THE ULTRASTRUCTURE OF THE FERTILIZED EMBRYO SAC OF PETUNIA

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SUMMARY

The ultrastructural changes of the embryo sac of *Petunia* during and after fertilization are described. The pollen tube enters the embryo sac by growing through the filiform apparatus and discharges its content into one of the synergids (penetrated synergid). The volume of the penetrated synergid increases and the cell bursts at its chalazal pole. The synergid and discharged pollen tube cytoplasm merge and subsequently degenerate. The degeneration is marked by the darkening of the cytoplasm and the disappearance of organelles. A complex rough endoplasmic reticulum and numerous small spheres remain discernible longest. Two degenerating nuclei are present in the penetrated synergid.

The ultrastructure of the zygote changes slightly during its early development. 50 hrs after pollination (10–15 hrs after fertilization) the nucleus does not contain chromatin clumps any more and is surrounded by a shell of plastids.

The primary endosperm cell shows a number of marked changes after the formation of its nucleus. The ribosomes become aggregated into large polysomes and the plasma matrix becomes homogeneously electron-dense. Both the mitochondria and dictyosomes change in ultrastructure and shape. Plastids, without starch, appear as the endoplasmic reticulum becomes very extensive. This ultrastructural differentiation indicates a changing metabolic activity.

1. INTRODUCTION

There exists a great variation in the way of fertilization among various species, according to the numerous light microscopical studies (*cf.* MAHESHWARI 1950, 1963). However, ultrastructural studies of *Torenia* (VAN DER PLUIJM 1964), *Gossypium* (JENSEN & FISCHER 1968), *Capsella* (SCHULZ & JENSEN 1968a), and *Zea* (DIBOLL 1968) established a common fertilization principle, i.e., the pollen tube enters the embryo sac through the filiform apparatus and discharges its content into one of the synergids.

COOPER (1946) reported that in *Petunia* the pollen tube enters the embryo sac between the apices of the synergids and the egg. The tube enlarges and forms two cone-like projections at its apex, one of which extends into the central cell and the tip of the other one is closely appressed to the egg. Cooper assumed that the male nuclei enter the embryo sac by way of these projections. Two X-bodies remain in the pollen tube and Cooper interpreted them as the cytoplasmic remnants of the male gametes.

The present paper describes the ultrastructural changes of the embryo sac of *Petunia* during fertilization and the early development of the zygote and primary endosperm cell. The ultrastructure of the mature embryo sac has been described

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previously (VAN WENT 1970a, b). The data collected differ significantly from those of COOPER (1946) and shed new light on the fertilization mechanism in *Petunia*.

2. MATERIAL AND METHODS

Flowers of *Petunia hybrida*, clone W 166k, were hand-pollinated with pollen of *Petunia hybrida*, clone T2U, and the anthers removed immediately after opening. The time at which pollen tubes arrive in the ovary was estimated by UV fluorescence microscopy (LINSKENS 1957) and varies from 35 to 40 hrs after pollination. In order to obtain fertilized ovules at various stages of development flowers were collected for fixation at 2 hrs intervals from 35 to 50 hrs after pollination.

The central placentas and attached ovules of the flowers were isolated and fixed in 5% glutaraldehyde (GA) in 0.1 M phosphate buffer at pH 7.2 for 3 hrs at 4 °C. Following GA fixation the material was washed overnight in 0.1 M phosphate buffer of pH 7.2 and subsequently cut into sections, 0.5–1 mm thick. The sections were post-fixed in either 5% $KMnO_4$ for 7 hrs or 2% OsO_4 for 24 hrs at room temperature and then washed in water. The ovules which had attached pollen tubes were isolated from the ovary sections, dehydrated in ethanol, and embedded in Epon via propylene oxide. A complete description of the embedding procedure was given previously (VAN WENT 1970a).

3. RESULTS

3.1. Pollen tube and synergids

The pollen tube enters the embryo sac by growing through the filiform apparatus (*figs. 1, 3*). The growth of the tube ceases and an opening is formed at its apex shortly after it has penetrated the cytoplasm of one of the synergids (penetrated synergid) (*figs. 2, 3*). The volume of the penetrated synergid increases greatly. The same holds for the amount of cytoplasm which the cell contains (*figs. 2, 7*). The large chalazal vacuoles disappear; only a few small ones remain. The cell bursts at its chalazal pole and part of its cytoplasm lodges itself between the central cell (primary endosperm cell) and the chalazal parts of the egg (zygote) and the other synergid (*figs. 2, 9*).

The cytoplasm of both the penetrated synergid and pollen tube show a similar ultrastructure (*figs. 2, 3, 4, 5*). Numerous mitochondria with long cristae oriented parallel to each other, and a few starch containing plastids are discernible during the earliest development of the fertilized embryo sac (*fig. 8*). Characteristic for the cytoplasm is the presence of a large number of small spheres, 0.2–0.3 μm in diameter. They have a dense center and are surrounded by a translucent layer after GA-OsO_4 fixation; however, they are stained homogeneously weak after GA-$KMnO_4$ fixation (*figs. 4, 8*). The spheres in the penetrated synergid eventually form larger aggregates. In the pollen tube the spheres fuse and form an additional wall layer (*fig. 4* double arrow). Sometimes the

tube, especially near its tip, becomes almost filled by this additional layer (*fig. 6*). The cytoplasm of both the penetrated synergid and the pollen tube also contains a complex rough endoplasmic reticulum (RER) (*figs. 4, 5*). At several places the intercisternal phase of this RER is translucent and continuous with the translucent layer of the above described spheres (*fig. 4* single arrow).

The penetrated synergid contains two degenerating nuclei (*figs. 7, 8*). One of them is spherical and lies halfway the synergid near the side; the other one is irregular in shape and lies at the chalazal pole of the cell.

The ultrastructure of the synergid which has not been penetrated by the pollen tube does not change within the period studied (from 35 to 50 hrs after pollination).

3.2. Zygote

The ultrastructure of the zygote changes only slightly during its early development. The polar distribution of the cytoplasm, characteristic for the mature egg (VAN WENT 1970b), is maintained, although some small vacuoles are formed below the nucleus. Nuclear bridges or other signs of incomplete nuclear fusion are rarely observed. The zygote nucleus has an average diameter of 9 μm and has a somewhat irregular shape (*fig. 9*). The chromatine clumps which were present in the mature egg nucleus disappear completely (*fig. 10*).

The number of organelles seems to increase slightly. Most of the plastids become concentrated in a shell around the nucleus (*figs. 9, 10*). There is an increase in the amount of lipid in the developing zygote. No changes in the composition of the polysome population are observed.

3.3. Primary endosperm cell

Incomplete fusion of the male and female nuclei was observed much more frequently in the primary endosperm cell than in the zygote. An initial stage of fusion of the polar nuclei and the sperm nucleus is shown in *fig. 11*. Serial sectioning of these nuclei revealed that they are connected by several narrow bridges and that in a number of other places only their outer membranes are continuous (*figs. 12, 13*). At the later stages of fusion there are only nuclear bridges of variable size.

The primary endosperm nucleus becomes convoluted and the ultrastructure of the cytoplasm changes rapidly as soon as the nuclear fusion is completed (*fig. 14*). The plasma matrix looses the spotty texture which was observed after GA-$KMnO_4$ fixation (*fig. 11*) and becomes homogeneously electron-dense. The numerous free ribosomes become aggregated into large polysomes (*fig. 15*). The mitochondria change in both shape and ultrastructure. They become large and irregular in shape, especially in the plasma region near the penetrated synergid (*fig. 7* insert). Large amounts of lipid are formed in the same plasma region. The changed mitochondria contain a translucent matrix and numerous randomly distributed cristae and tubuli.

Dictyosomes become numerous in the primary endosperm cell. The vesiculate type which was present originally (*fig. 11*) disappears completely. Ulti-

mately each dictyosome consists of 4–6 flat cisternae, approximately 0.8 μm long, which are swollen at their ends (*fig. 14*).

The endoplasmic reticulum (ER) becomes extensive. The ER cisternae are randomly distributed and their membranes are partly covered with ribosomes.

Whereas all plastids of the mature central cell contain starch (VAN WENT 1970b), in the developing primary endosperm cell there are also smaller ones which do not contain starch.

During the above described cytoplasmic differentiation a thick irregularly shaped wall is formed between the primary endosperm cell and the chalazal parts of the egg and synergids (*fig. 7*).

4. DISCUSSION

It has been pointed out previously (VAN WENT 1970a) that the synergids of *Petunia* may produce and secrete substances which direct the growth of the pollen tube (*diagram 1-A*). The present data show that one of the synergids is involved in the cessation of the tube growth, the opening and discharge of the tube, and the transport of the male gametes (*diagram 1-B, C*). In this respect, *Petunia* fits the pattern established for the other angiosperm species examined with the electron microscope: *Torenia* (VAN DER PLUIJM 1964), *Gossypium* (JENSEN & FISCHER 1968), *Capsella* (SCHULZ & JENSEN 1968a), and *Zea* (DIBOLL 1968). The penetrated synergid of *Petunia*, however, does not degenerate before the arrival of the pollen tube, as it does in *Gossypium* (JENSEN & FISCHER 1968) and in a number of other species (VAZART 1958).

The actual mechanism by which the pollen tube is opened is unknown. The impression is that in *Petunia* the pollen tube bursts after its entrance into the synergid cytoplasm. According to JENSEN & FISCHER (1968) the opening of the pollen tube in *Gossypium* can in no sense be described as passively bursting.

LINSKENS (1968) postulated that the sperm nuclei enter the egg and central cell by way of a cell fusion process, as shown in *diagram 1-D, E, F*. This hypothesis is supported by the observations in *Petunia:* 1. No remains of the cytoplasm or walls of the sperm cells are observed in the penetrated synergid; 2. No sperm cell walls are found in the zygote or primary endosperm cell; 3. No trace of penetrated synergid cytoplasm is found in the zygote or primary endosperm cell; 4. No ruptures or pores are observed in the walls of the zygote or primary endosperm cell. SCHULZ & JENSEN (1968a), however, reported the presence of a rupture in the common zygote-synergid wall in *Capsella*. They assume that the sperm nucleus enters the egg through this rupture.

The X-bodies in the penetrated synergid of *Petunia*, which COOPER (1946) interpreted as the cytoplasmic remains of the sperm cells, probably are the degenerating synergid and vegetative pollen tube nuclei. These degenerating nuclei at least are the only structures of comparable size and shape. The identification as nuclei has also been reported for the X-bodies of *Gossypium* (FISCHER & JENSEN 1969) and recently of barley (CASS & JENSEN 1970).

The reported observations on nuclear fusion in the primary endosperm cell

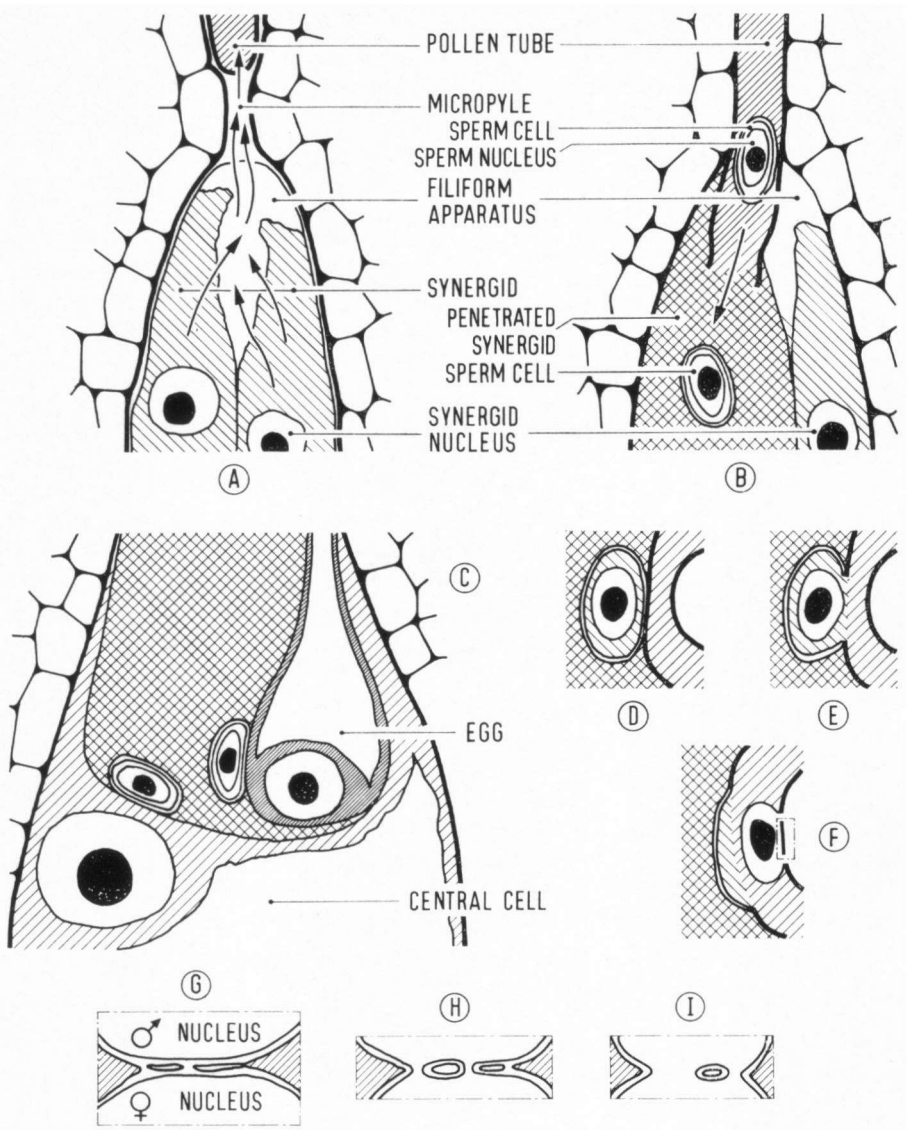

Diagram 1. Diagrammatic representation of the proposed way of fertilization in *Petunia: A*. The mature synergids produce and secrete substances which direct the growth of the pollen tube, *B*. the pollen tube grows through the filiform apparatus and discharges its content into one of the synergids, *C*. the penetrated synergid bursts at its chalazal pole and the sperm cells are transported to the egg and central cell, *D-E-F*. the sperm cells fuse with the egg and central cell resp. and their nuclei become located near the female nuclei, and *G-H-I*. the outer membranes of the male and female nuclei fuse at several places, followed by the fusion of the inner membranes. The formed nuclear bridges enlarge and coalesce.

of *Petunia* suggest the presence of a fusion mechanism similar to that proposed by JENSEN (1964). First the outer nuclear membranes fuse and become continuous in several places, followed immediately by the fusion of the inner membranes (*diagram 1-G, H, I*). The nuclear bridges formed in this way enlarge and coalesce.

JENSEN (1968), SCHULZ & JENSEN (1968b), and DIBOLL (1968) observed dramatic changes in the ultrastructure of the zygote after the formation of the zygote nucleus. Comparable changes are absent in the developing zygote of the *Petunia* clone described here, although the disappearance of the chromatine clumps in the zygote nucleus indicates that the physiology of the cell is changing.

The appearance of marked changes in the ultrastructure of the primary endosperm cell after nuclear fusion indicates that the cell becomes highly active. The responses to fertilization in the zygote and the primary endosperm cell are thus completely different. The presence of masses of free ribosomes in the mature central cell (VAN WENT 1970b) and the rapid formation of large polysomes after nuclear fusion suggest that the availability of a large protein-synthesizing machinery, at the time of fertilization, is involved in the rapid differentiation of the primary endosperm cell.

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KEY TO LABELING

D = dictyosome; dN = degenerating nucleus; ER = endoplasmic reticulum; FA = filiform apparatus; L = lipid; M = mitochondrium; N = nucleus; Nu = nucleolus; P = plastid; PEC = primary endosperm cell; PS = penetrated synergid; PT = pollen tube; RER = rough endoplasmic reticulum; S = synergid; Sph = sphere; St = starch; V = vacuole; Z = zygote.

LEGENDS

Fig. 1. Transverse section through the micropylar part of the fertilized embryo sac. GA-$KMnO_4$ fixation, × 4,500.

Fig. 2. Longitudinal section through the fertilized embryo sac. The pollen tube has discharged its content into the penetrated synergid. GA-$KMnO_4$ fixation, × 4,000.

Fig. 3. Longitudinal section through the micropylar part of the fertilized embryo sac. Arrow indicates the location of the pollen tube wall. GA-$KMnO_4$ fixation, × 7,000.

Fig. 4. Enlarged view of the pollen tube showing the complex rough endoplasmic reticulum, the spheres, the additional wall layer (double arrow), and the original wall. The intercisternal phase of the RER is continuous with the translucent layer of the spheres (single arrow). GA-OsO_4 fixation, × 50,000.

Fig. 5. Enlarged view of the penetrated synergid cytoplasm. GA-OsO_4 fixation, × 26,000.

Fig. 6. Transverse section through the micropylar part of the fertilized embryo sac. The pollen tube is almost completely filled by the newly formed translucent wall layer. GA-OsO_4 fixation, × 5,000.

Fig. 7. Transverse section through the fertilized embryo sac showing the greatly enlarged penetrated synergid. The mitochondria in the primary endosperm cell are large and irregular in shape (insert). GA-$KMnO_4$ fixation, × 3,700.

Fig. 8. Enlarged view of the penetrated synergid cytoplasm. Arrow indicates the location of the complex RER. GA-$KMnO_4$ fixation, × 13,400.

Fig. 9. Transverse section through the chalazal part of the zygote. GA-$KMnO_4$ fixation, × 6,500.

Fig. 10. Transverse section through the chalazal part of the zygote. GA-OsO_4 fixation, × 10,200.

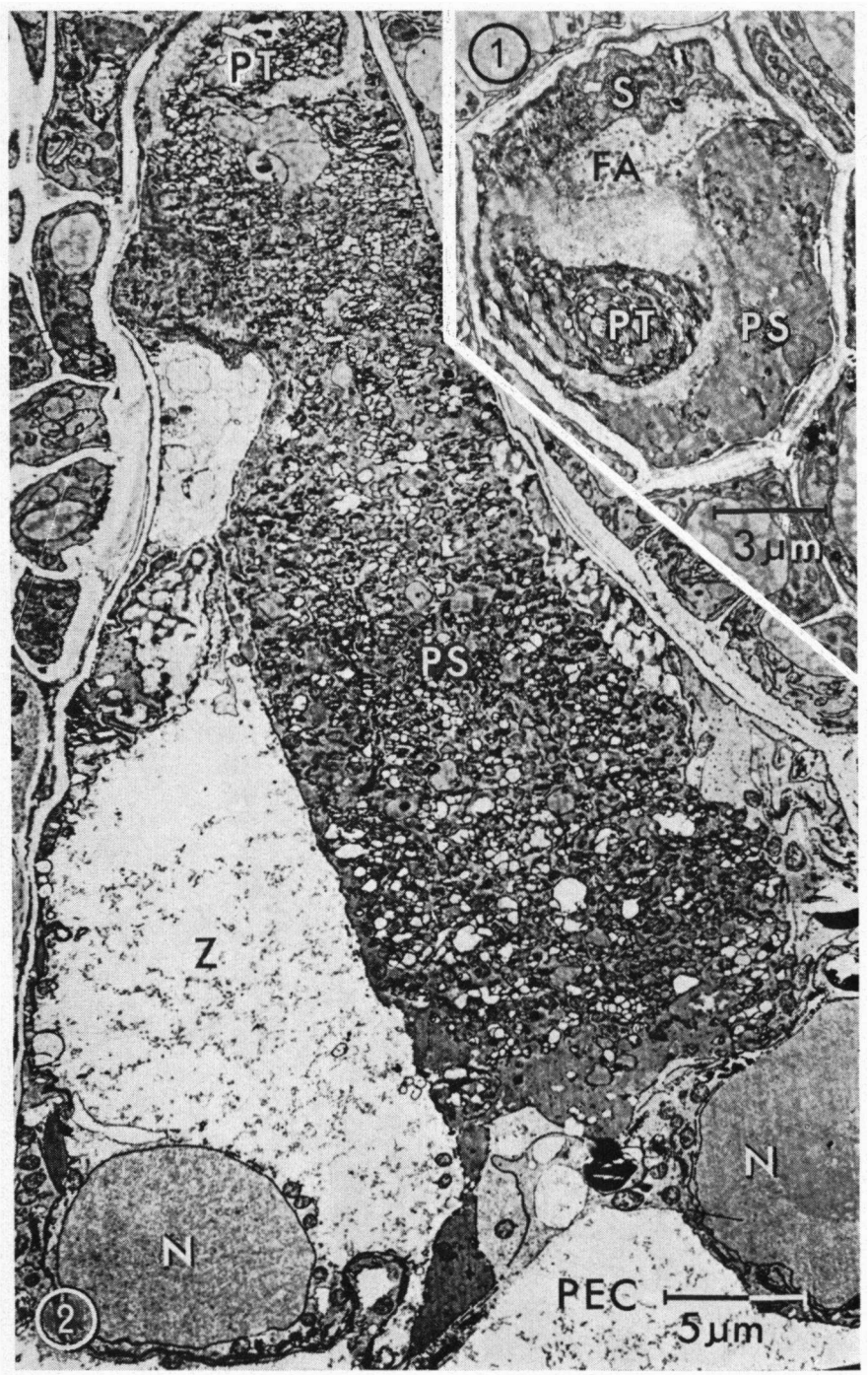
Fig. 11. The primary endosperm cell containing three fusing nuclei. GA-$KMnO_4$ fixation, × 7,000.

Fig. 12. Enlarged view of two fusing nuclei. The outer membranes of the nuclei are continuous. GA-$KMnO_4$ fixation, × 27,000.

Fig. 13. Enlarged view of two fusing nuclei which are connected by several narrow bridges. GA-$KMnO_4$ fixation, × 22,000.

Fig. 14. Part of the primary endosperm nucleus and associated cytoplasm. GA-$KMnO_4$ fixation, × 11,000.

Fig. 15. Enlarged view of the primary endosperm cell cytoplasm showing polysomal aggregation (arrow). GA-OsO_4 fixation, × 26,000.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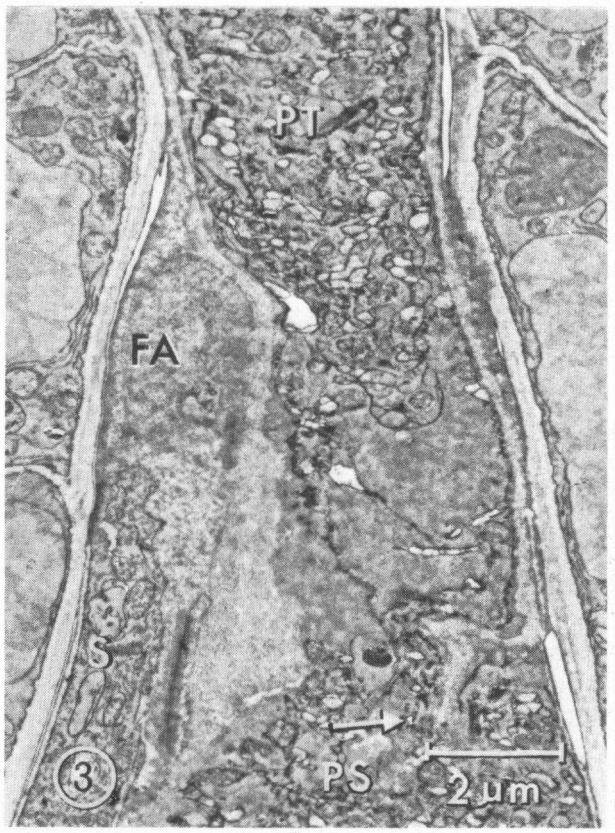
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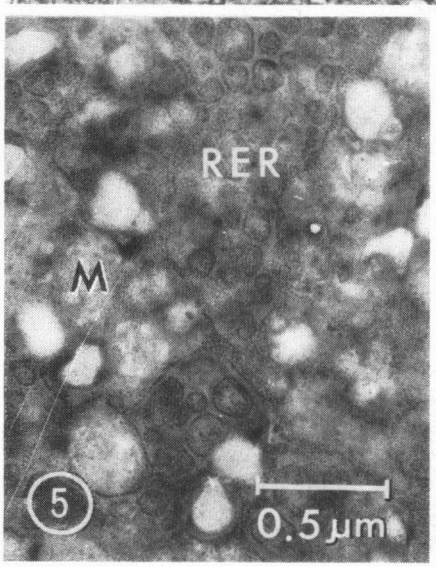
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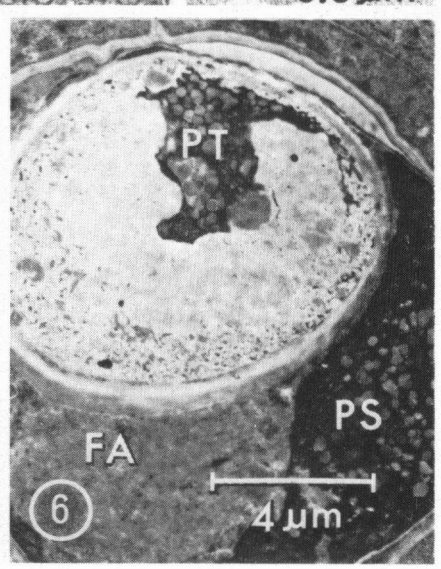
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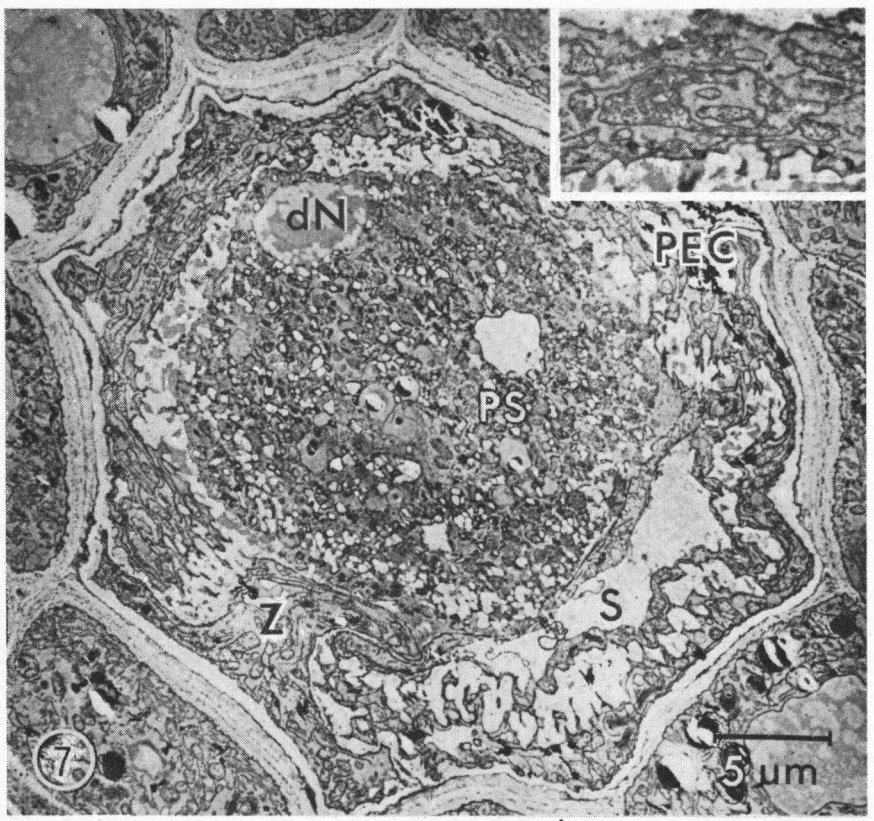
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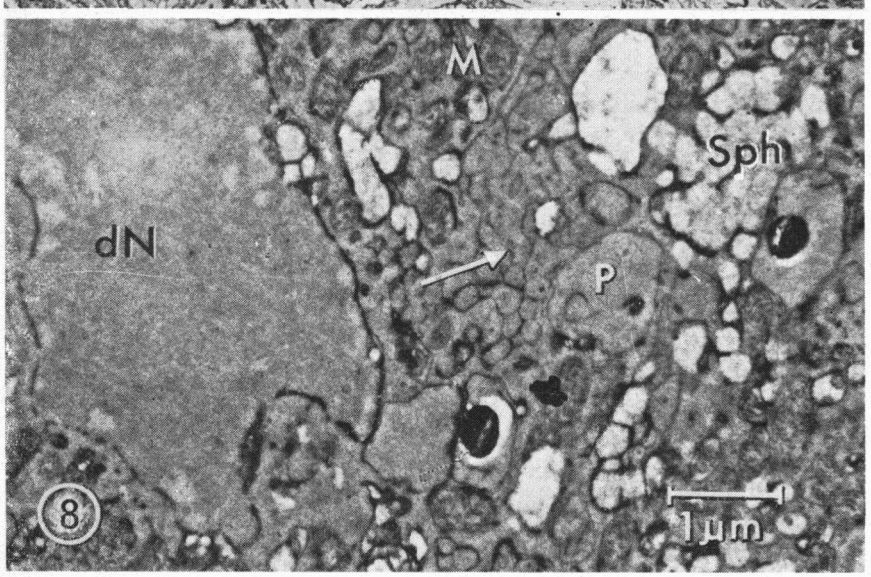
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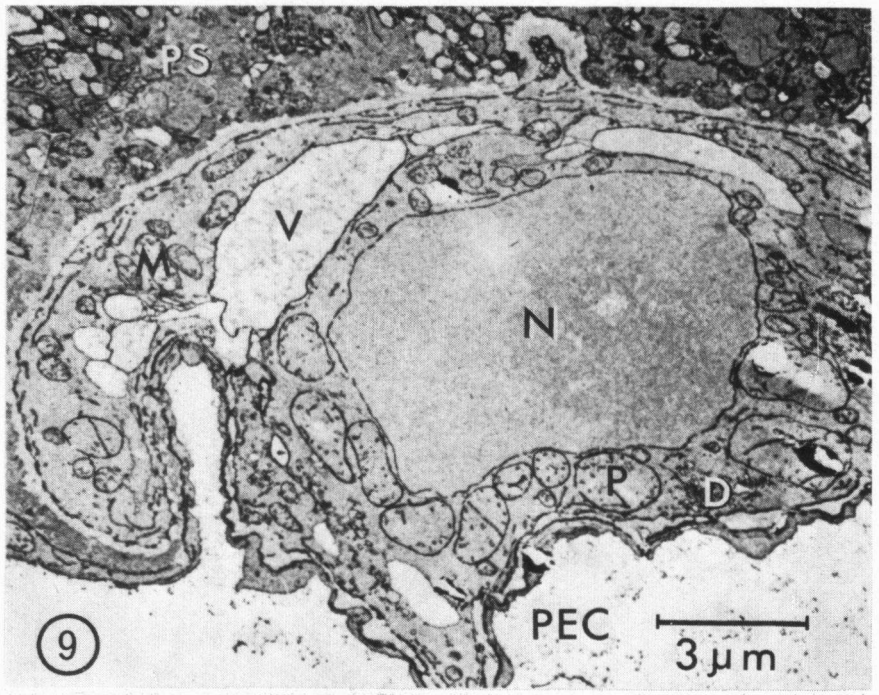
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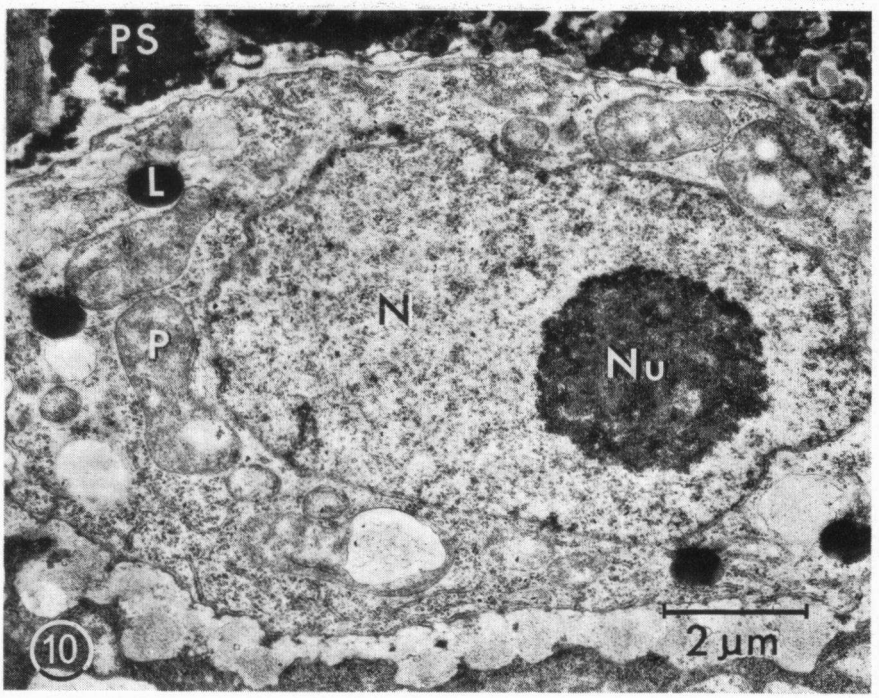
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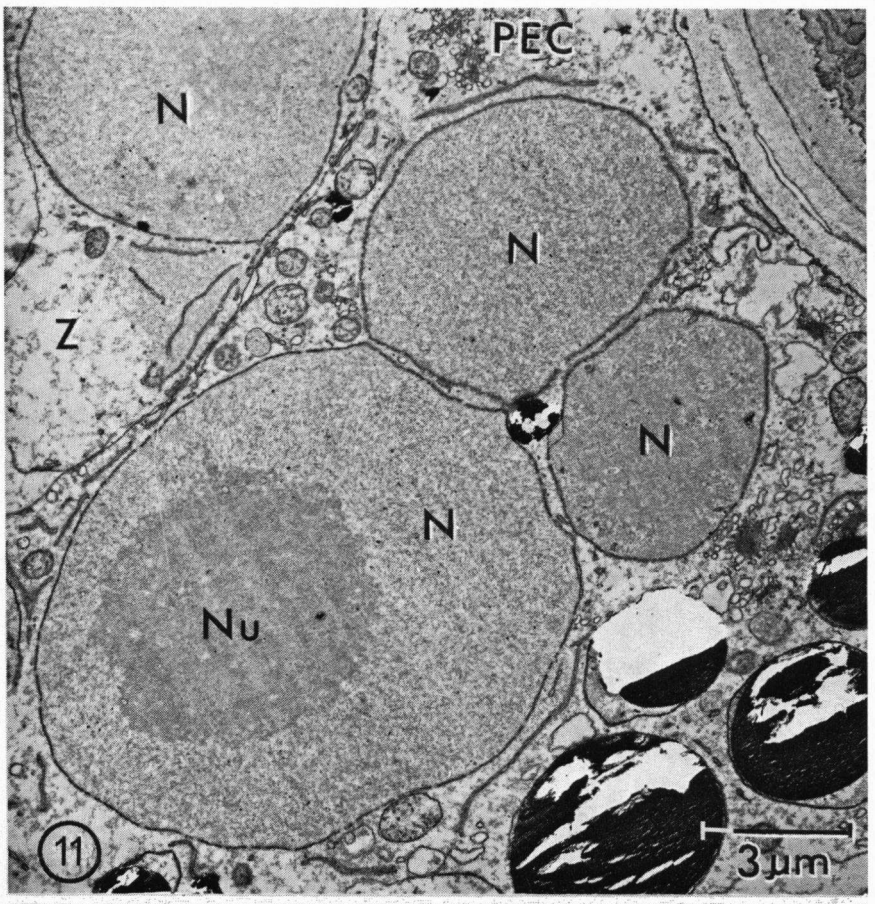
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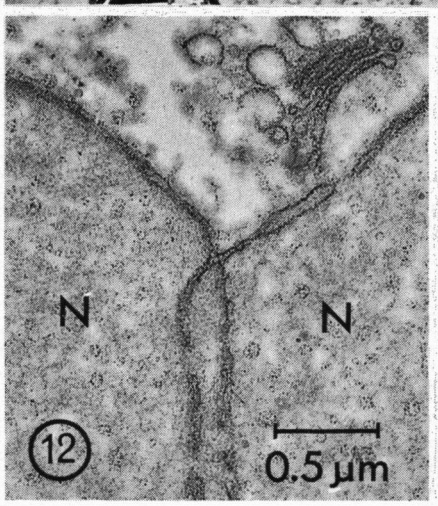
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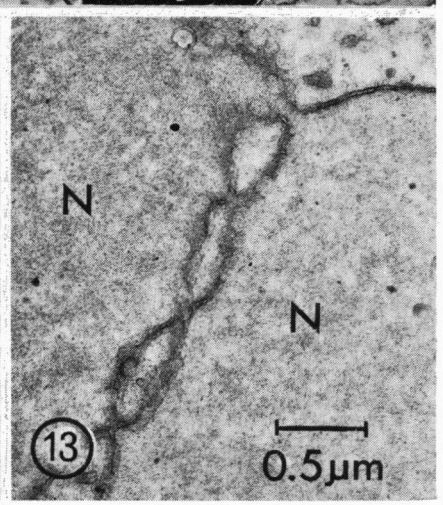
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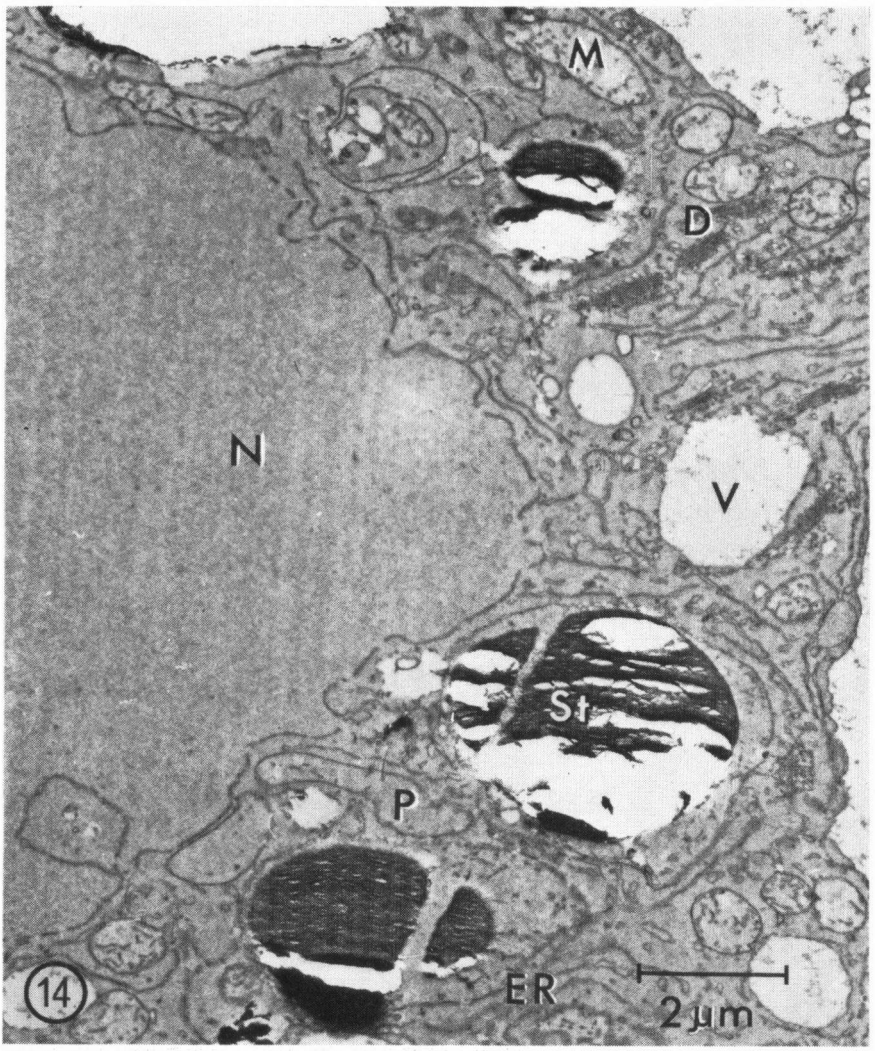
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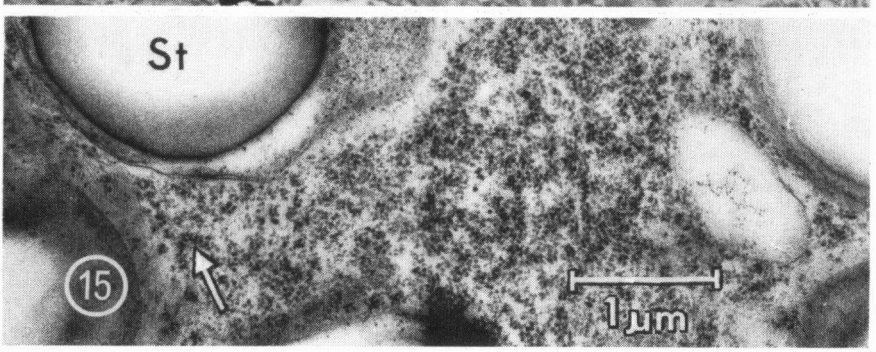
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