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Cotton Embryogenesis: The Pollen Cytoplasm

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Summary. The ultrastructure and composition of cotton (*Gossypium hirsutum*) pollen, exclusive of the wall, was examined immediately before and after germination. The pollen grain before germination consists of two parts: the outer layer and a central core. The outer layer contains large numbers of mitochondria and dictyosomes as well as endoplasmic reticulum (ER). The core contains units made of spherical pockets of ER which are lined with lipid droplets and filled with small vesicles; the ER is rich in protein and may contain carbohydrate while the vesicles are filled with carbohydrate. Starch-containing plastids are also present in the core as are small vacuoles. The cytoplasm of the pore regions contains many 0.5 μ spherical bodies containing carbohydrate. After germination the ER pockets open and the lipid droplets and small vesicles mix with the other portions of the cytoplasm. With germination the pore region becomes filled with mitochondria and small vesicles. The vegetative nucleus is large, extremely dense and contains invaginations filled with coils of ER. A greatly reduced nucleolus is present in the generative cell which is surrounded by a carbohydrate wall. The cytoplasm of the generative cell is dense and contains many ribosomes, a few dictyosomes and mitochondria, many vesicles of several sizes, and some ER. No plastids were identified. The generative nucleus is also dense with masses of DNA clumped near the nuclear membrane. An unusual tubular structure of unknown origin or function was observed in the generative cell.

Introduction

This paper is part of a continuing study of the embryogenesis of *Gossypium hirsutum* (cotton) and examines the cytoplasm of the pollen grain, exclusive of the wall, immediately before and after germination. Data are presented on the ultrastructure and composition of the pollen cytoplasm, vegetative nucleus, and the generative cell. There have been several ultrastructual studies of pollen grains (BOPP-HASSENKAMP, 1960; DIERS, 1963; LARSON, 1965; SASSEN, 1966); but no investigations relating structure and composition at this level have been reported. The pollen of cotton has several unique features which are reported here. One of these, the special ER pockets, is given more intensive examination in a paper by FISHER *et al.* (1968).

Materials and Methods

Pollen of *Gossypium hirsutum* L., cultivar M 8949, was collected in the morning soon after the flowers opened. The grains were fixed for electron microscopy in 6% glutaraldehyde in 0.06 M phosphate buffer for 3 hours, washed for 1 hour in water, fixed for 4 hours in 2% OsO_4, dehydrated in acetone and embedded in Epon. The 70% acetone step of dehydration contained 1% uranyl nitrate. Handling

of the pollen was facilitated by the use of 1 ml centrifuge tubes and a microcentrifuge. The sections were stained with REYNOLD's lead citrate and examined with a Zeiss EM-9 electron microscope.

Pollen was germinated using the method of BRONCKERS (1961) as given by MIRAVALLE (1965). This method involves exposing the grains to acenaphthene under controlled conditions. Germinated pollen was fixed by the same methods used with the ungerminated pollen.

Mature pollen grains were prepared for light microscopy as described by FISHER *et al.* (1968). Briefly, this consisted of freeze-substitution, embedment in polyvinylpyrollidone (PVP), and sectioning at 0.2 to 2.0 μ, depending on the purpose for which the section was to be used. Proteins were localized by staining with Aniline blue black or Coomassie Brilliant Blue R 250, insoluble carbohydrates by the periodic acid-Schiff's (PAS) reaction, and nucleic acids by Azure-B staining. Lipids were localized by Sudan-black-B staining of 2.0 μ sections of glutaraldehyde-fixed pollen embedded in a 2:1 mixture of glycol methacrylate (GMA) and ethylene glycol. These localization procedures are sufficiently precise for comparison with electron micrographs.

Carbohydrate localization on the ultrastructural level was achieved through the use of the periodic acid-silver (PA-silver) method as given by ROMBOURG (1967). The only modifications we made were to use gold grids and support films instead of floating the sections during the treatments. The application of this method to plant tissue has been discussed by PICKETT-HEAPS (1967) who used it primarily with $KMnO_4$-fixed tissue. For our purposes this fixative was not suitable, and we used glutaraldehyde (GA) as in the original procedure. Artifacts are caused by the fact that the GA will bind to the tissue, particularly to some proteins and chromatin, leaving free aldehydes which will react with the silver without previous oxidation. Controls are essential on which to base comparisons to determine the sites of material, presumably carbohydrate, which are reactive only after periodic acid oxidation. The major source of artifact in the present tissue was chromatin, nucleolar material, and, to a much lesser extent, cytoplasmic protein. Ribosomes, which have been reported to react after GA fixation, did not in the present case. Attempts to use aldehyde blacking agents after GA fixation and before periodic acid oxidation were unsuccessful for a number of reasons but primarily because those strong enough to completely block the reaction produced extensive damage to the tissue.

Results

The Cytoplasm of the Grain. The cotton pollen is spherical in shape, with a diameter of about 140 μ. The diameter of the cytoplasmic portion is about 130 μ; before germination it is loosely organized into two strata: an outer layer and a central core (Fig. 1). The outer layer is relatively thin (4—12 μ wide) and is characterized by the presence of large numbers of mitochondria and dictyosomes and the general absence of lipid bodies (Fig. 1). The mitochondria are spherical or rod-shaped and contain numerous long cristae which are arranged parallel to the long axis of the organelle (Fig. 1). The outer membranes of the mitochondria as well as the matrix are exceptionally electron-dense. The dictyosomes usually consist of five straight cisternae with a few vesicles clustered about the edges (Fig. 1). There are many 0.05 μ spheres in the cytoplasm similar to those associated with the dictyosomes. The endoplasmic reticulum (ER) is abundant, and the cisternae are highly distended and

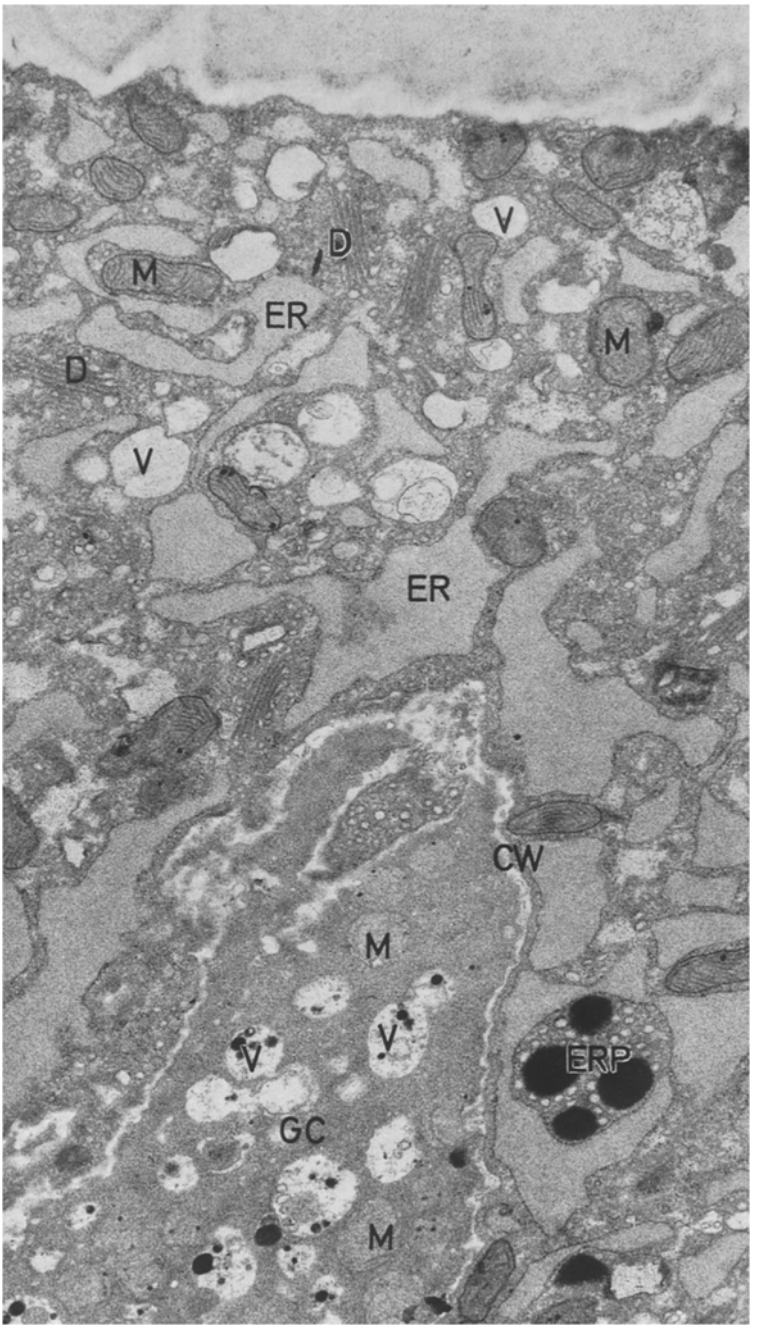


Fig. 1

appear filled with a dense material (Fig. 1). Ribosomes apparently pack the surface of the ER but, because of their spacing and the high density of the ground cytoplasm, they are not readily seen as distinct units. Single membrane bound vesicles, 0.3—0.5 μ in diameter, are also found in the outer cytoplasmic layer (Fig. 1). They may contain granular or membranous inclusions and are irregular in shape. The ground cytoplasm gives a mottled appearance as it is composed of alternate light and dark areas of amorphous material (Fig. 1). No membranes limit these areas.

The core cytoplasm occupies the bulk of the grain and surrounds the vegetative nucleus and the generative cell (Fig. 2). A conspicuous feature of the core cytoplasm is an unusual arrangement of ER, lipid bodies, and small vesicles (Figs. 1—4). The ER is folded in many places to form a hollow pocket (Figs. 3 and 4). Arranged against the inner wall of the pocket are a number of lipid bodies approximately 0.3 μ in diameter. Surrounding the lipid bodies and filling the center of the pocket are 0.05 μ vesicles presumably derived from the dictyosomes. These pockets may vary in size and shape, but they are remarkably similar in organization. Mitochondria and dictyosomes are never found in these associations. These organelles occur between the ER-lipid-vesicle combinations and are similar in character to those found in the outer layer (Fig. 3). The plastids are simple in structure and contain only minimal amounts of lamellae. In the core a few of the plastids contain a single large starch grain (Figs. 3 and 5). Occasionally a plastid with a starch grain will occur in the ER pockets. Large single membrane-bound vesicles are also present in the core and the ground cytoplasm appears mottled as it does in the outer layer.

When the ultrastructure observations are combined with the results of the cytochemical analysis of 1.5 μ Epon-, GMA- or PVP-embedded pollen, the composition of many parts of the pollen grain can be inferred. This is particularly true for the ER pockets found in the core. Sudan-black-B staining of GMA-ethylene-glycol embedded sections confirmed that the dark droplets in the electron micrographs are actually lipids. A weaker, but definite, sudanophilic area also occurs in the ER pockets inside the ring of lipid droplets. This latter staining is presumably due to the large amounts of membrane present in the numerous 0.05 μ

Fig. 1. Enlarged portion of an ungerminated pollen grain. The outer layer occupies the upper two thirds of the picture, the inner core portion the bottom third. The outer layer is particularly rich in mitochondria (*M*), dictyosomes (*D*), single membrane-bound vesicles (*V*). Small vesicles are numerous in both regions, and the ER is highly extended throughout the grain. An ER pocket (*ERP*) can be seen in the core portion. The tip of the generative cell (*GC*) is present in the section and the wall (*CW*), mitochondria (*M*), and vesicles (*V*) characteristic of that cell are visible. GA-Os; × 22,750

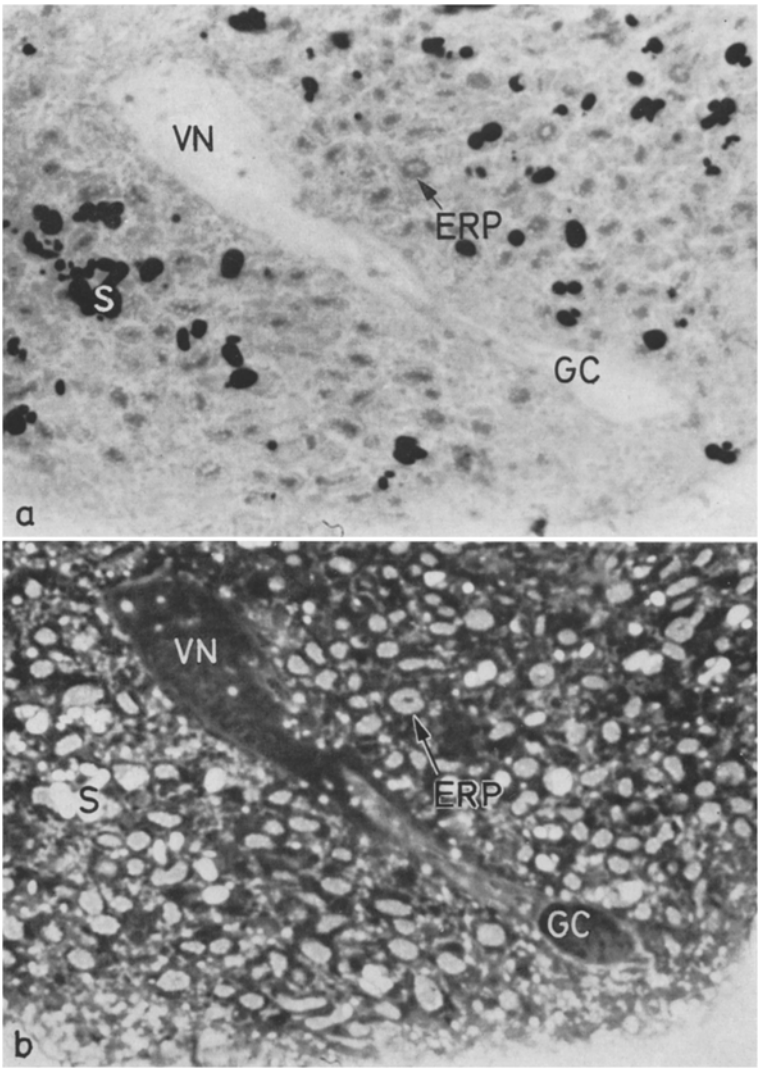


Fig. 2a and b. The generative cell (*GC*) and the vegetative nucleus (*VN*) surround core cytoplasm as seen in a 1 μ section of glutaraldehyde fixed pollen stained with PAS for carbohydrates (a) and Aniline blue black (b) for protein. The same section is seen in a and b. By comparing areas in the two parts of the plate the composition of the various parts of the cytoplasm can be deduced. The ER pocket (*ERP*) shows the PAS-positive material in a ring (a) surrounding and surrounded by ER-rich in protein (b). Starch (*S*) is also indicated. $\times 400$

Fig. 3. Enlarged view of core portion of pollen grain. Part of the generative cell can be seen containing the nucleus (*N*) and mitochondria (*M*). The cell is surrounded by a thin wall (*CW*). The cytoplasm of the grain contains ER with swollen cisternal

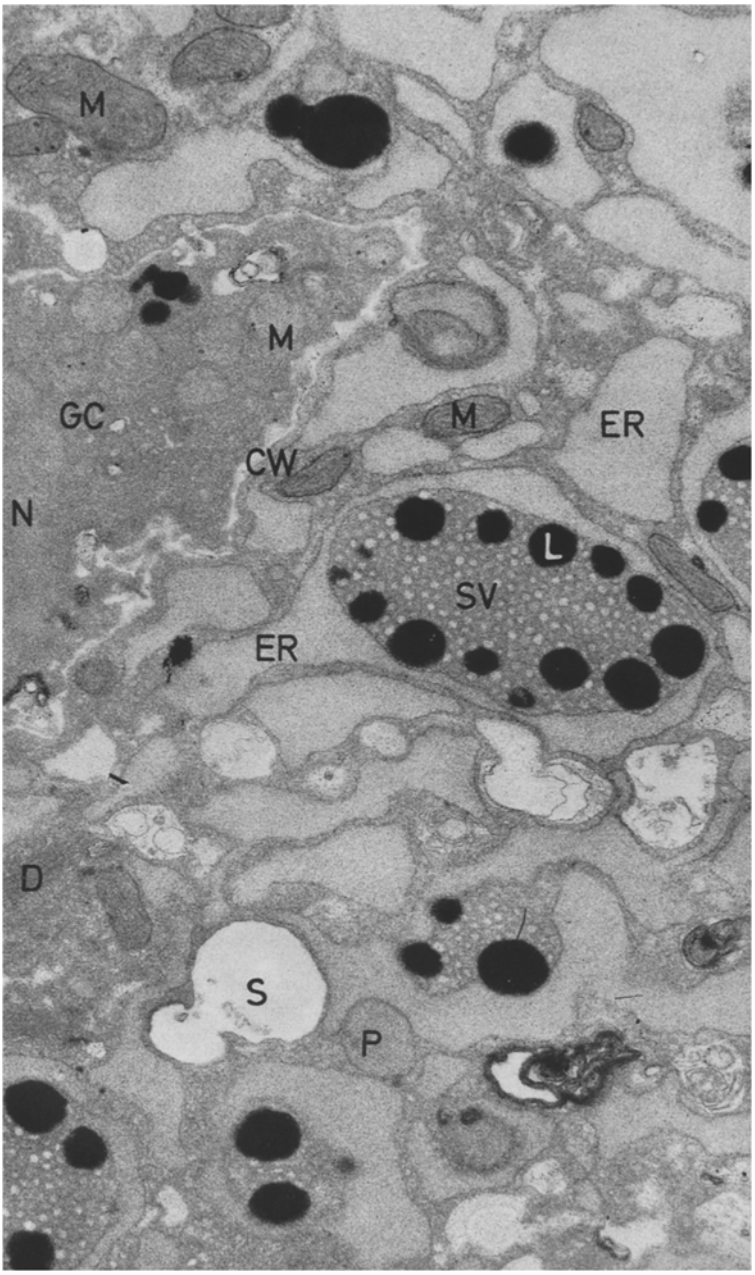


Fig. 3

regions. Well-developed mitochondria (*M*), dictyosomes (*D*), poorly developed plastids (*P*), and starch (*S*). An ER pocket composed of a surrounding wall of ER, lipid droplets (*L*) and numerous small vesicles (*SV*) can be seen. × 22,750

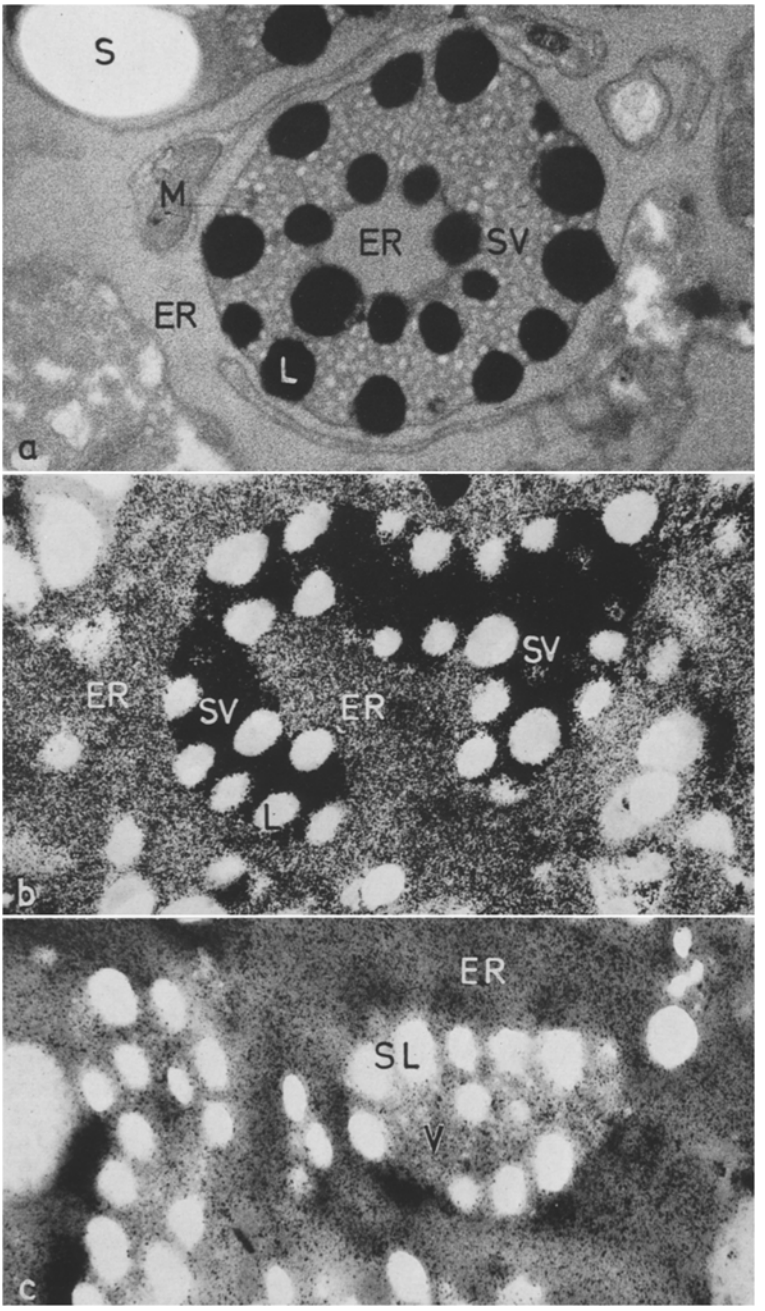


Fig. 4a—c

vesicles found in that area. All of the Sudan-black-B-positive material in the cytoplasm disappeared after acetone extraction.

The cisternal area of the ER is so large that it can readily be seen in the light microscope. These areas stain strongly with the protein stains (Fig. 2b) and lightly with the PAS reaction (Fig. 2a). The area not occupied by ER stains only slightly for proteins and carbohydrates. The center of the ER pockets are strongly PAS-positive (Fig. 2a) presumably due to the contents of the 0.05 μ vesicles present there.

The PA-silver results allow direct confirmation on the ultrastructural level of some of these conclusions. These preparations show (Fig. 4) a strong reaction in the center of the ER pockets associated with the 0.05 μ vesicles and a lighter although distinct reaction in the ER.

Many bodies which stain only with the protein stains are seen (Fig. 2b) in the cytoplasm and are presumed because of their size and distribution to be plastids and mitochondria. The latter are negative in the PA-silver reaction (Fig. 6). Starch is present in the plastids but when thin sections are stained with PA-silver (Fig. 5) the starch falls out and only a coarse precipitate of silver is seen. The PA-silver-treated plastids, however, do contain regions which are strongly PA-silver-positive (Fig. 5). These appear to be distinct from the starch grains in that they are usually smaller, are frequently crescent-shaped, and show no signs of dropping from the sections. The reaction is due to the presence of a periodic acid-oxidizable compound and is not a fixation artifact.

The starch, the ER pockets, and the ER itself account for most of the PAS-positive material in the cytoplasm although other structures also stain. Most of these are difficult to identify on the EM level. One exception is the pink-staining, PA-silver positive vesicles, 0.4 μ in diameter, clustered in the germ pores and scattered throughout the outer layer of the cytoplasm close to the intine (Fig. 6). Unidentified structures include irregular pink bodies, about 0.4 μ in diameter, distributed randomly throughout the cytoplasm.

A summary of the composition and structure of the ER pockets is given in Fig. 7.

Fig. 4. a A portion of the core cytoplasm showing an ER pocket. The pocket is spherical with a projection of ER into the center. The lipid (*L*) droplets and the small vesicles (*SV*) are clearly visible. Note that the mitochondrion (*M*) is outside the ER pocket. A starch (*S*)-containing plastid is present in another pocket. × 22,750. b A similar ER pocket after the PA-silver reaction. The portion occupied by the small vesicles (*SV*) is strongly positive while the ER is much less reactive. The tissue was fixed only in glutaraldehyde and the lipids (*L*) have been extracted during dehydration so that the space they occupied appears clear. × 22,750. c A control section on the PA-silver reaction. The section was placed in the silver regent without previous oxidation with periodic acid. While the general background precipitation is marked, there are still clear differences between it and the PA-silver (b). × 22,750

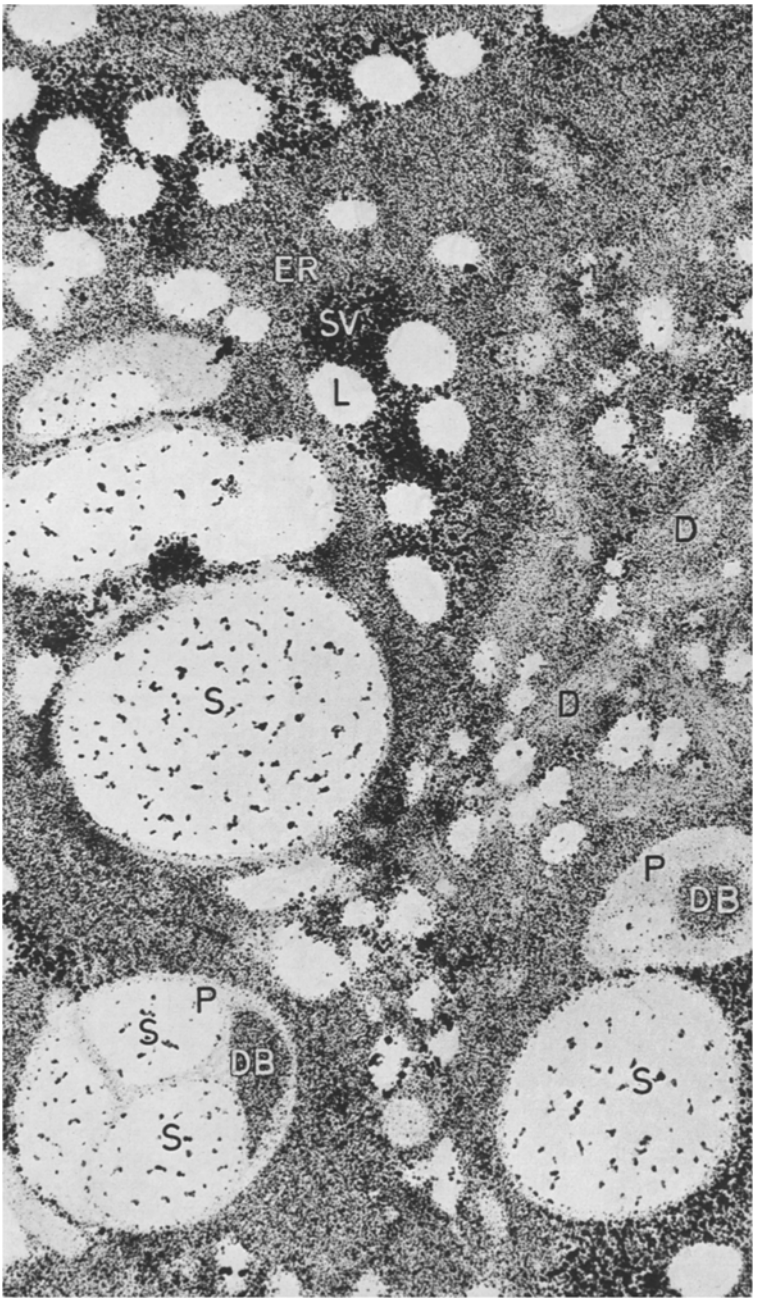


Fig. 5

Although there is very little qualitative variation from the above description from pollen grain to pollen grain, there are a number of quantitative differences. The amounts of starch and lipid, swelling of the ER, and the numbers of 0.5 μ PAS spheres in the outer cytoplasm and germ pores are widely, and independently, variable. The relative amounts of lipid in the ER pockets and that scattered as single droplets in the cytoplasm is also quite variable.

When the pollen germinates, the spherical ER pockets of the core open. The lipid droplets and small vesicles they contained mix with the plastids and the mitochondria of both the core (Fig. 8) and the outer layer (Fig. 9). The lipid bodies are frequently seen to be surrounded by a shell of small vesicles (Fig. 9). The ER in the center of the grain expands as do the single membrane bound vesicles. The latter enlarge to form considerable vacuoles and frequently contain filament-shaped deposits (Fig. 8). The density of the background cytoplasm decreases and ribosomes become clearly evident. The surface of the ER is literally packed with ribosomes (Fig 10). The dictyosomes are surrounded by a greater number of vesicles. The cytoplasm in the pores becomes a compact mass of mitochondria and various sized lipid droplets as well as small vesicles and some ER (Fig. 11).

The Vegetative Nucleus. The tear-shaped vegetative nucleus and the elongated generative cell are always found in close association in the pollen grain, either in a linear array (Fig. 2) or, more frequently, in a long crescent in the peripheral part of the inner cytoplasmic core. The nuclear membrane is highly convoluted and invaginations of coiled ER can be seen in section (Fig. 12). These are PAS positive (Figs. 2 and 13). Lipid deposits are frequently observed located in these invaginations (Fig. 12) and stain with Sudan-black-B. One arm of the nucleus is always found close to the generative cell (Figs. 2 and 13). The extreme density of the nucleus in the GA-Os fixed grains makes observations difficult and no internal structures have been seen, including a nucleolus. However, in the PA-silver preparations (Fig. 13) the vegetative nucleus appears clear and what apparently is a small nucleolus can be seen.

The Generative Cell. The generative cell is irregular and elongate in shape with its nucleus displaced away from the vegetative nucleus (Figs. 2 and 13). The general distibution of carbohydrates and protein is seen in Figs. 2 and 13. The generative cell is a definite cell although

Fig. 5. The core cytoplasm after the PA-silver reaction. The starch (*S*) has fallen out of the sections but the plastids (*P*) still contain strongly reactive dense bodies (*DB*). An ER pocket is seen with extracted lipid droplets (*L*) and accumulates of reactive small vesicle (*SV*). A cluster of dictyosomes (*D*) is present. The regions between the dictyosome cisternae rather than the cisternae themselves appear reactive. × 22,750

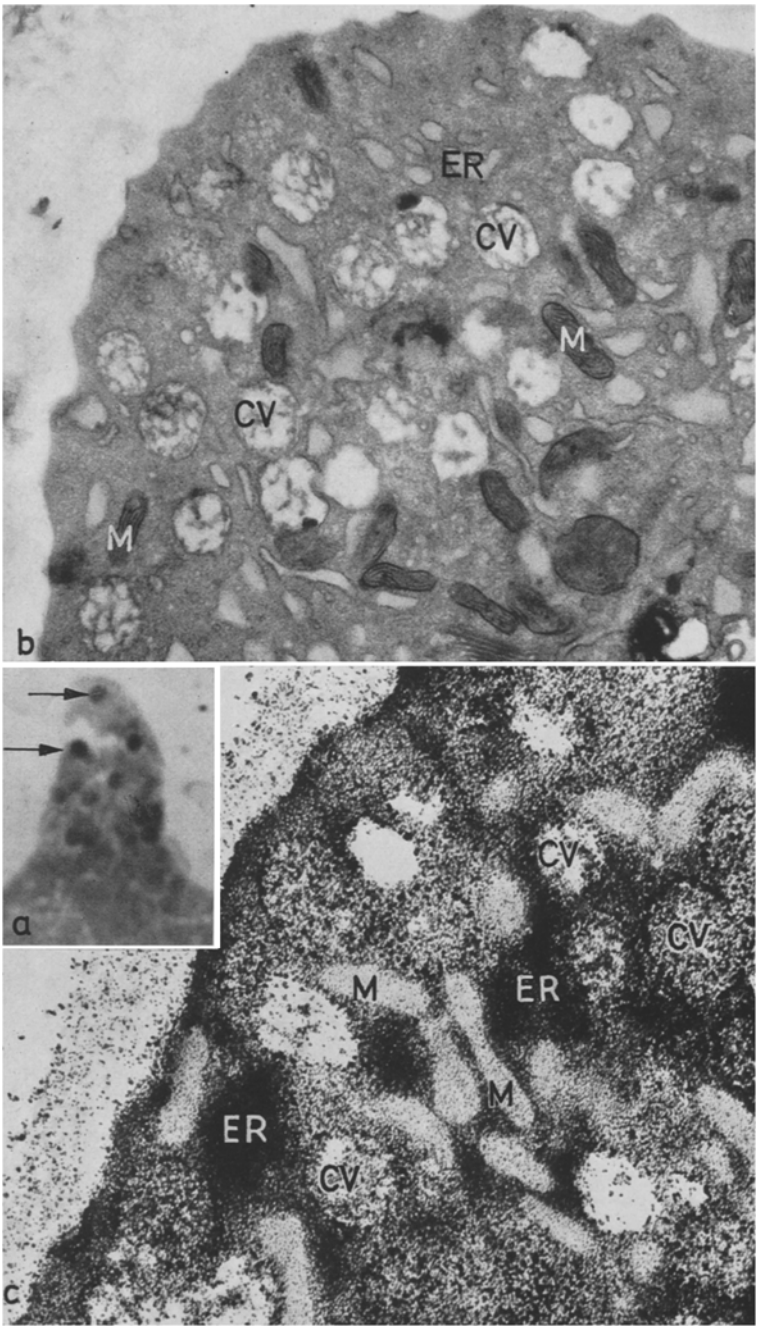


Fig. 6

a greatly reduced one. It is apparently almost always surrounded by a thin, birefringent, PAS-positive wall (Fig. 2) although it frequently would not be seen in light-microscope preparations, perhaps due to its extreme thinness. The reactivity of the wall can be seen best in the PA-silver preparations (Fig. 15).

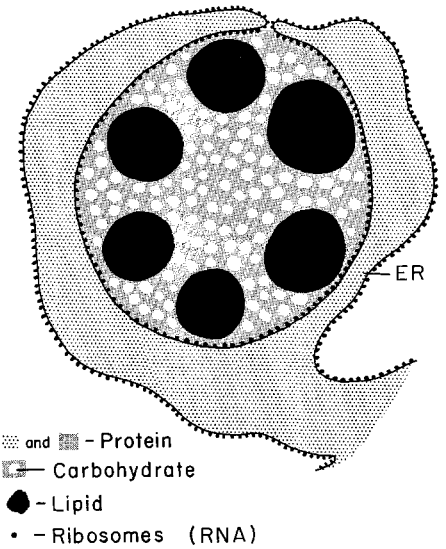


Fig. 7. Diagrammatic summary of the composition of the ER pockets as deduced from the combination of the ultrastructural and histochemical analysis of the pollen grain

The cytoplasm of the generative cell is extremely dense in appearance (Figs. 14 and 17) and in contrast to the weakly-staining vegetative cytoplasm gives a strong positive reaction for RNA when stained with Azure B. Ribosomes are numerous and loosely grouped into clumps (Fig. 17). Little ER is present, and most of what has been seen is arranged parallel to the plasma membrane. There are few dictyosomes present. Each usually has five cisternae, with a few small, associated vesicles (Fig. 17). Lipid droplets are present although few in number (Figs. 1 and 14). Extremely reduced mitochondria can be observed (Figs. 14 and 17) although clearly defined plastids seem to be lacking.

Fig. 6a—c. The pore region of an ungerminated pollen grain. a Lightmicroscopic preparation stained with PAS reaction and Aniline blue black for protein. Positive 0.5 μ spheres are seen throughout the cytoplasm (arrows). $\times$ 3,000. b Electron-microscope preparation of GA-Os fixed tissue. The 0.5 μ vesicles (*CV*) are believed to contain carbohydrate material. There are numerous mitochondria (*M*) and ER is distended. The ground cytoplasm is dense and contains numerous small vesicles. $\times$ 22,750. c Electron-microscope preparation of GA-fixed tissue after the PA-silver reaction. The 0.5 μ vesicles (*CV*) are strongly positive, as is the ER and cytoplasm, while the mitochondria (*M*) are negative. $\times$ 22,750

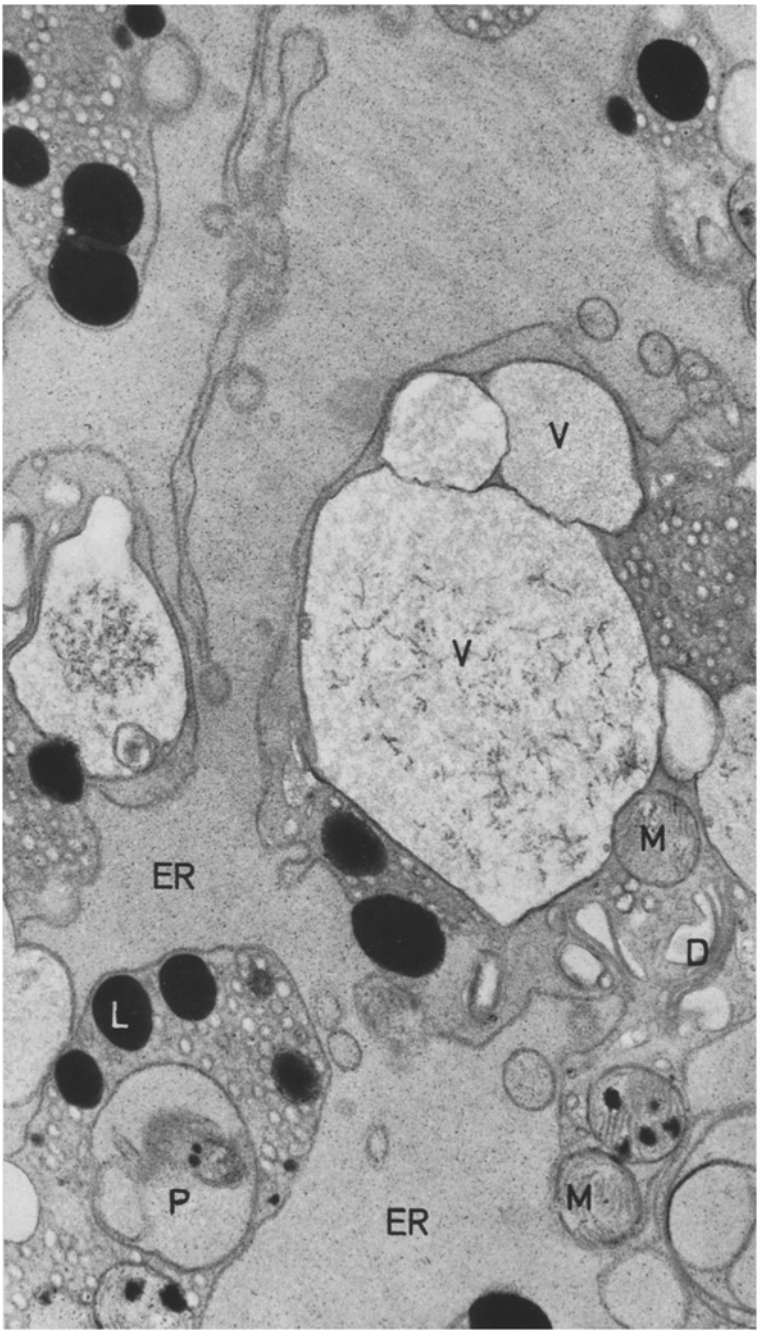
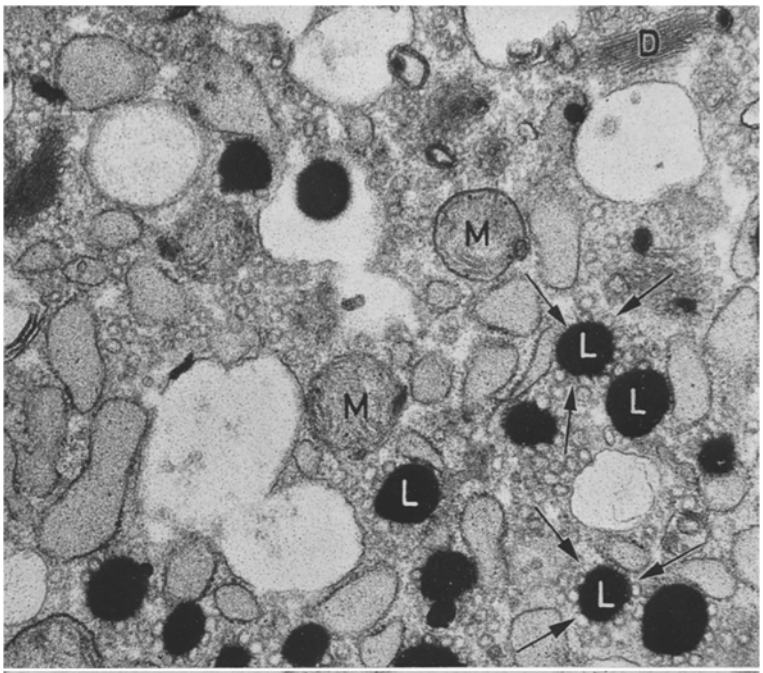


Fig. 8. Core region of pollen grain following germination. An ER pocket which has opened can be seen in the lower left-hand corner. Plastids (*P*), mitochondria (*M*), dictyosomes (*D*), lipid droplets (*L*) and small vesicles are all mixed together. The ER is highly distended and the vacuoles (*V*) are enlarged and contain fiberous and granular material. GA-Os; × 22,750



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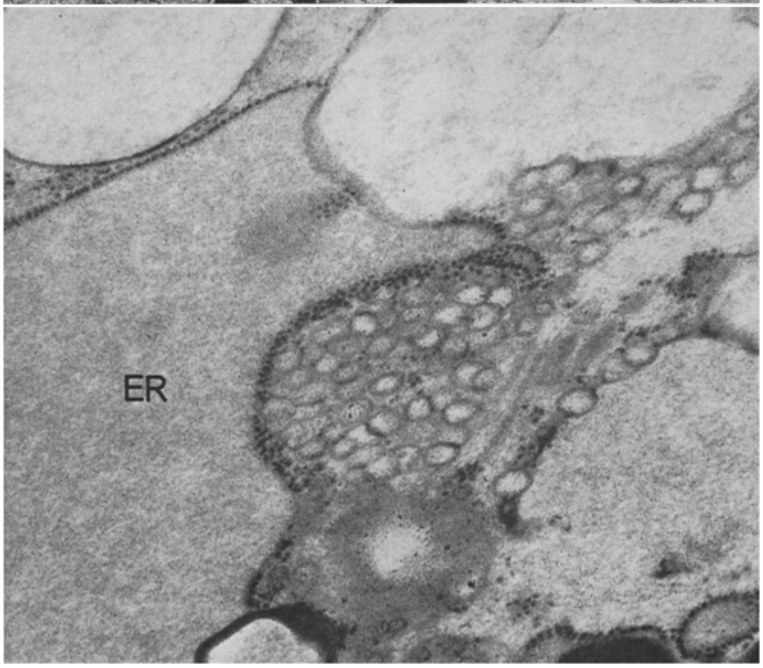

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Fig. 9. Outer region of pollen grain following germination. Lipid droplets (*L*) can be surrounded by a ring of small vesicles (arrows). Numerous mitochondria (*M*) and dictyosomes (*D*) can be seen. GA-Os; × 22,750

Fig. 10. Portion of a pollen grain following germination showing the large number of ribosomes packed along the surface of the ER. GA-Os; × 48,000

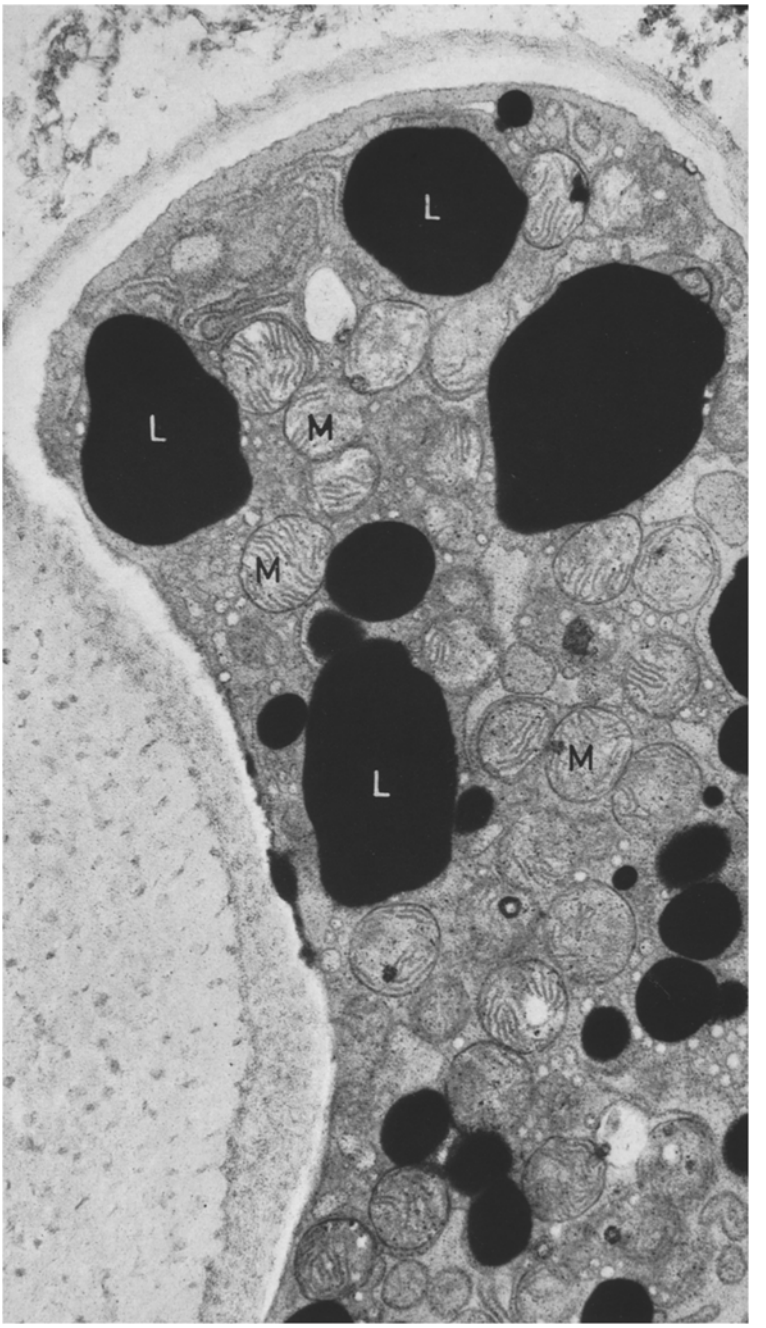

Fig. 11. A pore in a germinated pollen grain. The pollen tube has developed from another pore but all pores show a similar cytoplasmic arrangement. Highly conspicuous are the many large lipid droplets (*L*) and mitochondria (*M*). GA-Os; $\times$ 22,750

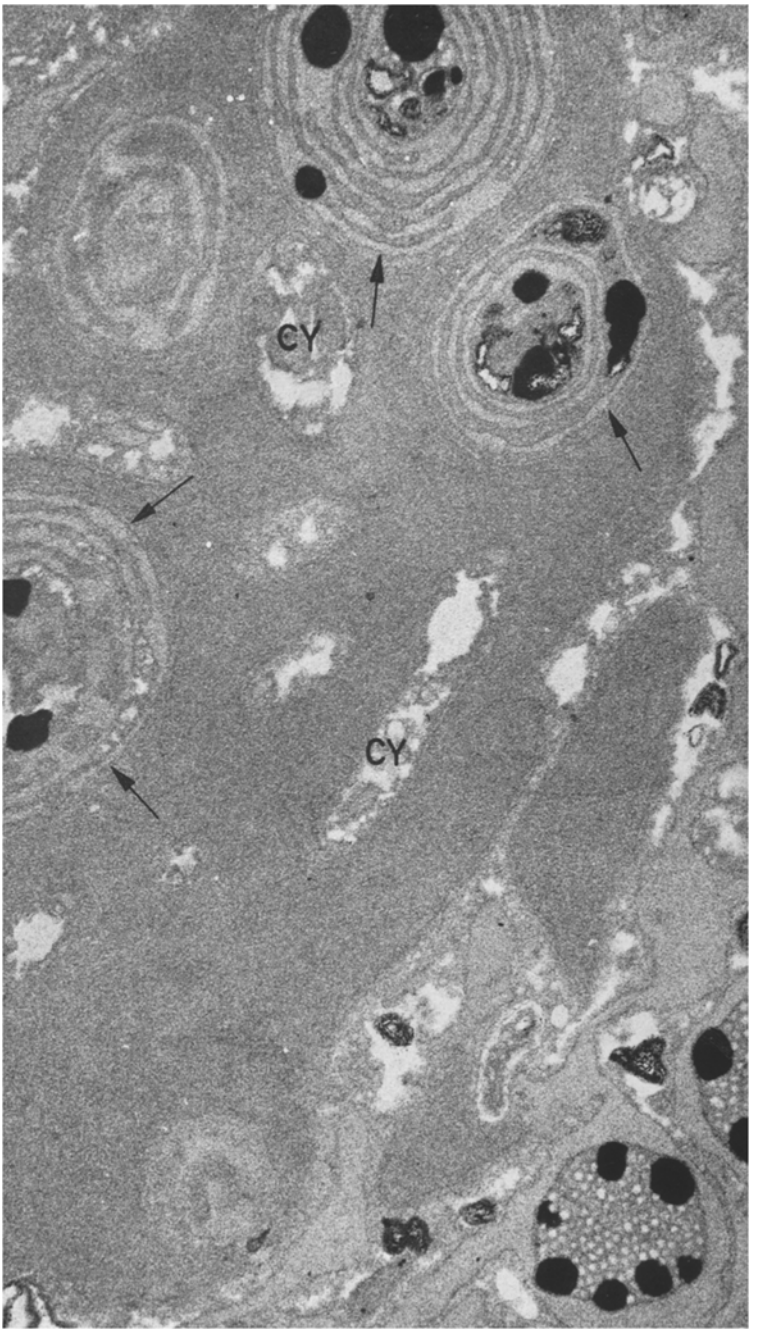

Fig. 12. A portion of the vegetative nucleus. The coils of ER and nuclear membrane (arrows) containing lipid deposites are characteristic of the vegetative nucleus. The deep invaginations of the nuclear membrane containing cytoplasm (CY) are also found in the vegetative nucleus. $\times$ 17,900

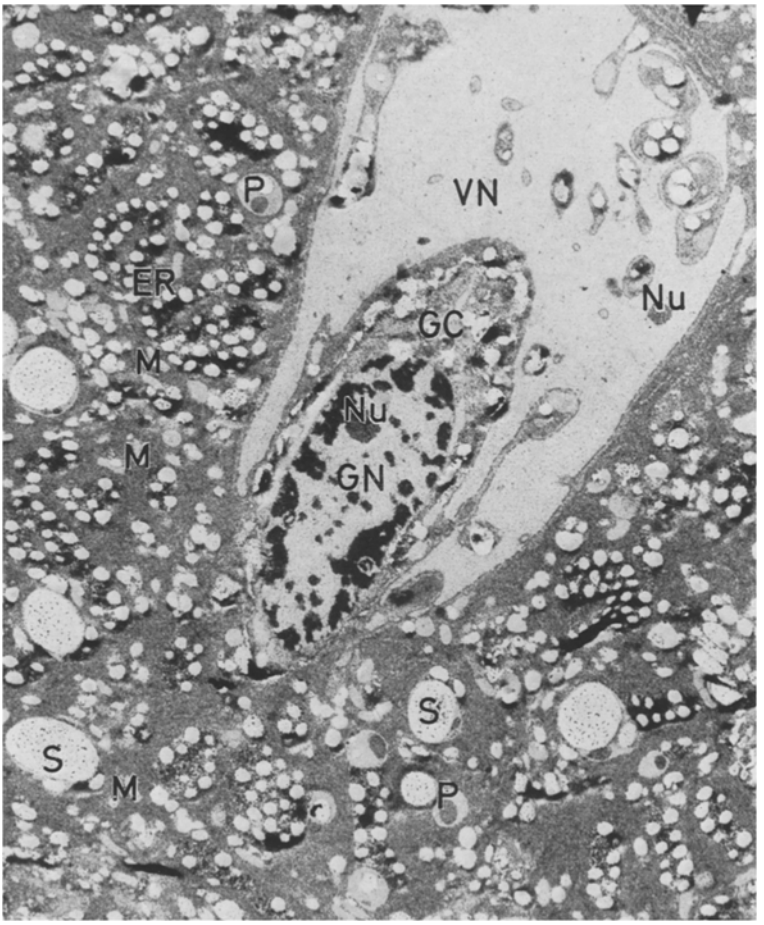

Fig. 13. The vegetative nucleus (*VN*) and the generative cell (*GC*) with the generative nucleus (*GN*) surrounded by core cytoplasm after GA fixation and the PA-silver reaction for carbohydrate localization. The nucleolar (*Nu*) and chromatin reactions are artifacts due to GA fixation. The general morphology of the vegetative nucleus is shown remarkably well in this preparation. The core cytoplasm contains many ER pockets, mitochondria (*M*), and plastids (*P*) containing starch (*S*). × 6,000

Fig. 14. The generative cell containing the generative nucleus (*GN*). A thin wall (*CW*) surrounds the cell and dictyosomes (*D*) and mitochondria (*M*) can be seen in the cytoplasm. At the lower end of the nucleus an arrangement of tubular elements (*TE*) can be seen. These are seen in higher magnification in longitudinal section in the insert. GA-Os; × 22,750. Insert, × 85,500

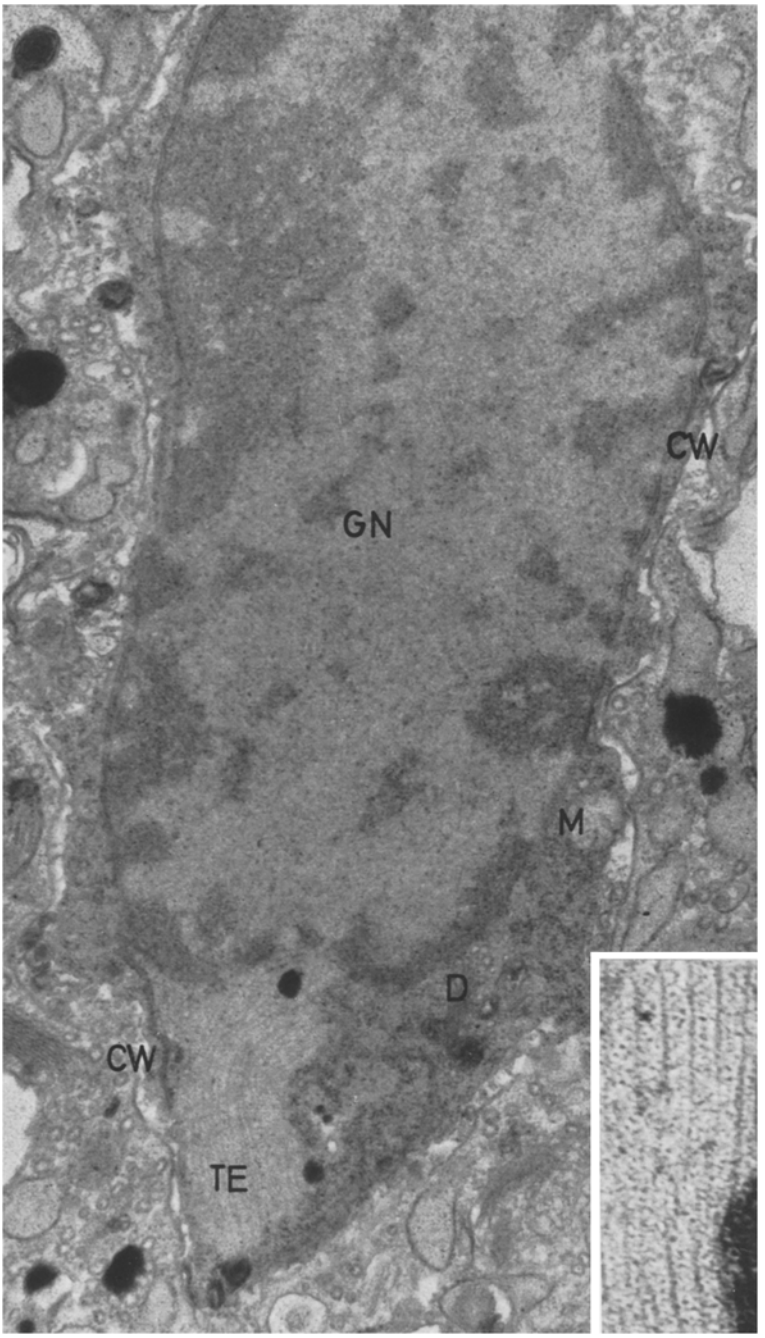

Fig. 14

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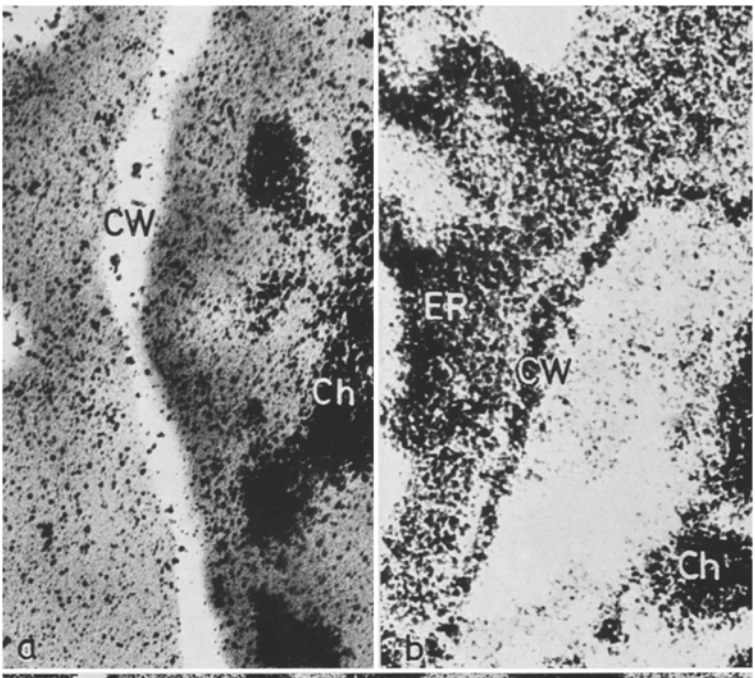

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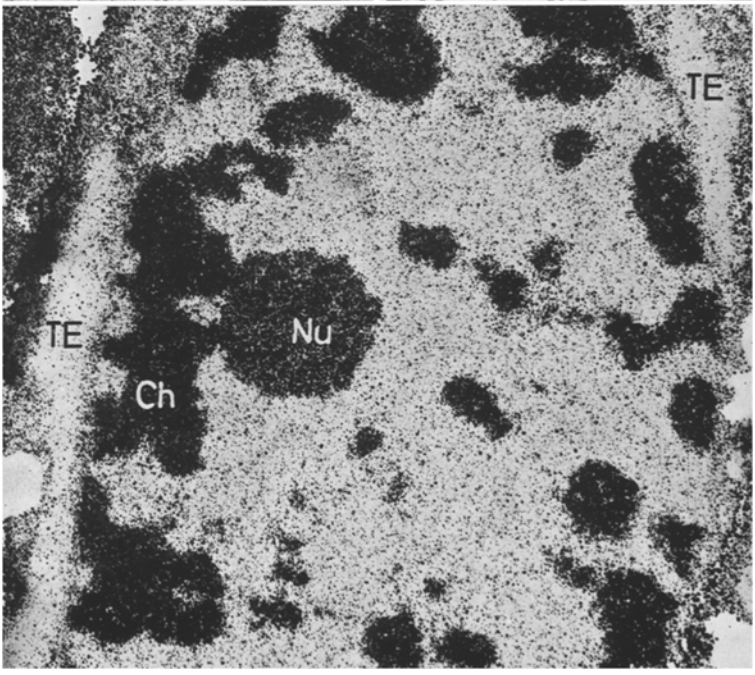

Figs. 15 and 16

Single membrane-bound vesicles, 0.2 to 0.5 μ in diameter, are found at one end of the cell (Fig. 2). These frequently contain lipid droplets and segments of membranes.

The generative nucleus is about 3×7 μ (Fig. 13). It is dense in appearance and the chromatin, which stains strongly for DNA with Azure B, is located near the nuclear membrane (Fig. 13). Many pores can be seen in the membrane. A small nucleolus is present.

An unusual structure composed of parallel strands of material has been observed in various parts of the cytoplasm of the generative cell (Fig. 14). In spite of their provocative position in Fig. 14, examination of the strands in a number of generative cells failed to show any special relationship to the nucleus. They occurred in all parts of the cell cytoplasm and did not, in any instance, penetrate the nuclear envelope. It was not possible to determine the total length of the strands. The structure is composed of alternating light and dark bands (Fig. 14) in a honeycomb pattern when seen in transverse section. The structure is negative in the PA-silver reaction (Fig. 16). The meaning of this structure is unknown, and no vestige of it is seen in the sperm (JENSEN and FISHER, 1968).

Discussion

In many of its ultrastructural characteristics cotton pollen is similar to other pollen examined with the electron microscope (DIERS, 1963; LARSEN, 1965; SASSEN, 1966). The large numbers of dictyosomes, the numerous mitochondria with many cristae, the great many small, spherical vesicles, the abundant ER and the poorly differentiated plastids are all characteristic of pollen.

There are also some unique features in the structure of cotton pollen. The most conspicuous of these involve the ER. The core cytoplasm of the cotton pollen grain contains a distended form of ER which is frequently invaginated to form pockets filled with lipid bodies and small vesicles. These pockets are most remarkable storage combinations. The location of the lipid bodies against the ER suggests that they are formed in association with the ER some time during the development of the pollen grain. The small vesicles are presumably produced by dictyosomes also during pollen development because no dictyosomes are ever found

Fig. 15a and b. The wall surrounding the generative cell is seen to be negative without periodic acid oxidation (a) and positive with such treatment (b) to the silver reagent used in the PA-silver test for insoluble carbohydrates. The chromatin of the generative nucleus is positive in both cases indicating the reaction is not due to the oxidation. The ER is also PA-silver positive. × 62,000

Fig. 16. The nucleus of the generative cell and portions of the surrounding cytoplasm containing the tubular elements (*TE*) after the PA-silver reaction. The tubular elements are negative while the chromatin and nucleolus are giving a false positive reaction. × 22,750

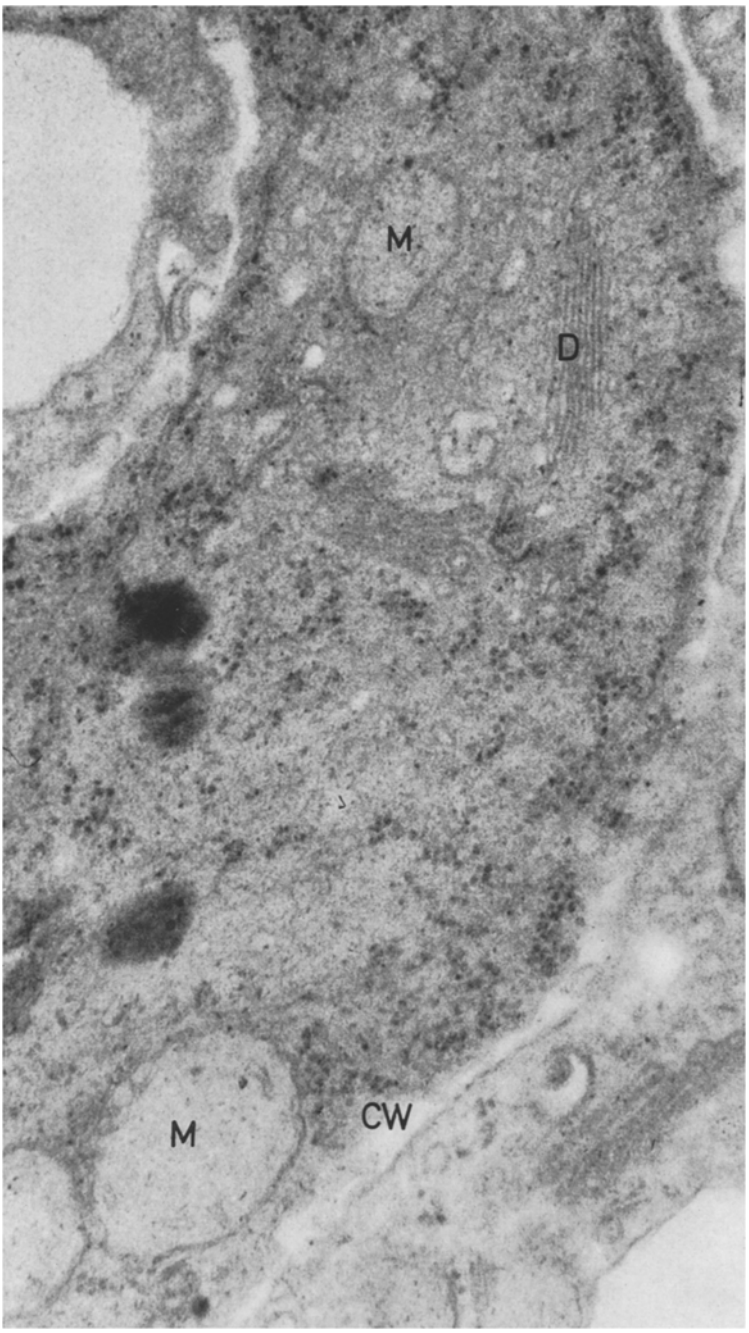

Fig. 17. A portion of the generative cell showing the nature of the mitochondria (*M*), dictyosomes (*D*), ribosomes and cell wall (*CW*). GA-Os; × 63,500

in the ER units themselves. The fact that no organelles, other than an occasional plastid, are observed within the ER pockets must have some functional meaning. Possibly such packaging of the lipid reserves by the ER prevents their premature oxidation by the mitochondria.

The plastids found in the pollen grain are simple in organization and contain fair amounts of starch. However, the reaction of structures within the plastids in the PA-silver test poses some questions. Loss of starch grains from the sections was expected on the basis of the work of PICKETT-HEAPS (1967), but there were, in addition, strongly reacting deposits which apparently differ from the bona-fide starch grains. These irregular-shaped masses of matter must be carbohydrate in nature and cannot be a fixation artifact as they are lacking in the control. They may represent young starch grains that do not drop out of the section, or some other type of storage deposits.

Another unique feature of the cotton pollen grain is the distended nature of the ER itself and the marked density of the cisternal phase. This suggests that the ER is involved in the storage of materials, mostly proteins, which are used in the growth of the tube. This is supported by the fact that in the young tubes the ER is distended and as the tube becomes longer the ER assumes the more common, narrow cisternal form (JENSEN and FISHER, unpublished data).

While the ER in the pollen tube may become less inflated during growth of the tube, the ER remaining in the grain itself becomes even more distended. At the same time the vacuoles in the grain enlarge many times. Both of these changes would have the effect of increasing the pressure on the cytoplasm in the grain and thus promote the rapid growth of the tube.

The ribosomal economy during germination and pollen tube growth is also of interest. The ER of the pollen is packed with ribosomes while relatively few are free in the ground cytoplasm. STEFFENSEN (1966) has shown that the vegetative nucleus in *Lilium* apparently does not produce ribosomal RNA during pollen tube growth, nor does it contain a nucleolus. The present data would indicate that a similar situation exists in cotton and that the ribosomes necessary for subsequent pollen-tube growth are present in the pollen grain before germination, mainly on the surface of the ER.

The data on the vegetative nucleus and the generative cell are, in general, similar to those reported by DIERS (1963), LARSON (1965) and SASSEN (1966). In all cases the vegetative nucleus is highly convoluted and devoid of internal structure except for perhaps a small nucleolus which may be disappearing since no nucleolus has been found in the vegetative nucleus in the pollen tube (JENSEN and FISHER, unpublished data). The coils of ER within the nucleus appear unique for cotton.

In all the species thus far examined with the electron microscope (BOPP-HASSENKAMP, 1960; DIERS, 1963; LARSON, 1965; SASSEN, 1966), the generative cell is indeed a true cell. Cotton is no exception and the present data strengthen the conclusion that this is the general case. Indeed, the generative cell is surrounded by a true wall as observed in *Petunia* by SASSEN (1966).

The unusual structure associated with the nucleus, the parallel rows of organized tubular units, is curious and no explanation is attempted for it.

Finally, with regard to the generative cell, there is the question of the presence of plastids. Both LARSON (1965) and SASSEN (1966) report difficulty in identifying plastids in the generative cell. The condition in the generative cell of cotton is similar and no plastid or plastid like organelles appear present. The possibility that some of the "mitochondria" are, in fact, much reduced plastids remains. However, the data suggest that at some time during its development the generative cell loses its plastids as the sperm which are produced by the generative cell lack plastids (JENSEN and FISHER, 1968).

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