J. Cell Sci. 3, 175–186 (1968)
Printed in Great Britain

THE ULTRASTRUCTURE AND ONTOGENY OF POLLEN IN *HELLEBORUS FOETIDUS* L.

II. POLLEN GRAIN DEVELOPMENT THROUGH THE CALLOSE SPECIAL WALL STAGE

P. ECHLIN AND H. GODWIN
Electron Microscope Laboratory, Botany School, University of Cambridge

SUMMARY

During the early stages of microsporocyte ontogeny in *Helleborus foetidus* L. there is protoplasmic continuity between the cells of the tapetum and between the individual sporogenous cells, but not between the two tissues. The plasma canals and plasmodesmata are progressively sealed off by the deposition of thick callose walls, so that by the first meiotic division, each pollen mother cell is isolated from its neighbours and from the surrounding tapetum. Callose is formed by dictyosomes in the individual pollen mother cells. The four meiocytes are separated by the deposition and coalescence of masses of callose forming in the cell plate area. The exine pattern is initiated at the surface of the young microspores while they are still invested with a thick wall of callose. Periclinally arranged endoplasmic reticulum lying just below the microspore cell membrane corresponds with the position of the furrows. The cell membrane in the interfurrow region thickens and becomes highly convoluted. A fibrous layer appears between the outer part of the convolutions and the callose, and locally it becomes less electron-dense at places that become filled with material of moderate electron density corresponding to the probacula; these in turn will become the bacula of the mature exine. In spite of an extensive examination of material prepared by a variety of techniques, no organelle or cytoplasmic component may be consistently associated with the positioning of the first signs of exine patterning.

INTRODUCTION

Using the techniques of light microscopy, fluorescent microscopy and staining, Waterkeyn (1962, 1964) was able to demonstrate that in *Helleborus foetidus* L. the development of pollen grains takes place from the spore mother cell stage to completed meiosis within thick envelopes of callose, the outermost enclosing the spore tetrad, and the innermost surrounding and separating the individual cells of the pollen tetrad. There seems little doubt that this is a constant characteristic of pollen grain ontogeny in the flowering plants, and Heslop-Harrison (1966*a*, *b*) has produced experimental evidence strongly suggesting that the callose layers act as a substantial physiological barrier isolating the dividing haploid cells from the environment. The present paper describes our attempt to follow by electron microscopy the structural changes accompanying pollen grain ontogeny through the whole period of investment by the callose special wall, a period of particular importance since the ultimate exine pattern of the pollen grain can be seen to have been laid down as

'primexine' before dissolution of the callose envelope. A preliminary report on this work has been presented elsewhere (Echlin, Godwin, Chapman & Angold, 1966).

MATERIALS AND METHODS

Intact flowers of *Helleborus foetidus* L. (Stinking Hellebore) were removed from plants grown in a Cambridge garden, and the stage of development of the anthers established by light-microscopic examination of aceto-carmine squashes. Intact anthers of the required stage were removed from the flowers, immediately placed in 5% glutaraldehyde in 0·1 M Sorenson's phosphate buffer (pH 7·0) and cut in half. The anthers remained in the fixative for 15 h at 4 °C. The tissue was then thoroughly washed in Palade's veronal-acetate buffer (pH 7·0) and post-fixed for 2 h at 4° C in 1% osmium tetroxide in Palade's veronal-acetate buffer (pH 7·0). Although this particular fixation régime proved to be adequate for all stages of development, an attempt was made to obtain more critical fixation of tetrads within the callose. The procedure closely followed the fixation régime previously outlined and involved a series of different concentrations of glutaraldehyde for varying times, followed by the appropriate post-fixation.

Although our findings are based on material fixed by the glutaraldehyde/osmium method, it was considered necessary to prepare material using permanganate as a fixative, as this has been the principal technique used by other workers who have studied pollen ontogeny at these early stages. Accordingly, anthers from the appropriate stages in development were placed either in 2% potassium permanganate in distilled water or 2% potassium permanganate in 0·1 M phosphate buffer (pH 7·0) for 2 h at room temperature.

All the tissues fixed by the various methods were dehydrated in a graded ethanol series, passed through three changes of 1:2 epoxy propane and embedded in Araldite without plasticizer. Thin sections were briefly stained with either lead citrate or potassium permanganate where appropriate, dried, coated with a thin layer of evaporated carbon and examined in AEI EM6 electron microscope.

The majority of the micrographs in this investigation were prepared by the 5% glutaraldehyde 1% osmium tetroxide technique outlined in the first part of this section.

RESULTS

We begin our discussion of the development of the pollen grain in *Helleborus foetidus* at the stage of sporogenous tissue surrounded by tapetal cells. (See fig. 1 in Echlin & Godwin, 1968.) The sporogenous cells have thin, evenly formed walls, which are penetrated in a few places by plasmodesmata. Whilst plasmodesmata occur between one tapetal cell and another, and between one sporogenous cell and another, they do not occur between the two respective tissues. The sporogenous cells, with the exception of the very prominent nucleus and nucleolus, contain few recognizable organelles. Mitochondria, in the absence of typical cristae, remain recognizable by their dense contents and a denser limiting membrane, whilst chloroplasts, although

possessing a dense limiting membrane, have relatively electron-transparent contents. There are a number of small dictyosomes and vesicles in the cytoplasm and the limiting cell membrane of the sporogenous cells appears highly convuluted. The endoplasmic reticulum is not readily recognizable at this stage and the ribosomes tend to be clumped in small groups. There is some evidence of discrete spheroidal bodies of medium electron density in the sporogenous cells, as well as in the tapetum, but they are considerably less apparent in the former tissue. Echlin & Godwin (1968) have already shown that similar spheroidal bodies arising in the tapetum of this species are the precursors of the Ubisch bodies that will eventually line the anther cavity.

The sporogenous cells undergo a number of mitotic divisions and give rise to the pollen mother cells (Fig. 1). During the early stages of the development the pollen mother cells resemble the sporogenous cells from which they arise, but as the tissue develops the pollen mother cells become larger than the surrounding tapetal cells. The tapetal/pollen mother cell wall gradually thickens, and the pollen mother cell membrane remains highly convoluted (Fig. 2); in some instances a distinct gap is present between the tapetal/pollen mother cell wall and the pollen mother cell cytoplasm. This gap appears to contain electron-transparent material of the same appearance and electron density as that found in discrete vesicles within the pollen mother cell cytoplasm (Figs. 2, 3), and later developmental stages lead us to regard this as callose. It is by no means certain whether callose, which is deposited immediately outside the developing mother cell, is formed in the dictyosomes or in the endoplasmic reticulum. Dictyosomes are abundant in the peripheral cytoplasm at this stage, and the endoplasmic reticulum is particularly difficult to demonstrate adequately because it is masked by labile cellular components which are preserved by the organic aldehyde fixative. The plastids and mitochondria still lack their respective membrane configurations. The grey spheroidal bodies are present in the pollen mother cells and as has been shown in the tapetal tissue (Echlin & Godwin, 1968) are surrounded by a zone of radiating ribosomes (Fig. 7). As the pollen mother cell develops these bodies may be seen to be closely associated with elements of the endoplasmic reticulum (Fig. 8).

The pollen mother cells increase in size and become progressively enveloped in the layer of callose that characterizes a well-defined stage of pollen grain ontogeny (Waterkeyn, 1962, 1964). The dictyosomes continue to be closely associated with the formation of the callose (Fig. 4), which becomes progressively more electron-dense and is deposited between the pollen mother cell wall and the cell membrane. The pollen mother cells still remain connected to each other by means of plasmodesmata (Fig. 3) and wide plasma canals (Figs. 4–6), although these too are finally disrupted, and by the beginning of the first meiotic division the pollen mother cells are completely isolated from each other and from the tapetum by the thick layer of callose.

The callose at this stage is homogenous except that the outer parts, nearer the original pollen mother cell wall, appear roughly granular and much less compact than the more recently formed callose adjacent to the microspores. It is considered that the callose, which is a straight chain β 1–3 glucan and an extremely hydrophilic material,

may be selective for the passage of materials of low molecular weight. It is clear from the work of Heslop-Harrison (1966*b*), and others, that nucleotides and nucleosides are not able to penetrate it, but no evidence is available concerning the passage of smaller molecules.

At the first meiotic division no cell wall is formed (Fig. 9). The microsporocyte quickly divides again to form the tetrad of haploid cells that develop into the mature pollen. Although no cell wall or cell plate is deposited between the two nuclei at the first division, the cytoplasm in the median region of the cell, nevertheless, has the characteristic appearance of a cell about to form a new cell wall (Fig. 9). Microtubules, presumably forming spindle fibres, are found in this median region and the mitochondria and plastids tend to be arranged parallel to the plane where cell division would be expected. Following the second division, however, the four daughter nuclei are tetrahedrally arranged and 'plaques cellulaires' (Waterkeyn, 1962) arise transversely to the axes joining the four nuclei. The separation of the cytoplasm does not involve formation of a cellulosic wall for the young microspores are next seen limited by a cell membrane, outside which is a layer of callose.

During the final separation of the microspores the dictyosomes again appear particularly active, and a large number of vesicles may be found in the immediate vicinity of the dictyosome cisternae. Numerous microtubules may be seen running at right angles to the 'plaques cellulaires', and vesicles of the same size and appearance as those associated with the periphery of the dictyosomes, collect along this central region (Fig. 10). These vesicles coalesce and as the coalescence proceeds outwards, so there is a gradual disappearance of the microtubules (Fig. 11). Mitochondria and free ribosomes are now abundant, and profiles of the endoplasmic reticulum are occasionally observed. Following the gradual coalescence of the dictyosome-derived vesicles there is an apparent formation of callose in them, until this material begins to form a layer between the cells (Fig. 11). A cell plate or pectic layer does not appear to be deposited. Towards the end of this process the dictyosomes are no longer visible, although the whole region is filled with vesicles believed to be derived from these organelles.

This cellular activity culminates in the total separation of the four haploid microspores (Fig. 12). As Waterkeyn (1962, 1964) has shown, each microspore is surrounded by an individual layer of callose, and each tetrad of four microspores is in turn surrounded by a common layer of callose, a layer originating from the callose layer initially surrounding the pollen mother cell. At the electron-microscope level it is difficult to tell these layers apart, but they are more readily resolved by fluorescence microscopy using suitable dyes such as aniline blue. The original cellulosic pollen mother cell wall is now very much reduced (Fig. 12), and in many instances has been broken by expansion of the developing tetrad. In some places the breaks appear to correspond to the sites of the plasma canals which originally interconnected the pollen mother cells. Until this stage the cytoplasm has been comparatively electron-transparent, but as development proceeds it becomes more electron-dense, due largely to an increased number of ribosomes. Grey spheroidal bodies are also recognizable (Fig. 13) alongside dictyosomes and mitochondria, and the central position in

the cell is occupied by the prominent nucleus and nucleolus. The cell membrane is closely apposed to the callose wall, and in the stages immediately after the individuality of the microspores within the tetrad has been established, the callose-bound tetrads are themselves packed tightly in the anther loculus. Soon there follows some separation of the tetrads (Fig. 12), although the individual microspores remain more or less firmly embedded in the callose. The apparent shrinkage of the callose from the pollen mother cell wall may be due partly to a gradual dissolution of the callose before its complete disintegration.

In the periphery of the microspore cytoplasm, a number of changes now occur that culminate in the formation of the template or pattern for the deposition of sporopollenin on the surface of the pollen grain. Previous examination of *H. foetidus* by light microscopy had established that development proceeded fairly rapidly at this stage, and it proved correspondingly difficult to determine the exact sequence of events. For example, it was found that, although there was a sequence of development in anthers across the flower, the stage of development within an individual anther might show slight variation along the long axis. Experience showed that these variations in development were small enough not to obscure the over-all sequence of events but at this phase of more rapid development the exact sequence of events was harder to ascertain.

The exine pattern in *H. foetidus* appears to arise as follows. There is initially a gradual increase in the number of dictyosomes in the periphery of the cytoplasm (Figs. 16, 17). Small vesicles, some of which appear to contain electron-dense material, are closely associated with the dictyosomes, and it is possible to trace these vesicles towards the edge of the cell (Fig. 17). Concomitant with the appearance of the dictyosomes and associated vesicles, there is an apparent increase in the thickness of the cell membrane at all places around the microspore (Fig. 19). At certain places around the microspore a layer of endoplasmic reticulum is found immediately below, and running parallel to, the microspore cell membrane, and it is believed that this region corresponds to the region where the furrow will eventually form (Figs. 20, 21). It is interesting to note that similar long profiles of endoplasmic reticulum appear deeper in the cytoplasm but are randomly orientated in relation to the microspore cell membrane. In a few instances during these early stages it is possible to see discontinuous pieces of endoplasmic reticulum lying parallel to, and immediately below, the cell membrane (Figs. 17, 18). This particular appearance of the endoplasmic reticulum is not a constant feature of the cell periphery at this, or any of the later stages.

The thickened cell membrane, in regions other than those associated with the long strands of endoplasmic reticulum, now appears convoluted (Fig. 22) and spaces appear between it and the callose wall. It is suggested that the callose has retracted slightly, as the edge of the callose nearest the developing microspore does not bear the impression of the convolutions, as might be expected if the thickened microspore membrane had been forced outwards at regular intervals. It is not clear whether these convolutions are formed from a physical disruption of the cell membrane or by extrusion at the surface of vesicles derived from within the cytoplasm. There is some evidence for the latter view, for in later stages of development there is a close similarity

between material in the spaces between the microspore cell membrane and the callose wall and the material in dictyosome associated vesicles (Fig. 20). Soon after the convolutions have appeared a thin layer of electron-dense fibrous material appears outside the cell membrane and lying along the top of the convolutions (Fig. 22).

The fibrous material appears to increase in density and this is accompanied by a further increase in the number of small vesicles derived from the dictyosomes which contain electron-dense material and are occasionally found in the space between the cell membrane and the fibrous layer (Fig. 17). When the fibrous material has reached a certain thickness it begins to fray or disappear in some places (Fig. 23) and these correspond to the places where the first elements of the primexine are deposited. At this particular phase the patterning of the whole pollen grain is apparently determined, and we have sought unsuccessfully to find any organelle or structure that might be causally concerned with the process. We may note, however, that at this stage of primexine determination microtubules may occasionally be seen radiating from the cell nucleus towards the periphery of the cell (Figs. 14, 15).

Where the layer of fibrous material frays and becomes less electron-dense (Fig. 23) gaps are formed in which there occur deposits of material of medium electron density (Fig. 24), in appearance not unlike the material found inside the grey spheroidal bodies. These deposits form the pro-bacula, the precursors of the bacula of the mature exine. The fibrous material becomes more electron-transparent, and expands to fill the whole space between callose and the cell membrane, the latter by this stage appearing considerably less convoluted.

In the regions of the furrow, no such development has taken place, and during the phases of primexine formation the apertural region has been delimited by a strand of endoplasmic reticulum lying immediately below the cell membrane (Fig. 24).

Throughout these changes the microspore cytoplasm has remained relatively unaltered, with the exception of a rather substantial increase in the number of dictyosomes. The nucleus and nucleolus continue to occupy a central region surrounded by mitochondria and plastids, which still fail to show their characteristic morphology, and are identified only by the differing density of their contents and the nature of their limiting membranes. The grey spheroidal bodies, which are such a characteristic feature of the tapetum, are present to a limited extent in the microspore cytoplasm throughout the formation of the primexine, but their position bears no evident relation to the arrangement of the pro-bacula along the surface of the developing microspore. The envelope of callose still firmly surrounds the microspore, although there is some evidence of dissolution on the face nearest the tapetum, and of tapetal cell wall breakdown. The tapetum at this stage begins to show signs of extrusion of the Ubisch bodies which, like the primexine, will eventually become coated with a layer of sporopollenin, but only after the callose has begun to break down.

DISCUSSION

Heslop-Harrison (1964), working with *Cannabis sativa* and *Silene pendula*, found that after the final mitosis in the archesporium, the peripheral archesporial cells showed plasmodesmatal links with the tapetal cells, and these in turn with the inner wall layers of the anther. We do not find interconnexions at such a late stage in development, and it would appear that very early in development of *Helleborus foetidus*, while the sporogenous tissue is still dividing mitotically to form the pollen mother cells, the presumptive tapetal cells and the presumptive microsporocytes are effectively isolated from each other. It should be pointed out, however, that during the very early stages, it is extremely difficult to differentiate between presumptive tapetal and microspore tissue; interconnexions such as those reported by Heslop-Harrison may exist to begin with, but if so, they are certainly lost by the time the pollen mother cells are being formed.

The phase of protoplasmic continuity between individual diploid sporocytes of *H. foetidus* persists for some time. Eschrich (1963) reported the presence of intercellular cytoplasmic connexions between pollen mother cells of *Cucurbita ficifolia*, although they did not contain nuclei. Weiling (1965*a*) found similar plasmatic connexions between pollen mother cells in *Lycopersicum esculentum* and *Cucurbita maxima* and was able to show the presence of cytoplasmic inclusions such as mitochondria and elements of endoplasmic reticulum within the larger channels. In a later paper Weiling (1965*b*) was able to observe that both nuclei and nucleoli were able to pass between cells via the plasma canals. In some instances he was able to observe cytomixis when the chromosomes were fully differentiated. Weiling concedes, that although cytomixis may under certain conditions be a normal process, it can be produced artificially, and may represent a pathological condition. The exact time of appearance of these intercellular connexions in tomato and squash is not clear, but plasmodesmata disappear when the thick callose wall forms around the pollen mother cell and the plasma canals are absent by the later stages in tetrad development. Heslop-Harrison (1966*a*) found similar massive cytoplasmic connexions between the meiocytes of several angiosperm species. Plasmodesmata occurred between all the cells of the archesporium, and between the tapetal cells and the wall cells. They appeared to be initiated in the early phases of meiosis and disappeared before meiosis II. In an earlier paper Heslop-Harrison (1964) considered that the original intercellular connexions, which persisted from the sporogenous cell stage, were quickly eliminated by the rapid growth of callose. As prophase advanced and with the deposition of callose, massive new protoplasmic connexions were established between the cells, apparently providing a passage for the movement of organelles and metabolites between the cells. By the time of the first meiotic division all these newly formed protoplasmic connexions were severed, and the cells were again totally isolated by the callose wall. Heslop-Harrison questions the earlier finding by Gates (1908) in which these connexions were thought to allow movement of nuclear material between meiocytes, and concludes, along with other more recent investigators, that nuclear translocation is an artefact of fixation and handling. It is similarly thought that the

studies of Takats (1959) on the movement of DNA surrounding *Lilium* prophase meiocytes may also represent an artefact due to wounding. It is quite clear from Heslop-Harrison's work that cytoplasmic organelles do pass from cell to cell *in vivo*: he suggested that the whole archesporium in a single loculus behaves as a giant coenocyte, and that the channels are associated with the movement of materials during meiotic prophase. Heslop-Harrison made the valid point that the sharing of a common cytoplasm before gene segregation presents no problems, but that when the new generation of haploid nuclei emerges, the expression of their genetic identity demands that each should act within an independent unit of cytoplasm. It is at this point that the special callose wall surrounding the microspores is completed.

Waterkeyn (1962, 1964) had adequately demonstrated with light microscopy the presence of a number of layers of callose surrounding, initially the pollen mother cell, and finally the individual microspores. Due probably to the limitations of light microscopy, Waterkeyn (1962) was unable to demonstrate callose before pachytene in prophase I. However, he showed that the two outermost layers of callose were deposited by the end of prophase I, the third and fourth layers between telophase I and prophase II, the fifth layer before cytokinesis, and the final layers following this process. It is clear that although the bulk of the callose deposition occurs during meiosis, the process has been initiated before reduction division begins in the pollen mother cells. We now find that before the cell membrane becomes highly convoluted callose deposition is initiated in the pollen mother cell, and in some instances separates adjoining cells. Skvarla & Larson (1966), working with *Zea mays*, found that callose deposition was preceded by withdrawal of the plasma membrane from the original pollen mother cell wall, and that vesicles and tubules of varying sizes and form were present within this zone. Eschrich (1962), in a study on the deposition of callose in *Cucurbita* and *Atropa*, found a substantial protein-lipid layer in the peripheral cytoplasm of the pollen mother cells during callose deposition, and concluded that this was connected in some way with callose deposition. The clear zone we find *outside* the cell membrane is much narrower than that described by Eschrich and does not have the staining characteristics of proteins or lipids. Larson & Lewis (1962) find that during microsporogenesis in *Parkinsonia* a callose layer is deposited against the original pollen mother cell wall and that following meiosis the continued deposition of this material successfully completes the cytokinesis.

A number of other workers have studied the formation of callose, and all, but especially Heslop-Harrison, draw attention to its function in isolating the microspore cytoplasm. Heslop-Harrison (1966*b*) in discussing the chemistry of the sporocyte wall comments on the fact that callose is very rapidly formed and destroyed, and that it has unusual permeability. He consideres that callose deposition is initiated during late leptotene of prophase I, the first deposits appearing initially at the corners of the cell, between the cell membrane and the pollen mother cell wall. He regards the dictyosomes as involved in formation and deposition of this material and suggests that the callose progressively severs the numerous cytoplasmic channels which connect the pollen mother cells.

Microspore formation in monocotyledons and dicotyledons differs in that cyto-

kinesis in the former group is of the successive type with cell plates forming at telophase I and telophase II, while in the latter, prophase II is in progress before telophase I is completed with no cell plate being formed in between. No plasmodesmata are ever formed between the daughter cells at either division, so that at the close of meiosis, each individual haploid microspore is wholly ensheathed in callose and isolated from its neighbours.

The dicotyledonous *Helleborus foetidus* shows the pattern typical of its group, although the cytoplasm in the median region of the cell at teleophase I shows the typical appearance of a cell about to initiate a cell plate.

Waterkeyn (1962), in his light-microscope studies of *H. foetidus*, found that cytokinesis was accompanied by phragmoplast formation and eventually by the formation of 'plaques cellulaires'. The daughter nuclei of the second division lie at the points of a tetrahedron, and the 'plaques cellulaires' accordingly occupy the six contact faces between the spores constituting a symmetric geometrical figure. It was sections of this figure that were described by Beer (1911) in *Ipomoea purpurea*, as tri-radiate lamellae. The problem remains as to how the components of the first 'plaques cellulaires' (first meiotic division) come to be rearranged in combination with 'plaques cellulaires' of the second meiotic division to form this final complex.

Waterkeyn (1962) concluded that cellular division proceeded by the fusion of the granular plaque which contains no pectins, callose or cellulose. This was then transformed to a continuous layer made of callose that was then covered by a second denser layer of the same material. Skvarla & Larson (1966), in their studies on *Zea mays*, find on the basis of ruthenium red staining that the cell plates which form at telophase I and II appear to be composed predominantly of pectic substances, and these form the framework upon which the internal callose walls are laid down. Our observations suggest that in *Helleborus foetidus* the earliest wall separating the microspores is formed by the aggregation of vesicles derived from dictyosomes and that it is of callose.

The formation of the primexine, the precursor stage to mature exine deposition, has engaged the attention of a number of workers in the past few years. It is clear, from these, as from our own studies, that pollen wall formation does not begin until the microspores are formed within the callose layers, and that the callose layers are not finally dissolved until the basic wall pattern has been laid down.

The first evidence of individual spore wall formation in *Helleborus foetidus* is a thickening of the cell membrane surrounding the microspore. Soon after this stage the membrane becomes highly convoluted and long profiles of endoplasmic reticulum may be found lying immediately below the surface at certain regions around the cell. The presence of endoplasmic reticulum in these regions is thought to delimit the region of the furrows in this tricolpate pollen grain, in the same manner as Heslop-Harrison (1963*a*, *b*) showed for the pore sites in the microspores of *Silene pendula*. Larson & Lewis (1962) found that shortly after cytokinesis in *Parkinsonia aculeata* the microspore membrane drew away from the callose wall and developed irregular convolutions, except in the regions corresponding to the position of future apertures, where it remained smooth. Horvat (1966), working with *Tradescantia paludosa*, found

a 'pellicle' formed between the special callose wall and the cytoplasmic membrane. This pellicle, which had a warty appearance, was thought to give rise to the foot layer, a stage of development which appears much later in the development of *Hellebore*. Horvat was unable to find involvement of endoplasmic reticulum at any stage during development, and the first appearance of any structure in the wall was considered to be little blebs of electron-dense material. Heslop-Harrison (1963*a*, *b*) did not describe the presence of convoluted membranes in *Silene*, but Skvarla & Larson (1966) describe in *Zea mays* the initial appearance of a highly convoluted cell membrane, which is followed by periclinal profiles of endoplasmic reticulum in the region of presumptive pore formation. Before seeking to attribute too comprehensive a role to the periclinal endoplasmic reticulum in determination of pore location, it would be well to recall the convincing evidence produced by Wodehouse (1935) that pore pattern in many types of pollen bears a very close relationship to the contact geometry of the tetrad and may be to some extent at least a haptotypic response rather than emphytic. We have in fact some evidence in *Helleborus foetidus* (unpublished results) that a pore may be initiated at the point of common contact between the four microspores within the tetrad.

Soon after the appearance of the endoplasmic reticulum in the presumptive furrow region in *H. foetidus*, the space between the callose and the crenellated cell membrane, in the inter-furrow regions, becomes progressively filled with electron-dense fibrous material, that may possibly be an artefact caused by the presence of derivatives of low molecular weight of the initial dissolution of callose. In some as yet unknown way certain parts of the fibrous region become less compact, and are progressively filled with some electron-dense granular material: these become the pro-bacula of the primexine. Heslop-Harrison (1963*a*, *b*) and Skvarla & Larson (1966) both consider that strands of endoplasmic reticulum lying at right angles to the surface of the microspores determine the position of the pro-bacula, but we were unable to find evidence of this in *H. foetidus* even when fixation in permanganate had given excellent preservation of endoplasmic reticulum. In the few instances where we have been able to demonstrate strands of endoplasmic reticulum at the surface of the microspore in the inter-furrow region, they have been arranged periclinally to the surface, not at right angles.

On the other hand, our micrographs show that microtubules occur in the developing microspore at this stage, and it is conceivable that in association with vesicles derived from the dictyosomes they play some part in determining the pro-bacular pattern, expecially since these organelles have been shown to be closely associated with initiation of cell walls in dividing parenchyma (Northcote & Pickett-Heaps, 1966; Pickett-Heaps & Northcote, 1966).

Beer (1911) long ago drew attention to the presence in developing microspores of *Ipomoea* of 'kinoplasmic radiations' from the nucleus and had speculated that they might be concerned with determination of the exine pattern. Recalling this Heslop-Harrison (1963*a*) was able to show in *Silene pendula* very striking radial extrusions of endoplasmic reticulum from the nuclear envelope. We have some evidence for comparable structures in *Helleborus foetidus* and although it has not been possible

to relate their disposition to elements of the primexine it remains an attractive conjecture.

Although the chain of processes concerned in the determination of exine pattern is still not apparent, it is evident that in *H. foetidus* the template has been laid down whilst the microspores are still enclosed within intact walls of callose. The probabilities are therefore strongly weighted in favour of the view that the exine pattern is genetically determined by the haploid microspore, and that the parent sporophyte (acting via the tapetum and locular contents) is unlikely to play any substantial part in this control, whatever part it may subsequently play in the deposition of sporopollenin and other materials in the developing spore wall.

The authors wish to express their most grateful thanks to Mr Brian Chapman, whose technical assistance throughout has been invaluable, and to Mr Paul Curtis for assistance with the photography.

REFERENCES

BEER, R. (1911). Studies in spore development. *Ann. Bot.* **25**, 199–214.

ECHLIN, P., CHAPMAN, B., GODWIN, H. & ANGOLD, R. (1966). The fine structure and development of the pollen of *Helleborus foetidus* L. *Sixth Int. Congr. Electron Microsc.*, Kyoto, 1966, vol. II, pp. 315–316.

ECHLIN, P. & GODWIN, H. (1968). The ultrastructure and ontogeny of pollen in *Helleborus foetidus*. I. The development of the tapetum and Ubisch bodies. *J. Cell Sci.* **3**, 161–174.

ESCHRICH, W. (1962). Elektronenmikroscopische Untersuchungen an Pollenmutterzellencallose. IV. Mitteilung über Callose. *Protoplasma* **55**, 419–422.

ESCHRICH, W. (1963). Cytoplasmabrücken zwischen den Pollenmutterzellen von *Cucurbita ficifolia* im Elektronenmikroskop. *Protoplasma* **56**, 718–722.

GATES, R. R. (1908). A study of reduction in *Oenothera rubrinervis*. *Bot. Gaz.* **46**, 1–34.

HESLOP-HARRISON, J. (1963*a*). An ultrastructural study of pollen wall ontogeny in *Silene pendula*. *Grana palynol.* **4**, 7–24.

HESLOP-HARRISON, J. (1963*b*). Ultrastructural aspects of differentiation in sporogenous tissue. *Soc. exp. Biol.* **17**, 315–340.

HESLOP-HARRISON, J. (1964). Cell walls, cell membranes and protoplasmic connections during meiosis and pollen development. In *Pollen Physiology and Fertilization* (ed. H. F. Linskens), pp. 39–47. Amsterdam: North Holland Publishing Co.

HESLOP-HARRISON, J. (1966*a*). Cytoplasmic connexions between angiosperm meiocytes. *Ann. Bot.*, N.S. **30**, 221–230.

HESLOP-HARRISON, J. (1966*b*). Cytoplasmic continuities during spore formation in flowering plants. *Endeavour* **25**, 65–72.

HORVAT, F. (1966). Contribution à la connaissance d'l'ultrastructure des parois du pollen de *Tradescantia paludosa* L. *Grana palynol.* **6**, 416–434.

LARSON, D. A. & LEWIS, C. W. (1962). Pollen wall development in *Parkinsonia aculeata*. *Grana palynol.* **3**, 21–27.

NORTHCOTE, D. H. & PICKETT-HEAPS, J. D. (1966). A function of the Golgi apparatus in polysaccharide synthesis and transport in the root-cap cells of wheat. *Biochem. J.* **98**, 159–167.

PICKETT-HEAPS, J. D. & NORTHCOTE, D. H. (1966). Relationship of cellular organelles to the formation and development of the plant cell wall. *J. exp. Bot.* **17**, 20–26.

SKVARLA, J. J. & LARSON, D. A. (1966). Fine structural studies on *Zea mays* pollen. I. Cell membranes and exine ontogeny. *Am. J. Bot.* **53**, 1112–1125.

TAKATS, S. T. (1959). Chromatin extrusion and DNA transfer during microsporogenesis. *Chromosoma* **10**, 430–453.

WATERKEYN, F. (1962). Les parois microsporocytaires de nature callosique. *Cellule* **62**, 225–255.

WATERKEYN, F. (1964). Callose microsporocytaire et callose pollinique. *Pollen Physiology and Fertilization* (ed. H. F. Linskens), pp. 52–58. Amsterdam: North Holland Publishing Co.

WEILING, F. (1965*a*). Zur Feinstruktur der Plasmodesmen und Plasmakanale bei Pollenmutterzellen. *Planta* **64**, 97–118.

WEILING, F. (1965*b*). Light und elektronenmikroskopische Beobachtungen zum Problem der Cytomixis sowie ihrer moglichen Beziehung zur Potocytose. Untersuchungen bei *Cucurbita* arten und *Lycopersicum esculentum*. *Planta* **67**, 182–212.

WODEHOUSE, R. P. (1935). *Pollen Grains*. London and New York: McGraw Hill.

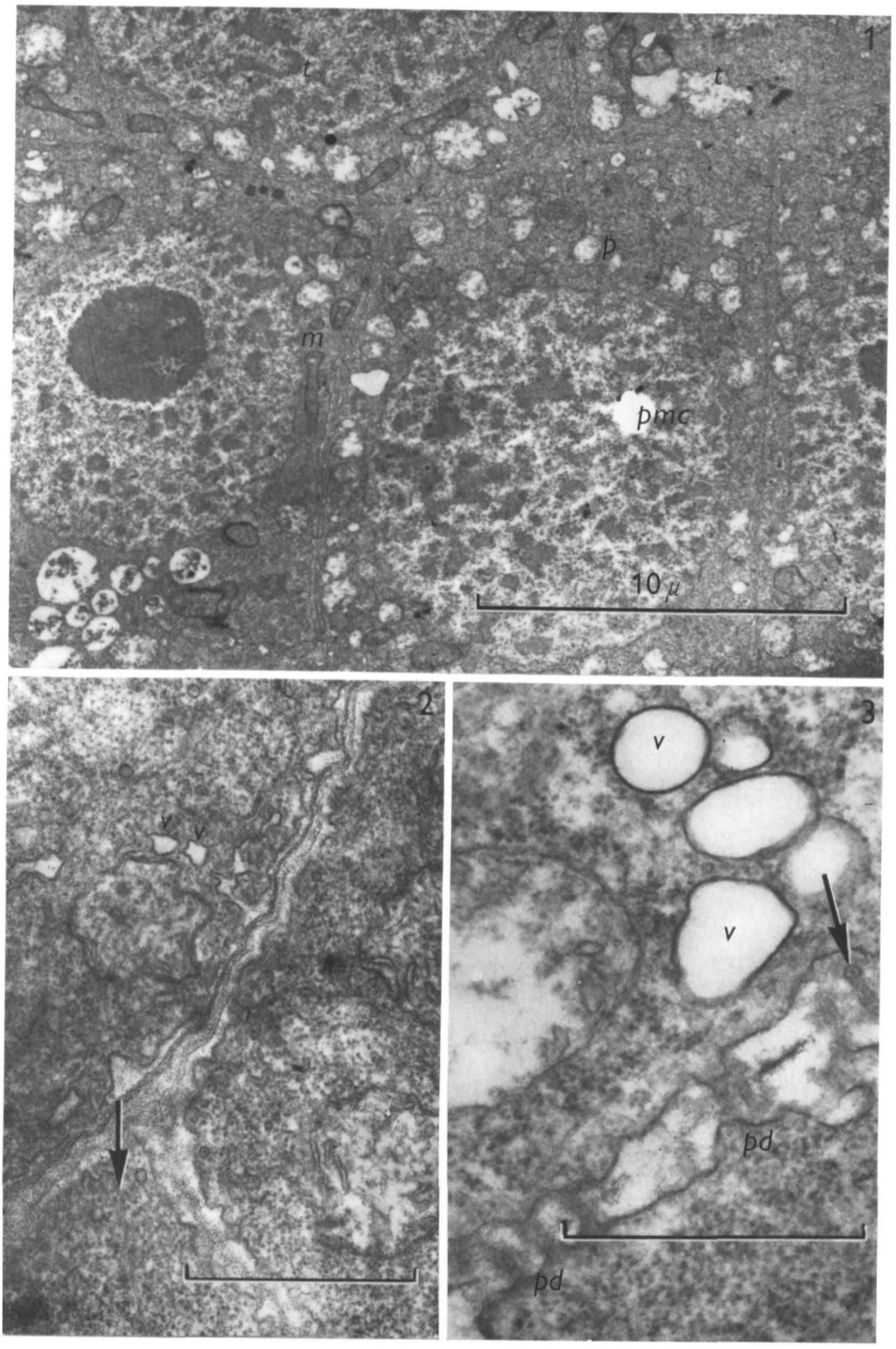
(*Received* 21 *July* 1967—*Revised* 18 *October* 1967)

The scale on the micrographs is equivalent to 1μ unless otherwise indicated.

Fig. 1. Pollen mother cells (*pmc*) surrounded by tapetal cells (*t*). Note the prominent nuclei, mitochondria (*m*), and plastids (*p*). 5 % glutaraldehyde/osmium. × 5800.

Fig. 2. Two adjacent pollen mother cells showing discrete vesicles (*v*) and microtubules (arrow) within the cytoplasm. Many ribosomes present at this stage. 5 % glutaraldehyde/osmium. × 36000.

Fig. 3. Two adjacent pollen mother cells of slightly later stage than Fig. 2. The large membrane-bound electron-transparent vesicles (*v*) are thought to contain callose material. Note the numerous plasmodesmata (*pd*), including one in cross-section (arrow). 5 % glutaraldehyde/osmium. × 48000.



P. ECHLIN AND H. GODWIN. II

(*Facing p.* 186)

Fig. 4. Two pollen mother cells, showing active dictyosomes (*d*), and callose (*c*) beginning to accumulate between the cells. The break in the cell wall (arrow) indicates the site of a plasma-canal between the two cells. 5 % glutaraldehyde/osmium. × 35 000.

Fig. 5. Intercellular connexion (plasma-canal) between developing pollen mother cells. Callose (*c*) has increased in density. 5 % glutaraldehyde/osmium. × 7500.

Fig. 6. Plasma-canal between two pollen mother cells. The pollen mother-cell wall (*pw*) is now reduced, and invested with callose (*c*). Note the mitochondrion (*m*) in the immediate vicinity of the plasma-canal. × 20 000.

Fig. 7. Grey spheroidal body associated with zone of radiating ribosomes 5 % glutaraldehyde/osmium. × 40 000.

Fig. 8. Grey spheroidal body at later stage of development, associated with endoplasmic reticulum. 5 % glutaraldehyde/osmium. × 18 000.

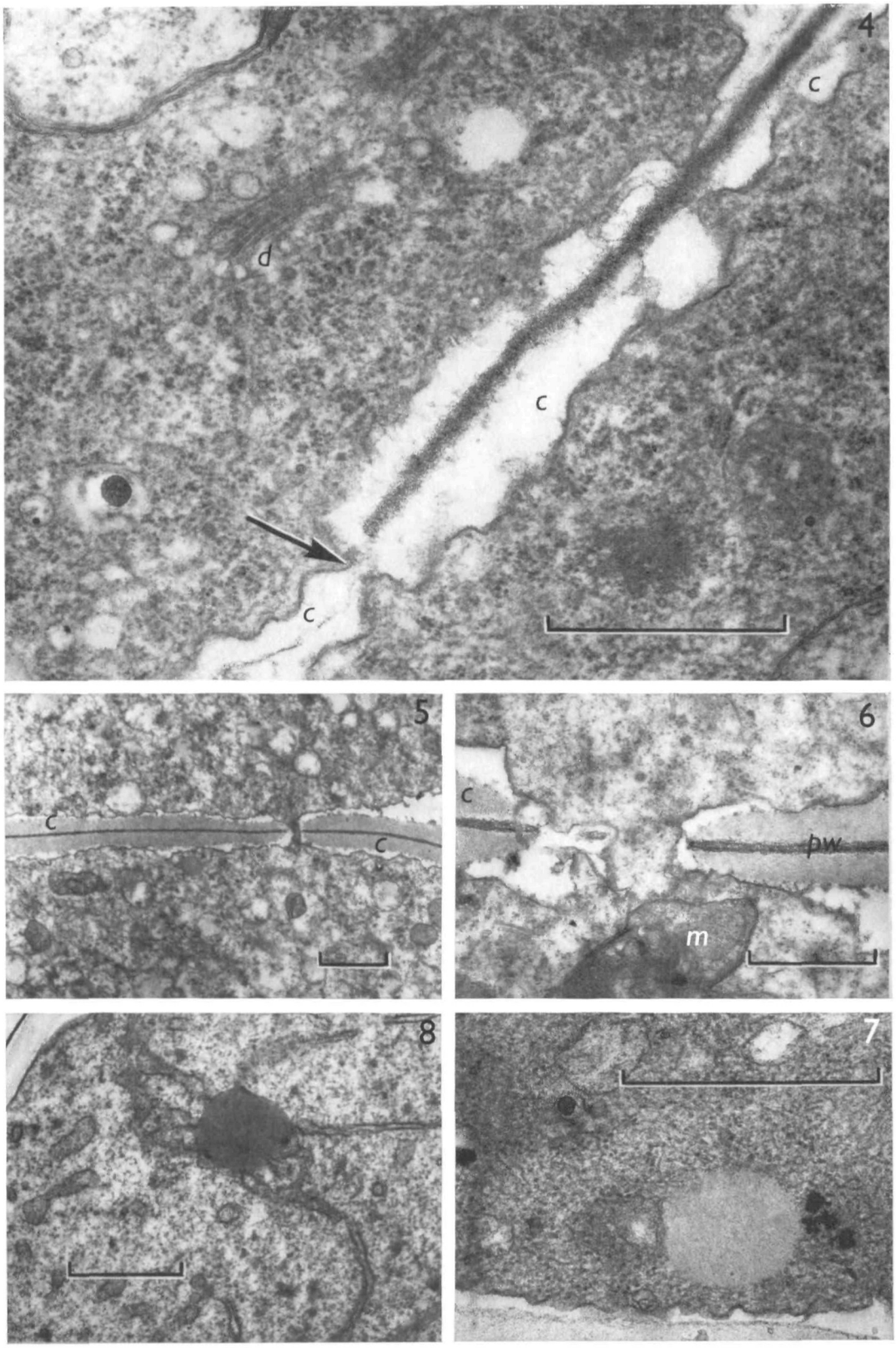
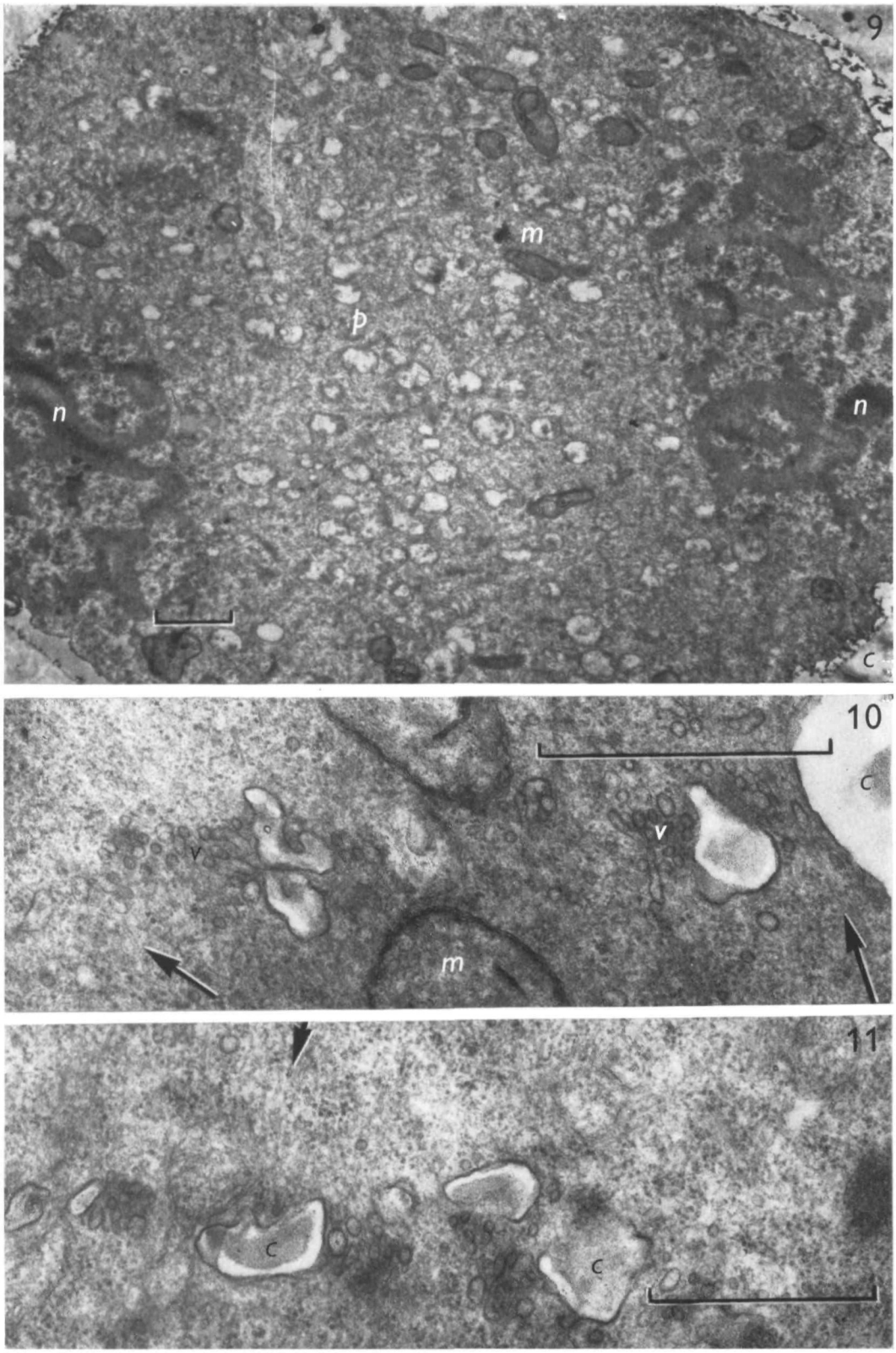

4
c
d
c
c
5
c
c
6
c
pw
m
8
7

Fig. 9. Meiocyte at end of first meiotic division. Note the two nuclear masses (*n*), and the plastids (*p*) and mitochondria (*m*) in the central region of the cell. There is some withdrawal between the surface of the cell and the callose (*c*). 5 % glutaraldehyde/osmium. × 7500.

Fig. 10. Central region of tetrad at end of second meiosis, showing gradual coalescence of dictyosome-derived vesicles (*v*). Microtubules (arrow) and mitochondria (*m*) are found in this region. Note close similarity between electron density of callose (*c*) and material within vesicles. 5 % glutaraldehyde/osmium. × 45 000.

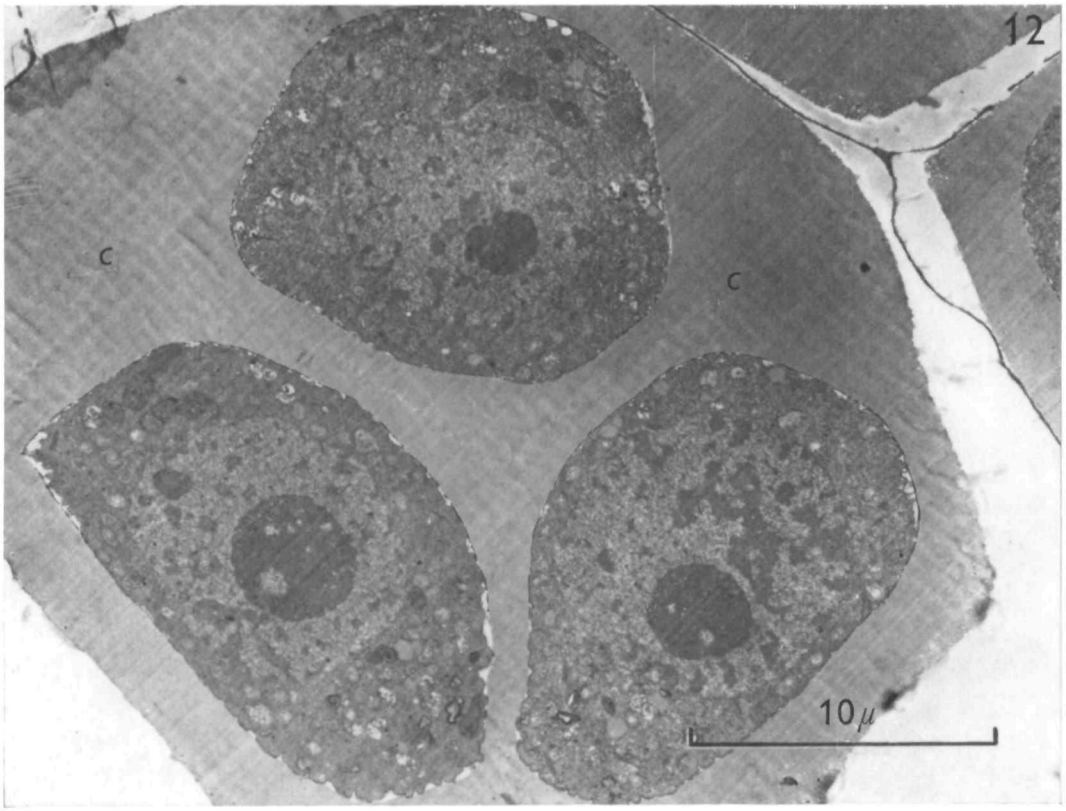
Fig. 11. Central region of tetrad at slightly later stage than Fig. 10. Some of the vesicles contain callose material (*c*). Microtubules (arrow) may be found between the vesicles. 5 % glutaraldehyde/osmium. × 35 000.

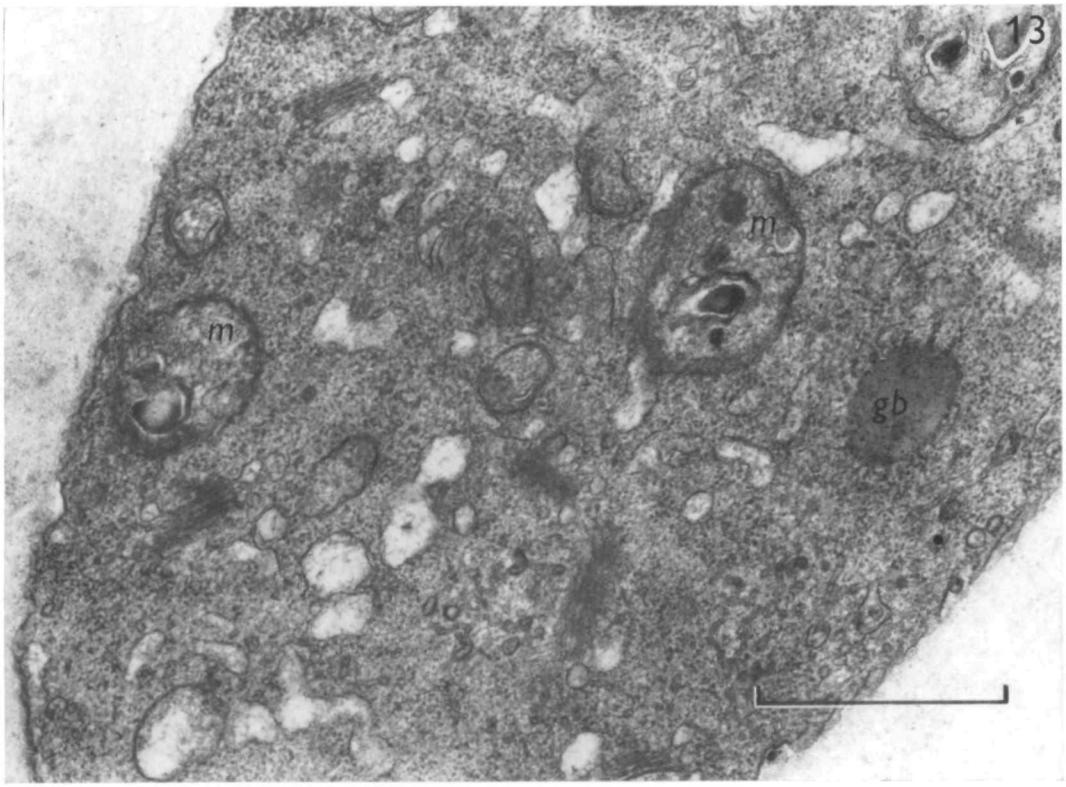


P. ECHLIN AND H. GODWIN. II

Fig. 12. Tetrad showing three of the microspores, all enveloped in a thick layer of callose (*c*). The original pollen mother cell wall is broken in a few places. 5 % glutaraldehyde/osmium. × 4000.

Fig. 13. General appearance of microspore cytoplasm, showing numerous dictyosomes and vesicles. Mitochondria (*m*) and grey bodies (*gb*) are also present, as are the numerous densely staining ribosomes. The cell membrane appears intact and no development of the primexine has taken place. 3 % glutaraldehyde/osmium. × 3200.





P. ECHLIN AND H. GODWIN. II

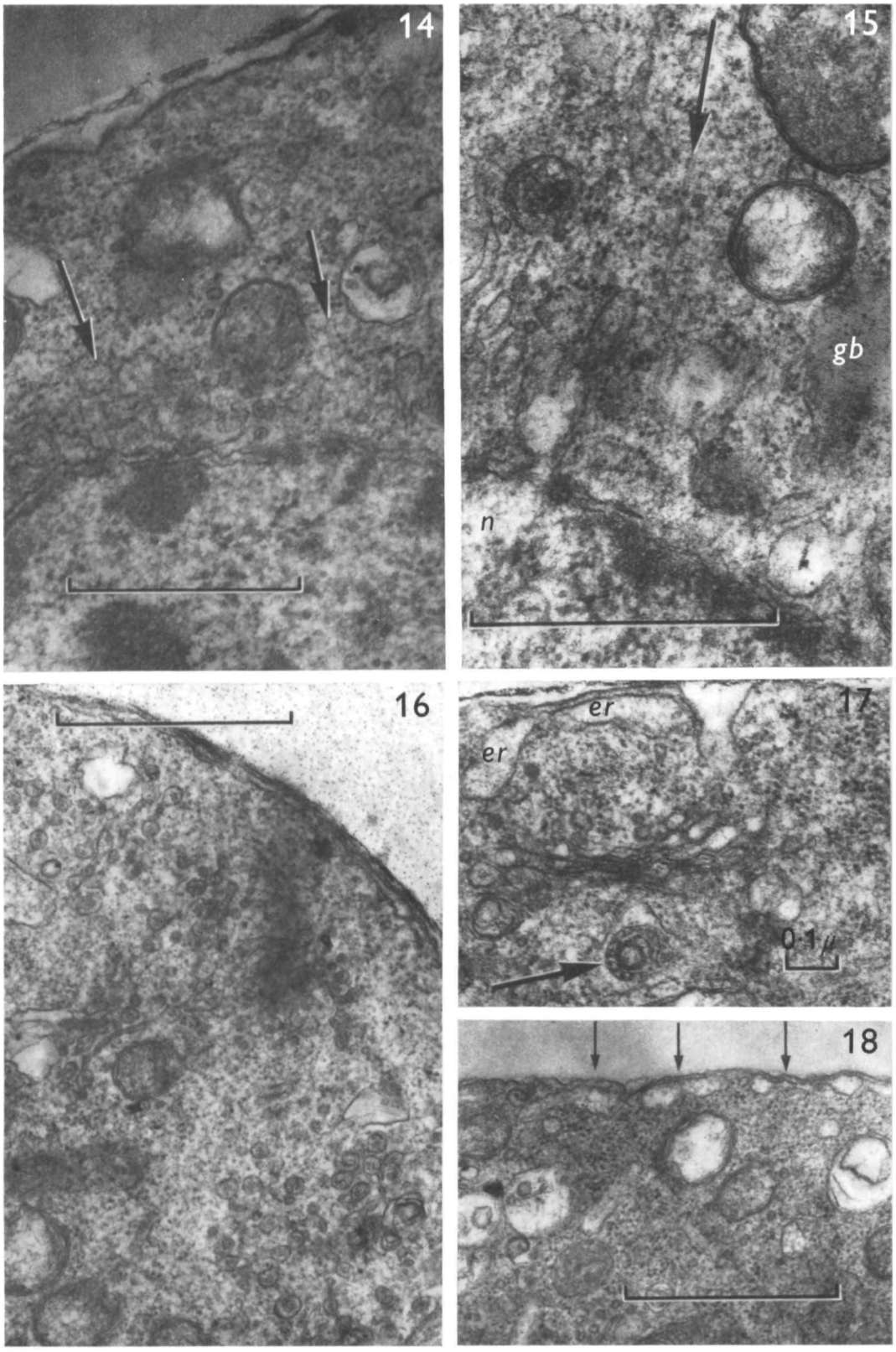
Fig. 14. General appearance of microspore cytoplasm at a later stage than Fig. 13. Note microtubules (arrow) radiating from the nucleus. 3 % glutaraldehyde/osmium. × 36 000.

Fig. 15. General appearance of cytoplasm at a similar stage to Fig. 14. Note the microtubules (arrow) radiating from the nucleus (*n*), and the prominent grey body (*gb*). 3 % glutaraldehyde/osmium. × 48 000.

Fig. 16. Periphery of microspore cytoplasm showing numerous dictyosomes. Note the dense fibrous material in the small vesicles associated with the dictyosomes. 3 % glutaraldehyde/osmium. × 36 000.

Fig. 17. Dictyosome at edge of developing microspore. Note strands of endoplasmic reticulum (*er*), and coiled arrangement of a number of ribosomes (arrow). × 47 000.

Fig. 18. Association of fragments of endoplasmic reticulum (arrows) at the periphery of the microspore. 3 % glutaraldehyde/osmium. × 32 000.



P. ECHLIN AND H. GODWIN. II

Figs. 19–24. Represent the stages in development of the primexine while the microspore is still within the callose.

Fig. 19. Cell surface of undifferentiated microspore showing initial thickening of the cell membrane. 3 % glutaraldehyde/osmium. × 36 000.

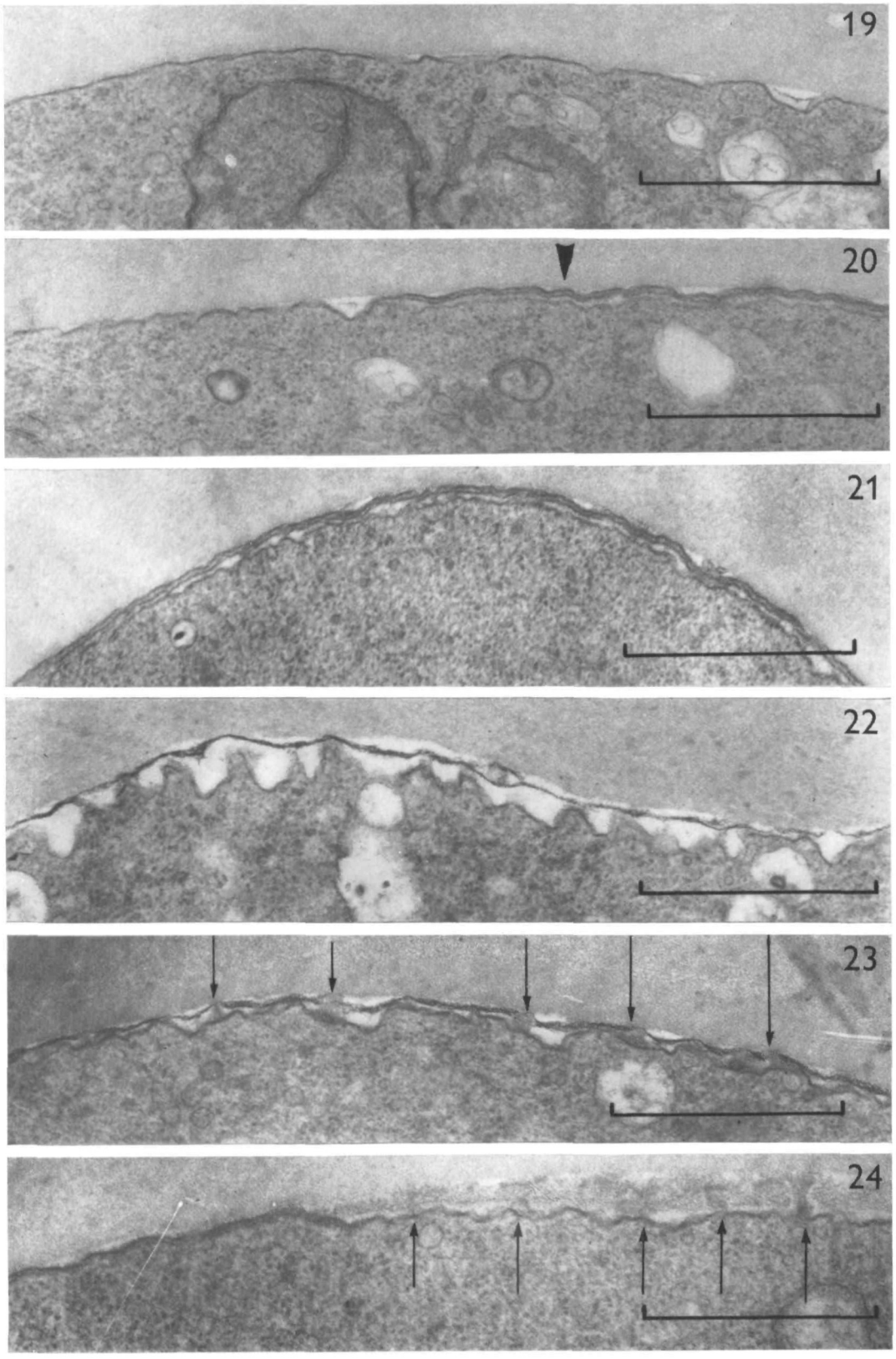
Fig. 20. Development of endoplasmic reticulum (arrow) just below cell membrane in presumptive furrow region, but not in the inter-furrow region. Note the large vesicle containing material of same electron density at the space between the callose and microspore. 3 % glutaraldehyde/osmium. × 36 000.

Fig. 21. Endoplasmic reticulum lying below the surface of the microspore in the presumptive furrow region. 3 % glutaraldehyde/osmium. × 36 000.

Fig. 22. Convolutions in the microspore membrane, fibrous layer beginning to form between callose and tips of the cytoplasmic projections. 5 % glutaraldehyde/osmium. × 36 000.

Fig. 23. Incipient loss in density in certain regions (arrows) of the fibrous layer, coupled with deposition of material of moderate electron density. 5 % glutaraldehyde/osmium. × 36 000.

Fig. 24. Final development of primexine. Future bacula will occur at places marked by arrows. Note the endoplasmic reticulum lying below the surface in the furrow region, coupled with the complete lack of primexine development in this region. 5 % glutaraldehyde/osmium. × 36 000.


19
20
21
22
23
24