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FINE STRUCTURE OF PETUNIA POLLEN GRAIN AND POLLEN TUBE

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ABSTRACT

Electron microscopic investigation of germinating *Petunia* pollen has yielded new data concerning fine structure of the pollen tube wall and of the protoplasm. The cell wall reveals two layers, an inner one containing cellulose microfibrils, embedded in a matrix, and an outer one consisting of acid resistant material. The multi-net growth theory is applicable to the growth of the pollen tube.

Round the generative nucleus, there is a generative cytoplasm containing organelles. This cytoplasm is separated from the vegetative cytoplasm by a cell wall-like structure. So we ought to speak of a generative cell.

The vegetative nucleus is lobed and is surrounded by a nuclear envelope containing pores.

The Golgi apparatus produces vesicles which accumulate in the tip of the pollen tube and probably have a function in building up the cell wall.

INTRODUCTION

Though there are many investigations concerning the morphology of pollen grain and pollen tube as seen with the light microscope, there are relatively few studies concerning fine structure of the cell contents and pollen tube wall. Of germinating pollen grains two important aspects can be studied:

First the ultrastructure of the plasma particles and of the generative cell. The generative nucleus is often said to be surrounded by cytoplasm and a plasma membrane and for this reason we should talk about the generative cell instead of the generative nucleus (a.o. MAHESHWARI, 1949). But other investigators (a.o. VENEMA and KOOPMANS, 1962) ignore this cell concept. It is evident that this should be studied by means of the electron microscope.

CHARDARD (1958) was the first to investigate the plasmatic fine structure of pollen grains, but not after germination. Although he gives no clear details concerning a membrane around the generative cytoplasm, he nevertheless speaks of the generative cell. The cytoplasm of the generative cell is very poor in organelles and he does not find plastids and mitochondria. LARSON and LEWIS (1961, 1962) give a survey of the ultrastructure of germinated pollen grains, but they do not take into account the nuclei and the cell wall. LARSON (1963) gives more details of the generative cell. He finds a cytoplasm around the generative nucleus and an empty space between this cytoplasm and that of the vegetative cell. Both cytoplasms are surrounded by

a plasma membrane. He states that there is no real cell wall around the cytoplasm of the generative cell. BOPP–HASSENKAMP (1960) studied *Lilium* pollen with the electron microscope, but the nature of the wall around the cytoplasm of the generative nucleus is not clearly shown. DIERS (1963) recently investigated the generative nucleus in *Oenothera* pollen. He found around the nucleus a cytoplasm with plasma organelles. He states that this cytoplasm is surrounded by a real cell wall. As concerns *Petunia*, SCHLÖSSER (1961) finds with the light microscope that the generative nucleus is sickle-shaped with a length of 10–15 micron and a width of 3–5 micron. He did not look for cytoplasm belonging to the generative nucleus. The vegetative nucleus was seldom seen and if seen it seemed to him merely a cluster of little granules.

The second important aspect of pollen tubes that can be studied with the electron microscope is the cell wall growth at the tip of the tube. It may give us more information about tip growth in general. This has been studied by means of the electron microscope in roothairs (SIEVERS, 1963a, 1963b). He found a correlation between the Golgi bodies and the vesicles which he saw in great number in the tip of the roothair. He shows that the vesicles move towards the wall and that their contents coalesce with the wall. He supposes that the contents may be a pectic substance. At the Symposium on Pollen Physiology and Fertilization at Nijmegen, ROSEN (1964) and SASSEN (1964) gave a paper in which they showed the relation between Golgi vesicles and cell wall growth in pollen tube tips. In our laboratory fine structure of the pollen tube is studied in connection with the incompatibility process in the styles of *Petunia*.

MATERIAL AND METHODS

Petunia pollen were germinated in vitro at 27 degrees C in 10 % sucrose solution with 0.01 % boric acid. Fixation was carried out with potassium permanganate at room temperature. The material was embedded in Epon 812 and thin sections were made on a Porter and Blum ultra microtome. Monitor slides of 2 micron thickness were cut to be observed with the phase-contrast microscope. For cell wall investigation the germinated pollen were treated with a mixture of hydrogen peroxide and acetic acid and in some cases with ethanolamine. These preparations were suspended on formfar coated grids and shadowed with chromium, palladium or platinum.

OBSERVATIONS

1. *Cell wall*

To the exine of the pollen wall not much attention has been paid because in the case of *Petunia*, this exine has been studied by MÜHLETHALER (1955). Permanganate stains the exine very well, as is to be seen in figures 1 and 2. The intine is electron transparent (figs. 1,

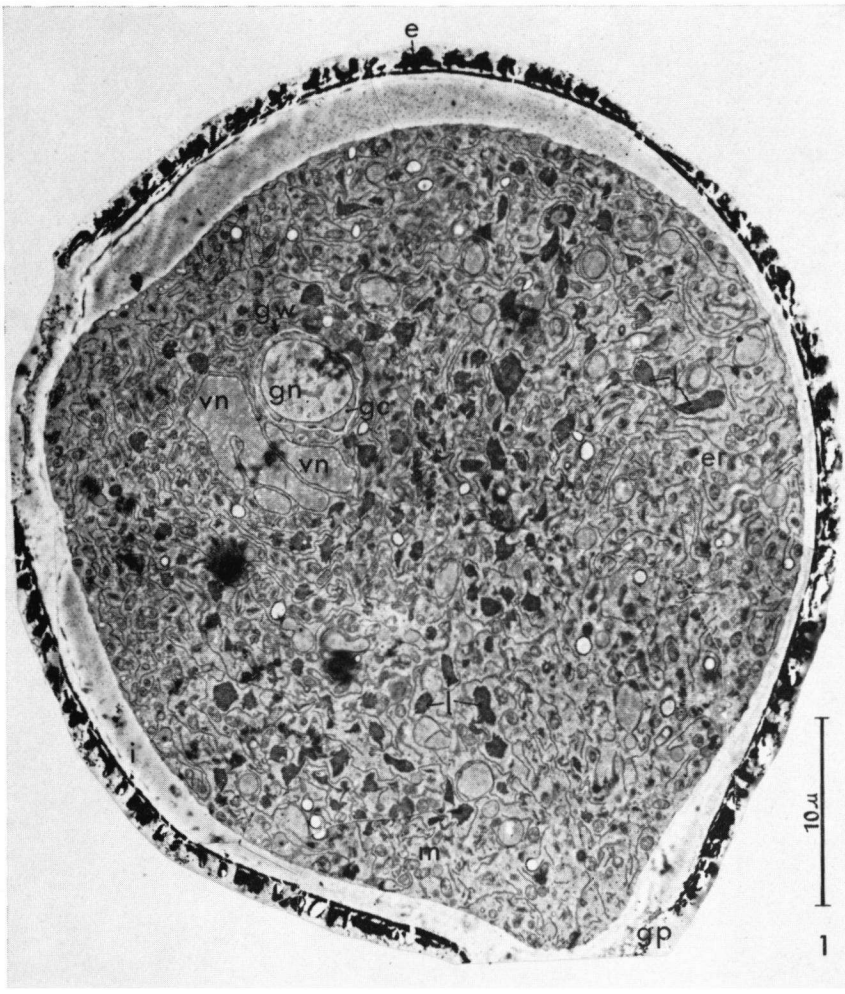

PLATE 1

Fig. 1. Section through a pollen grain with generative cell and vegetative nucleus.

Explanation of figures 1–18

Key to labeling

dm – dense material
e – exine
er – endoplasmic reticulum
g – Golgi-apparatus
gc – generative cell
gn – generative nucleus
gp – germ pore
gv – Golgi-vesicle
gw – wall around gen. cell
i – intine
l – lipid material
m – mitochondrion
ne – nuclear envelope
p – pore in nuclear envelope
pl – plastid
pm – plasma membrane
v – vesicle
va – vacuole
vn – vegetative nucleus
w – tube wall

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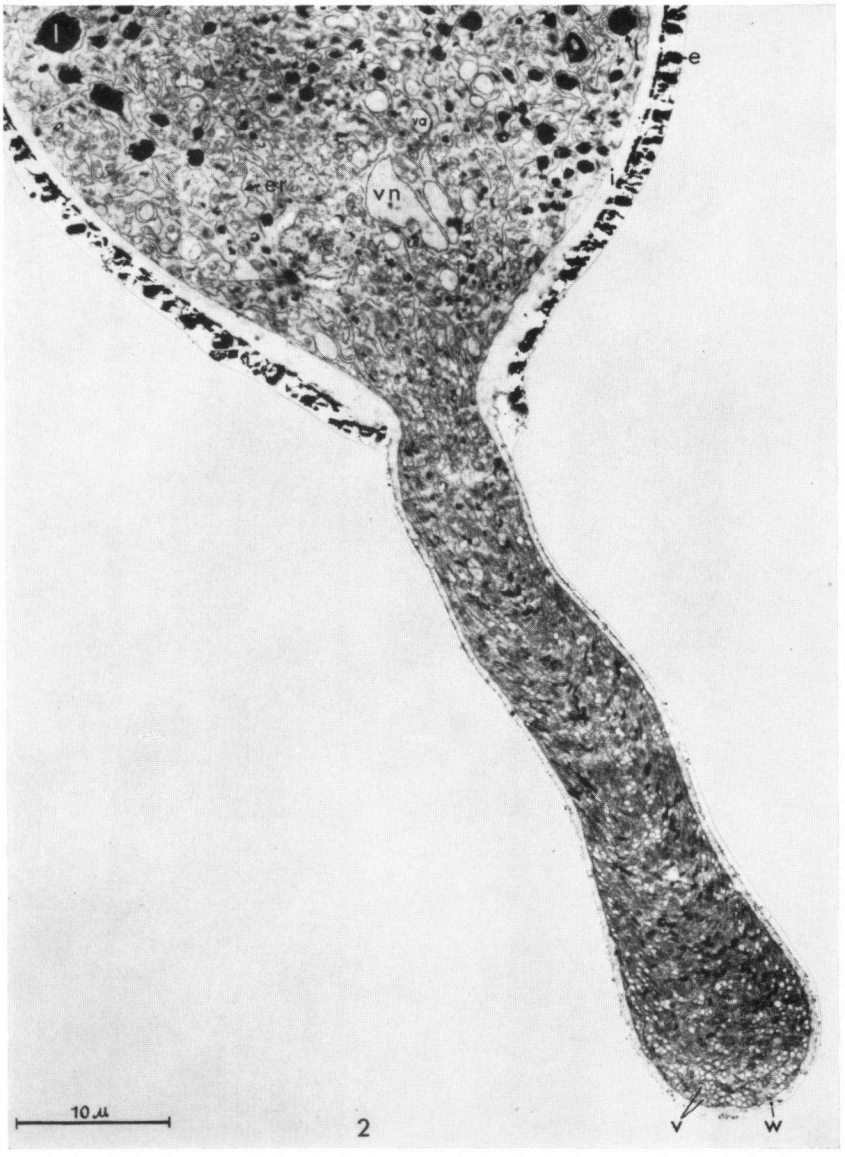


PLATE 2

Fig. 2. Part of a pollen grain with germ tube. Note the vesicles in the tip of the tube.

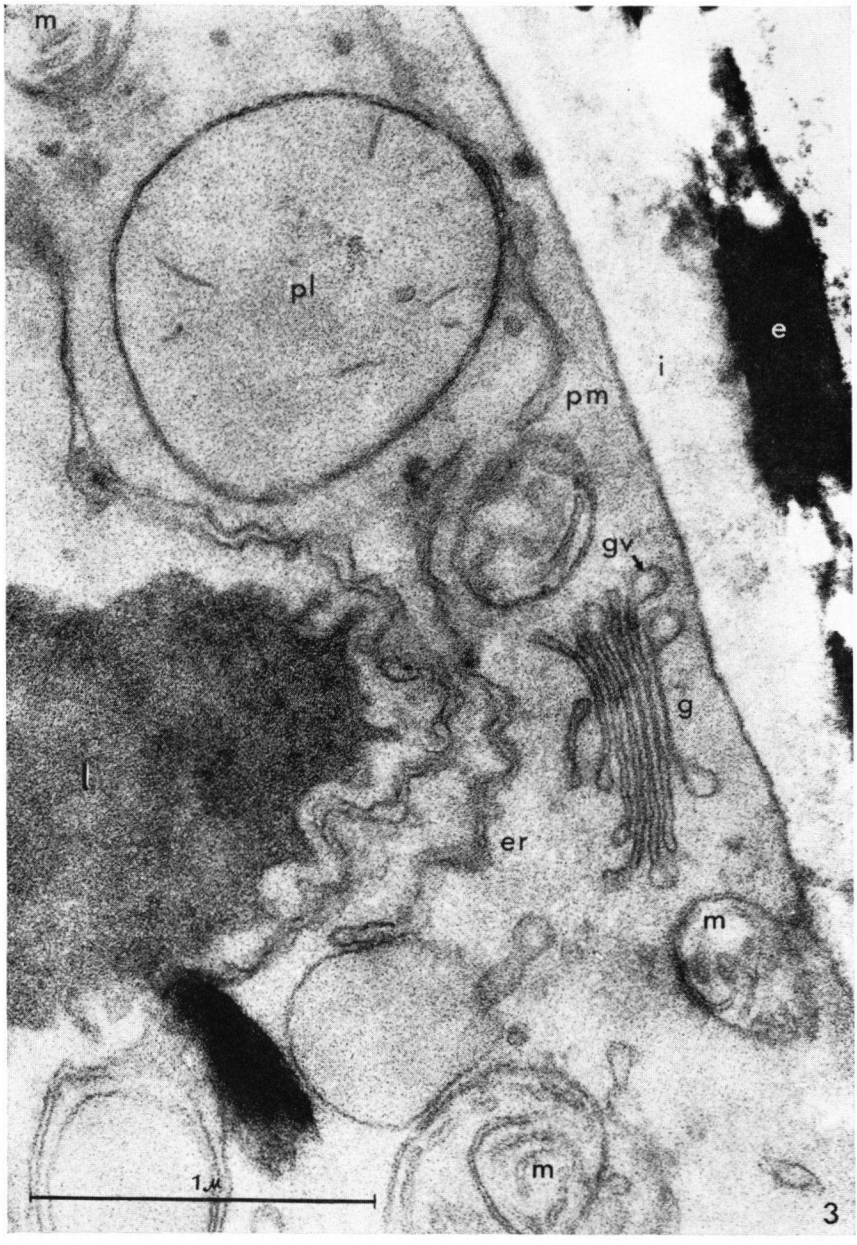
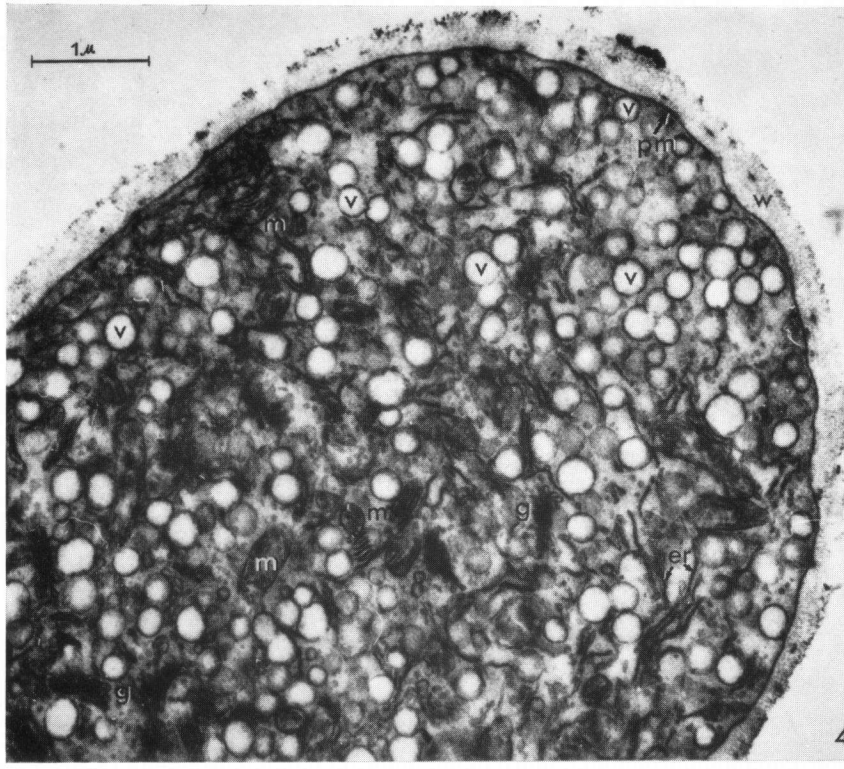


PLATE 3

Fig. 3. Enlargement of part of the pollen grain. The structure of the Golgi apparatus is clearly to be seen.



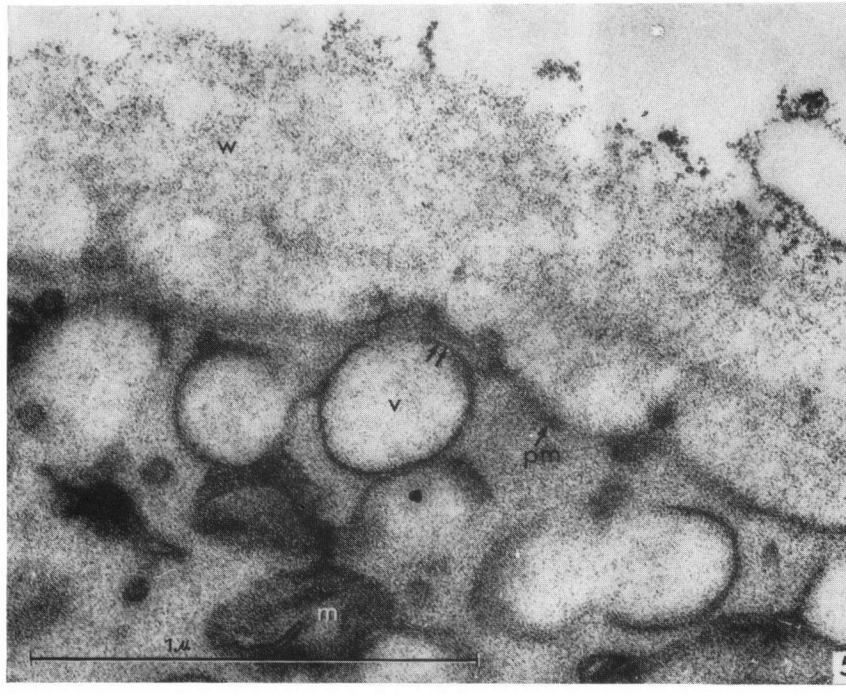


PLATE 4

Fig. 4. Tip of the pollen tube with vesicles, which are involved in the formation of the tube wall.

Fig. 5. Enlargement of the tip wall showing the loose outer structure. The vesicles are surrounded by a unit membrane (see double arrow).

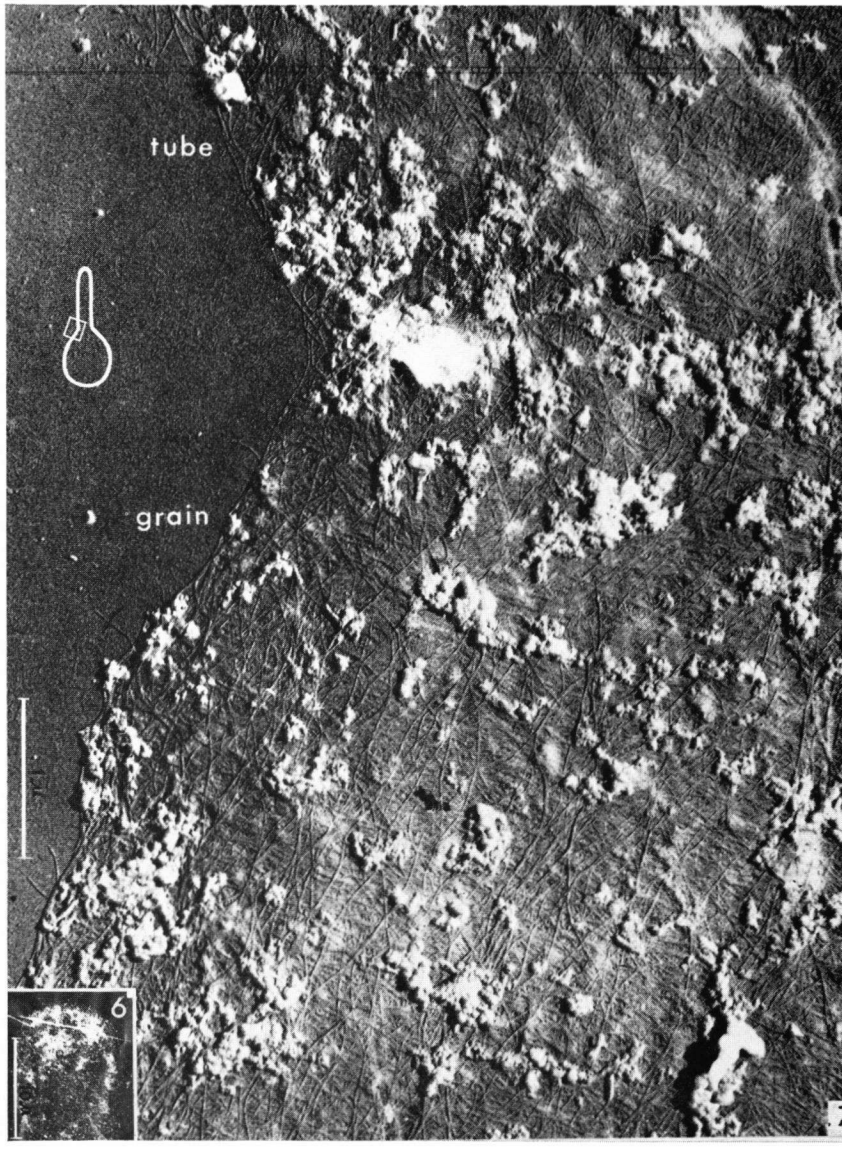


PLATE 5

Fig. 6. Pollen tube treated with hydrogen peroxide and acetic acid for 72 hours at 60° C. White areas on the pollen tube and especially on the tip are remnants of the acid resistant layer.

Fig. 7. Part of pollen grain and tube at higher magnification. The same treatment as in Fig. 6. It is evident that the cellulose layer is covered by the acid resistant layer.

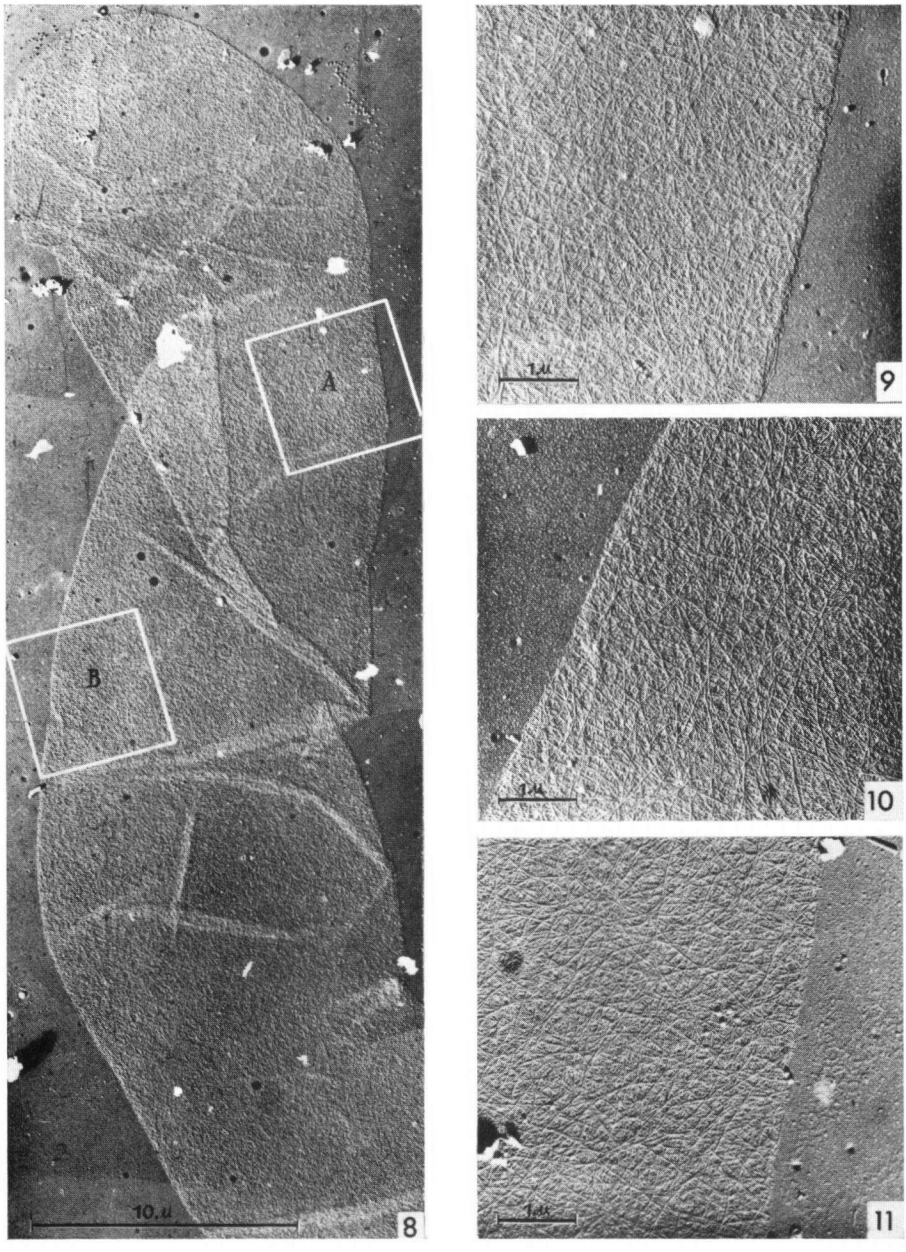


PLATE 6

Fig. 8. Pollen tube treated with ethanolamine. Cellulose skeleton clearly visible. Figs. 9, 10 and 11. Details of pollen tube in Fig. 8. Fig. 9 is an enlargement of Fig. 8A. Fig. 10 is an enlargement of Fig. 8B. Fig. 11 is an enlargement of a lower part of the tube which is not to be seen on Fig. 8. On these micrographs the fibrilar orientation is to be seen.

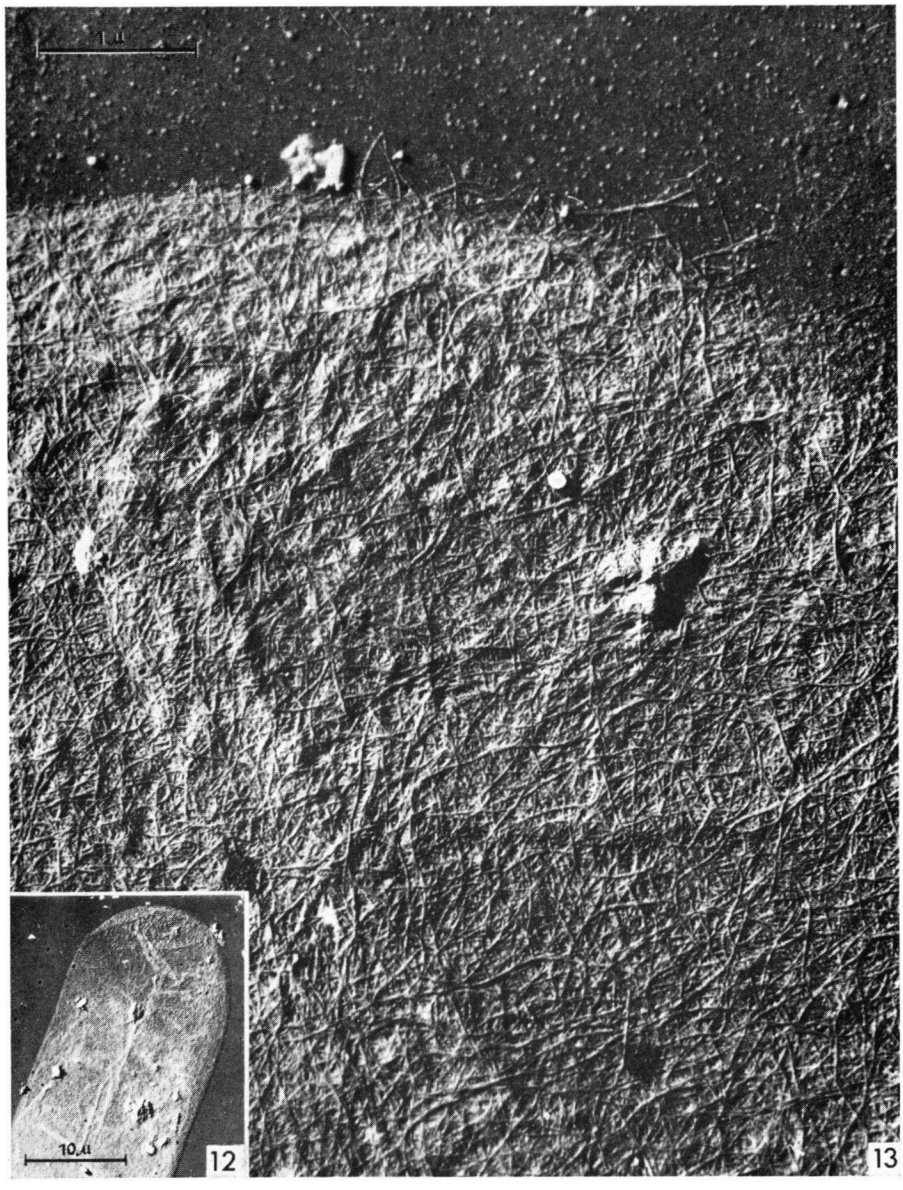


PLATE 7

Fig. 12. Tip of a pollen tube treated as in Fig. 8.
Fig. 13. Detail of Fig. 12 showing the microfibrils at the tip of the pollen tube.

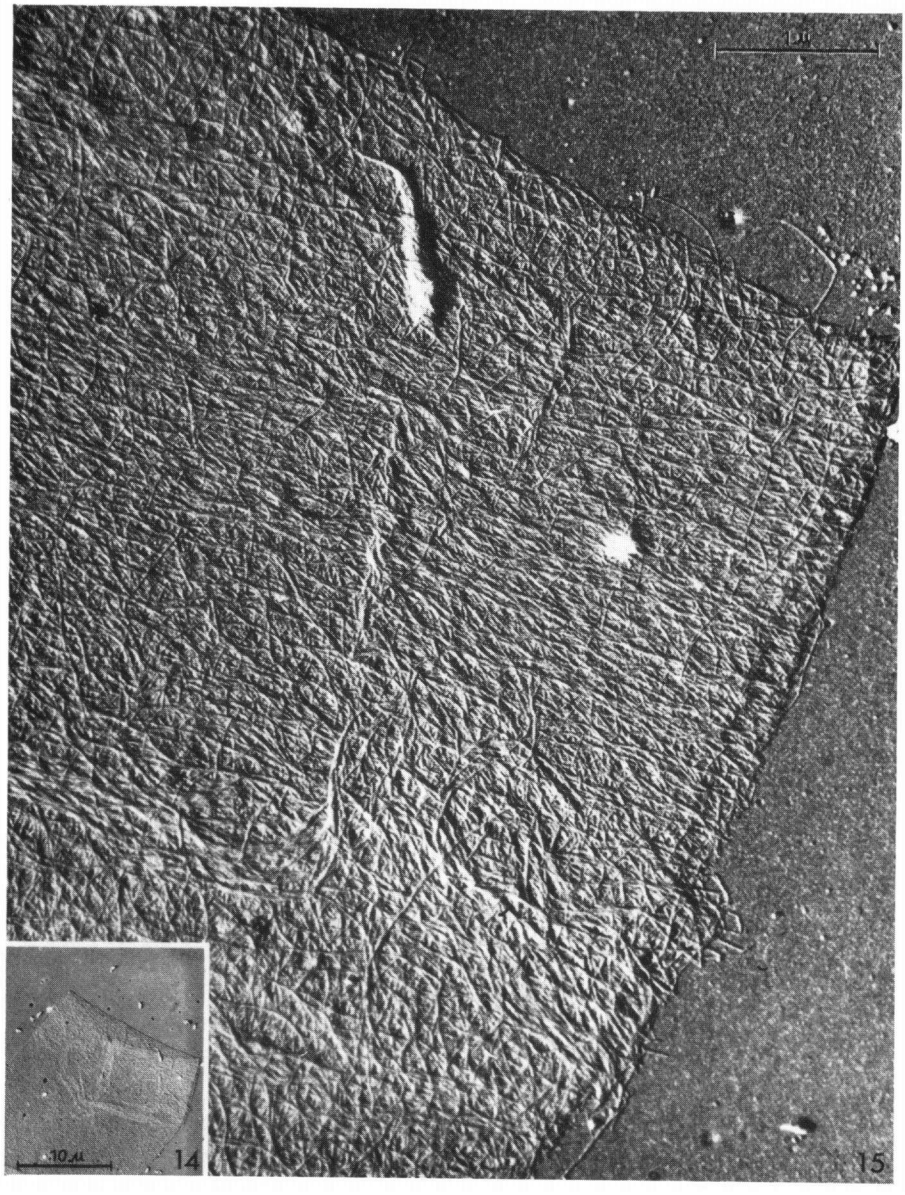


PLATE 8

Figs. 14 and 15. Wall of a pollen tube which is opened at the tip and folded back like a cuff. The microfibrils at the inside are orientated perpendicular to the long axis of the tube.

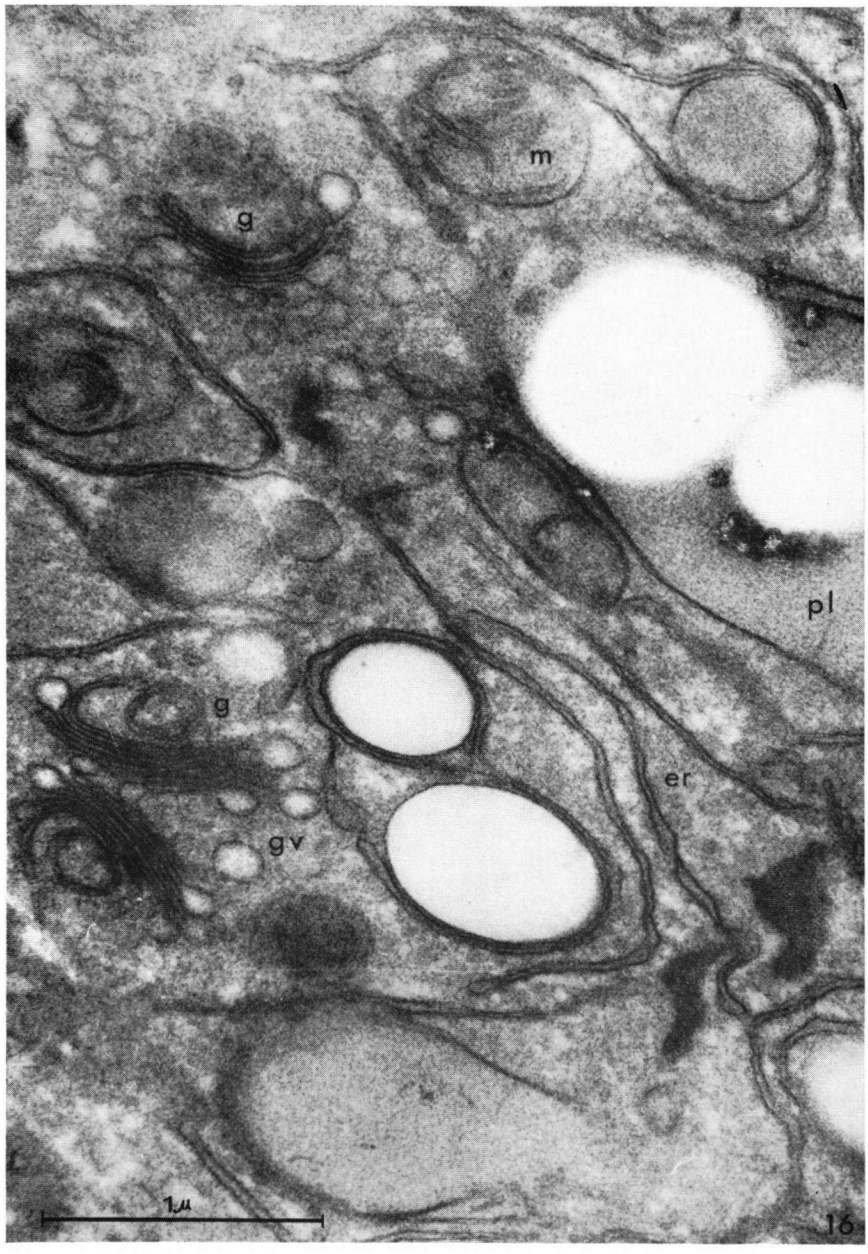
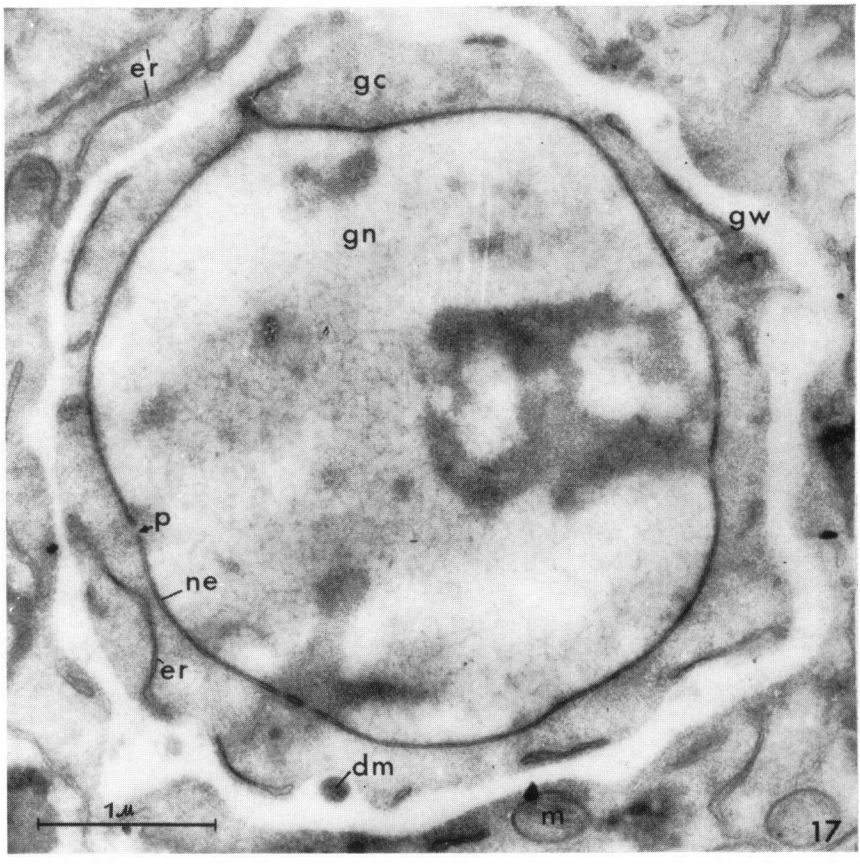


PLATE 9

Fig. 16. Section through the pollen grain. It shows Golgi bodies and vesicles which apparently are pinched off from the Golgi cisternae.



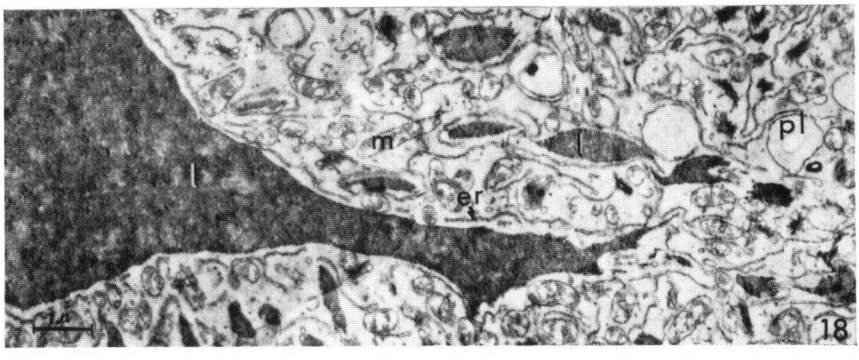


PLATE 10

Fig. 17. Generative cell with cell wall and nucleus. Note the dense material in the wall.

Fig. 18. Lipid material dividing up into smaller parts.

2, 3), a common feature of plant cell walls in sections after permanganate fixation, but sometimes one can discern two parts (figs. 2, 3). The outer part of the tube wall and the whole wall at the tip of the tube is very loose and lacks a sharp outer boundary (figs. 2, 4, 5).

If we treat the germinated pollen with a mixture of hydrogen peroxide and acetic acid at boiling temperature, we see that both grain and tube consist of a cellulose skeleton covered with remnants of a layer which is built up of an unknown but acid resistant substance, especially at the tip of the tube (figs. 6, 7). With ethanolamine this layer can be removed. Then the whole cellulose skeleton of pollen grain and tube is visible (fig. 8). The microfibrils at the outside of the tube have not always a fixed position, but we can see that there are two preferent directions, which form an angle of approximately 45 degrees with the long axis of the tube (figs. 8, 9, 10, 11). Also at the tip a meshwork of microfibrils is to be seen (figs. 8, 12 and 13). They are directed at random. Sometimes we observed tips, which were open and the wall of which was folded back like a cuff (figs. 14, 15). In this particular case we saw the microfibrils at the inside of the tip and they were perpendicular to the long axis of the tube.

2. *The cell contents*

a. *Plasma membrane and endoplasmic reticulum.* The cytoplasm of the pollen grain and pollen tube is separated from the wall by a plasma membrane (fig. 3), which is often seen in connection with the endoplasmic reticulum. Sections of the endoplasmic reticulum are found throughout the cytoplasm of the pollen grain and tube (figs. 3, 16).

b. *Nuclei.* In *Petunia* pollen we can discern two different nuclei, the vegetative and the generative (fig. 1). The vegetative nucleus is surrounded by a nuclear envelope with pores in it (fig. 1). This nucleus is generally lobed by infoldings of the nuclear envelope. For this reason one often sees more separated parts in sections. The generative nucleus has also a nuclear envelope containing pores (fig. 17). This generative nucleus is surrounded by cytoplasm and a wall-like structure, which is electron transparent. It is, however, not an empty space, sometimes we can see electron dense structures in it (fig. 17). In the cytoplasm of the generative cell, as we shall call it henceforth, we can see organelles like mitochondria, endoplasmic reticulum and also Golgi bodies.

Until now plastids have not yet been seen, but then we did not specially look for them. The endoplasmic reticulum in the cytoplasm of the generative cell is connected with the nuclear envelope and runs parallel to the cell wall of the generative cell (fig. 17).

c. *Mitochondria.* Mitochondria of a normal type and shape are found in great number in the pollen grain and tube. They consist of an outer membrane and an inner one, which have infoldings: the cristae mitochondriales (figs. 3, 16). These are often arranged parallel to each other.

d. *Golgi apparatus.* The Golgi apparatus is well developed in both grain and tube. It is composed of a stack of flat sacs, mostly 5–7, which bear vesicles at their edges (figs. 3, 16). We find these vesicles all over the grain and tube, but in large numbers at the tip of the tube (figs. 2, 4, 5). The contents of the vesicles are electron transparent after potassium permanganate fixation.

e. *Plastids.* We find relatively few plastids in the grain and the tube. They are surrounded by a double membrane. In the plastids we find starch grains or only membrane-like structures (figs. 3, 16).

f. *Lipid bodies.* Lipid is found in the form of one or more droplets in the mature pollen grain. During germination these droplets divide into a lot of small particles (figs. 1, 2). This lipid material is involved in metabolism and perhaps plays a part in building up the membrane system of the cytoplasm (fig. 18).

The organelles in the fronter part of the tube are arranged in curves (fig. 2). This shows clearly that plasm stopped streaming immediately after fixation started. The vesicles in the tip are not involved in this flow pattern.

Discussion

Electron microscopic investigations show that both the intine of the pollen grain and pollen tube wall of *Petunia* consist of a cellulose skeleton, which is covered by a layer of material that is fairly resistant to oxidizing and acid substances. After a long period of treatment with hydrogen peroxide and acetic acid remnants of this material are still to be found on this cellulose layer. Between the cellulose microfibrils this material is absent. The matrix components like pectin and possibly callose are removed as a result of the above-mentioned treatment. After treatment with ethanolamine this outer layer disappears completely. From data of Mühlethaler and Linskens (1956) it appears that also the walls of pollen tubes of *Petunia*, grown in vivo, after treatment with 5 % sodium hydroxide and 5 % hydrochloric acid, contain other substances apart from cellulose. On the other hand it appears from investigations carried out by Kroh (1964) concerning the penetration of pollen tubes into the stigma-papillae of *Brassica nigra* that, after a short treatment with hydrogen peroxide and acetic acid, the walls of the pollen tubes only consist of cellulose.

Rowley (1959), too, distinguishes two layers in the intine of the pollen grain but attributes this layer-structure to poor fixation. This is not the case here.

The microfibrils in the cellulose skeleton are directed at random in the pollen grain and in the tip of the tube. On the tube itself a clear orientation is perceptible in two directions, both at angles of approximately 45 degrees to the main axis of the pollen tube. This is in contradiction with the work of O'Kelley and Carr (1954), who are of opinion that the microfibrils of the pollen tube walls are orientated mostly at right angles to the main axis of the tube. The

same authors do not find microfibrils at the tip of the pollen tube. Instead, they say, the cellulose appears to be made up of short, irregularly distributed units. Our study has clearly revealed that the tip of the pollen tube, too, contains long microfibrils that are composed of cellulose.

In some cases the pollen tube is seen to be open at the tip as a result of the treatment. We have often observed that the cell wall was then folded back like a cuff. In these cases the inside of the cell wall can be studied. Right under the tip the microfibrils then turn out to be transversely orientated to the cell axis. So this is likely to be the direction in which they were laid down during the growth of the wall. This datum, combined with the direction of the microfibrils on the pollen tube fits in with the multi-net growth theory forwarded by ROELOFSEN (1959).

This study does not prove that the cell wall-like structure which surrounds the cytoplasm of the generative cell, is made up of the same substances that other plant cell walls consist of. In our opinion the electron transparent layer is by no means an empty space, brought about by fixation and embedding of the material. Indeed in many places electron dense material is seen in the wall, which is otherwise structure-less. Plasma membranes on the inner and outer side of the wall have been observed and turn out to consist of a unit membrane.

LARSON (1963) observed the same structures but concludes from this that there can be no question of a cell wall. In his opinion the generative cell does have a plasma membrane of its own and between this membrane and the plasma membrane of the vegetative cell he observes a space in which also some structures are to be seen. DIERS (1963) has investigated pollen tubes of *Oenothera* with the electron microscope and arrives at the conclusion that there is a real cell wall round the generative cell.

Apart from the evidence obtained with the electron microscope there are other arguments that point to a cell wall-like structure, whose rigidity gives shape to the generative cell. Generative cells are often described as sickle-shaped or spindle-shaped. If such a shape is to be preserved, both during the presence of the generative cell in the germinating pollen grain and in the pollen tube, there should be something to maintain this shape. In our opinion a real cell wall is the cause of this rigidity. Yet another argument can be derived from the work of HOFMEISTER (1956). He plasmolyzes the pollen grain of *Haemanthus brachyphyllus* and finds a clear zone round the cytoplasm of the generative cell. In our opinion this can only be explained by assuming that the cytoplasm of the generative cell is surrounded by a rigid wall which is permeable to the plasmolyticum. The generative nucleus of *Petunia* pollen grains, too, is described as sickle-shaped (a.o. SCHLÖSSER, 1961). Consequently he did not see the generative nucleus but the generative cell. In our investigation the sickle shape was observed. In transverse sections round shapes are often seen as well, of course. Meanwhile it is not yet an established fact that this cell wall round the generative cell is a general phenomenon.

Some investigators, among whom VENEMA and KOOPMANS (1963) and SCHLÖSSER (1961) are hardly able to observe the nuclear envelope round the vegetative nucleus with the phase-contrast microscope or after staining. This is probably a consequence of the many infoldings that the vegetative nucleus appears to possess. However, a real nuclear envelope containing pores is present around the vegetative nucleus in the *Petunia* pollen grain.

It is now generally accepted that the Golgi apparatus is capable of pinching off vesicles at the edges of the flat sacs. The function of these vesicles in the cells varies (MOLLENHAUER and WHALEY, 1963). They are especially found in secreting cells (SCHNEPF, 1961, 1963), where they are supposed to be involved in the secretion process, or they have a function in the formation of the cell plate and cell wall (WHALEY and MOLLENHAUER, 1963). The vesicles of the Golgi apparatus are also involved in the formation of the cell wall of the outer root cap cells (MOLLENHAUER, 1961).

In an electron microscopic investigation of roothairs, SIEVERS (1963a, 1963b) observes an accumulation of vesicles in the tip, the contents of which are supposed to coalesce with the cell wall. We, too, observe a large accumulation of vesicles in the tip of the pollen tubes. The contents of these vesicles are electron transparent in this case. The interpretation concerning the origin and the function of the vesicles in *Petunia* pollen tubes is in agreement with that of ROSEN (1964), who investigated the pollen tubes of *Lilium* with the electron microscope. Further research, however, will have to establish the contents and how they are incorporated in the cell wall.

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REFERENCES

BOPP-HASSENKAMP, G. 1960. Z. Naturforsch. **15b**: 91.
CHARDARD, R. 1958. Rev. Cytol. Biol. Végét. **19**: 223.
DIERS, L. 1963. Z. Naturforsch. **18b**: 562.
HOFMEISTER, L. 1956. Protoplasma **46**: 367.
KROH, M. 1964. Pollen Physiology and Fertilization. Amsterdam. North-Holland Publishing Co.
LARSON, D. A. and C. W. LEWIS Jr. 1961. Amer. J. Bot. **48**: 529.
——— and ———. 1962. Fifth Int. Congress. E. M. **2**: 11. New York and London.
———. 1963. Nature **200**: 911.
MAHESHWARI, P. 1949. Bot. Rev. **15**: 1.
MOLLENHAUER, H. H., W. G. WHALEY and J. H. LEECH. 1961. J. Ultrastruct. Res. **5**: 193.
——— and ———. 1963. J. Cell Biol. **17**: 222.
MÜHLETHALER, K. 1955. Planta **46**: 1.
——— und H. F. LINSKENS. 1956. Experientia **12**: 253.

O'KELLEY, J. C. and P. H. CARR. 1954. Amer. J. Bot. **41**: 261.
ROSEN, W. G. 1964. Pollen Physiology and Fertilization. Amsterdam. North-Holland Publishing Co.
ROELOFSEN, P. A. 1959. The Plant Cell Wall. Berlin-Nikolassee.
ROWLEY, J. R. 1959. Grana Palynologica **2**: 3.
SASSEN, M. M. A. 1964. Pollen Physiology and Fertilization. Amsterdam. North-Holland Publishing Co.
SCHLÖSSER, K. 1961. Z. Bot. **49**: 266.
SCHNEPF, E. 1961. Z. Naturforsch. **16b**· 605.
————. 1963. Naturwissenschaften **21**: 674.
SIEVERS, A. 1963a. Protoplasma **56**: 188.
————. 1963b. Z. Naturforsch. **18b**: 830.
VENEMA, G. and A. KOOPMANS. 1962. Cytologia **27**: 11.
WHALEY, W. G. and H. H. MOLLENHAUER. 1963. J. Cell Biol. **17**: 216.