid, complete closure being effected at 10^{-3} M. Progressive saturation of the mesophyll intercellular spaces occurred with increasing abscisic acid concentrations and appeared to be virtually total at 10^{-3}M. No apparent quantitative or qualitative differences were observed in the stained ion-adsorbent bodies (Fig. 11.7; statistical analyses are presented in Appendix 4.13).

Abscisic acid inhibits stomatal opening in the light in isolated whole leaf segements. The reduced stomatal openings at higher concentrations may be enhanced by progressive saturation of the intercellular air spaces. This effect requires further clarification. There was apparently no change in the size or the density of staining of the ion-adsorbent bodies although Mansfield & Jones (1971) have successfully demonstrated a reduction in guard cell potassium, in the related species Commelina communis, with abscisic acid treatment using the basic Macallum technique (Macallum, 1905). These observations do not necessarily conflict with those of Mansfield & Jones (op. cit.) since the modified Macallum stain was designed to demonstrate ion-adsorbent bodies although it could also indicate potassium localisations (vide Chapter 2).

Effect of water stress and photoperiod

Experiments were conducted to determine whether changes in the size of the ion-adsorbent bodies of their adsorbent capacity could be correlated with changes in stomatal aperture using the modified Macallum technique (vide Chapter 2). However, no definite changes in the state of the bodies could be detected between light and dark treated leaves, or in leaves under varying degrees of water stress induced by natural wilting and by treatments with graded mannitol

osmotica. The photomicrographic evidence obtained was very similar to that illustrated for Experiment 11.12 in Fig. 11.7.

Effect of endogenous stomatal movements

In the previous experiments, attempts were made to correlate changes in the ion-adsorbent bodies with stomatal movements induced by abscisic acid and wilting. Using viscous flow porometry (vide Chapter 2), attempts were also made to correlate longer term changes in stomatal aperture with changes in the ion-adsorbent bodies.

Typical porometer data obtained simultaneously from leaves on four separate plants of Commelina communis are presented in Fig. 11.8. The results, in this case, have been expressed in terms of percentage viscous conductance and percentage viscous resistance. In this way, small changes in stomatal aperture when the stomata are almost closed are best expressed in terms of viscous resistance, whilst those occurring when the stomata are more fully open are best expressed in terms of viscous conductance. The plants were initially pretreated with a 14 h photoperiod (10.00 - 24.00 h B.S.T.) followed by 10 h darkness for 7 days prior to the porometer cups being fitted. Stomatal behaviour was monitored subsequently for 7,5 days.

Night opening of stomata occurred in leaves b,c, and d during the first period of darkness, but did not reappear in any leaf during the second period of darkness. The subsequent opening in light was delayed, in the absence of night opening, by between 10 and 30 minutes (c.f. Martin & Meidner, 1972). Maximum stomatal opening usually occurred about 1 h after the onset of the photoperiod and was characterised by an 'overshoot', during which the stomata open, then

close for a short period of time, before opening to achieve steady state opening after 2 to 2,5 h (c.f. Martin & Meidner, 1972, 1975). The four leaves were then exposed to continuous darkness for 34 h, during which time all leaves showed a fluctuation in aperture, but with major peaks corresponding to night opening during the period of subjective day (i.e. that period during which the plant would normally experience day conditions). During the photoperiod which followed this extended dark period, the stomata showed a delayed opening response, without the normal overshoot phenomenon. During the following 10 h dark period, night opening occurred in all leaves. This was followed by a period of continuous light, during which the stomata exhibited circadian opening and closing cycles with a period of ca. 27,5 h, although the amplitude of successive cycles damped with time (c.f. Martin & Meidner, 1971).

Using similar treatments, epidermal strips were removed during the light and the dark phases of the stomatal rhythm and treated with the modified Macallum stain (vide Chapter 2). No correlation could be found between stomatal aperture and the size or degree of staining of the ion-adsorbent bodies.

ION ADSORBENT CAPACITY OF THE ION-ADSORBENT BODIES

Epidermal strips

Experiment 11.14. The capacity of ion-adsorbent bodies in isolated epidermal strips of Commelina communis to adsorb various cations from an incubating medium.

Abaxial epidermal strips of Tradescantia pallidus were removed during the photoperiod and incubated in 2 cm^3 aliquots of 5% (w/v) aqueous solutions of nickel sulphate, $NiSO_4$, ferrous sulphate, $FeSO_4$, ferric chloride, $FeCl_3$, lead nitrate, $Pb(NO_3)_2$, cobaltous sulphate, $CoSO_4$, cupric sulphate, $CuSO_4$, mercuric chloride, $HgCl_2$, silver nitrate, $AgNO_3$, and 1% gold chloride, $AuCl_3$ at 137 J m^{-2} s^{-1}. After 0,5 h, the strips were washed for 10 s in absolute ethanol, floated on 5% ammonium sulphide for 2 minutes, before being re-washed in ethanol and mounted in water for microscopic examination.

The experiment was repeated on tissue removed 1 h prior to the onset of the photoperiod and incubated in the dark. Strips incubated on distilled water acted as controls in both experiments.

The controls in both light and dark treatments showed no signs of any precipitation. All the treated strips, except those floated on $AuCl_3$, showed distinct heavy metal precipitations in the polar regions of the stomata. There was no apparent difference between comparable light and dark treatments. Very weak polar precipitations were obtained with $AuCl_3$ after an extended incubation of 6 h. Sample light micrographs are illustrated in Plates 11.1 and 11.2.

The cationic fractions of all the metal salts used in this experiment became adsorbed onto the ion adsorbent sites. The bodies do not appear to be specific and can accomodate monovalent (Ag^+), divalent (Ni^{++}, Fe^{++}, etc.) and trivalent (Au^{+++}, and Fe^{+++}) cations.

Excised leaves

Experiment 11.15A. The capacity of ion-adsorbent sites in excised whole leaves of Tradescantia x andersoniana to adsorb various

cations from the transpiration stream.

Whole leaves were excised from plants 1 h before the onset of the photoperiod and transferred rapidly to phials containing 5 cm^3 aliquots of the experimental solutions used in the previous experiment. The leaves were placed in an environmental chamber for 16 h at 37 $J\ m^{-2}\ s^{-1}$. The experiment was repeated on leaves excised from dark treated plants and were incubated in the dark. Distilled water controls were used. After 16 h, epidermal strips were removed from the abaxial leaf surfaces at least 1 cm above the level of the experimental solutions and the presence of cations visualised with 5% ammonium sulphide.

The controls showed no sign of precipitation, whilst all the treated leaves, from both light and dark treatments, exhibited heavy metal accumulations on the ion-adsorbent bodies. All the treated leaves were severely wilted. Sample micrographs are illustrated in Plates 3.13, 11.3, and 11.4.

The heavy metal cations were assumed to be transported to the ion-adsorbent bodies via the cut petiole but the exact pathway cannot be determined from this experiment. As in the previous experiment, the ion adsorbent bodies were apparently non selective.

Experiment 11.15B.

This experiment was a repeat of the previous one (11.15A) carried out to define the water pathway utilised by the heavy metal ions more clearly.

6 cm long epidermal strips lifted off the abaxial leaf surface at the base of the lamina but were left attached to the leaf

distally. The cut petiole was placed in a phial containing distilled water whilst the semi-detached epidermis was placed in an adjacent phial containing a heavy metal solution. The exposed inner surfaces of the free epidermis and adjacent mesophyll were lightly coated with liquid paraffin to prevent dessication. After 16 h incubation, the epidermis was detached for a further 2 cm and two epidermal strips prepared. One was taken from the freshly detached area and the other was from the area of previously detached tissue just above the level of the experimental solution. The heavy metals were visualised with 5% ammonium sulphide.

The results were identical to those obtained in the previous experiment (11.15A), and imply that the metal cations were adsorbed directly from the epidermal transpiration stream.

Discussion

The foregoing experiments show the substomatal ion-adsorbent sites can adsorb a wide variety of cations of varying valencies. Experiment 11.14 shows that these cations can be adsorbed directly from an exogenous source, whilst Experiment 11.15 indicates that they can be adsorbed from the transpiration stream.

In Chapters 6 and 7, evidence was offered which advances the concept that the ion-adsorbent bodies are largely pectinaceous. Pectic substances are well known for their ability to adsorb a wide variety of cations (Kertesz, 1951) and particularly those of heavy metals. This adsorption is achieved by the ionisation of the acid radical in polygalacturonic acid (Fig. 7.4) and the subsequent ionic attachment of free cations to the carboxyl anion. Therefore, if the bodies are

largely composed of pectin, the results observed in Experiments 11.14 and 11.15 are to be expected.

The fact that the heavy metal solutions are carried through the epidermal tissue in the transpiration stream (Experiment 11.15B) agrees with the findings of Tanton & Crowdy (1972), Gaff et al. (1964), Burbano et al. (1976), Pizzolato et al. (1976), and Byott & Sheriff (1976) whose evidence suggests that this pathway is an important element in the transpiration stream. Their interpretation of water pathways, identified with heavy metal precipitates, has been elaborated on by Sheriff & Meidner (1974) and Meidner (1975) using alternative techniques. However, the results of Experiment 11.15 do not indicate that the substomatal ion-adsorbent bodies are preferential sites of transpirational loss necessarily since Experiment 11.14 shows that cations are adsorbed onto these bodies non-selectively. The present findings suggest that the ion-adsorbent bodies could play an important role in extracting potentially toxic metal ions from the transpiration stream. However, Lane & Martin (1977) have found that lead uptake in whole radish plants is largely prevented from reaching the leaf tissues by an apparent barrier at the suberised endodermis, and any lead passing this tissue is liable to become adsorbed onto exchange sites in the vascular tissue.

RELEVANCE OF THE MACALLUM STAIN TO HISTOCHEMISTRY OF ION-ADSORBENT BODIES

This investigation has yet to provide conclusive evidence that potassium is associated with the ion-adsorbent bodies, and no

correlation has been made between either their size or density of staining and stomatal aperture. The results obtained with the Macallum stain (Macallum, 1905) may indicate no more than the adsorption of cobalt onto the bodies (Experiments 11.14 and 11.15), rather than the presence of potassium. However, since the bodies appear to be so non-specific in respect of what cations can be adsorbed, there is a strong possibility that potassium is associated with the structures (Braconnot, 1825, found that pectic acid will readily adsorb potassium).

However, if a potassium positive reaction is to be obtained with the Macallum stain, the potassium must be in a free form since the triple salt cannot be formed if only bound potassium is present. Since the polar bodies are ostensibly binding sites, it is unlikely that a potassium positive reaction at these sites is to be expected. Indeed, this may be the reason why, in this investigation, attempts to correlate stomatal aperture with Macallum stain reactions have been so unsuccessful.

It is believed that if the ion-adsorbent bodies do play a direct role in stomatal functioning, and if they are involved in stomatal potassium fluxes, alternative techniques will have to be employed to determine their functional significance. Of the techniques, currently available, for potassium analysis, X-ray microanalysis is considered to be the most suitable and accurate for studies relating to the ion-adsorbent bodies.

Fig. 11.1.

Buffer effects on stomatal behaviour in isolated epidermal tissue.

Each value represents the mean of 120 stomatal apertures (20 x 2 replicates on 3 ocassions). Strips removed 1 h before the onset of the photoperiod; incubation period - 3 h. p-t = stomatal aperture prior to treatment.

a. pH effect on light incubated Commelina communis ($137\ J\ m^{-2}\ s^{-1}$). Vertical bars represent standard deviation. Optimal opening effect observed at pH 5.5.

b. Buffer concentration effect on dark incubated Commelina communis. Optimal opening effect at 5 - 10 mM. The stomatal opening observed is not light dependant, but must be ion stimulated.

c. pH effect on light incubated Polypodium vulgare ($137\ J\ m^{-2}\ s^{-1}$). Vertical bars represent standard error. Very poor stomatal opening was achieved; optimal at pH 5,5/6,0. The opening observed at low pHs believed to result from toxic effects.

d. pH effect on light incubated Polypodium vulgare ($137\ J\ m^{-2}\ s^{-1}$). Very poor stomatal opening; optimal at pH 5,5/6,0. Opening at pH 8,0 probably resulting from toxic effects.

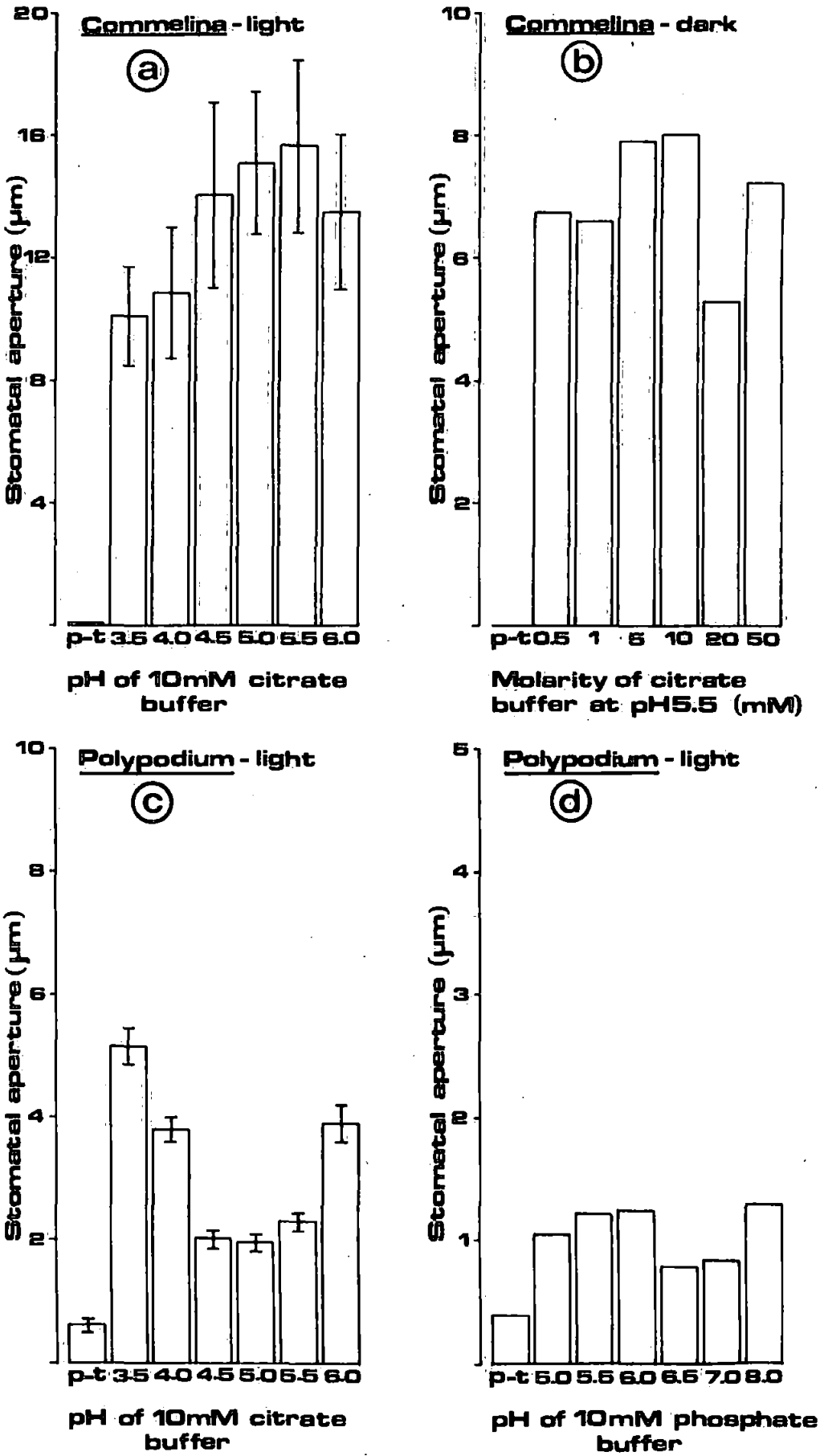

Commelina - light
a
Stomatal aperture (µm)
20
16
12
8
4
p-t 3.5 4.0 4.5 5.0 5.5 6.0
pH of 10mM citrate buffer
Commelina - dark
b
10
8
6
4
2
p-t 0.5 1 5 10 20 50
Molarity of citrate buffer at pH5.5 (mM)
Polypodium - light
c
10
8
6
4
2
p-t 3.5 4.0 4.5 5.0 5.5 6.0
pH of 10mM citrate buffer
Polypodium - light
d
5
4
3
2
1
p-t 5.0 5.5 6.0 6.5 7.0 8.0
pH of 10mM phosphate buffer

Fig. 11.2.

The effect of light on stomatal behaviour in isolated epidermal strips of Commelina communis.

Epidermal strips removed 1 h before the commencement of the photoperiod; incubated on 10 mM citrate buffer (pH 5.5). 20 stomatal apertures measured from each light treatment every 15 minutes for 6 h. Values represent average of 120 readings (20 x 2 replicates on 3 ocassions). Dark treatment value at 3 h extrapolated from Experiment 11.2.

At light intensities of up to 100 J m^{-2} s^{-1}, the major cause of stomatal opening must arise from the buffer ions per se (i.e. ion stimulated opening). At highest light intensity investigated (137 J m^{-2} s^{-1}), 80% of the observed stomatal response is due to ion stimulated opening (vide Expt. 11.2 and Fig. 11.1b)

Stomatal opening progressively increases with time (linear logarithmic relationship) for the first 3 h; thereafter steady-state opening maintained. The curves have been fitted by hand.

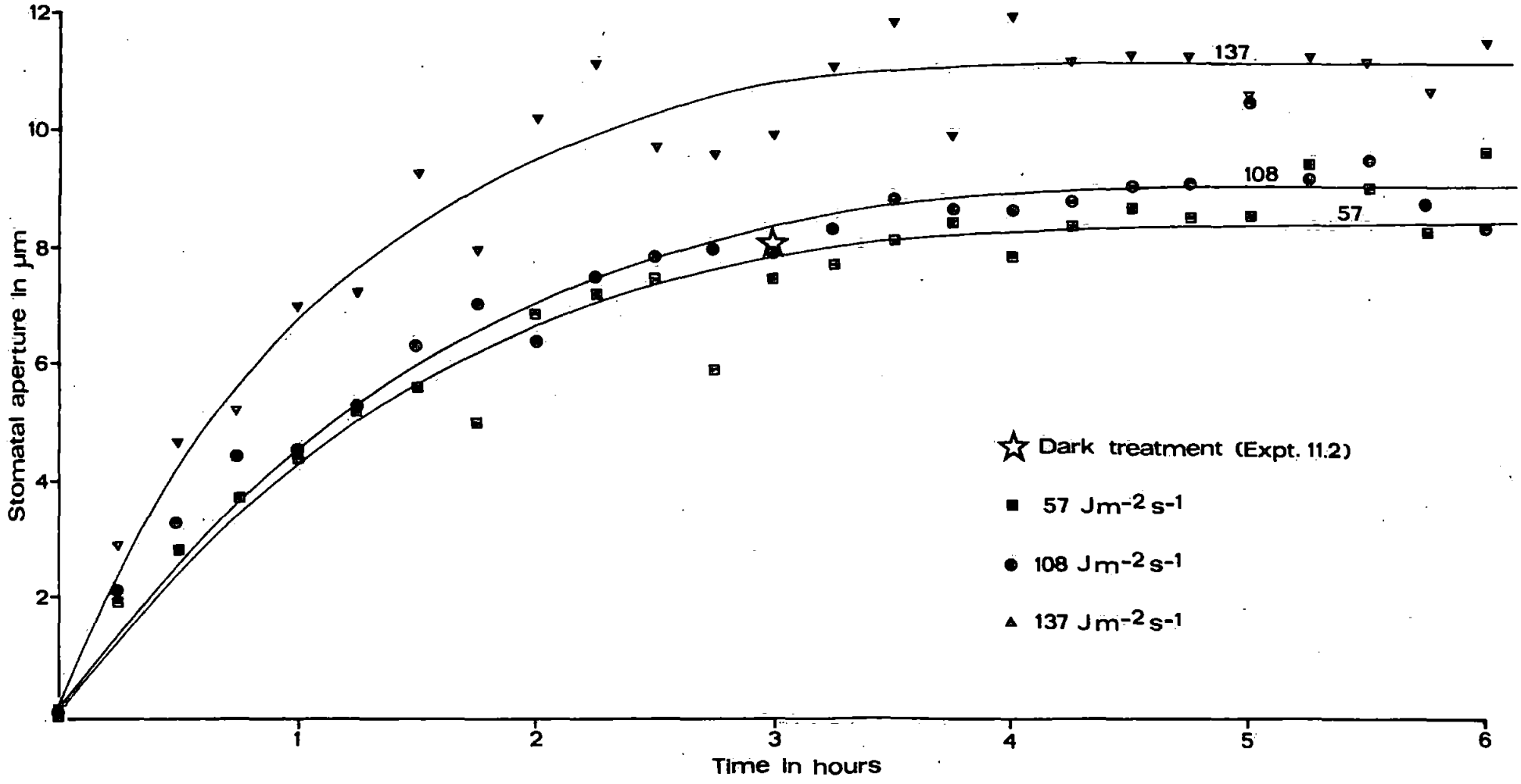

Stomatal aperture in µm
12
10
8
6
4
2
1
2
3
4
5
6
Time in hours
137
108
57
Dark treatment (Expt. 11.2)
57 Jm-2 s-1
108 Jm-2 s-1
137 Jm-2 s-1

Fig. 11.3.

Effects of chlorogenic acid on stomatal behaviour in isolated epidermes of Commelina communis.

Each value represents the mean of 120 stomatal apertures (20 x 2 replicates on 3 ocassions). Incubation time - 3 h. p.t. = stomatal aperture prior to the treatments; C = stomatal aperture of control (buffer only) at the end of the treatment period.

a. Concentration effect on stomatal opening at 137 J m^{-3} s^{-1}. Epidermal strips removed 1 h prior to the commencement of the photoperiod. Vertical bars represent standard error. Progressive inhibition of stomatal opening obtained with increasing chlorogenic acid concentrations; optimal inhibition at 10^{-3} M. Stomatal response at 10^{-2} may result from toxic effects (requires confirmation).

b. Concentration effect on opening in the dark. Epidermal strips removed 1 h prior to the photoperiod commencing. Slight inhibition observed between 10^{-5} and 10^{-3} M but inhibitory effect of chlorogenic acid is greatly reduced in the absence of light (c.f. Fig. 11.3a)

c. Concentration effect on open stomata at 137 J m^{-2} s^{-1}. Epidermal strips removed from leaves whose stomata had been induced to open in the light (vide Chapter 2). Results indicate that chlorogenic acid does not promote stomatal closure. The 50% increase in stomatal aperture over pretreatment levels cannot be attributed to chlorogenic acid since it also occurs in the control. The enhanced opening observed could result from the collapse of the subsidiary cells (pH effect, vide Expt. 11.1). Alternatively, or concomitantly, extra ions could be taken up from the buffer to stimulate further opening.

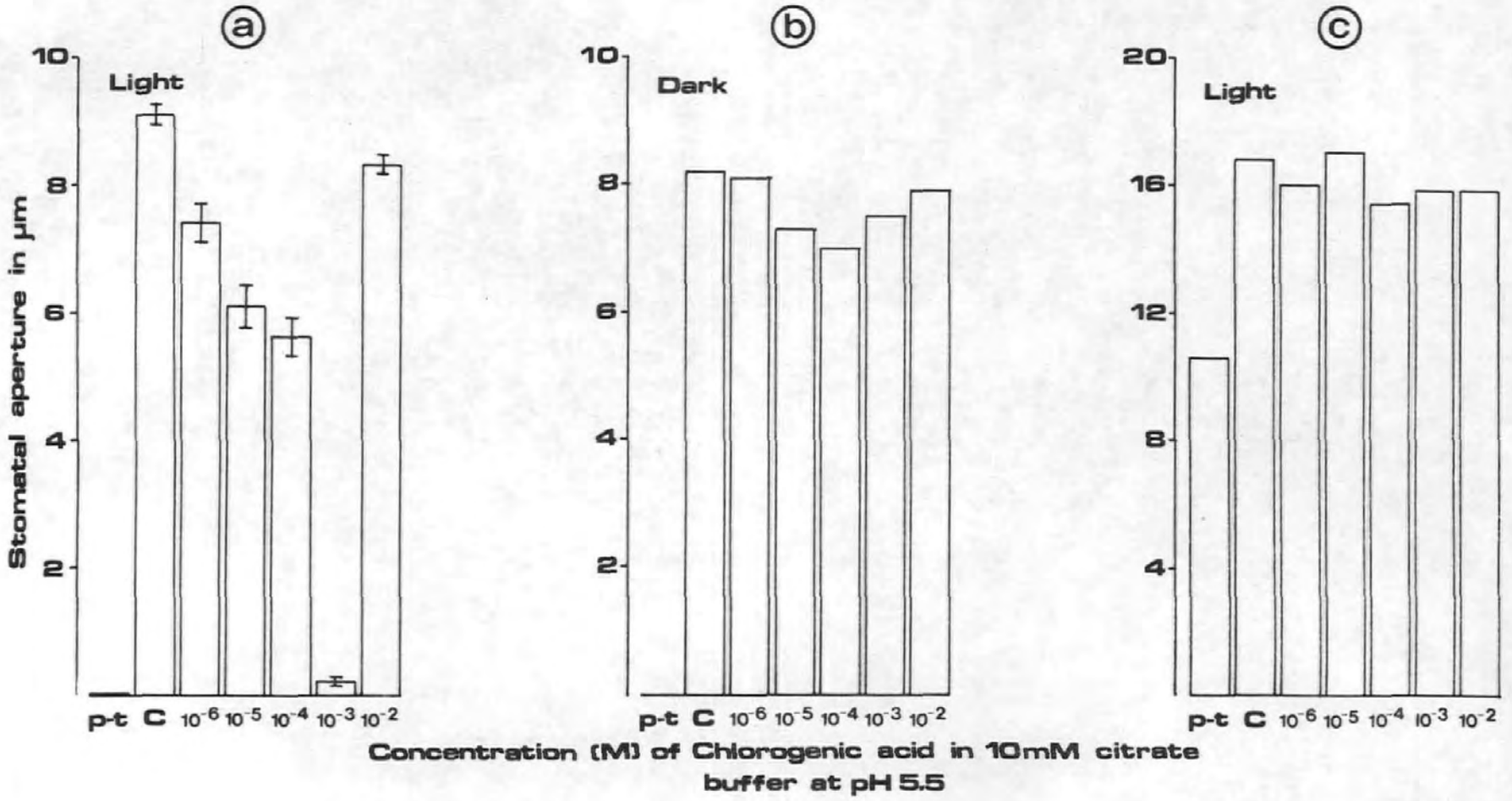

a
b
c
Light
Dark
Light
Stomatal aperture in µm
10
8
6
4
2
20
16
12
p-t
C
10-6
10-5
10-4
10-3
10-2
Concentration (M) of Chlorogenic acid in 10mM citrate buffer at pH 5.5

Fig. 11.4.

Effect of ferulic acid on stomatal behaviour in isolated epidermal tissue of Commelina communis.

Each value represents the mean of 120 stomatal apertures (20 x 2 replicates on 3 ocassions). Incubation period - 3 h. p.t. = stomatal aperture prior to incubation; C = stomatal aperture of control (buffer only) at the end of the treatment period. N.B. at 10^{-2} M, ferulic acid crystalised out at the experimental temperature (20^{o}C).

a. Concentration effect on stomatal opening at 137 J m^{-2} s^{-1}. Epidermal strips removed 1 h prior to the commencement of the photoperiod. Vertical bars represent standard error. Progressive inhibition of stomatal opening occurred with increasing concentrations of ferulic acid; optimal inhibition at 10^{-3} and 10^{-2} M.

b. Concentration effect on stomatal opening in the dark. Epidermal strips removed 1 h prior to the onset of the photoperiod. Progressive inhibition of stomatal opening achieved with increasing concentrations of ferulic acid; optimal inhibition at 10^{-3} and 10^{-2} M. The apparently incongruous results obtained at 10^{-4} M require confirmation.

c. Concentration effect on open stomata at 137 J m^{-2} s^{-1}. Epidermal strips removed from leaves whose stomata had been induced to open in the light (vide Chapter 2). Results indicate that ferulic acid does not stimulate stomatal closure. The enhanced opening over pretreatment levels is not associated with the ferulic acid treatment and is thought to result for the same reasons as given in the legend of Fig. 11.3c. The 10^{-2} results are probably indicative of toxic effects.

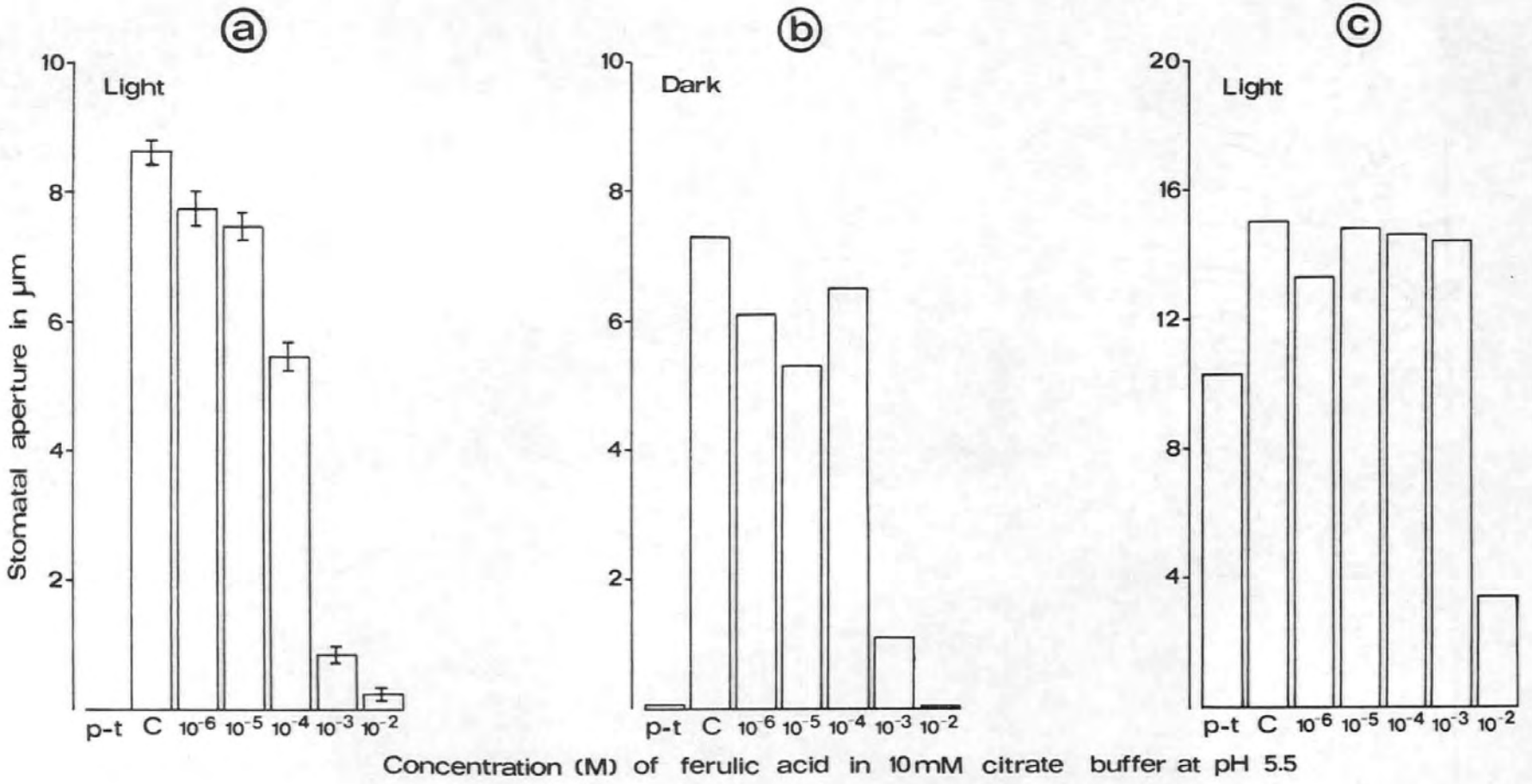

a
b
c
Light
Dark
Light
Stomatal aperture in µm
10
8
6
4
2
20
16
12
p-t
C
10-6
10-5
10-4
10-3
10-2
Concentration (M) of ferulic acid in 10 mM citrate buffer at pH 5.5

Fig. 11.5.

Effect of ferulic acid on stomatal opening in isolated epidermal tissue of Polypodium vulgare.

Each value represents the mean of 120 stomatal apertures (20 x 2 replicates on 3 ocassions). Incubation period - 3 h, at 137 J m^{-2} s^{-1}. p.t. = stomatal aperture prior to treatment; C = stomatal aperture of control (distilled water only) at the end of the treatment period. The epidermal strips were removed 1 h before the onset of the photoperiod.

Ferulic acid appears to stimulate stomatal opening at all the experimental concentrations; optimal opening occurs at 10^{-4} and 10^{-3} M.

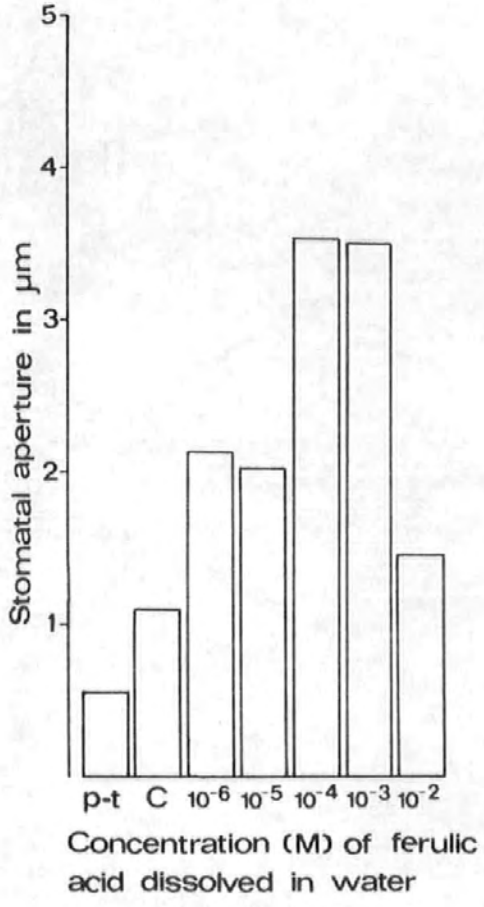

5
4
3
2
1
Stomatal aperture in µm
p-t
C
10-6
10-5
10-4
10-3
10-2
Concentration (M) of ferulic acid dissolved in water

Fig. 11.6.

Stomatal behaviour in a variety of isolated leaf tissues of Commelina communis when floated on distilled water.

The tissues were all removed 1 h before the commencement of the photoperiod and floated on distilled water for 3 h at 137 J m^{-2} s^{-1}.

From this preliminary study it can be seen that optimal response was achieved from lower epidermal semi-leaf discs floated in a carbon dioxide-free atmosphere, closely followed by whole leaf discs under the same conditions. Isolated epidermal strips do not open on distilled water at all.

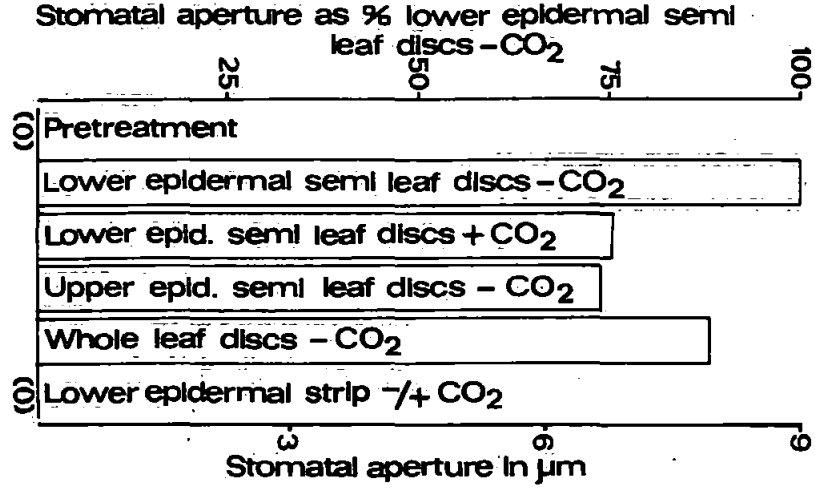

Stomatal aperture as % lower epidermal semi leaf discs – CO_2
25
50
75
100
(0)
Pretreatment
Lower epidermal semi leaf discs – CO_2
Lower epid. semi leaf discs + CO_2
Upper epid. semi leaf discs – CO_2
Whole leaf discs – CO_2
Lower epidermal strip –/+ CO_2
(0)
3
6
9
Stomatal aperture in µm

Fig. 11.7.

Effect of abscisic acid on stomatal opening and ion-adsorbent bodies in Tradescantia pallidus.

Each point represents the mean of 90 stomatal apertures (30 x 3 replicates); the vertical bars represent standard error.

The micrographs, above each treatment, indicate the state of the ion-adsorbent bodies after the 4 h incubation period as determined with the modified Macallum technique.

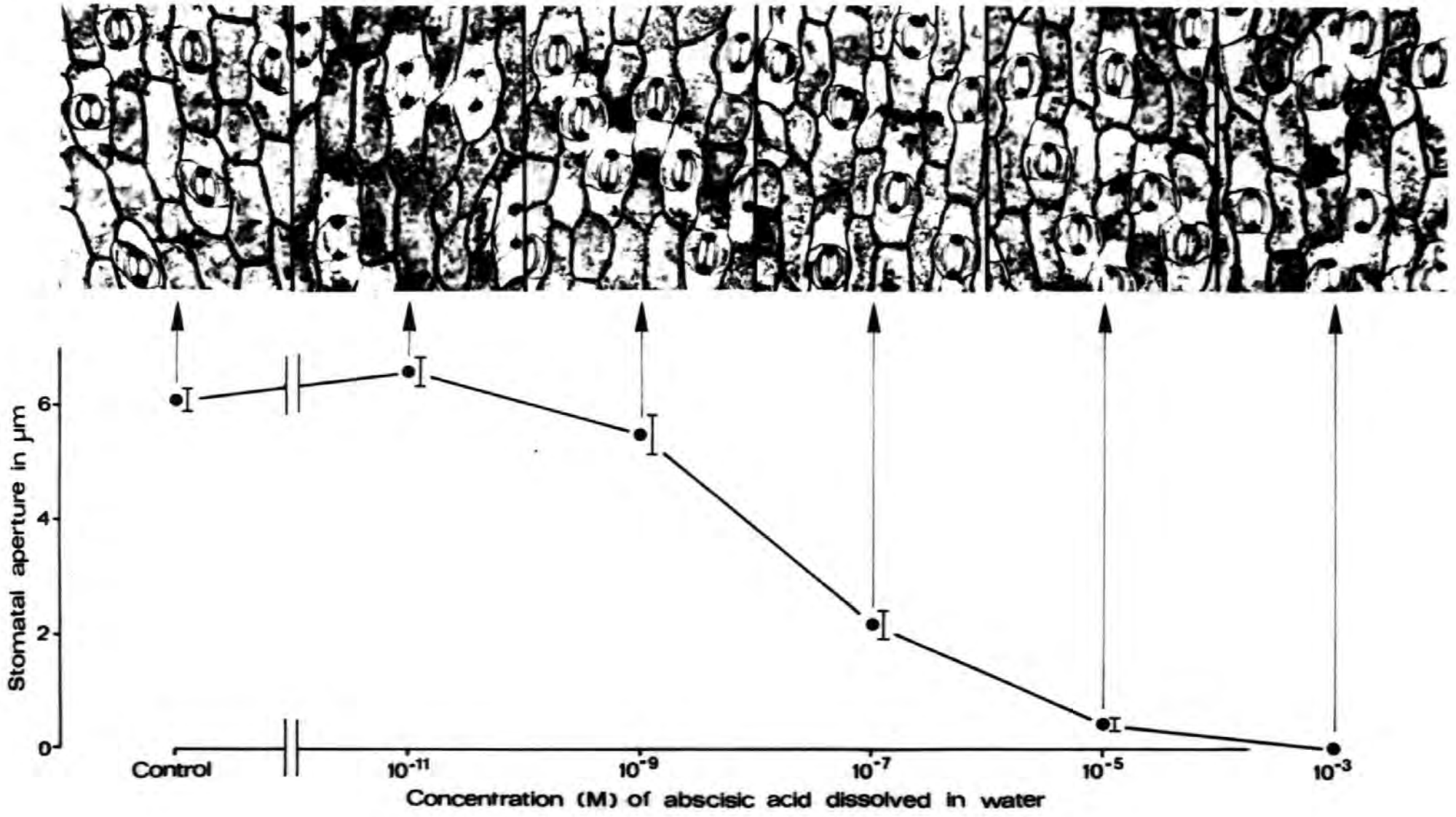

Stomatal aperture in µm
0
2
4
6
Control
10-11
10-9
10-7
10-5
10-3
Concentration (M) of abscisic acid dissolved in water

Fig. 11.8.

Sample viscous flow porometry results from Commelina communis.

The figure records the stomatal responses of stomata from four separate plants simultaneously for a period of 7,5 d following a 5 d entraining period. The entrained (subjective) light regime of 10 760 lx / 37 J m^{-2} s^{-1} between 10.00 and 24.00 h B.S.T. was maintained during the experimental period (black and white bar along the base of the figure) although different light regimes were imposed on the actual plants as indicated by the black shading in the body of the figure. R.H. was maintained at 60%, and temperature was constant at 20°C. The stomatal response of each leaf (a, b, c, and d) is expressed in both terms of percentage conductance (upper traces) and percentage resistance (lower traces).

Features exhibited by the traces include night opening, a characteristic overshoot on stomatal opening prior to the achievement of steady state opening, and circadian cycling under both continuous light and dark treatments. These features are described in the body of the thesis.

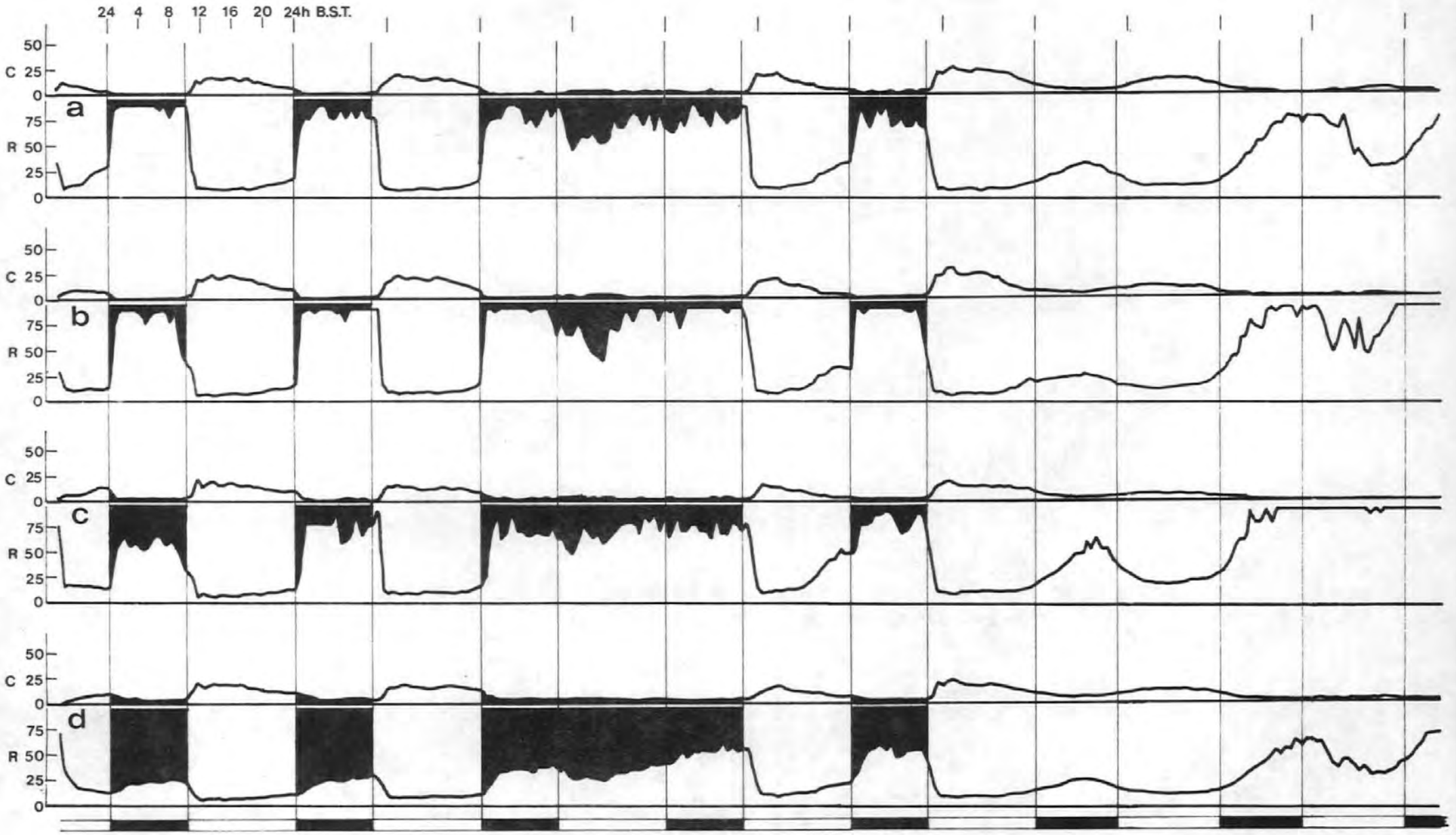

24 4 8 12 16 20 24h B.S.T.
C
R
50
25
0
75
a
b
c
d

Plate 11.1.

Heavy metal uptake by isolated epidermes of Tradescantia pallidus, I.

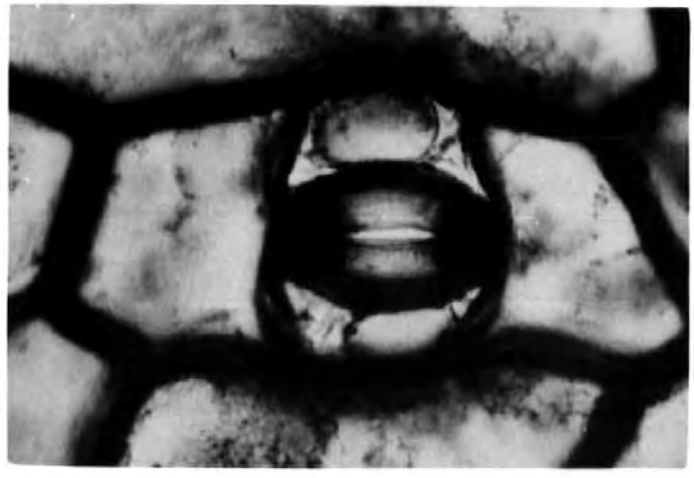
A. $NiSO_4$

B. $FeSO_4$

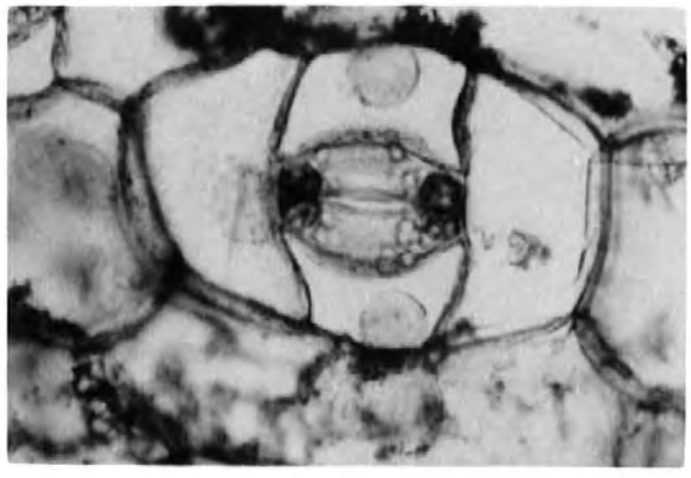
C. $Pb(NO_3)_2$

Light micrographs, x 550.

A



B



C

Plate 11.2.

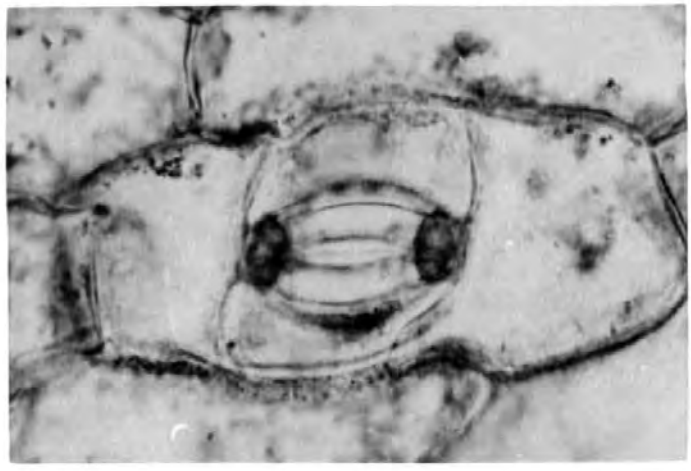
Heavy metal uptake by isolated epidermes of Tradescantia pallidus, II.

A. $CuSO_4$

B. $HgCl_2$

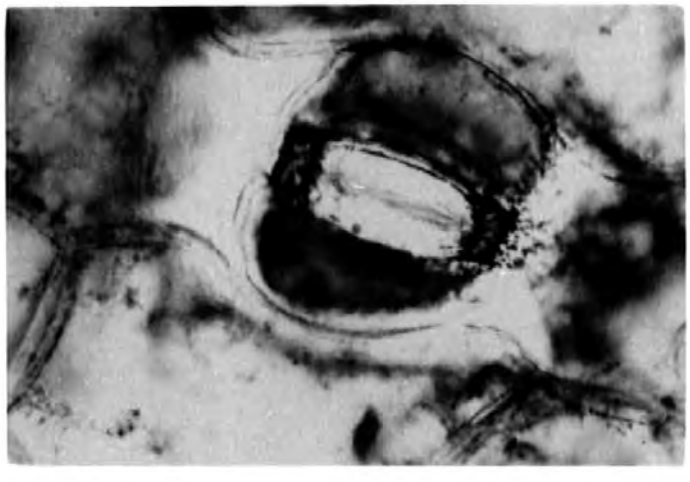
C. $AgNO_3$

Light micrographs, x 550.



A

B



C

Plate 11.3.

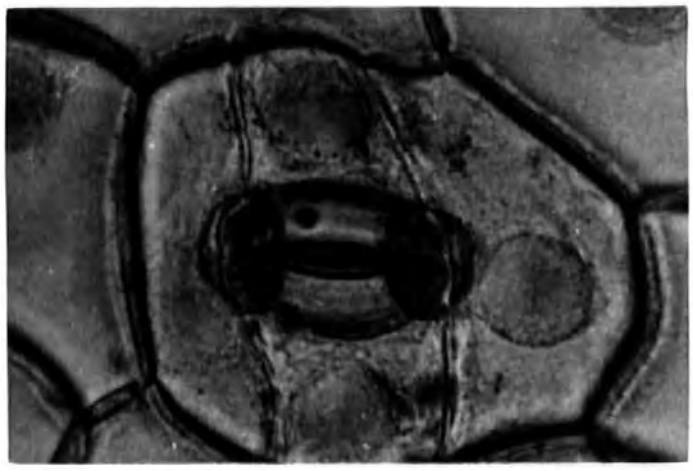
Heavy metal uptake in the transpiration stream of Tradescantia x andersoniana, I.

A. $HgCl_2$

B. $AuCl_3$

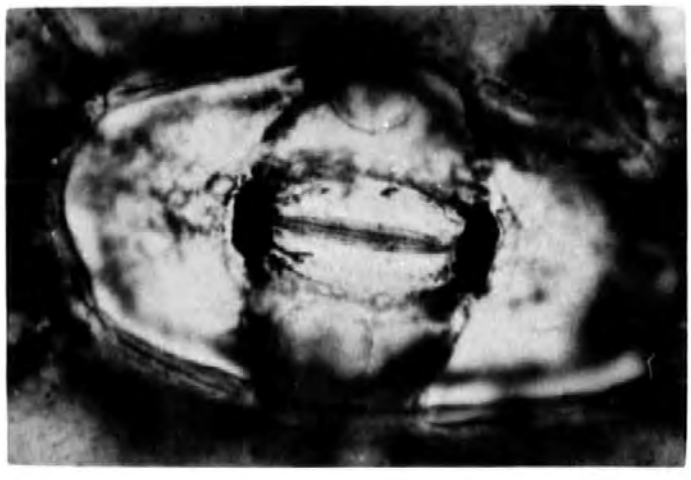
C. $AgNO_3$

Light micrographs, x 550.



A

B



C

Plate 11.4.

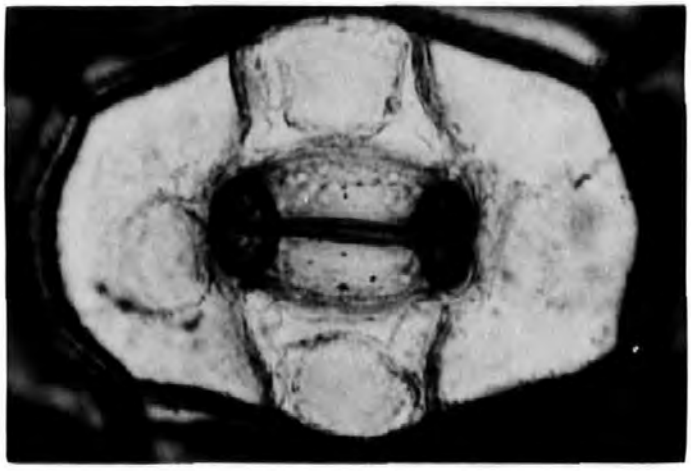
Heavy metal uptake in the transpiration stream of Tradescantia x andersoniana, II.

A. $Pb(NO_3)_2$

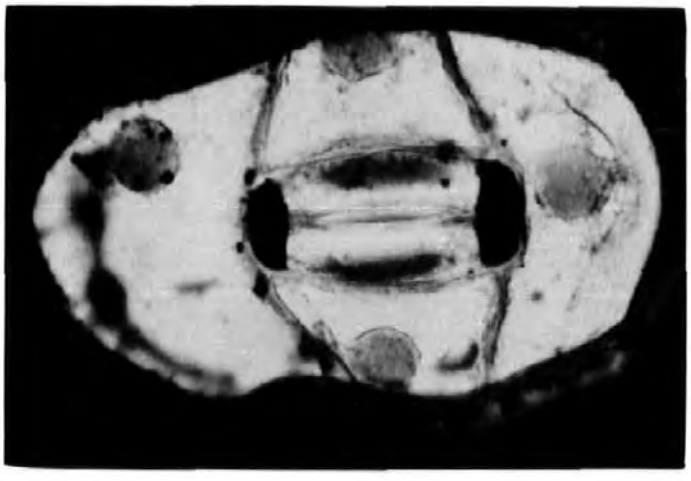
B. $NiSO_4$

C. $CuSO_4$

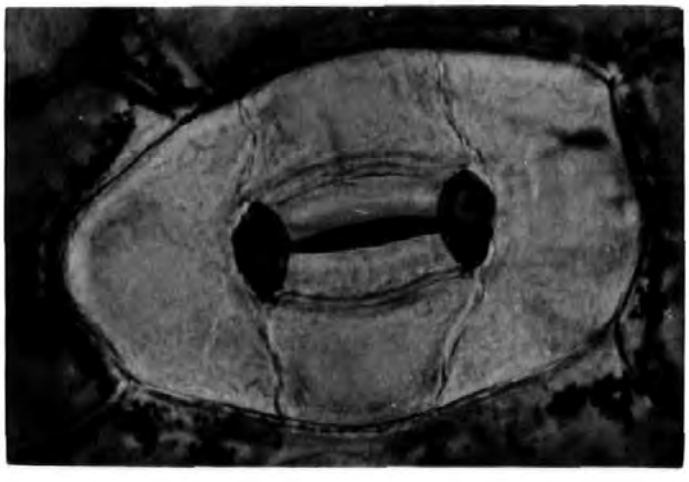
Light micrographs, x 550.



A



B



C

CHAPTER 12

X-RAY MICROANALYSES

INTRODUCTION

In view of the fact that substomatal ion-adsorbent bodies can adsorb a wide variety of cations non-specifically (Chapter 11), and that the Macallum stain is based on cobalt, which is known to become readily adsorbed onto the sites, it was decided to analyse potassium levels in the bodies and surrounding tissues by electron microprobe analyses. It was also decided to use this technique to establish whether the bodies can adsorb anions, notably chloride, which has been implicated in stomatal ion fluxes (Raschke & Fellows, 1971; Willmer & Pallas, 1974; Penny et al., 1976; Raschke, 1976). Like potassium, the standard histochemical stain for chloride is based on a cation, silver (Gersch, 1938; Gomori, 1952), which is readily adsorbed onto the bodies (Chapter 11).

The X-ray microanalyses were carried out on fresh material which was frozen in liquid nitrogen immediately before analysis (vide Chapter 2). It is considered that this very brief preparative procedure minimises ion loses when compared with the preparative procedures involving complete dehydration of the tissues as employed by Sawhney & Zelitch, 1969; Humble & Raschke, 1971; Willmer & Pallas, 1974, and Dayanandan & Kaufman, 1975.

EXPERIMENTAL

Ion adsorbent capacity of ion adsorbent bodies

Experiment 12.1. The capacity of ion adsorbent bodies to adsorb cobalt, potassium, and chloride ions from an incubating medium.

This was a similar experiment to Experiment 11.14 (in part) except that the epidermal strips were incubated on 5% cobalt chloride and potassium chloride for ca. 15 minutes under a 100 W tungsten lamp. After incubation, the strips were mounted with the side adjacent to the mesophyll uppermost on an aluminium stub and analysed on the cryostage.

Spectral analyses of K_α emissions obtained are presented in Figs. 12.1, and 12.2. The ion-adsorbent structures become very rich in chloride after the cobalt chloride treatment, whilst it is present within the guard cells in barely detectable quantities. There is no appreciable difference in the cobalt levels within the ion-adsorbent bodies and guard cell lumen after the cobalt chloride treatment. By comparison both potassium and chloride are taken up from the potassium chloride solution, but there are higher levels of both ions present at the ion-adsorbent sites than in the guard cells.

The results largely confirm the findings of Experiment 11.14, and also provide evidence that both potassium and chloride are adsorbed by the ion-adsorbent bodies. The failure to demonstrate preferential adsorption of cobalt by the polar bodies, as is found with histochemical staining, is not unexpected since the beam voltage (15 KeV) is below the optimal level required to stimulate K_α emission by cobalt. It is interesting that the chloride, in potassium chloride, is preferentially adsorbed by the polar structures whilst it is, apparently, not taken up

into the guard cells, although no explanation for this can be offered at present. These analyses were replicated on different epidermal strips and similar results were obtained.

Experiment 12.2. The capacity of the ion-adsorbent bodies to adsorb cobalt and chloride from the transpiration stream.

As in Experiment 11.15A, leaf material was incubated in 5% (w/v) cobalt chloride for ca. 90 minutes under a 100 W tungsten lamp. An epidermal strip was then removed and analysed on the cryostage.

Spectral analyses of K_α emissions are presented in Fig. 12.3. The results are almost identical to those obtained in Experiment 12.1 (c.f. Fig. 12.1). Again, chloride does not appear to be taken up into the guard cells, but, unlike the spectra obtained in Experiment 12.1, there does appear to be significantly more cobalt present in the polar structures than in the guard cells.

Experiment 12.3. The relationship between cobalt and potassium levels in Macallum-treated epidermal strips.

Epidermal strips from light-exposed P. vulgare pinnae were treated with 7,5% (w/v) modified Macallum stain (Chapter 2) and stored overnight in absolute ethanol before analysis on the cryostage the following morning. Three stomatal complexes were analysed at four locations, viz. the polar structures, the walls of the stoma, the body of the guard cell, and the body of the subsidiary cell.

The results are presented diagrammatically in Fig. 12.4, in which the actual cobalt and potassium peaks are reproduced exactly, but have been cut off basally at the bremsstrahlung (background noise

level). The relative amounts of the elements present correspond to the area under the peaks and not the height of the peaks. Direct comparison between the absolute amounts of cobalt and potassium present are not permissible, since they have different optimal excitatory levels.

Relatively speaking, the amount of potassium in the body of the guard cells greatly exceeds that of cobalt; the cobalt exceeds the potassium in the polar structures and subsidiary cells (except D_3); the amounts of potassium and cobalt are subequal in the lips of the stoma. Most cobalt is found in the ion-adsorbent structures and subsidiary cells, and the least in the body of the guard cells. The distribution of potassium is exactly the converse, with most occurring in the body of the guard cell and the least in the polar structures and subsidiary cells.

These results are extremely difficult to interpret. It must be emphasised that the triple salt resulting from the Macallum treatment (vide Chapter 2) is virtually insoluble in absolute ethanol, whilst any free potassium remaining in the tissue after staining would probably have been leached out during storage in ethanol. It could be argued that the polar bodies and subsidiary cells have been 'drained' of potassium as it was taken up by the guard cells during stomatal opening. The moderate levels of potassium in the region of the the differentially-thickened ridges of the stoma may indicate a store of the ion bound either naturally or as the triple salt in the apoplast of the cell wall where it would be readily available for uptake into the guard cells when required. The high cobalt levels in the polar structures are consistent with the results of previous experiments (Experiments 11.14, and 11.15). No correlation can be found between the relative levels of cobalt and potassium and the ratios between the two varies with the site of analysis. It is not possible, therefore, to evaluate

the specificity of the Macallum stain for potassium as had been hoped. More meaningful results could be obtained using freshly stained epidermes. However, even then, interpretation would be difficult as there are probably other exchange sites within the stomatal complex which are capable of adsorbing cobalt (e.g. starch grains; Plate 4.15).

X-ray analyses of fresh stomatal complexes

Experiment 12.4A. The distribution of elements in the guard cell complex of fresh tissue, I.

Microanalyses were performed on the ion-adsorbent body and body of the guard cell illustrated in Plate 5.8A. The plant from which the tissue was taken had been exposed to normal sunlight between 06.30 and 10.00 h B.S.T., but subsequently had been kept in very subdued light of the electron microscope suite for 2 h prior to examination on the cryostage. The tissue had been kept in subdued light since, at the time, a search was being made for ion-adsorbent bodies and microanalyses had not been envisaged. The stomata were presumed to be almost closed judging from Plate 5.5, which was taken from the same plant just prior to electron probe microanalyses.

The K_α emission spectra obtained are illustrated in Fig. 12.5. The most noticeable feature is that the ion-adsorbent body is particularly rich in potassium compared with the guard cell body which contains relatively little. This is to be expected if the ion-adsorbent bodies act as the immediate sink/source of the potassium required for stomatal movements. If the assumption that the stomata were virtually closed is correct, it is likely that the potassium which

had been taken up by the guard cells during earlier opening between 06.30 and 10.00 h, would have been released from the guard cells during closure in the subdued light conditions prevailing in the electron microscopy suite. The fact that potassium is present in considerable quantities in the ion-adsorbent bodies could indicate that this structure is the immediate sink for potassium during stomatal closure.

Experiment 12.4B. The distribution of elements in the guard cell complex of fresh tissue, II.

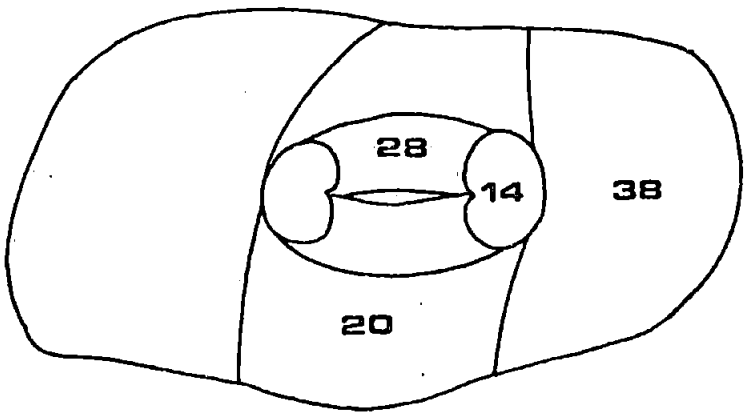
This is a repeat of the previous experiment, but carried out on plant tissue which had been given a light pretreatment (i.e. it had been placed on a window sill in the electron microscopy suite). The tissue was analysed from the internal surface aspect.

The relevant emission spectra from the polar bodies, body of the guard cell, polar subsidiary cell, and lateral subsidiary cell are illustrated in Fig. 12.6. No definite conclusions can be drawn about the role of light-stimulated ion fluxes associated with stomatal opening from these isolated spectra, especially since the tissue had been subjected to such a crude pretreatment. Significantly, the only element which seems to vary appreciably between the different probe areas is potassium. The percentage distribution of potassium between the four sites analysed is illustrated diagramatically in Fig. 12.7, overleaf.

From this figure, it can be seen that the potassium levels in the polar subsidiary cells > guard cells > lateral subsidiary cells > ion-adsorbent sites. It is assumed that the stomata were open at the time of analysis, and that the ion-adsorbent bodies act as the

Fig. 12.7.

The percentage distribution of potassium in the stomatal complex analysed in Fig. 12.6.



immediate sink/source for potassium during stomatal movements, the following interpretation could be placed on these values.

Potassium could be taken up by the guard cells from the ion-adsorbent bodies to bring about a reduction of osmotic potential to cause the stomata to open. This would mean that the potassium level in the ion-adsorbent bodies would fall whilst that of the body of the guard cell will rise. The potassium levels of the lateral subsidiary cells are also lower than those of the guard cells, which means that the latter will have an osmotic advantage and can expand into the lumen of the subsidiary cell. The high potassium level found in the polar subsidiary cell could have a dual effect. In the first instance, since their osmotic potential is presumably lower than that of the guard cells, they could act to prevent any tendency for the guard cells to distend longitudinally and could, in addition, enhance the lateral

expansion of the guard cells into the lateral subsidiary cells by applying a positive pressure to the polar cell walls of the guard cells. Secondly, it is becoming apparent that the polar subsidiary cells are particularly important in the stomatal mechanism. In Chapter 3, it was shown that the trabeculae connect the polar bodies to the polar subsidiary cells, and that cobalt-rich (indicative of potassium ?) vesicles occur in these cells which have been observed in direct contact with the region of the ion-adsorbent body.

These electron microprobe analyses confirm that the polar subsidiary cells are particularly rich in potassium. Therefore, these cells may be an ultimate source/sink of potassium involved in stomatal movements. It has already been established (Chapter 4) that it is very unlikely that the guard cells are symplastically continuous with the subsidiary cells. If there is a potassium flux between the guard and subsidiary cells, then it must involve an apoplastic pathway. It does seem plausible, therefore, that the ion-adsorbent bodies act as an immediate sink/source for the potassium involved in stomatal movements. Their strategic position, next to the potassium-rich polar subsidiary cells, suggests that excess potassium accumulating at the bodies during stomatal closure could be passed to the polar subsidiary cells. During stomatal closure, the ion-adsorbent bodies could retain a reservoir of potassium which could provide the ions required for 'night-opening' (vide Porometry - Chapter 11). Similarly they could provide the potassium required for normal stomatal opening, so that as the bodies become depleted of potassium during opening, they could be replenished with ions from the vesicular system via the trabecular system of the polar subsidiary cells. This interpretation is not proven, but is consistent with the state of the potassium distribution

observed in the present X-ray microanalyses. Such an interpretation would establish the functional significance of the ion-adsorbent sites as a reservoir of ions for stomatal movements.

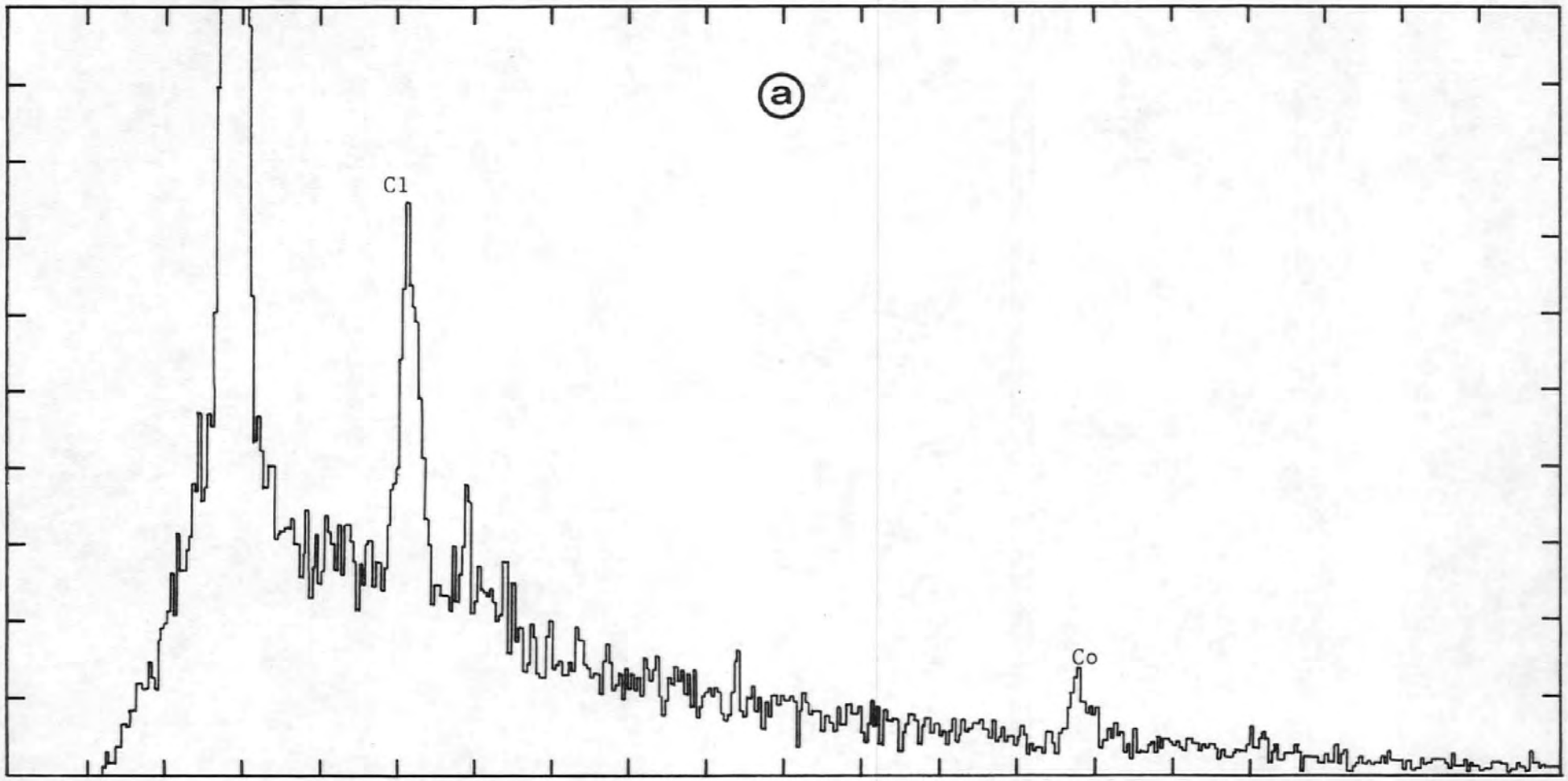
Fig. 12.1.

X-ray microanalyses of isolated epidermis of Tradescantia pallidus incubated on a 5% solution of cobalt chloride.

Analyses were carried out at 15 KeV for 100 s; take off angle - 35°. The peak to the extreme left of the spectra is of extraneous aluminium.

a. Spot analysis on ion-adsorbent body.

b. Spot analysis on guard cell.


ⓐ
Cl
Co

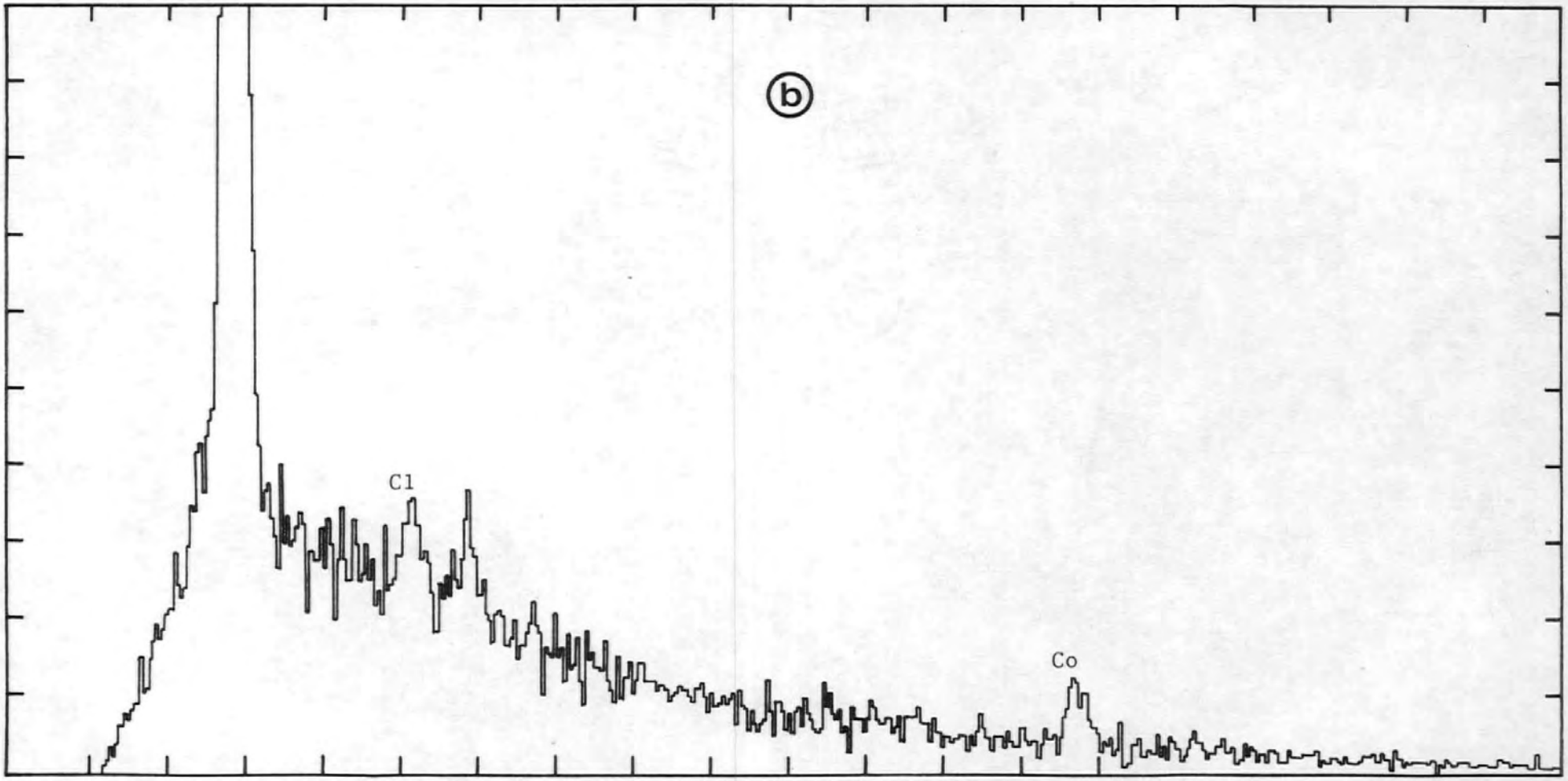

b
Cl
Co

Fig. 12.2.

X-ray microanalyses of isolated epidermis of Tradescantia pallidus incubated on 5% solution of potassium chloride.

Analyses were carried out at 15 KeV for 100 s; take off angle - 35°. The peak to the extreme left of the spectra is of extraneous aluminium.

a. Spot analysis on ion-adsorbent body.

b. Spot analysis on guard cell.

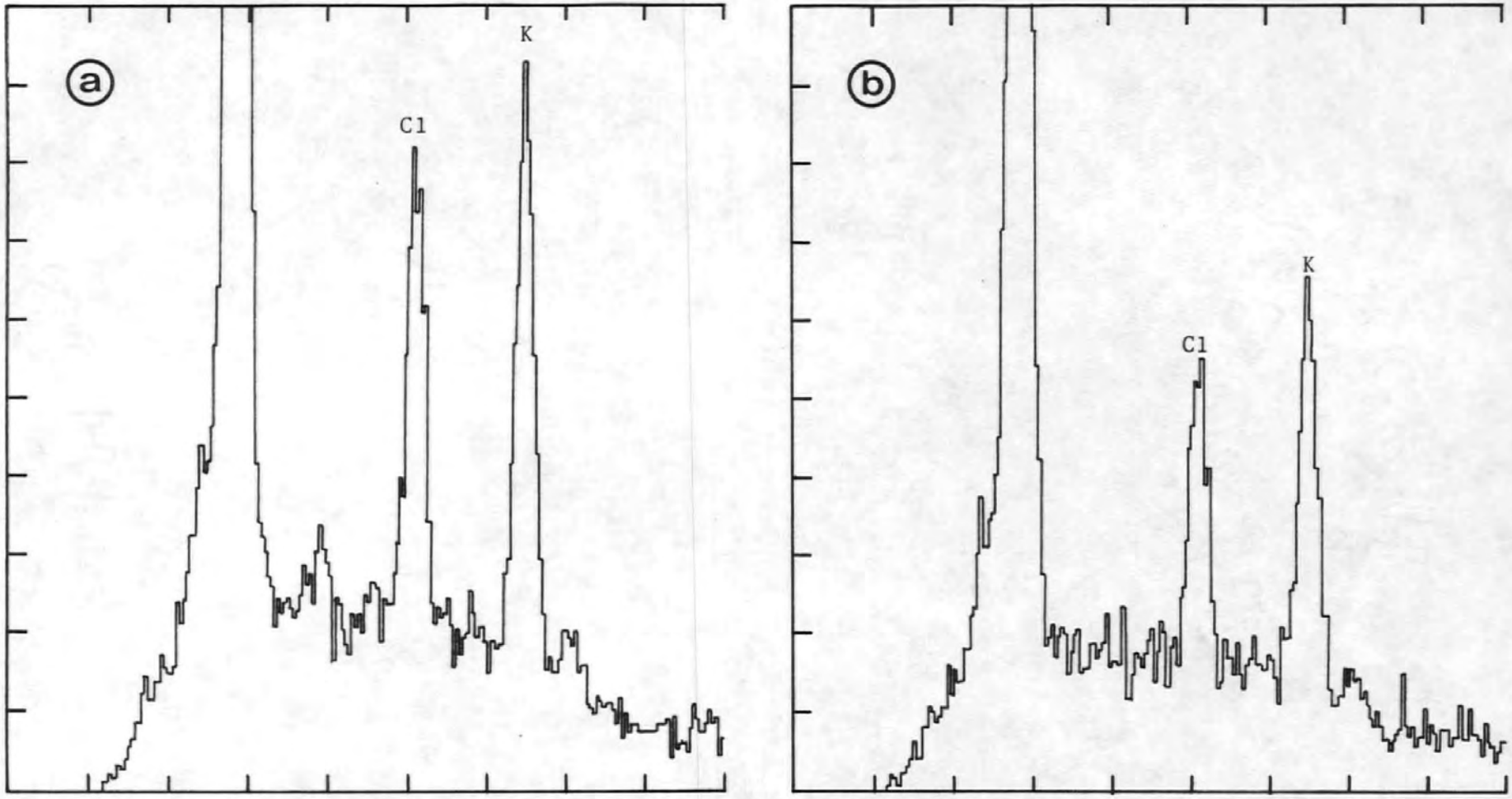

a
Cl
K
b
Cl
K

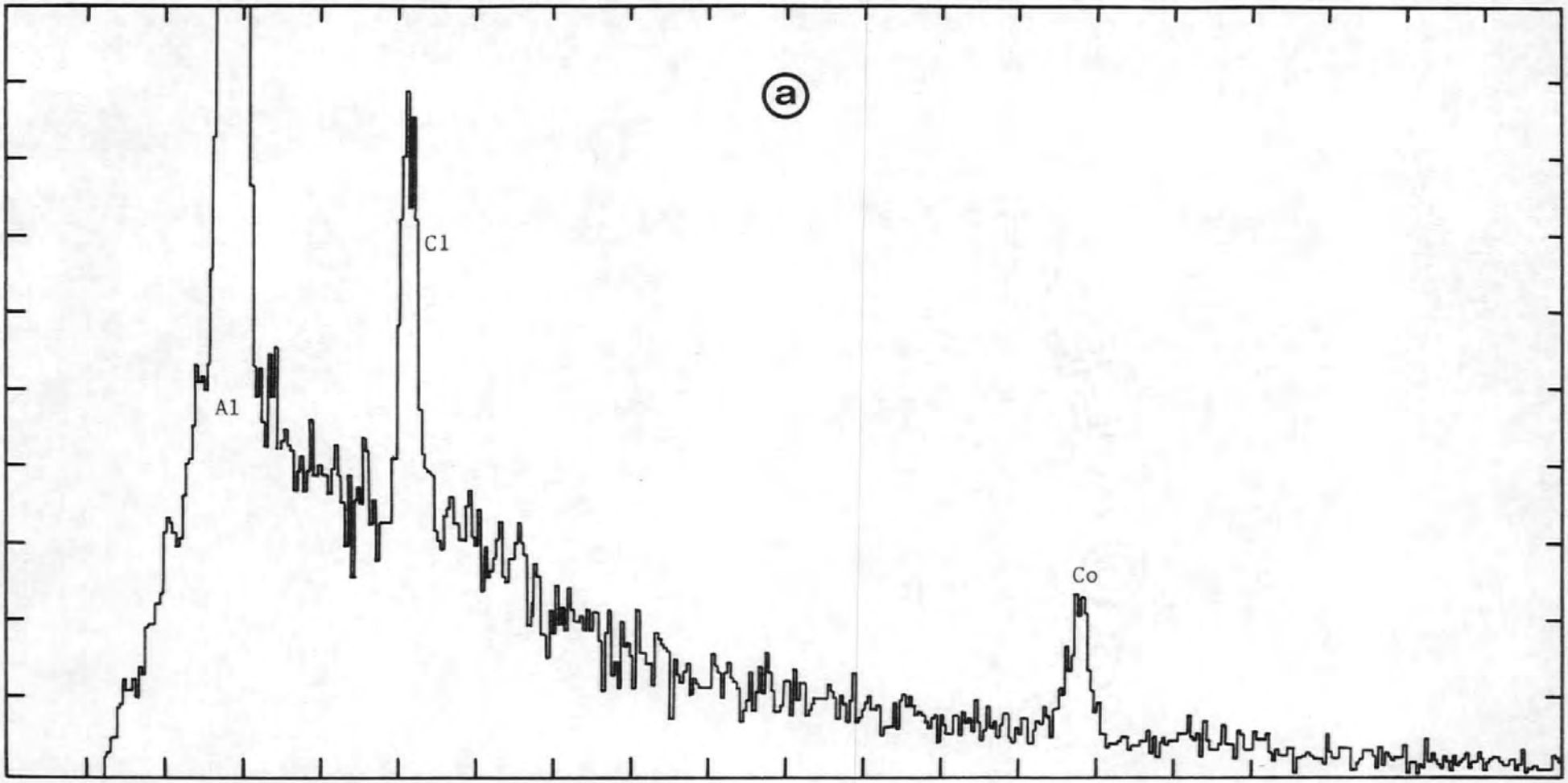
Fig. 12.3.

X-ray microanalyses of isolated epidermis of Tradescantia pallidus fed with 5% cobalt chloride through its transpiration stream.

Analyses were carried out at 15 KeV for 100 s; take off angle - 35°.

a. Spot analysis on the ion-adsorbent body.

b. Spot analysis on guard cell.


a
A1
C1
Co

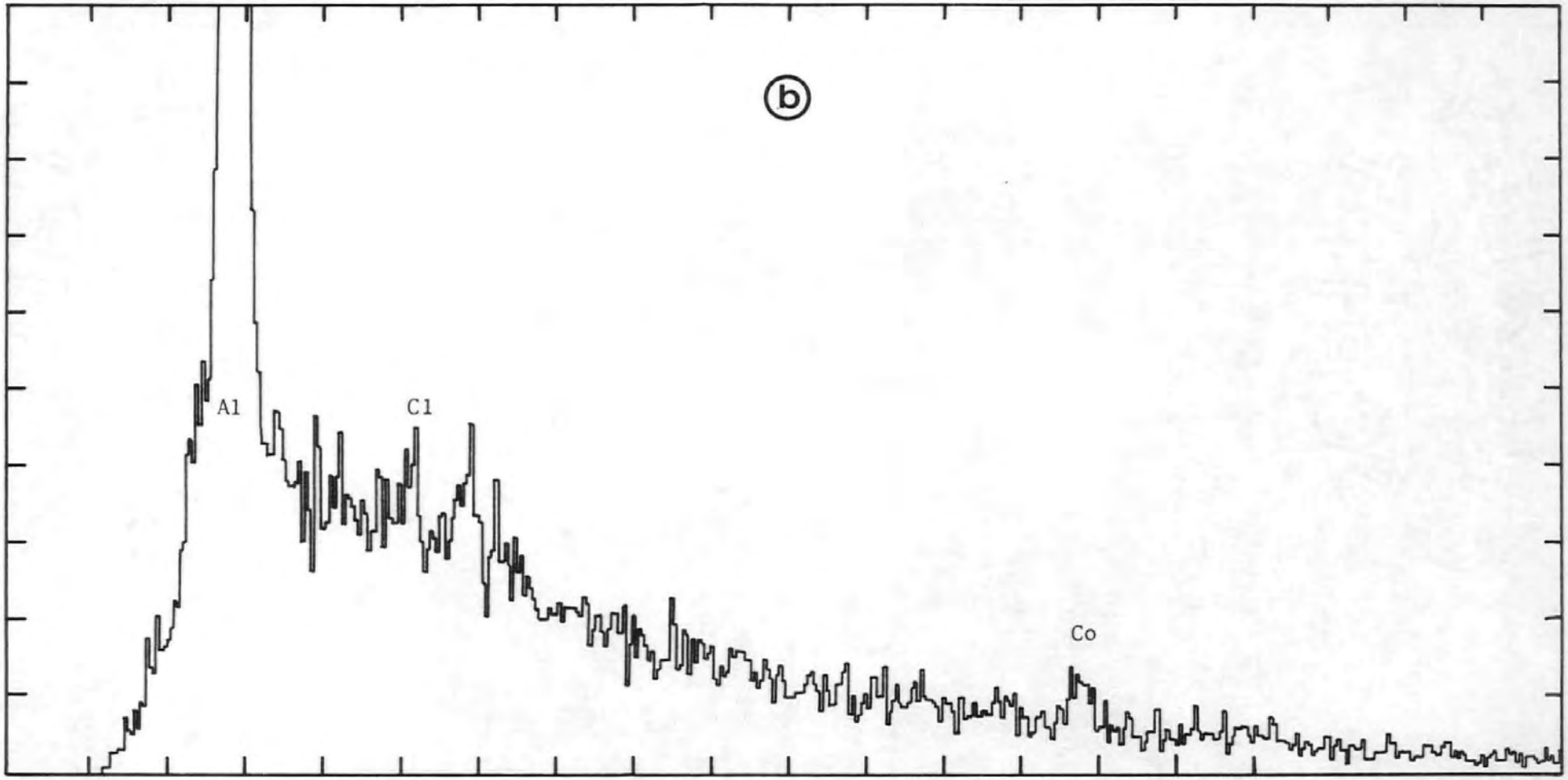

b
A1
C1
Co

Fig. 12.4.

X-ray analyses of isolated epidermes of Polypodium vulgare treated with 7,5% modified Macallum stain.

The analyses are from four locations in the guard cell complex:

A - Walls of the stoma,

B - Body of the guard cell,

C - Ion-adsorbent body, and

D - Subsidiary cell.

The subscript numeral following the above codes relates to the sample number - three complexes were analysed at 18 KeV for 100 s with a take off angle of 35^{o}.

The peaks have been cut off at the bremsstrahlung basally. The cobalt peaks are black; the potassium peaks white. The cobalt and potassium peaks are not directly comparable since they have different critical excitation levels.

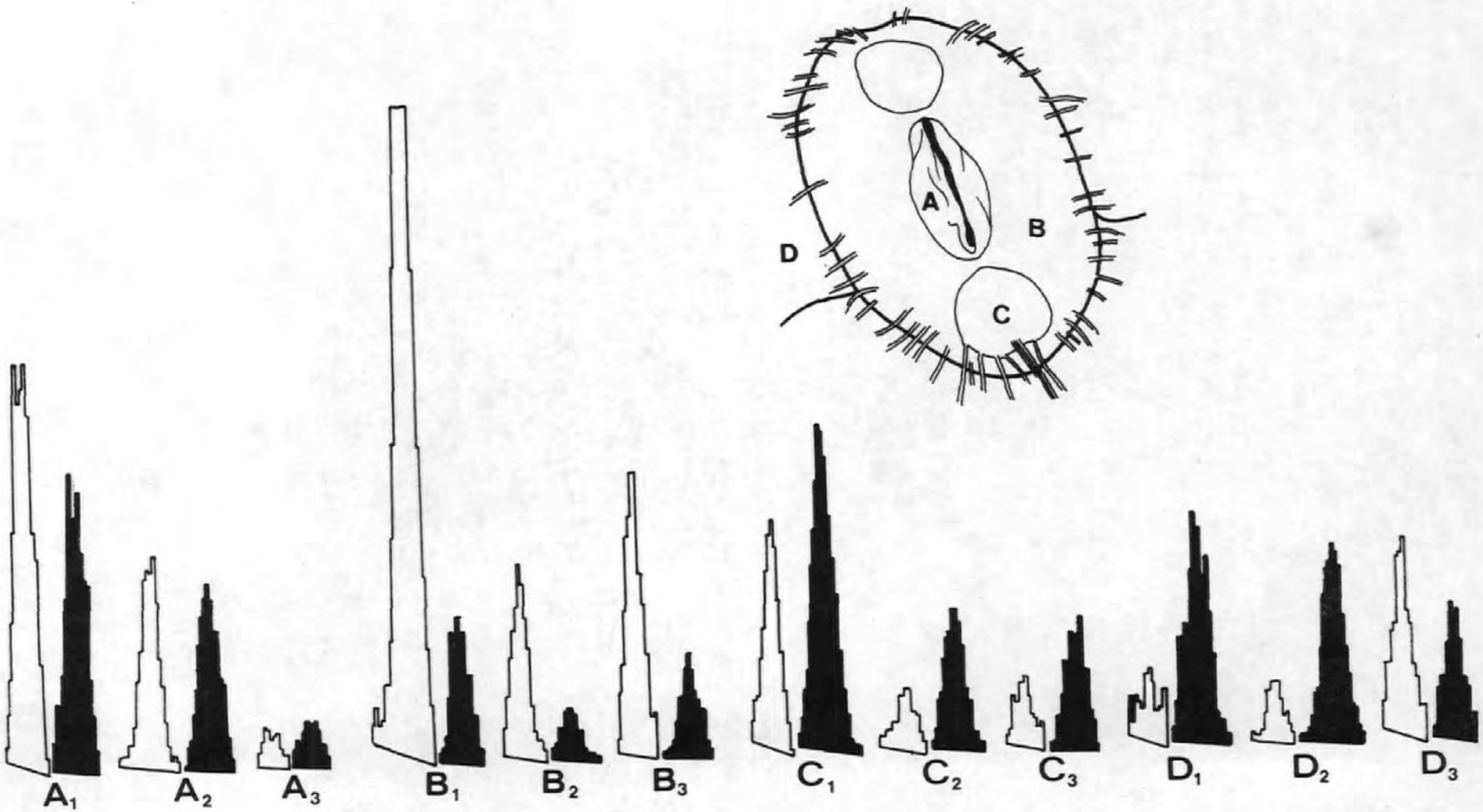

A
B
C
D
A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

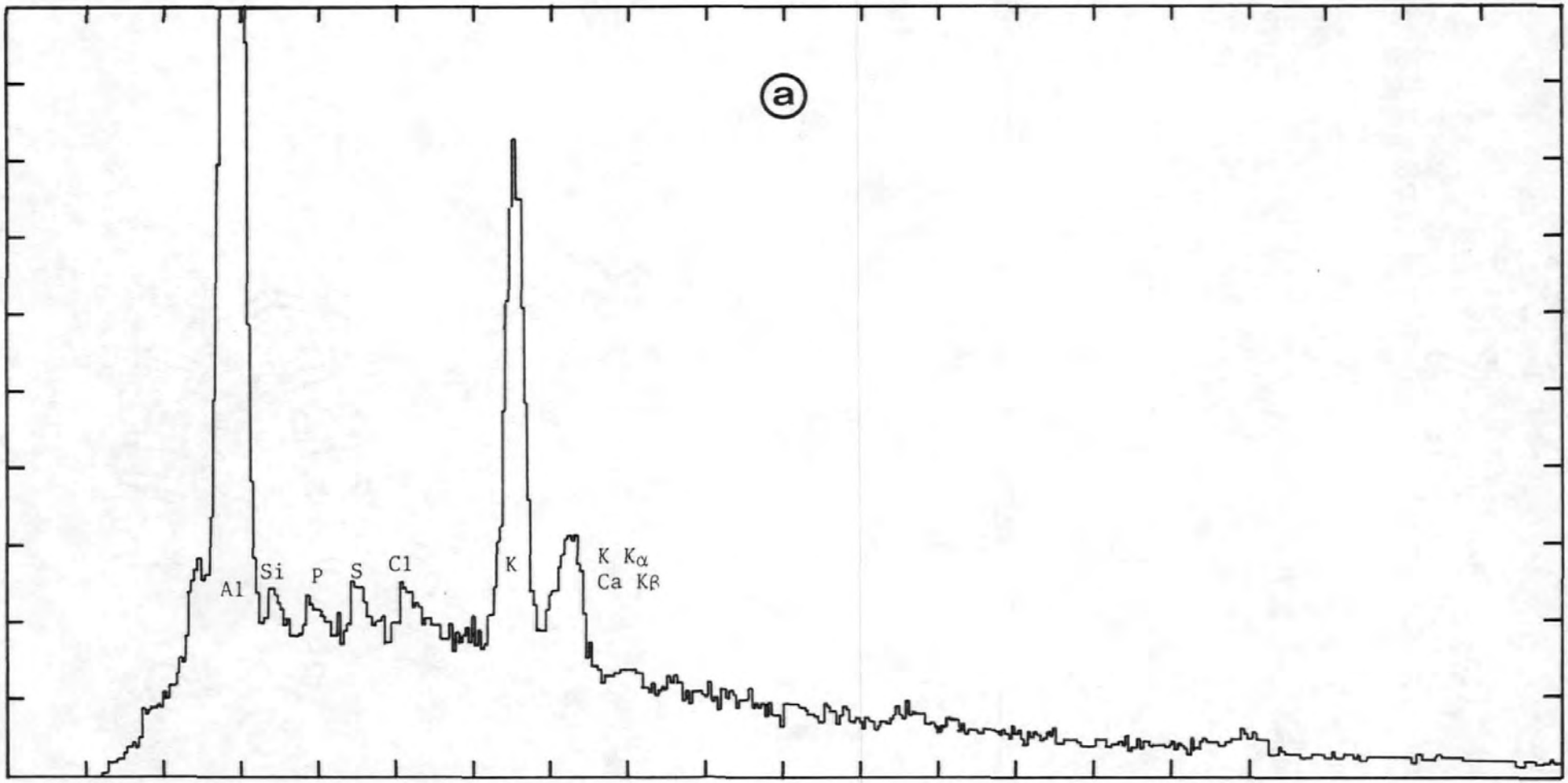
Fig. 12.5.

X-ray analyses on fresh stomatal complex of Tradescantia pallidus, I.

This is a spectral analysis of the specimen illustrated in Plate 5.8A. Analyses were carried out at 15 KeV for 100 s; take off angle - 35^{o}. The aluminium peaks are of extraneous origin.

a. Spot analysis of the ion-adsorbent body.

b. Spot analysis of the guard cell.


a
Al
Si
P
S
Cl
K
K Kα
Ca Kβ

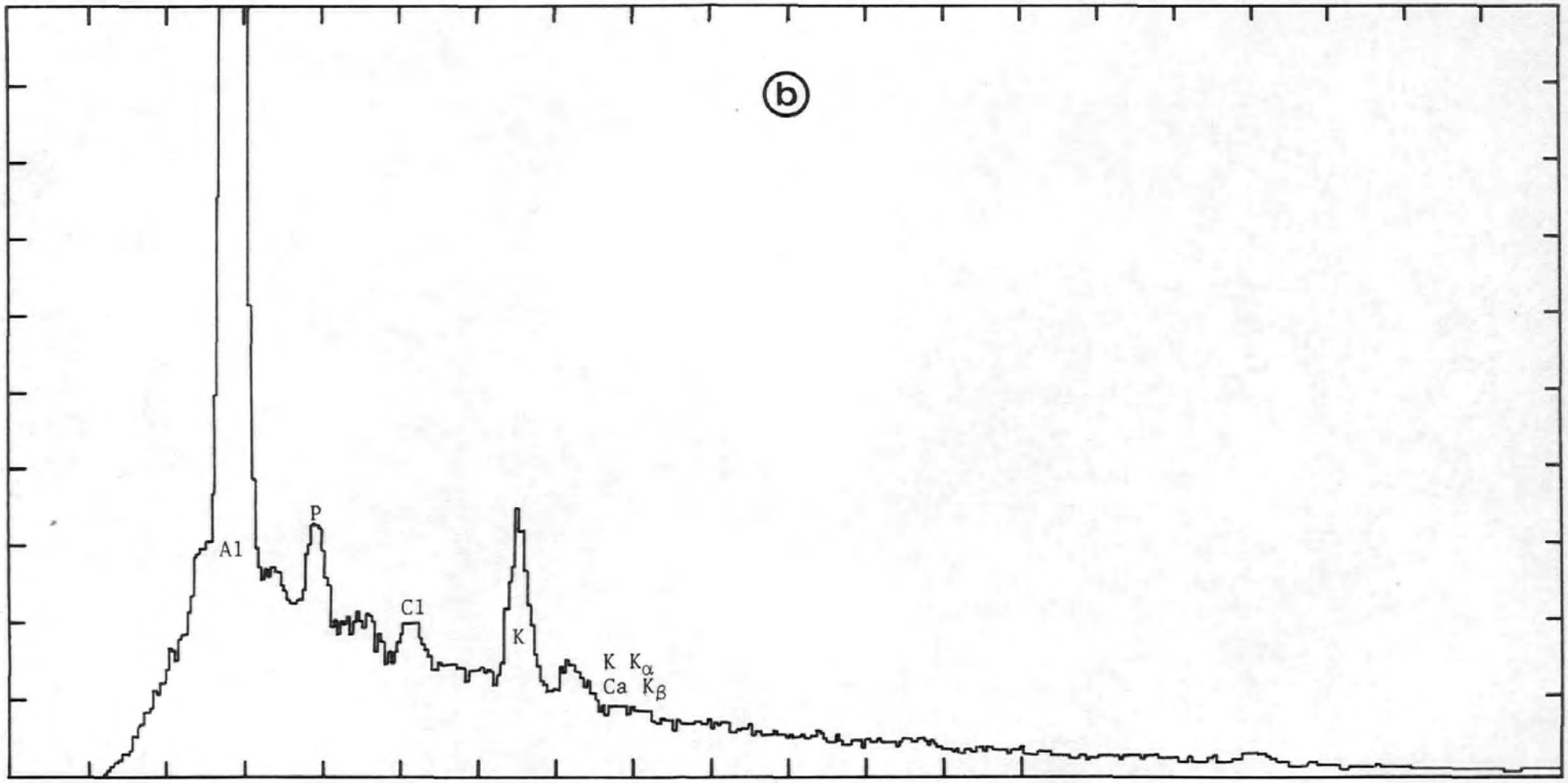

b
Al
P
Cl
K
K Kα
Ca Kβ

Fig. 12.6.

X-ray analyses on fresh stomatal complex of Tradescantia pallidus, II.

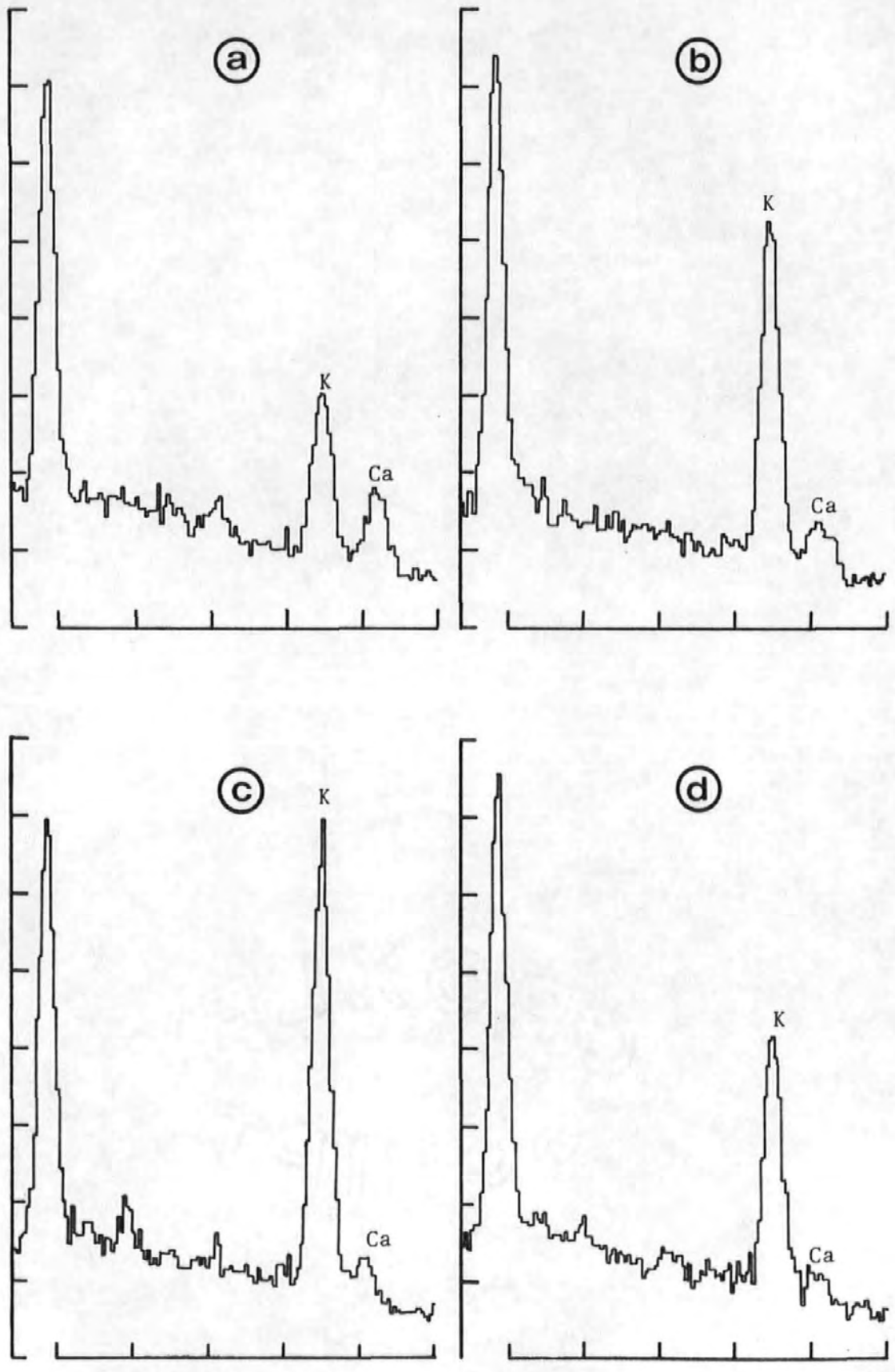
Analyses were carried out at 15 KeV for 100 s; take off angle - 35°.

a. Ion-adsorbent body.

b. Guard cell.

c. Polar subsidiary cell.

d. Lateral subsidiary cell.


a
K
Ca
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CHAPTER 13

GENERAL DISCUSSION ON SUBSTOMATAL ION-ADSORBENT BODIES

The work presented in this thesis has involved a wide range of techniques which have been directed towards the elucidation of the structure and function of localisations of Macallum's histochemical stain for potassium (Macallum, 1905) at the poles of guard cell complexes in certain plants.

The anatomical and morphological studies indicate that the polar localisations of the Macallum stain are consistent with the presence of an extracellular body situated between the guard cell wall and the underlying endocuticle on the inner face of the guard cell complex. The gross morphology of the polar structures has been shown to differ between Polypodium vulgare and Tradescantia pallidus, and a brief survey of the plant kingdom indicates that further types may exist in Dianthus and Maranta. In P. vulgare, the polar body is attached to the external face of the lower periclinal walls of the guard cell complex, at either pole, so that it lies below the complex and is covered by the endocuticle. In Tradescantia spp. the polar body does not appear to be associated with any particular cell wall, but is located within the intercellular space between the pole of the guard cell complex and the adjacent subsidiary cells. As in P. vulgare, the body is covered beneath by the endocuticle. An additional feature found in Tradescantia is that the lower periclinal walls of the guard cell complex contain a system of intramural lamellar spaces which appear to

be filled with a potassium-rich medium. In both species, the polar bodies are associated with endocuticular trabeculae which may act as apoplastic channels linking the guard cells with the surrounding epidermal tissue. These structures are of particular interest since no evidence has been found to show that the guard cells are connected symplastically to adjacent cells with plasmodesmata. Consequently, they may play an important role in intercellular transport to and from the guard cell complex.

Ultrastructural studies indicate that, in P. vulgare, the polar bodies are of a hollow sac-like structure whose walls are contiguous with the outer layers of the cell wall. A brief investigation into the chemical nature of the bodies confirms this view and suggests that they are largely pectinaceous. This is substantiated by a study of the morphogenesis of the bodies in P. vulgare in which they were found to be formed at an early stage of stomatal ontogeny and appear to result from the migration of superficial elements of the developing lower periclinal cell walls.

Functional studies on the polar bodies indicate that they are capable of non-selectively adsorbing a variety of ions of different valencies. It is because of this characteristic that the structures are referred to as substomatal ion-adsorbent bodies. The close proximity of the bodies to the stomatal complex means that they must have at least an indirect effect on transpiration and the stomatal mechanism. The bodies are capable of adsorbing certain ions from the transpirational stream when the ions are supplied at supra-physiological levels. Their involvement in the stomatal mechanism is still unsubstantiated since it has been impossible to correlate stomatal

movements with potassium levels in either the ion-adsorbent bodies or the guard cells using either the basic Macallum stain (Macallum, 1905) or its modified form (Chapter 2). Preliminary investigations on the potassium levels using X-ray microanalysis are more promising. Using this technique it was possible to show that the polar bodies adsorb potassium and chloride ions from a bathing medium and/or the transpiration stream (Chapter 12).

As redundancy is rare in nature it is possible that the ion-adsorbent bodies described here fulfil some function(s) in the plants which possess them. Their intimate association with the guard cell complex suggests some involvement in stomatal movements. Although considerable information has been obtained on the physical and chemical nature of the ion-adsorbent bodies in the present study, few of the observations relate directly to their physiology and this obviously makes it difficult to produce a definitive model for their function. Undoubtably a plethora of plausible models could be produced and by judicial, or biased, use of the literature some evidence to support these models could be produced. Such an exercise is pointless and, therefore, only three models, which at least seem plausible have been selected for detailed consideration.

HYPOTHETICAL ROLE AS HUMIDITY SENSORS

The stomata of P. vulgare have been shown to respond very rapidly to changes in humidity (Losch, 1977). Losch predicts that the guard cells in this species possess some system capable of acting as a very sensitive humidity sensor. It is possible that the bodies described here are the morphological expression of such a system.

This could act in the following way. Transpirational water loss is known to be maximal from areas immediately adjacent to the stomata (Meidner, 1975; Sheriff & Meidner, 1975; Byott & Sheriff, 1976). The topography and the physical state of the bodies, which are located in this critical position, could accentuate such loss making them prime sites for transpiration. This is perhaps substantiated by the ice deposits observed at these sites (Plate 5.7B).

Under conditions of low relative humidity, high evaporative loss will lead to the concentration and accumulation of solutes at these sites, which will tend to reduce, or at least delay, further transpirational loss. Thus even relatively small increases in overall evaporative loss could lead to a considerable increase in solute accumulation at these sites. Certainly these sites have been shown to accumulate many different solutes (Chapters 11 and 12). Such accumulation would cause the solute potential to fall in this critical region of the apoplast leading to osmotic loss by the guard cells and a tendency towards stomatal closure, decreasing further transpirational losses. Under conditions of high humidity, the above tendencies would be reversed.

By exaggerating changes in solute accumulation associated with changes in transpirational loss at these critical sites, the guard cells would be provided with a system capable of sensing small changes in humidity and triggering the rapid stomatal response observed by Losch (1977). Whether these bodies could act in a similar way in plants other than Polypodium is far from clear, but it could be anticipated that they would always tend to react to conserve water at times of high evaporative loss.

However, the above system assumes that the accumulating solutes are free to exert an osmotic effect. Adsorption by the bodies could effectively reduce, if not completely negate, the effect although it could be argued that selective adsorption of ions (potassium) known to stimulate opening could enhance stomatal closure under conditions of low humidity whilst selective release at higher humidities could support opening. The most serious critiscism of such a system is that the work carried out so far indicates that accumulation at these sites results only from adsorption and does not arise from a passive deposition accompanying evaporative loss. For example, preliminary investigations indicate that lead-EDTA chelate, which is uncharged, does not accumulate at these sites and unless future work can show that various uncharged solutes can be accumulated here, the above hypothesis must be discounted.

HYPOTHETICAL ROLE IN DETOXIFICATION

The capacity of ion-adsorbent bodies to adsorb a wide range of ions (Chapters 11 and 12) would tend to protect the delicate stomatal mechanism from possible harmful effects of potentially toxic ions carried in the transpiration stream, or adsorbed from foliar pollutant contact. This may be of considerable importance since several elements including cobalt (Meidner & Willmer, 1975), iron and tin (Harsh & Sen, 1974), and aluminium (Schnabl & Zeigler, 1975; Schnabl, 1976) have been shown to modify stomatal responses.

HYPOTHETICAL ROLE AS POTASSIUM RESERVOIRS

There is a considerable body of evidence implicating potassium in the stomatal mechanism (vide Chapter 1). Whilst Raschke

& Fellows (1971) conclude that the ion fluxes associated with stomatal movements are restricted to the guard and subsidiary cells in maize, and possibly other graminaceous types, the evidence of Penny & Bowling (1974) and Willmer & Pallas (1974) suggests that more extensive fluxes exist within the stomatal complex and surrounding epidermal cells in Commelina communis.

Ion movements between the guard and immediately adjacent epidermal cells must pass through the apoplast because of the absence of symplastic channels (i.e. plasmodesmata, Chapters 1 & 4). The apoplastic location of the ion-adsorbent bodies at the poles of the guard cell complex, places them in a strategic position for direct participation in the potassium fluxes associated with stomatal movements. Since the ion-adsorbent bodies are capable of holding considerable quantities of ions, including potassium (Chapter 12), their most likely function would be to act as dynamic reservoirs for the ions involved in these fluxes.

In the Commelinaceae, there may be at least two elements associated with stomatal ion fluxes. One, which has been evaluated by Penny & Bowling (1974) and Willmer & Pallas (1974), may be involved in long distance transport. The other, centred on the ion-adsorbent bodies, may be involved in short distance transport. The two elements probably work in concert, with the short distance element functioning within the framework of the long distance element; the ion-adsorbent bodies fulfilling the function of an ion reservoir. The advantage of such a system would be that the bodies act as an immediate sink/source for short distance ion fluxes into and out of the guard cells which would be particularly useful for transient stomatal movements. In

Vicia faba, which lacks substomatal ion-adsorbent sites, Raschke (1976) estimates that the apoplast of the epidermis is much too small to act as the total source of potassium to support stomatal opening but, in plants which possess them, the ion-adsorbent bodies would represent a considerable expansion to this apoplastic reservoir.

Concomitant with the potassium uptake required for stomatal opening, organic acids are imported or, more likely, synthesised within guard cells to maintain electroneutrality. Malate and aspartate are considered to be the most important counterions (Allaway, 1973; Pallas & Wright, 1973) with malate counterbalancing approximately 50% of the potassium in the open guard cells of Vicia faba (Allaway, 1973). That malate synthesis occurs in guard cells was substantiated by the work of Willmer et al. (1973a) who detected high levels of phosphoenolpyruvate carboxylase activity in C. communis epidermes, and Willmer et al. (1973b) who found that high levels of malic enzyme and NADP-malate dehydrogenase also occurred in these tissues. Furthermore, it was demonstrated that protons were excreted in sufficient quantities from guard cells to balance the influx of potassium/sodium ions during stomatal opening (Raschke & Humble, 1973; Raschke & Pierce, 1973).

It is suggested that a potassium/proton exchange could occur at the binding sites of the ion-adsorbent body, and that it is the excretion of protons by the guard cells that initiates the release of potassium and thus stimulates the production of a pool of readily available potassium to support stomatal opening.

In addition to these short distance ion exchange events, the long distance element of the ion flux system could be initiated by

active transport of potassium out of the subsidiary and epidermal cell symplasts into the apoplast. The potassium released from these sites would be transported to the guard cells via the apoplast and an uptake of potassium in excess of proton output could contribute to the rise in pH of the guard cells which accompanies stomatal opening (Meidner & Mansfield, 1968). Any potassium not taken up by the guard cells could be bound onto the ion-adsorbent body, although the major phase of potassium replenishment would be expected to occur with the efflux of ions from the guard cells during stomatal closure.

In addition to a role in actual stomatal movements, the bodies could be involved in Spannungsphase (Stalfelt, 1929), which is the preparative phase preceding opening in the diurnal rhythm of stomatal movements. The existence of Spannungsphase, in Tradescantia virginiana (= x andersoniana), has recently been confirmed by Meidner & Edwards (1975) and Edwards et al. (1976), who suggest that preparatory processes could include a 'softening' of the guard cell walls resulting from the exchange of potassium for calcium in the cell walls. During Spannungsphase, bound calcium could be exchanged for potassium from the ion-adsorbent body, a situation which is possibly reflected in Fig. 12.6a, in which the body contains a significant level calcium. This exchange may be particularly important in Tradescantia since it is suggested (Chapter 3) that the intramural lamellae which exist within the guard cell walls may be pectin-rich. In this way the ion-adsorbent body could be instrumental in rendering the guard cell walls more flexible in preparation for stomatal opening.

In the foregoing discussions the hypothetical role of the the ion-adsorbent bodies has been considered in general terms in

in respect of established aspects of stomatal behaviour. However, it is possible that the ion-adsorbent bodies could be involved in a more direct shuttle of ions to and from intracellular sites of accumulation within subsidiary cells.

The polar subsidiary cells have been shown to be closely associated with the bodies in the Commelinaceae by virtue of their location (Chapters 3, 4, and 5), potassium-rich vesicles (Chapter 3), trabeculae (Chapters 3, and 5), and by X-ray microanalyses (Chapter 12). These cells contain a relatively higher concentration of potassium than the inner lateral subsidiary cells when the stomata are closed, and a relatively lower concentration when the stomata are open (Penny & Bowling, 1974). This is rather difficult to interpret when it is considered that the polar subsidiary cell interface with the guard cell complex is extremely small when compared with that of the inner lateral subsidiary cell/guard cell interface. If ions flux equally in all directions into and out of the guard cell complex, one would expect the polar subsidiary cell potassium levels to be much higher when the stomata are open, and lower when the stomata are closed (i.e. the reverse situation to that found by Penny & Bowling, 1974). This anomalous situation could indicate that the polar subsidiary cells play a more specialised role in stomatal movements than other subsidiary cells.

FURTHER STUDIES

The prime aim of the next phase of research into substomatal ion-adsorbent bodies must centre on their hypothesised role in stomatal functioning as reasoned in the preceding discussion. Their suggested role as ion reservoirs can only be substantiated by

very accurate analyses and it is believed that only X-ray microanalytical techniques can provide sufficiently accurate data to quantify ion fluxes associated with stomatal movements. The first step in any further work must be the comparison of potassium levels within the constituent cells of the stomatal complex and the ion-adsorbent bodies when the stomata are fully open, when they are fully closed, and during the opening process. Results from such analyses should unequivocably confirm, or repudiate, the hypothesis proposed in the preceding discussion. If confirmation is obtained, detailed analyses should then follow into specific aspects of stomatal behaviour in order to build up a comprehensive picture of the role of ion fluxes in stomatal movements. Such studies should include the determination of ion fluxes in relation to stomatal movements induced by a variety of chemical and environmental agents.

However, it must be borne in mind that such studies, useful as they might be, should be paralleled by similar analyses in plant species which apparently lack the structures. In this way, it should be possible to determine what advantage(s) substomatal ion-adsorbent bodies confer on those species which possess them, and at the same time may lead to the identification of the structure(s) and/or system(s) which replace them in other species.

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APPENDIX 1

HISTOCHEMICAL PROCEDURES

2.1. Tetraphenylboron method for potassium (Collewijn, 1963)

1. Treat sections with 2% aqueous sodium tetraphenylboron at 22°C for 5 minutes.
2. Wash in 3 changes of distilled water.
3. Stain in 1% Chloroauric acid for 10 minutes.
4. Wash in 3 changes of distilled water.

2.2. Macallum's stain for potassium (Macallum, 1905)

Dissolve 20 g cobalt nitrite and 35 g sodium nitrite in 75 cm^3 of dilute acetic acid (10 cm^3 of glacial acetic acid diluted to 75 cm^3). After the evolution of nitrogen peroxide ceases, filter, and make up to 100 cm^3. The double salt solution should be stored in a refrigerator.

1. Rinse the tissue briefly in ice-cold water.
2. Stain in ice-cold solution of double salt (see above) for 30 minutes.
3. Wash thoroughly in ice-cold water for 30 minutes.
4. Precipitate the triple salt in 5% ammonium sulphide for 5 minutes.
5. Wash thoroughly in ice-cold distilled water for 5 minutes.

N.B. The times of the various treatments are purely arbitrary, but see Chapter 2.

2.3. Modified Macallum stain.

1. Rinse tissue in absolute ethanol for 10 s.
2. Stain in 7,5% aqueous sodium cobaltinitrite solution for 10 minutes.
3. Wash thoroughly in absolute ethanol for 60 s.

4. Precipitate the cobalt in 5% ammonium sulphide for 2 minutes.

5. Wash thoroughly in absolute ethanol for 60 s.

6.1. Gilson fixative for ectodesmata (Schnepf, 1959)

1. Epidermal tissue fixed in Gilson's fixative for 12 h at 38°C.

2. Washed in 30% ethanol for 45 minutes.

3. Stained for 10 minutes in 16% potassium iodide.

7.1. Isolation of endocuticle.

Epidermal strips were carefully floated with their sides adjacent to the mesophyll uppermost on concentrated nitric acid. The acid was than carefully brought to the boil and allowed to simmer. Periodically the epidermes were carefully removed on a glass slide, transfered to water and washed thoroughly, and examined under the microscope. With care, it has been found possible to obtain very good results despite the crudity of the method. It has the advantage of being very rapid.

7.2. Nile Blue procedure for total lipids (Cain, 1947)

1. Stain in 1% Nile blue at 37°C for 30 s.

2. Differentiate in 1% acetic acid at 37°C for 30 s.

3. Wash in distilled water.

7.3. Sudan dyes for total lipids (Baker, 1947; Gomori, 1952)

1. Place sections in 50% ethanol for 3 minutes.

2. Stain in Sudan IV or Sudan black B (saturated solutions in 70% ethanol) for 15 minutes.

3. Differentiate in 50% ethanol for 60 s.

7.4 Toluidine blue (Drawert, 1942)

A 0,01% aqueous solution was used which was adjusted to to pH 3,9 with hydrochloric acid.

7.5. Pectinase.

A 2% aqueous pectinase solution was used at room temperature. The pectinase used was Polygalacturonase, Poly-α-1,4 galacturonide glycanohydrolase purified from Aspergillus niger (Sigma Chemical Co., Kingston-upon-Thames, Surrey; cat. no. P.4625).

7.6. Periodic Acid-Schiff's stain for total carbohydrates (Hotchkiss, 1948; McManus, 1948).

1. Sections placed in 0,5% aqueous periodic acid solution for 0,5 h.
2. Wash in running water for 10 minutes.
3. Stain in Schiff's reagent for 15 minutes.
4. Rinse sections in water.
5. Treat with 2% sodium bisulphite for 90 s.
6. Wash in running water for 7 minutes.

7.7. Ruthenium red method for pectic substances, (Johansen, 1940).

Sections stained in a 0,02% aqueous solution of ruthenium red until it is visible in the cell walls.

7.8. Hydroxylamine - ferric chloride method for pectin, (Reeve, 1959).

1. Place tissue in 5 drops of alkaline hydroxylamine solution for 5 minutes.
2. Add 5 drops of 1 part concentrated hydrochloric acid and 2 parts of 95% ethanol.
3. Remove excess solution.
4. Flood section with 10% solution of ferric chloride in 60% ethanol conatining 0,1 N hydrochloric acid.

8.1. Acetic orcein stain for chromosomes, (Jensen, 1962).

A 1% orcein solution in 45% acetic acid was used.

12.1. Chloride histochemical stain, (Gersch, 1938; Gomori, 1952).

1. Place tissue in a 0,5% solution of silver nitrate in 95% ethanol for 0,5 h at 5°C.
2. Wash in distilled water for 30 s.
3. Treat with 0,5% nitric acid for 5 minutes.
4. Develop in Acutol.
5. Fix in Fix-Sol.

APPENDIX 2

TRANSMISSION ELECTRON MICROSCOPE TECHNIQUES

Typical fixation and embedding programme.

a. Tissue vacuum infiltered with 1,5% glutaraldehyde in 0,025 M cacodylate buffer at pH 7,0 for 1 h at room temperature.

b. 1,5% glutaraldehyde in 0,025 M cacodylate buffer at pH 7,0 for 1 h at $0^{\circ}C$.

c. 6,0% glutaraldehyde in 0,025 M cacodylate buffer at pH 7,0 and $0^{\circ}C$ overnight.

d. Wash in 0,025 M cacodylate buffer for 20 min.

e. Wash in 0,025 M cacodylate buffer for 20 min.

f. Wash in 0,025 M cacodylate buffer for 20 min.

g. 2% osmic acid in 0,025 M cacodylate buffer at pH 7,0 for 2,5 h.

h. Wash in 0,025 M cacodylate buffer for 5 min.

i. Wash in 0,025 M cacodylate buffer for 5 min.

j. Wash in 0,025 M cacodylate buffer for 5 min.

k. 50% acetone for 15 min.

l. 70% acetone overnight.

m. 90% acetone for 15 min.

n. 100% acetone for 1 h.

o. 100% acetone for 1 h.

p. Infiltrate 1 Spurr resin : 3 acetone for 1 h.

q. Infiltrate 2 Spurr resin : 2 acetone for 1 h.

r. Infiltrate 3 Spurr resin : 1 acetone for 1 h.

s. Infiltrate with pure Spurr resin for 1 h.

t. Infiltrate with pure Spurr resin for 1 h.

u. Leave in pure Spurr resin overnight.

v. Cure in fresh Spurr resin for 8 h at 70°C.

Spurr Resin (Spurr, 1969).

Spurr resin (Spurr, 1969), supplied by Taab Laboratories, Reading.

epoxy resin	ERL 4206	10,0 g
additive	DER 736	6,0 g
hardener	N.S.A.	26,0 g
accelerator	S-1	0,4 g

Post-microtomy stains for thin sections.

Uranyl acetate (Brody, 1959).

The sections were stained with a saturated aqueous solution of uranyl acetate in the dark for 30 min. After staining the sections were thoroughly washed in distilled water.

Reynolds' lead citrate (Reynolds, 1963).

a. Lead citrate prepared by mixing 1,33 g of lead nitrate, 1,76 g of sodium citrate, and 30 cm^3 of distilled water.

b. Mixture shaken for 1 min; allowed to stand for 30 min with intermittent shaking.

c. 8 cm^3 of N sodium hydroxide was then added and the solution made up to 50 cm^3 with distilled water. Centrifuged if necessary.

The sections were stained for 10 min in the presence of sodium hydroxide pellets in a petri dish. The sections were washed thoroughly in

distilled water after staining.

Post-microtomy stains for thick sections.

Either 0,5 % methylene blue dissolved in 1% boracic acid or 1% aqueous safranin was used. The thick sections were covered by a drop of either of these stains and then placed under a mercury lamp for 1 min. Superfluous stain was then removed by careful washing with distilled water.

Formvar coating of grids.

A 0,5% solution of polyvinyl formal in chloroform was poured into a clean glass dish. A clean microscope slide was washed in water and dried off with Velin tissue (Taab Laboratories, Reading) until it 'squeaked'. The slide, held at one end with forceps, was then lowered into the formvar solution and immediately withdrawn and excess solution allowed to drip off one end. The slide was then dried under a lamp and the extreme edge of one face of the slide was scored with a needle. The formvar enclosed by the scored edges was then floated off in a dish of distilled water and grids placed upside down over the formvar skin. The grids plus formvar coating were then picked up with a piece of suitably-sized thin card. The formvar-coated grids were finally coated with carbon in an Edwards E306 Vacuum Coating Unit (Edwards High Vacuum, Crawley).

APPENDIX 3

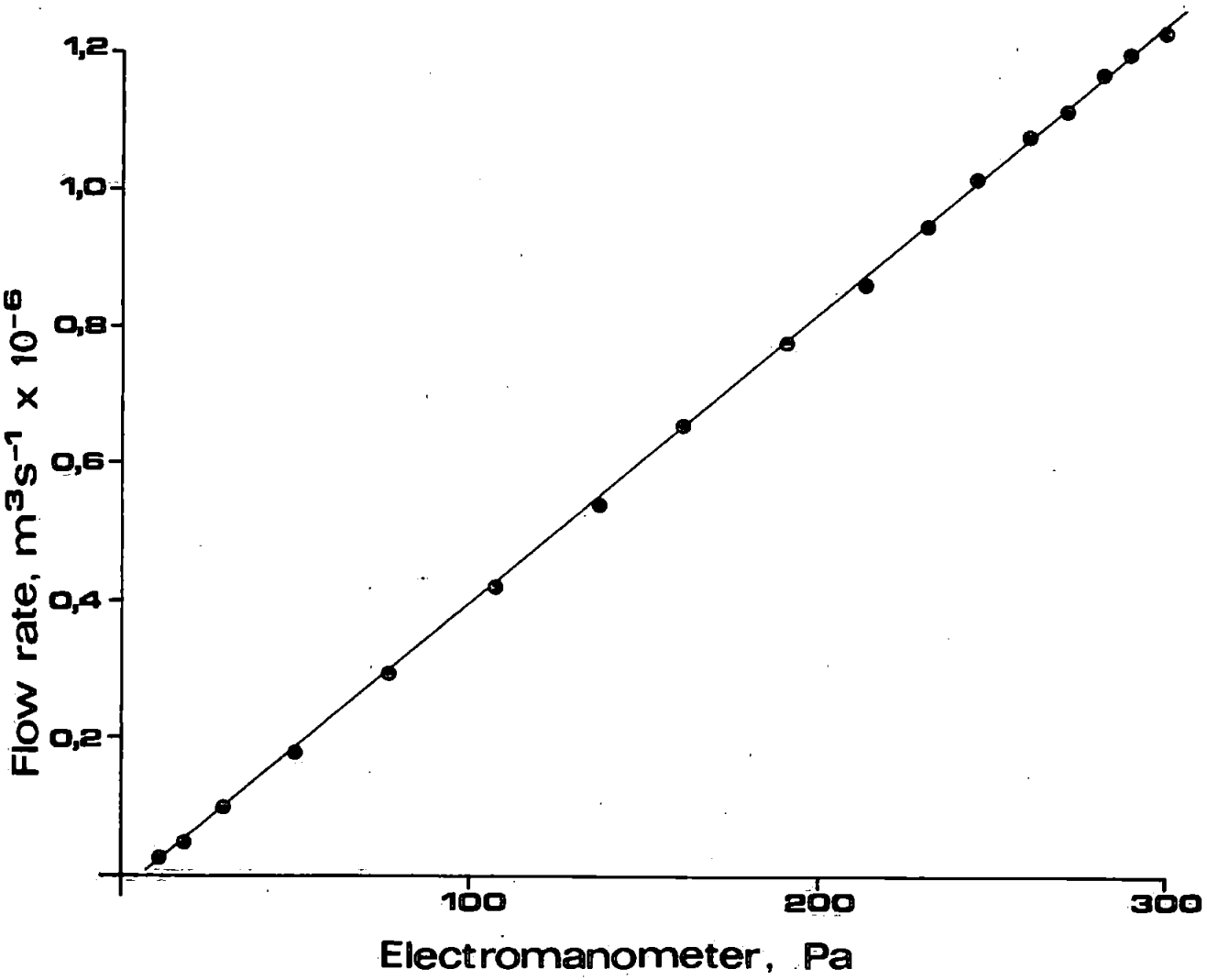
POROMETRY CAPILLARY RESISTANCES

The capillary resistances were constructed from a variety of broken thermometers and polarograph capillaries to approximate 0,1, 1, and 10 Gregory and Pearse unit resistances (Gregory & Pearse, 1934). The internal dimensions of the capillaries were determined by filling each with a short column of mercury which was measured with Vernier callipers and weighed. The radius of the capillaries were then determined from the formula (Gregory & Pearse, 1934):

$$\pi r^2 \times \text{length of column} = \frac{\text{weight of mercury}}{\text{density of mercury}}$$

Having established the internal dimensions, the length of each capillary to be cut off to give the desired resistances in Gregory and Pearse units was calculated from Heath's determination (Heath, 1939) that 1 Gregory and Pearse unit equals the unit resistance of a cylindrical tube for which $\frac{1}{r^4} = 3{,}77 \times 10^8 \text{ cm}^{-3}$.

A typical calibration curve for a resistance approximating 0,1 Gregory and Pearse units is illustrated in Plate A.3.1., overleaf.


1,2
1,0
0,8
0,6
0,4
0,2
Flow rate, m^3s^{-1} x 10^{-6}
100
200
300
Electromanometer, Pa

APPENDIX 4

STATISTICAL ANALYSES

In the statistical analyses below, the figures quoted for means, standard deviations, and standard errors are in μm. Probabilities are denoted by asterisks:

* less than 5%

** less than 2,5%

*** less than 1%

**** less than 0,1%

4.1. Experiment 11.1 (20 x 6 replicates)

Treatment	Mean	Standard deviation	Coefficient of variation
Pre-treatment	0,37	0,57	155,22
pH 3,5	10,55	1,64	15,57
pH 4,0	10,84	2,11	19,48
pH 4,5	14,13	3,04	21,51
pH 5,0	15,49	2,43	15,12
pH 5,5	15,74	2,84	18,01
pH 6,0	13,48	2,52	18,69

4.2. Experiment 11.2 (20 x 6 replicates)

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test
Among samples	406,89	5	93,38	104,1****
Within replications	640.36	714	0,90	
Total variation	1107,25	719		

4.3. Experiment 11.3 (20 x 6 replicates); citrate buffer.

Treatment	Mean	Standard error	Coefficient of variation
Pre-treatment	0,62	0,11	137,92
pH 3,5	5,15	0,31	65,93
pH 4,0	3,79	0,21	61,64
pH 4,5	2,03	0,15	80,39
pH 5,0	1,98	0,14	77,82
pH 5,5	2,32	0,15	71,55
pH 6,0	3,90	0,16	45,15

4.4. Experiment 11.3 (20 x 6 replicates); phosphate buffer.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test
Among samples	2,24	5	0,45	4,08
Within replications	78,39	714	0,11	
Total variation	80,63	719		

4.5. Experiment 11.4 (30x 3 replicates)

Treatment	57 J m^{-2} s^{-1}		108 J m^{-2} s^{-1}		137 J m^{-2} s^{-1}	
Treatment period	Mean	Standard error	Mean	Standard error	Mean	Standard error
0 h	0,2	0,07	0,1	0,06	0,1	0,06
0,25 h	2,0	0,20	2,1	0,17	2,9	0,29
0,50 h	2,9	0,14	3,3	0,23	4,7	0,27
0,75 h	3,8	0,16	4,5	0,17	5,2	0,29
1,00 h	4,4	0,18	4,5	0,17	7,0	0,40
1,25 h	5,2	0,16	5,3	0,19	7,2	0,41
1,50 h	5,5	0,16	6,3	0,21	9,3	0,36
1,75 h	5,1	0,21	7,0	0,20	8,0	0,44
2,00 h	6,9	0,20	6,4	0,16	10,2	0,30
2,25 h	7,5	0,19	7,2	0,13	11,1	0,46
2,50 h	7,5	0,30	7,9	0,21	9,7	0,44
2,75 h	6,0	0,18	8,0	0,23	9,6	0,40
3,00 h	7,5	0,12	7,9	0,20	9,9	0,33
3,25 h	7,8	0,22	8,3	0,17	11,0	0,39
3,50 h	8,2	0,21	8,9	0,22	11,8	0,38
3,75 h	8,4	0,38	8,7	0,18	9,9	0,52
4,00 h	7,9	0,31	8,6	0,23	11,9	0,36
4,25 h	8,4	0,21	8,8	0,18	11,2	0,51
4,50 h	8,7	0,22	9,1	0,24	11,3	0,35
4,75 h	8,5	0,22	9,2	0,19	11,3	0,50
5,00 h	8,6	0,23	10,6	0,23	10,7	0,36
5,25 h	9,5	0,24	9,3	0,22	11,3	0,36
5,50 h	9,1	0,27	9,6	0,25	11,2	0,37
5,75 h	8,3	0,26	8,8	0,23	10,7	0,43
6,00 h	9,7	0,23	8,4	0,21	11,5	0,36

4,6. Experiment 11.5. (20 x 6 replicates)

Treatment	Mean	Standard error	Coefficient of variation
Pre-treatment	0,0	0,02	1095,45
Control	9,1	0,16	18,86
10^{-6}	7,4	0,29	43,31
10^{-5}	6,1	0,36	65,32
10^{-4}	5,6	0,32	61,55
10^{-3}	0,2	0,06	319,31
10^{-2}	8,3	0,16	20,95

4.7. Experiment 11.6. (20 x 6 replicates)

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test
Among samples	11,60	5	2,32	3,23
Within replicates	513,19	714	0,72	
Total variation	524,79	719		

4,8. Experiment 11.7. (20 x 6 replicates)

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test
Among samples	14,40	5	2,88	3,10
Within replicates	662,91	714	0,93	
Total variation	677,31	719		

4.9. Experiment 11.8. (20 x 6 replicates)

Treatment	Mean	Standard error	Coefficient of variation
Pre-treatment	0,0	0,00	?
Control	8,6	0,17	21,31
10^{-6}	7,7	0,24	34,24
10^{-5}	7,5	0,22	32,08
10^{-4}	5,5	0,21	43,05
10^{-3}	0,9	0,11	147,46
10^{-2}	0,2	0,06	266,84

4.10. Experiment 11.9. (20 x 6 replicates)

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test
Among samples	479,39	5	95,88	**** 116,22
Within replicates	589,00	714	0,82	
Total variation	1068,39	719		

4.11. Experiment 11.10. (20 x 6 replicates)

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test
Among samples	1040,11	5	208,02	**** 218,1o
Within replicates	680,71	714	0,95	
Total variation	1720,82	719		

4.12. Experiment 11.11. (20 x 6 replicates)

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test
Among samples	51,98	5	10,40	**** 63,58
Within replicates	116,75	714	0,16	
Total variation	168,74	719		

4.13. Abscisic acid; (p. 254). (30 x 3 replicates)

Treatment	Mean	Standard error
Control	6,11	0,68
10^{-11}	6,59	0,70
10^{-9}	5,52	0,99
10^{-7}	2,20	0,79
10^{-5}	0,45	0,24
10^{-3}	0,01	0,03