Structural studies of protein deposition in developing legume seeds

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STRUCTURAL STUDIES OF PROTEIN DEPOSITION IN DEVELOPING LEGUME SEEDS

by

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To my Parents

Declaration:

The work described in this thesis has been carried out by the undersigned at the Department of Botany, University of Durham, and has not been previously submitted for any other diploma or degree.

Signed .....

Date .....

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ABSTRACT

A comparative study of storage protein deposition has been made in developing seeds of a range of legumes. Varieties of Pisum sativum, Vicia faba, Vigna unguiculata, and Phaseolus vulgaris have been examined. Particular attention has been paid to:

(i) morphological and cytological changes during the course of seed development,

(ii) the structural relationship of the developing seed to its maternal tissue with regard to nutrient transport,

(iii) the structure of the developing embryo with regard to possible pathways for nutrient transport,

(iv) the cytology and ultrastructure of storage protein deposition with the cotyledon leaves.

The results illustrate differences in seed coat structure which could influence the rate of nutrient supply during embryo development and features of embryo structure related to nutrient uptake. Evidence is presented which illustrates uniform storage protein deposition throughout the cotyledon parenchyma tissues of the developing legume seeds examined.

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ABBREVIATIONS

PB	Protein Body
Pb	Protein
CC	Columnar Cells
CHR	Chromatin
COT	Cotyledon
CS	Cell Space
CW	Cell Wall
D	Dictyosome
DB	Dark Body
E	Embryo
EMB EPID	Embryo Epidermis
EMB SA BAN WALL	Embryo Sac Boundary Wall
ENDO	Endosperm
EP	Epidermis
ER	Endoplasmic Reticulum
ES	Embryo Sac
F	Funicle
M	Mitochondria
N	Nucleus
NE	Nuclear Envelope
NU	Nucleolus
OEP	Outer Epidermis
PD	Plasmodesmata
R	Ribosomes
PC	Pod Chlorenchyma

S	Starch
SC	Seed Coat
SP	Seed Pod
VB	Vascular Bundle

INTRODUCTION

The early study of plant material consisted of gross chemical analysis of plant organs and the microscopic investigation of the tissues and cells (Wood (1945), Chibnall (1939), Onslow (1931), Steward & Durzan (1965)). The importance of nitrogen in plant nutrition was known from empirical practices in agriculture and the preoccupation with the maintenance of fertility in soils ((Chibnall (1939), and Stewart & Durzan (1965)). A realisation of the importance of plant nitrogen in animal nutrition has led to considerable investigation of plant metabolism and in particular that related to the development, quality and germination of seeds (Chibnall (1939), Pearson (1933), Rest & Vaughan (1972), and Dieckert & Dieckert (1972).

Early attempts to explain the physiological basis of nitrogen revolved around those compounds that were present in sufficient quantities to be separated and measured with the techniques then available. Research centred on the Gramineae and the Leguminoseae because of their economic importance, and the fact that legumes required no nitrogenous manure yet contained large quantities of nitrogen led to the discovery of nodulation and the development of microbiological techniques that were to provide much of the present knowledge of biosynthetic pathways

Mottier (1921), Onslow (1931), Flinn & Pate (1968), Marinos (1970) and Paramonova (1975)),Boulter (1977, 1981).

An alternative line of study, that of microscopy, led to the recognition of general patterns of morphological development although the importance of the sub-cellular particles,only just discernible by

light microscopy, remained obscure until the development of the more sophisticated techniques of electron microscopy and organic fractionation and biochemistry. These are now providing important information on the functional organisation within the cell. It is interesting to note that one of the earliest recognised subcellular particles was the 'aleurone grain', the intercellular site of storage of reserve protein in seeds. Seed development has been studied ultrastructurally throughout this sequence of events in only a few species of legumes, namely Pisum sativum, Bain & Mercer (1966), Vicia faba, Briarty, Coult & Boulter (1969), Phaseolus vulgaris, Opik (1968), Briarty (1973), Lupinus multiflorus, Arachis hypogoea, Altschul et al. (1966).

The seed reserve proteins have an important nutritional role during seed germination and, in the case of harvested seed, in animal and human diets.

Leguminous seeds have become an important source of man's protein (Protein Advisory Group (1973)) and consequently have been the subject of much research interest.

Leguminous seeds comprise an embryonic axis (radicle and plumule) with two cotyledon leaves contained within a seed coat. The cotyledon leaves contain the food reserve as starch, protein and lipid with relative quantities varying considerably with species, for example soya beans contain 25% of reserves as starch, 50% as protein, and 25% as lipid reserve, whereas cow pea seeds contains 60% of starch, 25% of protein and 1% of lipid reserve.

The importance of legume protein in animal diets has led to the biochemical and nutritional qualities being extensively examined and

associated with this physiological and structural aspects of legume seed development have also been examined.

Early studies of legume embryogenesis include the microscopic studies of Pisum sativum by Cooper (1938a, 1938b) and Reeve (1948).

Cooper described in detail the development of the embryo of Pisum sativum at the early stage. He showed the zygote divides transversely to form a two celled proembryo, the two cells being approximately equal in size. The basal cell now divided longitudinally to form twin suspensor cells and the apical cell divides transversely to form an apical embryo mother cell and a middle cell. The middle cell divides longitudinally to form a two celled middle piece. The nuclei of the two basal and two middle cells divide to form multilinucleate suspensor cells having respectively sixty-four and thirty-two nuclei each. The embryo develops in a typical manner from the apical embryo mother cell. The two multinucleate basal cells elongate and push the embryo and middle piece to the region of the bend of the campylotropous ovule. The suspensor cells disintegrate shortly after differentiation of the cotyledon, epicotyl and hypocotyl. The cotyledons act as storage organs in the mature seed, the endosperm being completely assimilated in the course of embryo development. Reeve (1948) found that the epicotyl apex in the embryo of Pisum was initiated by periclinal divisions in surface cells before the cotyledons appear. A simple apical dome with one or two tunica layers and a shallow corpus is developed during early cotyledonary growth. Also the development of procambium from the meristem of the cotyledons begins with the formation of cell walls in planes at right angles to those of rib-meristem activity. In Pisum, the hypocotyl does

not elongate appreciably before the epicotyl apex is well established or until the columnella tiers are well defined and fracalcium is clearly differentiated in the cotyledons.

During seed development the deposition of reserve protein has been reported to occur in the cell vacuoles of cotyledon storage parenchyma cells of legumes. The formation of these vacuolar protein bodies has been described for a range of legumes, for example Pisum sativum (Varner & Schidlovsky, (1963), Bain & Mercer (1966a), Phaseolus vulgaris Opik, (1968), Vicia faba Briarty, Coult & Boulter (1969), Graham & Gunning, (1970), Swift & Buttrose (1973), Vigna unguiculata Harris & Boulter, (1976). Evidence from Vicia faba cotyledon cell fractionation studies suggested that storage protein is localized within protein bodies (Varner & Schidovsky, (1963)), and this was supported by Graham & Gunning (1970) who showed, by microscopic fluorescent labelling studies with Vicia faba, that the storage proteins legumin and vicilin were contained within the protein bodies.

The development of legume cotyledon has been described in Pisum sativum (Bain & Mercer, (1966a) and Vicia faba (Briarty et al., (1969)), as consisting of four phases: (i) cell division: (ii) cell expansion: (iii) reserve biosynthesis: (iv) seed maturation and dormancy. These authors and others (Briarty, (1973), Phaseolus vulgaris; Harris & Boulter (1976), Vigna unguiculata; and Opik (1968), P. Vulgaris) have reported changes in vacuolar size and number during the development of legume cotyledon. Changes in the vacuolar system and cytoplasm of pea cotyledon parenchyma cells, during the period of storage protein deposition were reported by Craig, Goodchild & Hardham (1979) who used material grown under

carefully controlled conditions. They found that after cell expansion (phase (ii)) cotyledon parenchyma cells each contain 1 or 2 large vacuoles that were replaced by progressively smaller vacuoles during the next 10 days of development, that is, during the period of protein synthesis (phase (iii)). Stainable material that could be histochemically identified as protein appeared on the inner surface of the tonoplasts 8 days after flowering. The vacuoles became smaller and more frequent during embryo development and the amount of proteinaceous material within each vacuole increased until at 16 - 20 days after flowering the vacuoles were densely packed with protein and described as protein bodies. It is generally agreed that the vacuoles, which are present from an early stage in seed development, fill with protein and form the protein bodies of the mature seed. (Opik, (1968); Briarty, Coult & Boulter (1969)). In the case of Vicia faba, there is very strong evidence that the globulin proteins vicilin and legumin (Danielson (1952)) occur only in the protein bodies (Graham & Gunning (1970)) and constitute 90% of their total protein (Morris, Thurman & Boulter (1970)). The site of synthesis of the proteins remained, however, uncertain. The suggestion of Morton and Raison (1964) that the protein bodies are equipped with their own synthesizing machinery has been discounted (Wilson (1966)). There was very strong circumstantial evidence linking the formation of rough endoplasmic reticulum (RER) with the synthesis of the storage protein of V. faba, (Payne & Boulter (1969)).

In 1970, Bailey, Cobb & Boulter, using EM autoradiography, showed that grain counts associated with uptake of labelled amino acids by Vicia faba cotyledons were initially found over the endoplasmic

reticulum and were subsequently associated with the protein bodies. It was concluded that globulin protein is synthesized by the ribosomes of the endoplasmic reticulum and then moved to the protein bodies. From their pulse chase experiments it was suggested that the process took 25 minutes. Whilst the results of Bailey, Cobb and Boulter (1970) implied that protein synthesized at the RER was subsequently accumulated in the protein bodies, Bollini & Chrispeels (1979) showed that reserve protein was synthesized at the RER and it was not until 1980 that Baumgartner, Tokyasu & Chrispeels showed immunocytochemically that the protein synthesized and accumulated in this manner was actually a reserve storage protein. They used an EM immunocytochemical approach with antibodies to the 7S storage protein phaseolin applied to freeze-fixed and sectioned tissue from developing cotyledons of Phaseolus vulgaris. It was found that phaseolin was localised in the lumen of the cisternal ER, ER vesicles and protein bodies. Using high antibody concentrations which inevitably increase some non-specific labelling, label was also found associated with Golgi bodies.

The origin of the protein bodies and their relationship to large vacuoles of early development has been a matter of some debate (Pernollet, 1978). During cotyledon development and the accumulation of storage proteins the population of vacuoles within the storage parenchyma cells changes dramatically from a few large vacuoles to numerous small ones. There are two apparently distinct hypotheses to explain how these vacuolar changes occur. The first postulates that two distinct populations of vacuoles exist. The large empty vacuoles of early development stage which degrade are replaced by protein-containing vesicles and also vacuoles that are derived from the

endoplasmic reticulum (ER) or dictysome vesicles (Khoo & Wolf (1970), Neumann & Weber (1978)). Harris (1979) has suggested that the previously described small, ER-derived vesicles, considered to be of importance in protein transport, are not in fact vesicles but profiles of large ER tubules connected to the rough ER.

The second hypothesis postulates the depostion of protein directly into large vacuoles, which fragment during protein accumulation to give rise to a population of small protein bodies (Bain & Mercer, (1966a); Opik, (1968), Briarty et al., (1969), Craig, Goodchild & Millerd,(1980)).

Craig, Goodchild & Hardham (1979) reported the changes in vacuolar system and cytoplasm of cotyledon parenchyma cells during the period of storage protein deposition. They showed that eight DAF the parenchyma cells each contain one or two large vacuoles that are supplanted by progressively smaller vacuoles during the next 10 days of development. Stainable material that can be histochemically identified as protein appears on the inner surface of the vacuoles' tonoplast 8 DAF. These vacuoles became smaller and more frequent during development and the amount of proteinaceous material within each vacuole increases until 20 days after flowering. They become packed with protein and are described as protein bodies. Craig, Goodchild & Millerd,(1980), used serial sectioning studies of pea to establish the three-dimensional morphology of the vacuoles and protein bodies at various stages of pea cotyledons development and also to study the relationship between these structures. During phase (ii) (at 12 days with their growth condition) there are one or two vacuoles with very complex protrusions developing from a larger central

vacuolar volume.

Sectioning such a complex vacuole shows an apparent population of smaller vacuoles, however each of the protrusions were found to be interconnected. Within 5 days, however, the complex vacuole had given rise to numerous small vacuoles which were mostly discrete, i.e. not inter-connected. By 20 DAF the smaller vacuoles were full of storage protein and filled a major part of the cell volume. A small number of irregularly shaped protein bodies were still present and serial sectioned showed that these arose by sectioning interconnected protein bodies. The results from this work support the idea that the majority of the final protein body population is derived from subdivision of the primary central vacuole.

A combination of both large vacuole auto-division and the generation of new vesicles/vacuoles from ER/dictyosomes has been suggested for protein body ontogeny in cowpea (Harris & Boulter, (1976)), and lupin (Davey & Van Standen, (1978)) and soya (Bong et al., (1980)).

Harris & Boulter (1976) showed that the cells of the cotyledon were highly vacuolate prior to storage protein deposition, with a thin lining of cytoplasm to the cell wall and few transcellular strands of membrane across the vacuole(s). They studied the size changes in total population of vacuoles and vesicles and the size of developing protein bodies during cell differentiaton and concluded that a major part of the protein bodies were derived by enlargement, possibly by coalescing of smaller cytoplasmic vesicles, although sub-division of the original vacuole could account for some protein body formation.

Davy & Van Standen (1978) suggested that proteins forming the major storage product in mature white lupin cotyledonary cells were

deposited in vacuoles (protein bodies) which occupy a large volume of the cell at maturity.

They suggested that proteinaceous aggregates may fuse to form the protein vacuoles, although there was also protein deposition taking place at the tonoplast.

Whilst aspects of embryo development have been studied for a range of legume seeds, questions relating to attendant aspects of nutrient supply from plants to developing seeds have received rather less attention.

Bain & Mercer in 1966, described the ultrastrutural aspects of the development of pea cotyledons including the protein deposition, but the pathways between the developing system and its sources of nutrient were not investigated. Hardham (1976) studied these pathways and showed that the amount of vascular tissue in the funicle and the ovule of developing pea seeds increases markedly between the time of cotyledon initiation and the commencement of storage protein synthesis. Synthesis and deposition of a large number of transfer cells was associated with the sieve elements in the pod and funicle, but not in the ovule. The number of xylem and sieve elements in the funicle decreased as the strand passed from pod to the ovule. Two strands of phloem tissue were found to branch from the main vascular bundle in the ovule and to sweep across the base of the seed and around either side of the region of the embryo sac which contains the radicle. Two distinct pathways are available for metabolic movement into the developing embryo, either via the suspensor or directly across the intervening space between the endothelium of the embryo sac and the epidermis of the embryo. The role of the endosperm is important,

particularly in the young embryo where some or all of the suspensor cells are found to develop wall ingrowths and become transfer cells (Gunning & Pate, (1974)).

While the role of the endosperm, contained in the space between embryo and embryo sac wall, was thought to be passive, some evidence has been obtained to suggest a more active role for this tissue. Chen & Gibson (1974) have shown, in crosses between Trifolium nigrescens and T. occidentale at different ploidy levels, that where the balance of the genomes appeared to be adequate for embryo development, failure of the endosperm was often the primary cause of seed abortion. It appeared that food reserves built up in the endothelium, and when the endosperm degenerated it blocked the process of food transfer between endothelium and embryo. Hybridisation between Pisum sativum and Vicia faba produced a fertile cross but abnormal and limited endosperm development was suggested to be the reason for subsequent embryo collapse (Gritton & Weizbicka, (1975)). The amount as well as the nature of endosperm formed might also be an important limiting factor. Marinos (1970) demonstrated that the endosperm of Pisum sativum has highly mobile tissue forming an ensheathing wall around the embryo and suspensor which connected them to the boundary walls and actively supplied nutrients to the embryo. Paramonova (1975) described further structural modifications in Pisum sativum which appeared to assist nutrient movement into the embryo.

The outer cell wall of the epidermis contained numerous pores and canals, 0.1 to 0.5 μm in diameter, into which the endosperm might penetrate during seed maturation (Paramonova 1975).

This thesis will explore, in a general way the possible

relationships of the developing structures associated with storage protein deposition in a range of legume seeds. We have made a comparative study of protein deposition in a range of developing legumes with known differences in types of storage protein (albumin, globulin, etc.) The sequential stages of development of each legume were studied to determine any gross differences in the pattern of protein deposition within the cotyledons. Such differences might be expected since different patterns of protein mobilization occur during germination.*

Parenchyma cells of legume seeds/cotyledons were examined by light and electron microscopy with attention to both the synthesis and deposition of storage protein and also the possible routes for influx of protein precursors. In particular we have investigated:

(i) morphological and cytological changes in a range of legumes during the course of seed development;

(ii) the structural relationships of the developing seed to its maternal tissue with regard to nutrient transport;

(iii) the structural relationships within the developing embryo with regard to nutrient transport, for example, the structural differences and changes in the adaxial and abaxial cotyledon tissues during embryo development;

(iv) the ultrastructure of storage protein deposition including the roles of the endoplasmic reticulum and Golgi apparatus.

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* The storage proteins of cowpea and mungbean are first hydrolysed in those cells furthest from the cotyledon vascular bundles with a progressive formation of 'islands' of reserve material around the veins (Chrispeels, Baumgartner and Harris (1976)). In pea and V. faba, however, reserve mobilisation is initiated throughout the cotyledon tissues.

In order to investigate and compare the sequential development a range of legumes was grown either in soil in the greenhouse, or grown with nutrient solution under controlled environmental conditions in a growth room.

The legumes examined were; Pea (Pisum sativum) variety Feltham First; Cow Pea (Vigna unguiculata) variety. TVU 76 (Prime) TVU 1190E and TVU 57. New Era. TVU 37 Pale Green. 11. VITA3 and 4557 VITAS. Black Bean (Phaseolus Mungo) variety VAR 519N and Beans (Vicia faba) variety Sudanese Triple White and Maris Bead. The two varieties of V. faba were chosen for comparison because of the marked difference in the rate of seed development.

PEA:

Pisum sativum variety Feltham First was grown either in a greenhouse or grown under controlled environmental conditions in the growth room. In the greenhouse the seeds were sown in 5" plastic pots containing J. Arthur Bowers standard compost. The temperature was kept between 10 deg. C (min.) and 38 deg. C (max.) and humidity was never less than 55% to give optimum growing conditions. Seeds were sown at one per pot. Elatus bieb and P. abyssinicum were grown similarly.

There was variation in the rate of plant growth and date between flower opening and seed maturity. In summer time from germination to flowering was 20 days and from then to seed maturity the period was between 25 to 30 days. During spring, plant growth and seed development take longer with a period of 30 - 45 days between flowering and seed maturity.

For the greenhouse grown material because of the variations in time to maturity

seeds were harvested and their stage of development assessed by fresh weight rather than days from flower opening.

Figure 1 shows the fresh weight of pea at different stages of development at greenhouse and also shows the weight of dry seed at some early stage of development. The plants received constant attention regarding health and conditions.

For growth in controlled environmental conditions seeds of Pisum sativum var. Feltham First (Suttons Seeds Ltd. Reading Berks., U.K.) were germinated in alkathene polyethylene granules (I.C.I. Plastics Division, Welwyn Garden City, Herts., U.K.), in a dark spray room at 28 deg.C with water misting for 5 minutes in every hour for 4 - 5 days. Seedlings were then transported to 2 l water culture bottles of nutrient. The solution contained 154 ppm nitrogen, 200 ppm calcium, 40 ppm sodium, 193 ppm chloride, 54 ppm phosphorus, 36 ppm magnesium, 8 ppm sulphur, 0.064 ppm copper, 0.066 ppm zinc, 0.55 ppm manganese, 0.019 ppm molyb denum, 0.33 ppm borate and 5 ppm iron. Controlled environmental conditions were 16 h day at 890 lux per square metre and temperatures were 28 deg. C at day and 23 deg. C at night with humidity 75 - 80%.

Seeds took 21 days to start flowering and another 21 days for seed development to maturity. Flowers were labelled on the day of opening and cotyledon age refers to days after day of flower opening; so for example day 8 refers to the 8th day after the flower opened. Figure 2 shows the dry weight of seeds at different stages of development and Fig. 3 shows the weight of seed pod at different stages of development.

For microscope work (light and E.M.) pods of the required age were picked, the peas were removed and the cotyledons separated from the testa. These cotyledon were used for experimentation within 10 minutes of removal from the plant in the case of material from the greenhouse, and within 5 minutes for material from the growth room.

COW PEA

Vigna unguiculata var. TVU 76 Prime, TVU 1190E, TVU 57 New Era, TVU 37 Pale Green, 1190E VITA 3 and 455T VITAS were grown in the greenhouse. Seeds were sown in 5" plastic pots containing J. Arthur Bowers standard compost. The temperature was kept between 10 deg. C (min) and 38 deg. C (max) and humidity was never less than 55% to give optimum growing conditions. Seeds were sown at one per pot. There were variations in the rate of plant growth and date between flower opening and seed maturity. In summer the time for flowering was 6 weeks and the period between flowering and maturity was 30 days. Figs. 4 and 5 show the fresh and dry weight of cow pea at different stages of development in a greenhouse. During spring, plant growth and seed development take longer with a period of 8 weeks between flowering and seed maturity. Results of TVu76 are presented here.

For the greenhouse because of the variation in time to maturity seeds were harvested and their stage of development assessed by fresh weight rather than days from flowers opening. The plants received constant attention regarding health and general conditions.

BROAD BEAN:

Vicia faba var. Sudanese Triple White and Maris Bead were grown in a greenhouse. Seeds were sown in 5" plastic pots containing J. Arthur Bowers standard compost. The temperature was kept between 10

deg. C (min) and 38 deg. C (max) and humidity was never less than 55 % to give optimum growing conditions. Seeds were sown at one per pot. The two varieties of V. faba were chosen for comparison because of the marked difference in the rate of seed development. Sudanese Triple White was faster with regard to rate of flowering (22 days) and maturity (56) days whereas V. faba var. Maris Bead was slower for both flowering (28 days) and seed maturity (68 days). Also there were variations in the rate of plant growth between different times of the year. Figures 6 and 7 show the fresh weight and dry weight of Sudanese Triple White and Maris Bead at different stages of development in a greenhouse.

Fixation, dehydration and embedding for light and transmission electron microscopy.

For the light and TEM examination of the range of legumes studied, pods of the required age were picked, the seeds removed and the cotyledons separated from the testa. The cotyledons were then sliced in 1.5% formaldehyde (made from paraformaldehyde) and 2.5% glutaraldehyde in 0.05M sodium cacolylate buffered at pH 7.0. The cotyledons were cut to approximately 1-2mm^3 for tissue from more mature seeds, but for the younger embryos larger tissue pieces were fixed and in the case of 3 - 6 d seeds the developing embryo was fixed intact. The fixation time varied depending upon the size and nature of the tissue (Mollenhauer and Totten (1971), Horowitz (1981)). It was found that short (2-4h) fixations gave best results for younger seed tissues, however, with increasing maturity fixation times were prolonged up to 10h to give optimum results. The osmolarity of the aldehyde buffer was varied to give an optimum apparent fixation. The osmolarity was varied using sucrose with the

ionic concentration remaining constant. After washing with cold, distilled water, or buffer, for 15 to 30 minutes a post-fixation with 1% aqueous osmium tetroxide was used. The period of osmium post-fixation was similarly varied with shorter (1-3h) times used for less mature tissues and up to a maximum of 8h for mature cotyledon tissue. A brief (5 minutes) wash with cold distilled water was used before subsequent dehydration.

The resins used for embedding were hydrophobic, which meant that the specimens had to be progressively and completely dehydrated before resin infiltration. Dehydration was routinely performed with a graded series of ethanol solutions. Two 15 minute incubations in 25%, 50%, 75%, 95% and 100% dry ethanol were used. The 100% ethanol was dried over molecular sieve. Originally propylene oxide was included in the final stage in the dehydration procedure. It was found, however, that such a step was frequently associated with more numerous fixation artefacts, particularly with tissues of younger embryos, and the use of propylene oxide was discontinued.

The dehydrated tissue was progressively infiltrated with Spurr (1969) resin using one of two methods. Either, a 1/1 dry 100% alcohol/resin mixture was used for 3h followed by subsequent infiltration in pure resin for a further 3-6h, or, tissue was placed in the 1/1 mixture and left on a rotator (Taab Laboratories,(U.K.)) overnight with the specimen vial uncapped. The alcohol evaporated progressively so that by morning the specimens were in pure resin. The specimens were infiltrated with fresh resin for a further eight hours before being placed in moulds, in fresh resin, and then into the oven. The resin was polymerised at 70 deg C for a minimum of 8h and

usually overnight. The Spurr resin used was made either from bulk components or from premix kits. When bulk components were used the proportions mixed were Resin (VCD) 10ml, Hardener (NSA), 20ml, Flexibiliser (DER736) 6ml, and Accelerator (S1, DMAE) 0.4ml.

Morphological examination of specimens embedded for light and electron microscopy.

After cutting off the resin the blocks were trimmed with a razor blade and the area to be sectioned was selected. To select cells exactly, especially when a precise level of differentiation has to be studied both thick sections for survey by light microscopy and thin sections for electromicroscopy have to be cut.

Thick sectioning and staining with toluidine blue for light microscopy.

Thick (1-2 μm) sections were cut using a LKB Ultrotome with glass knives and were collected with a loop or hair brush, and sections were transferred on a drop of water to a glass slide and allowed to dry. The sections could be studied directly by phase contrast or preferably after staining.

The most convenient and rapid stain is toluidine blue. The stain is made by dissolving 1g toluidine blue and 1g of sodium borate (Borax) in 100 ml water or in a 2% solution of sodium carbonate and filtering. For staining, one large drop of the solution was placed on the sections on the glass slide. The staining is progressive and directly perceptible. The time of staining is shortened if the slide is warmed. The stain is drained off and the slide washed with water. The sections are allowed to dry at room temperature and may be observed in the microscope directly or after application of mountant and cover slip.

The light microscope used for general observation was a Vickers M75, and also the light micrographs included in this thesis were taken using a Leitz Orthomat TW with a fluorescence attachment. The filter combinations employed were matched to the fluorochromes used (see results). Results from light microscopy were recorded on Ilford FP4 (125 ASA) film. Negatives were printed on appropriate grades of veri brome (Kodak).

Sectioning and staining for electron microscope.

Sectioning of various thicknesses (between 50 - 100μm were cut on an LKB Ultrotome using glass knives. They were picked up on formar coated grids. Sections were stained by floating grids on a drop of a saturated aqueous solution of uranyl acetate for 15 - 30 mins at room temperature followed by washing with a jet of distilled water, and then further staining on a drop of lead citrate (Reynolds 1963) for 10 - 15 mins, followed by washing as before.

The electron microscope used was a Philips EM400 using accelerating voltage of 60-100kv. Micrographs were recorded on Kodak 4489 film and printed on Kodak or Ilford paper (usually grades 2 or 3).

Scanning Electron Microscopy (SEM)

Tissues from the legume seeds were examined by SEM either directly, without treatment in any way, after application of a thin metallic coat, or after critical point drying and coating.

Direct examination of uncoated specimens was limited to some pod tissues which had sufficient structural rigidity to prevent collapse in the microscope column vacuum. Generally, however, such examinations were difficult to record because of surface charging and

thus coating was frequently employed.

These samples were coated with gold, or gold/palladium in a sputter coater (Polaron Instruments Inc. type E5100) with a thickness of 2 Å being deposited as a conducting film to prevent surface charging.

With the more delicate tissue, critical point drying was required to maintain structure as in vivo when the samples were placed in the coating unit, and microscope column vacuum. For critical point drying samples were fixed in 2.5% glutaraldehyde, 1.0% formaldehyde, 0.1% sodium cacodylate at pH 7, and left overnight at room temperature. For dehydration samples were passed through an acetone series of 6%, 10%, 15%, 25%, 50%, 70%, 90%, and pure acetone, for 15 minutes at each stage. When the samples were in pure acetone, this was changed to new acetones 2 times more at 15 minute intervals. The samples were then critical point dried. The idea of critical point drying is that all liquid (acetone) is removed from the sample , without incurring any of the associated effects of surface tensions, which causes the structure of the sample to be destroyed.

In order to critical point dry, 2 suitable "carrier" liquids have to be found. The most common liquids are acetone and carbon dioxide, which is a liquid at high pressures under normal temperature and at 42 deg C and 1200 psi and also is miscible with acetone.

The samples were transferred to the critical point dryer which is simply a machined cylinder with inlet, outlet and exhaust valves capable of withstanding up to 2000 psi. The samples were put into a metal "boat" containing acetone (the samples should not be allowed to dry out at all at this stage) which was then inserted into the

cylinder. This was then sealed tight.

Before turning on the cold inlet valve the excess acetone was allowed to drain away. The inlet valve was turned on and the cylinder was flooded with liquid CO_2 at 800 psi and room temperature (i.e. 20 deg C). The exhaust valve was then opened for about 5 minutes to allow a thorough 'flushing' with CO_2. The exhaust and CO_2 inlet valves were closed tightly, and the sample was left for 1 hour (longer for thicker samples). The above procedure was then repeated. After this all valves were closed and the cylinder temperature was raised using warm air circulation. The temperature was allowed to rise slowly to about 40 deg C and 1200 psi. At this stage all the CO_2 should turn to a gas. The water circulation was turned off and the outlet valve opened slowly to let the gas out gradually until it had all been released to atmospheric pressure. The critically point dried samples were then removed and transferred to dry containers. The samples were sputter coated as above before examination in the SEM.

Coating

The dried specimens were stuck onto stubs with silver paint. These were then transferred to a sputter coater. A sputter coater was used to put a thin layer of gold onto the specimens, in order to make it electron conductive. (Most biological specimens are very poor electron conductors). This is done in order that electrical charge does not build up on the specimens (due to poor conductivity). In order to transfer the gold to the specimen, an inert carrier gas is required under low pressure. The gas is usually argon. The argon gas carries gold molecules to the specimen when excited by an electric field. The specimens were put into the sputter coater and the vacuum

pump switched on. The leak valve was opened to release air and carry a stream of argon through the apparatus. Thiswas done a number of times to ensure that the atmosphere within the coater was only argon. The coater was then left until the pressure was reduced to between 0.01 and 0.02μm. Once this has been achieved the current between target and specimen was switched on and raised to between 12-14 μmA. The unit should then glow due to the fluorescence of the argon. The electric current was left on for between 2-3 minutes, and then turned off. The pump was turned off and the system flooded with argon gas for a few moments prior to removal of the coated specimen.

Specimens were examined using a Cambridge Stereocan 600 at 7.5kV. Results were recorded on Ilford FP4 (125 ASA) film with a magnification marker in place.

Stereology

Stereological principles provide efficient and reliable tools for the determination of quantitation parameters of tissue structure on sections (Weibel, (1973); Briarty (1973); Kistler and Scherle (1966). These principles allow for the estimations of volumetric ratios, surface area, surface to volume ratios, etc.. The internal structure of tissues or cells can be investigated only after destruction of the tissue. Usually this is done by sectioning of fixed and embedded tissues. By sectioning, the integrity of the tissue is preserved in two of the three dimensions of space while the third dimension is sacrificed for the benefit of resolution. Each structure of the tissue is usually cut at random by a nonorientated section and the n-dimensional structure of the tissue is represented on the sections by an (n-1) dimensional image. Thus, volumes are seen

as areas, surface as lines, and lines as points. Quantitative occurrence of images in the sections can be correlated with the structural composition of the tissues or cells (Fig.8a).

In order to investigate the volume of cells in a range of legumes, material was fixed in 2.5% glutaraldehyde, 1.0% formaldehyde, 0.1m sodium cacodylate at pH (7.0 - 7.2) for 14 hours, and post-fixed with 1% aqueous osmium tetroxide. After ethanol dehydration the specimens were embedded in resin before sectioning. The thickness of the section was 1 micron and they were stained with toluidine blue for 20 seconds. The sections were photographed and the following matters were studied.

The experiment tissue was at three stages of development. We chose 16 pictures with 6 measurements using point counting for volume, and line counting for surface area and cell to cell and cell to intercelluler space comparisons. Measurement were made using a multi purpose grid system with 84 lines and results calculated using established procedures (Weibel , (1973) and Weibel, Kistler & Scherlg, (1966)).

Uptake of fluorescent tracers

Pea seeds, at different stages of development, were isolated with a small portion of pod wall tissue including the main vascular traces. The cut surfaces of the pod were immersed in a solution of either 0.1% fluorescein or 0.01% ANS (Gates and Oparka, 1982) with the seed supported by a Parafilm membrane which prevented direct contact between the seed coat and fluorochrome solution. The vials with seed and fluorochrome were placed in a covered tank with moist filter paper to reduce dehydration from the seed coat epidermis (Fig.8b). After various incubations the seeds were sectioned and examined using a Nikon Diaphot with epifluorescent illumination. Results were recorded on Kodak Photomicrography film.

Fig.1: Shows the increase in the fresh and dry weight of pea at different stages of development, when grown in greenhouse. (P.sativum var. Feltham First)

Fig.2: Shows the fresh and dry weight of pea at different stages of development, when grown in controlled environment. (P.sativum var.Feltham First)

Fig.3: Shows the fresh weight of seed pod at different stages of development.

Figs.4,5: Show the fresh and dry weight of cowpea at different stages of development in the greenhouse.

Fig.6: Shows the fresh and dry weight of Sudanese Triple White at different stages of development in the greenhouse.

Fig.7: Shows the fresh and dry weight of Maris Bead at different stages of development in the greenhouse.

Fig.8a.. Shows the measurements used with the multipurpose system with 84 lines.

Fig. 8b Shows covered tank used to reduce dehydration from the seed coat epidermis.

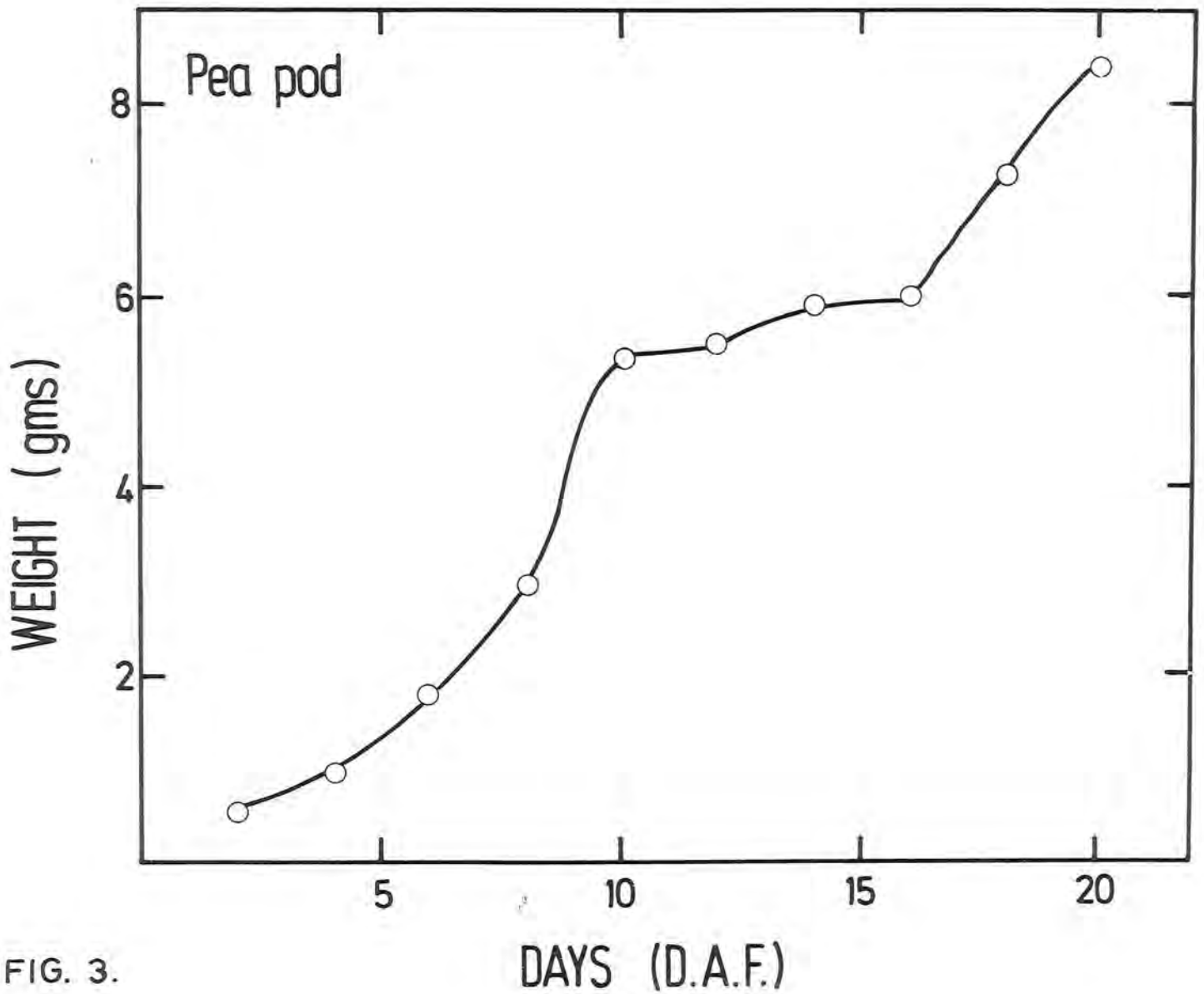


FIG. 3.

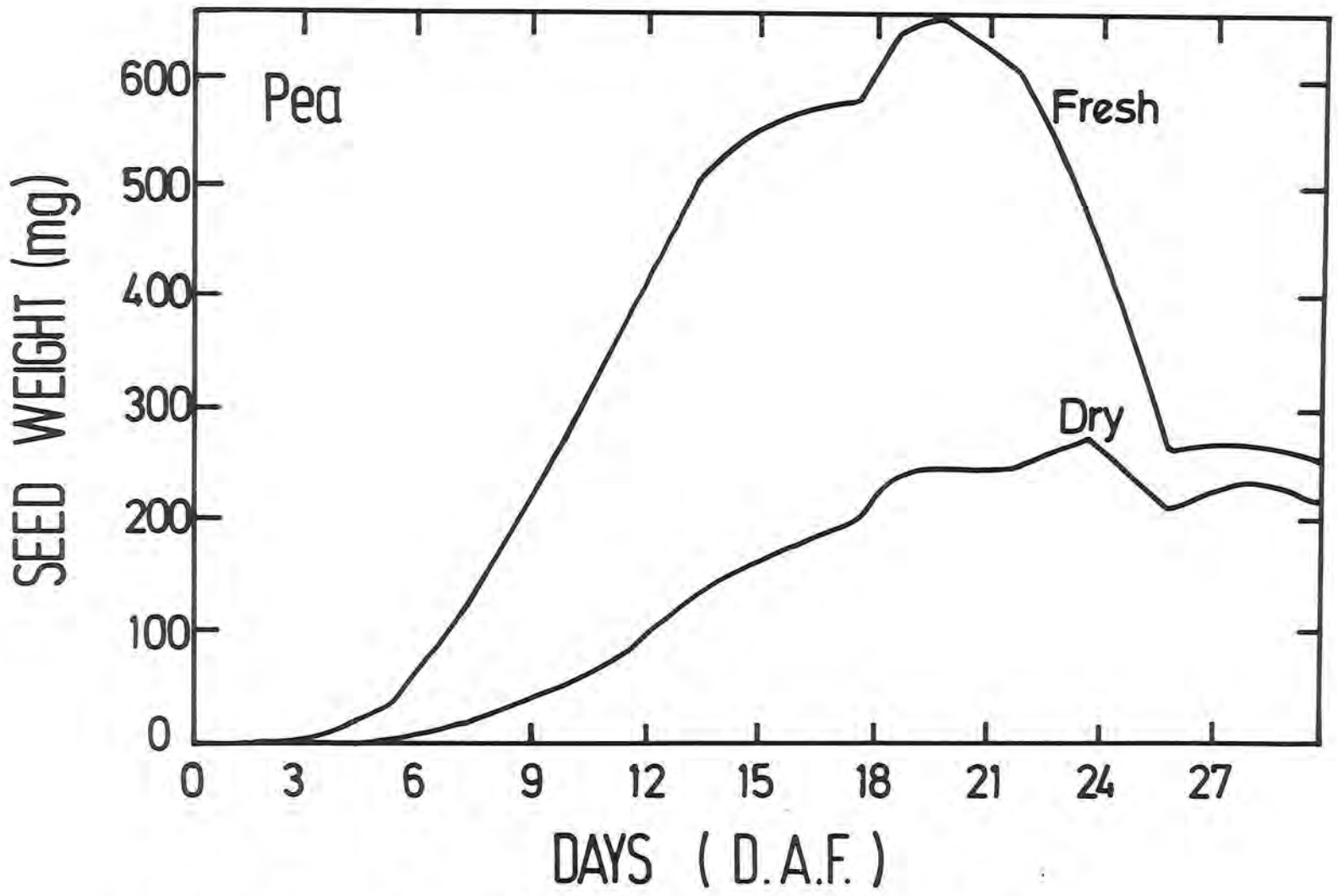


FIG. 2. Growth of seeds in growth house.

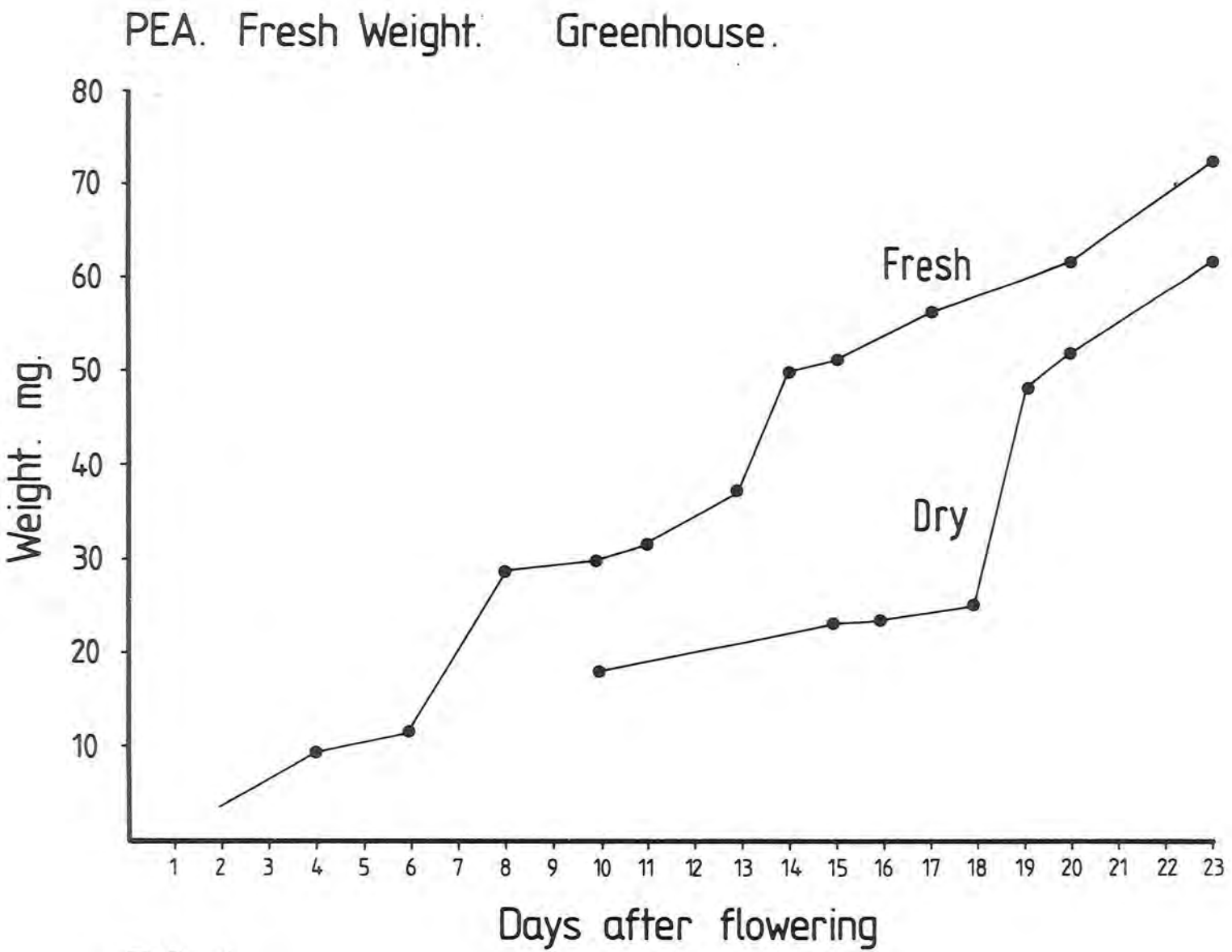

PEA. Fresh Weight. Greenhouse.
Weight. mg.
80
70
60
50
40
30
20
10
Fresh
Dry
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Days after flowering

FIG. 1.

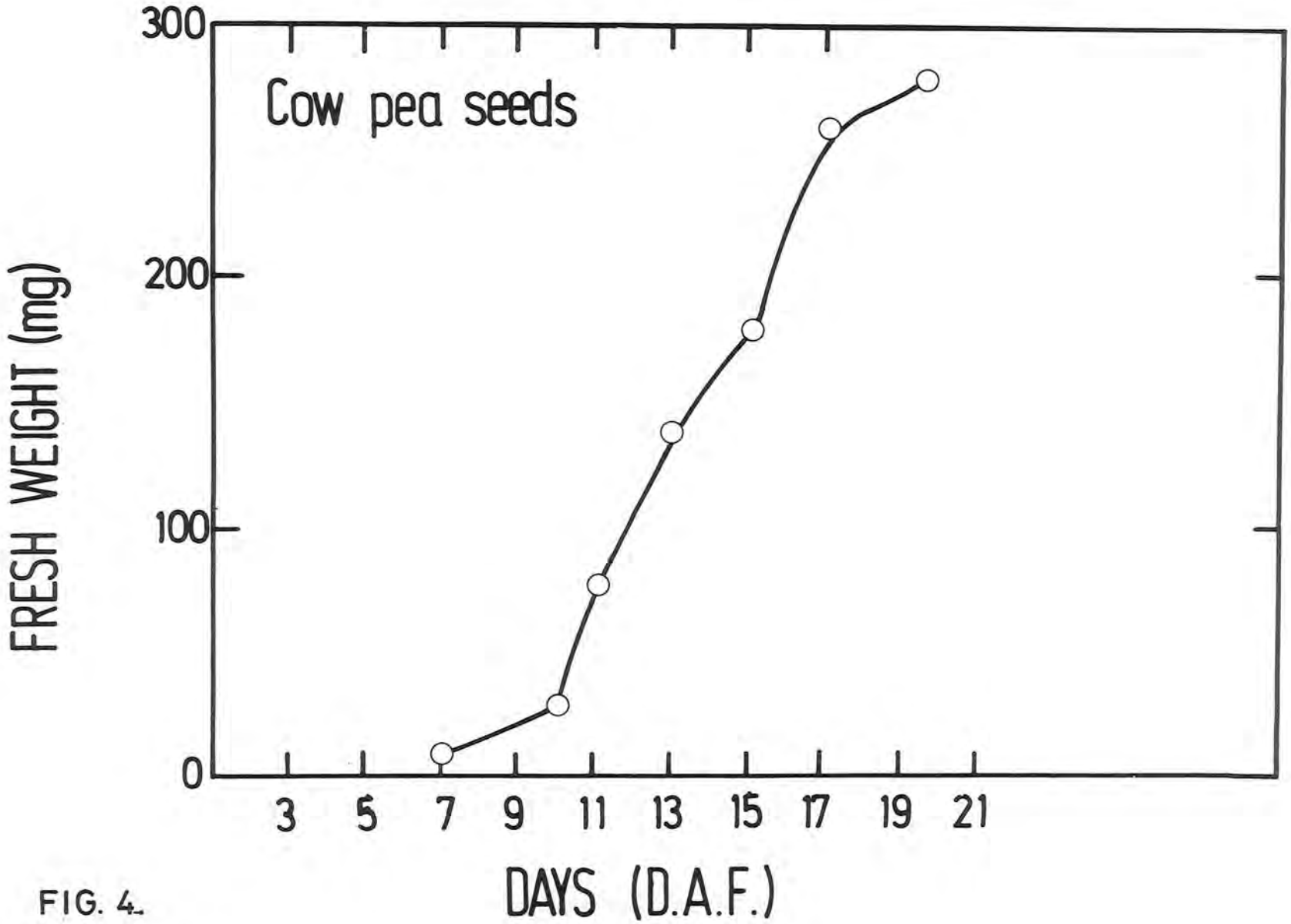

Cow pea seeds
FRESH WEIGHT (mg)
300
200
100
0
3
5
7
9
11
13
15
17
19
21
DAYS (D.A.F.)

FIG. 4.

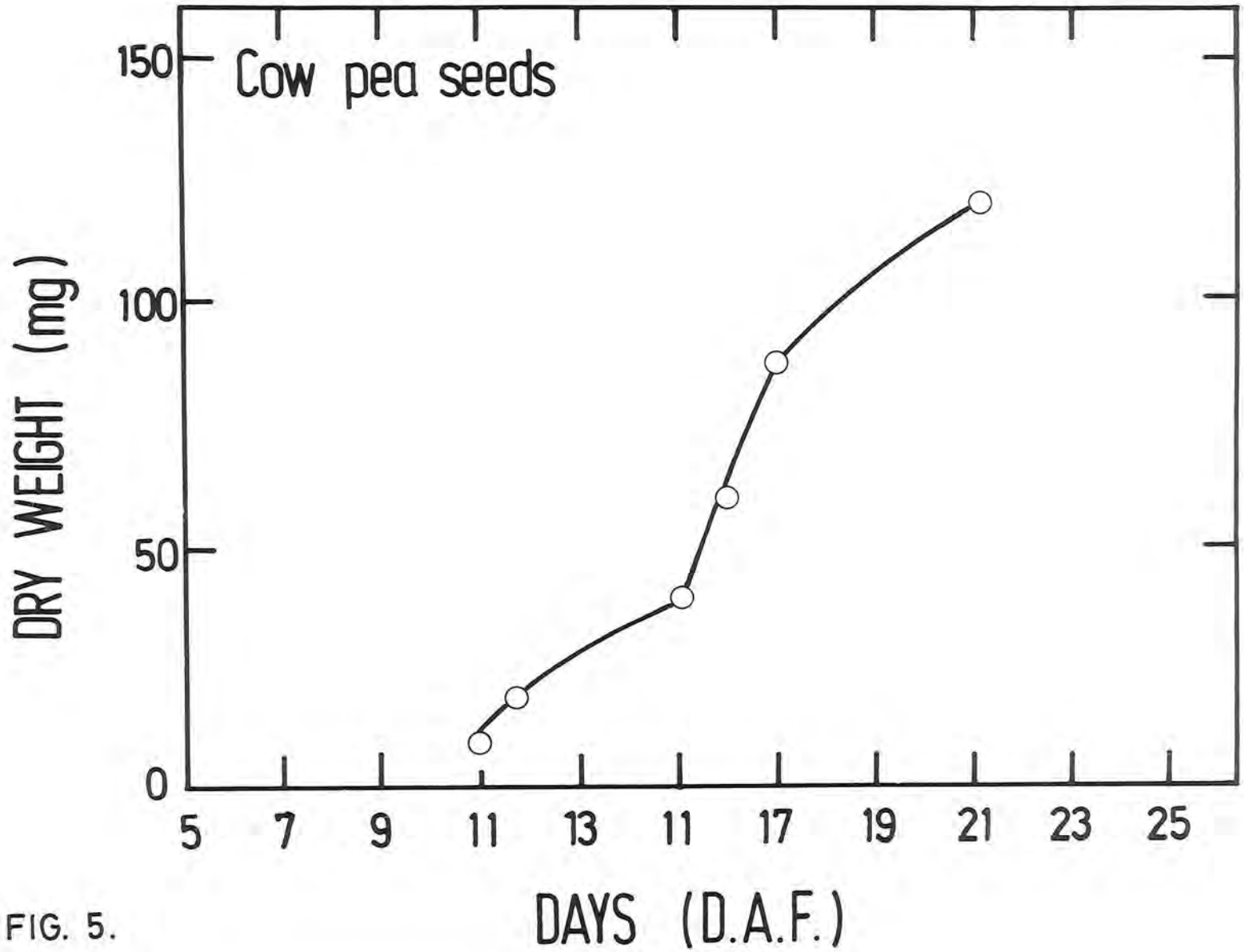

Cow pea seeds
DRY WEIGHT (mg)
150
100
50
0
5
7
9
11
13
11
17
19
21
23
25
DAYS (D.A.F.)

FIG. 5.

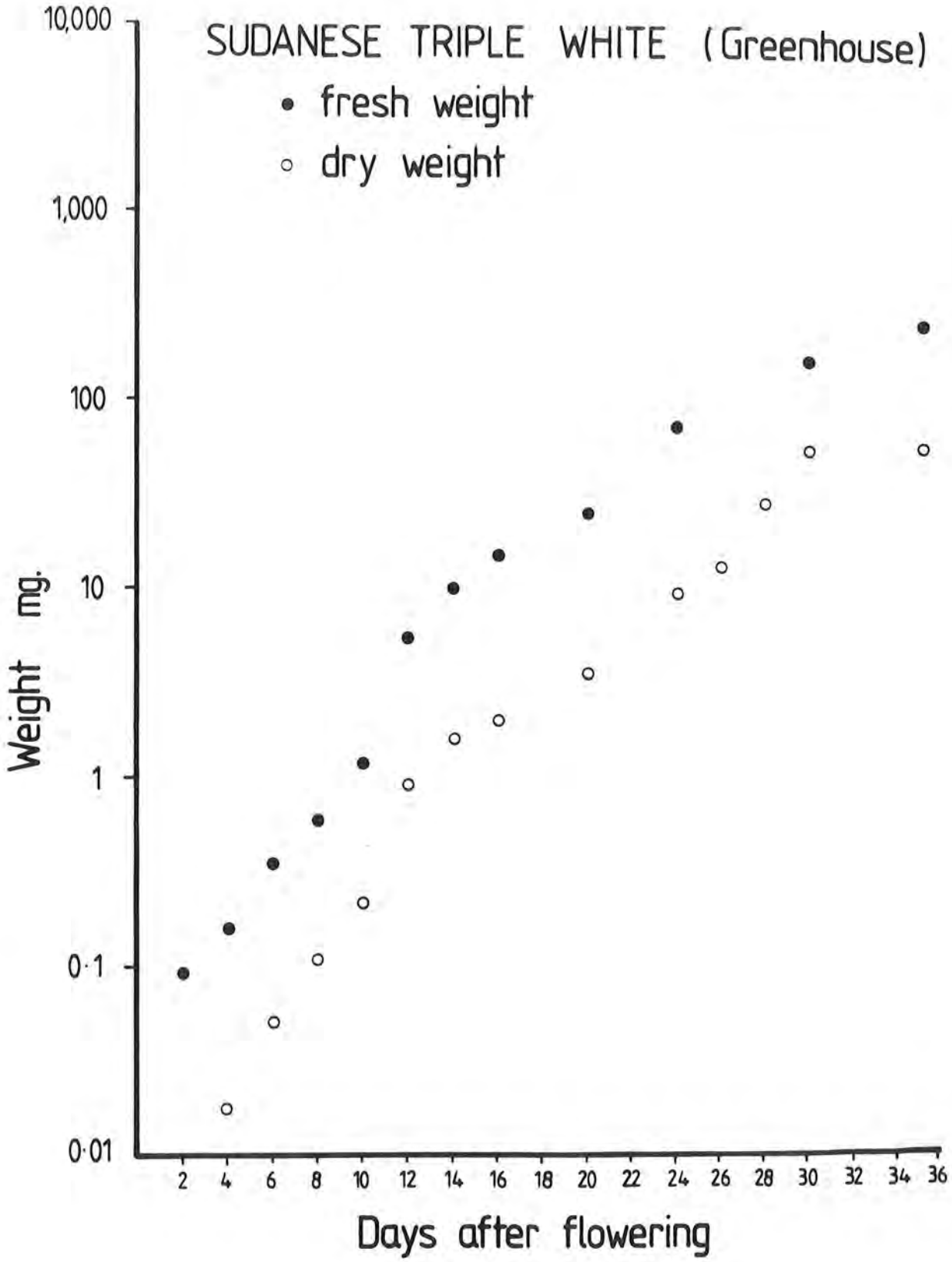

SUDANESE TRIPLE WHITE (Greenhouse)
• fresh weight
○ dry weight
10,000
1,000
100
10
1
0·1
0·01
Weight mg.
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Days after flowering

FIG. 6

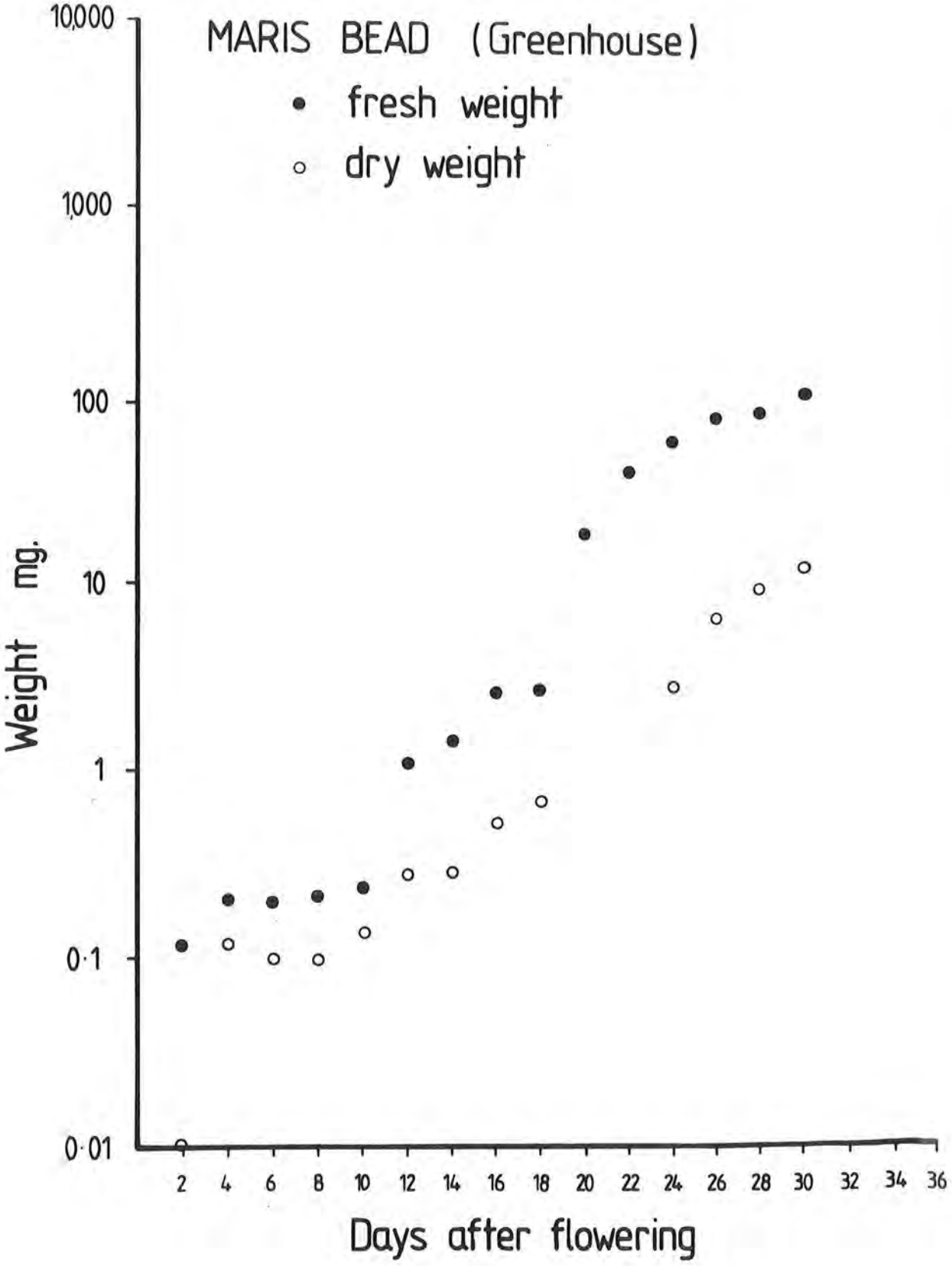

MARIS BEAD (Greenhouse)
fresh weight
dry weight
10,000
1,000
100
10
1
0·1
0·01
Weight mg.
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
Days after flowering

FIG.7

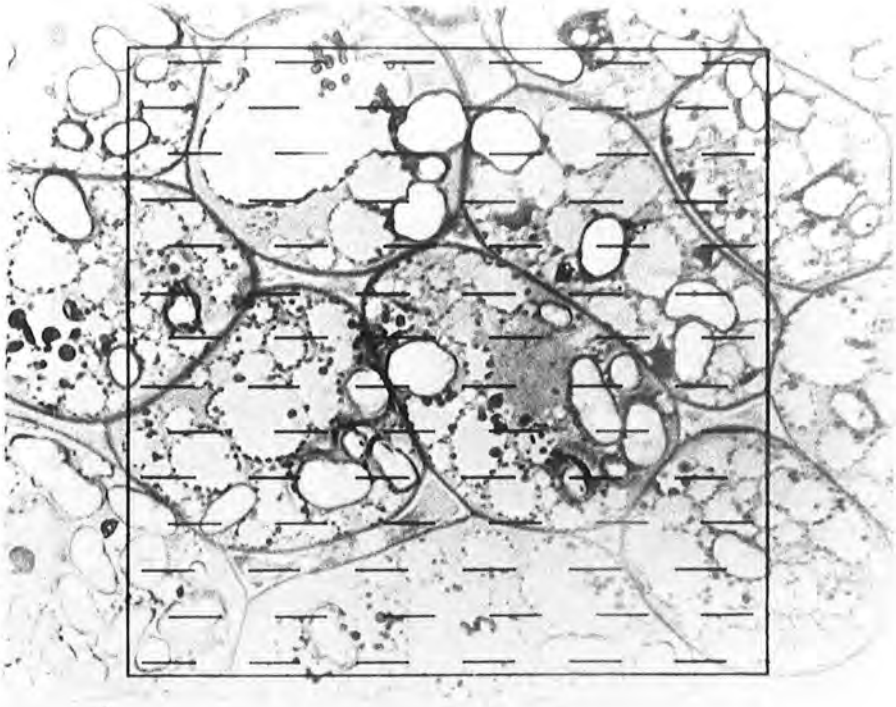


FIG. 8 a.

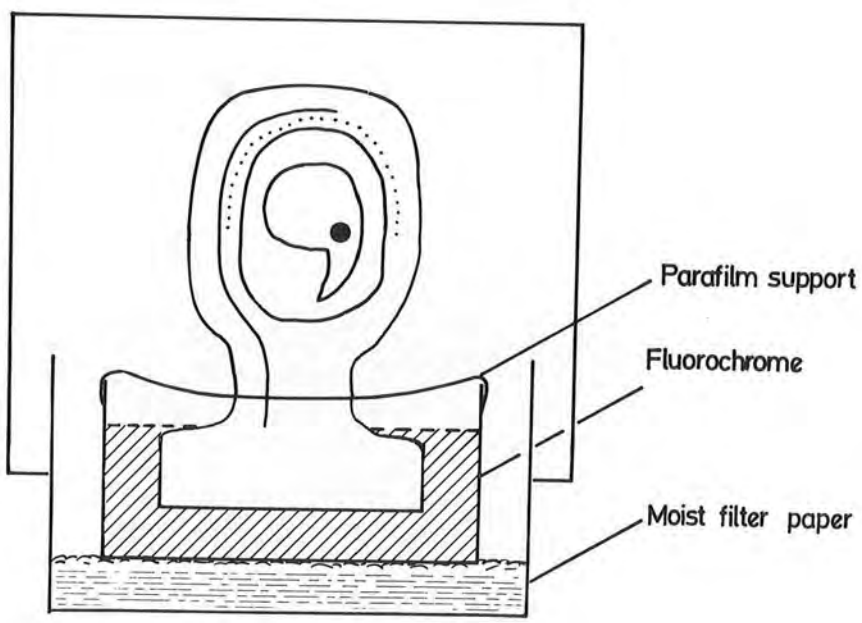


FIG. 8 b.

1. Development of Pisum sativum: pods and seeds

1.1) Light and scanning electron microscopy of pods.

Seed pods of both cultivated and wild type peas have been studied by light and scanning electron microscopy to determine any gross changes in structure during seed development that might be related to the structural development of the pea seeds.

The pea varieties examined were the wild types Elatus bieb (J181) and Pisum abyssinicum (J110) with rough surfaced pods and Pisum sativum (var. Feltham First), a cultivated pea developing a smooth pod. The differences in overall pod form during development are shown in Fig. 9 illustrating the increase in pod size in the cultivated variety. The pod wall of P. sativum is comparatively more fleshy although during the latter stages of seed maturity the pod walls progressively dry and will finally split to allow dispersal of seed if a crop is not harvested. Scanning electron microscopy was used to examine the surface structure of the fleshy inner layers of the pod wall at the early stages of pea seed development. Fig. 10 illustrates the sectioned pod wall at 4 DAF. Most of the wall is composed of thin-walled parenchyma cells although there is a marked inner epidermis as well as an outer epidermis of small cuboid cells with a cuticle. The two major vascular bundles are seen running along the pod as well as a number of inner veins in the parenchyma tissue many of which are located close to the inner epidermis.

By 6 DAF the seeds have started to enlarge within the pod although at this stage the seed embryo is still very small and undergoing a phase of very active cell division. The distribution of pod wall tissues with seed coat attached to funicle is shown in Fig.

11.

From 8 DAF the seed undergoes a phase of rapid enlargement with development of a dicotyledonous embryo. Fig. 12 shows the extent of cotyledon development within the seed at 13 DAF and the pod wall surrounding the thin seed coat. By this time the pod wall has differentiated into a number of distinguishable layers which are illustrated in Fig. 13. The outer epidermis has a thick cuticle on a layer of columnar epithelial cells and within these a few layers of closely packed small cuboid cells. Some of the thin-walled parenchyma cells have expanded although others have been crushed and the tissue adjacent to the inner epidermis has developed marked wall thickening, particularly associated with the pod wall veins. Light microscopy of a section of pod wall at 8 DAF is illustrated in Fig. 14.

SEM examination of tissue similar to that of Fig. 14 is shown in detail in Fig. 15 which illustrates the three-dimensional forms of the cell layers illustrated in Fig. 14. The developing seed coat is also shown (bottom left) and attached funicle.

The inner surface of the seed coat is relatively smooth at 4 DAF (Fig. 16) although a number of protrusions and pocket-like structures are apparent. As the inner epidermal layers differentiate to form the structure shown in Fig. 13 (at 13 DAF) the inner surface has become a maze of ridges and cracks.

The changes in pod wall structure during seed development have significant influence in consideration of the pod wall, firstly as a potential reservoir for nutrient supply for early embryo development and secondly as a barrier to dehydration of the developing seeds. Dehydration is considerable during the early and mid phases of seed

development but is an essential part of the later phase of seed maturation.

Development of the vascular supply from pod to developing seed has been examined in detail by Hardham (1976) and is not repeated here although we have examined the movement of tracers through the vascular supply network from the pod to seed (see section 1.6).

1.2 Light and scanning electron microscopy of seed coats.

The structure of the seed coat of a number of legumes was studied in relation to its function and with particular emphasis on pea P. sativum. The seed coat may have a role in determining the size of seed and importance in controlling the exchange of water and nutrient between seeds and their environment.

During the development of the pea embryo the surrounding seed coat undergoes considerable change. SEM studies appeared to show that the inner epidermis of the seed coat was undergoing considerable change during the course of seed development. However these results were a little misleading as indicated by Figure 17 which appears to show a breakdown of the innermost layer of the inner epidermis when the embryo was at the globular stage (6 DAF). Examination of sectioned material by light microscopy shows, however, that the highly convoluted layer remains intact at this stage, bounded by the embryo sac boundary wall.

Figures 19 to 21 are light micrographs showing the changes in structure of Pisum sativum during the period 8 - 16 DAF. They show the different layers of the seed coat cells, the epidermal layers, parenchymatous layers and vascular bundle tissues.

The differentiation of the seed coat epidermis is clearly illustrated. Fig. 19 to 21 show that the percentage concentration of the cells in the palisade layers is higher than other layers, therefore the cell to cell distance and the intercellular space is not large. The differentiation of such a columnar epithelial layer is usually associated with development of tissues involved in high rates

of absorbtion or secretion. This differentiation occurs prior to, and during, the maximal rates of storage reserve synthesis within the embryo. However it is also apparent that by 20 DAF the seed coat epidermal cells have developed considerably thicker walls and a cuticular layer (Fig. 21).

The parenchymatous layer of cells also shows considerable changes during the period 8 - 20 DAF. The cells enlarge with the development of large intercellular spaces. As the cells enlarge the plastids develop numerous starch grains in each cell. These develop in all three distinguishable layers of the parenchyma cells (Fig. 20 and 21). Figures 23 to 26 show a series of sections through the top half of a pea seed at 4 DAF. Figure 23 is a section through the seed coat just below the apex opposite to the hilium end. A vascular bundle runs from the funicle up one side of the seed, around the top and part way down the opposite side (Fig. 26). This vascular bundle is shown twice in the section as are the parenchymatous cells of the seed coat which have yet to develop into the three clearly distinguished layers evident later in development (Fig. 19 to 21).

Figure 19 shows a section taken at the position indicated in Fig. 26. At the centre of the section the large nuclei of the endosperm are seen lining the embryo sac boundary wall. Strands of endosperm run from the boundary sac layer into the main cavity of the embryo sac (Fig 25) and are seen lying against the embryo (globular at this stage) in Fig 26. Evidence of the beginnings of differentiation of the seed coat parenchyma layers is also present in Fig. 26. The vascular bundle on the right hand side is seen to get progressively smaller through the series of micrographs. Fig. 27 shows the position

of sections in Figs. 23 – 26.

Within the next 2 - 3 days the seed coat undergoes considerable structural change, as shown in Figs. 28 and 29. These sections are comparable in position to those shown in Figs. 23 and 24 of the younger seed. The micrographs of the older tissues show that the vascular bundles running through the seed coat have increased in size (Fig. 28 and 29). This increase is largely by phloem and vascular - parenchyma division and at this stage little increase in xylem differentiation is evident (Fig. 30). The seedcoat parenchyma has, however, differentated. It shows distinct layers by this time; an outer layer of enlarging, mostly spherical cells with intercellular spaces, a middle layer of smaller, most closely packed spherical cells and a layer of elongated cells adjoining the embryo sac but with large, very conspicuous intercellular spaces (Fig. 31). The embryo sac, although highly convoluted, is completely intact and contains granular material of endosperm origin.

Vertical sections through part of the seed at 6 DAF are shown in Figs. 32 and 33. Fig. 32 shows the enlarging globular embryo positioned near the top of the embryo sac. There is a reduction in the middle layer (small, closely-packed cells) of the seed coat parenchyma between the vascular bundle at the top of the seed and the embryo. Although the cellular endosperm is only seen as a thin lining to the embryo sac in Fig.32, the presence of amoeboid strands of tissue throughout the upper part of the embryo sac is shown in a further section of tissue (Fig. 33).

Fig.9: Shows the increase in pod size and seed number in a cultivated variety.

Fig.10: Scanning electron micrograph illustrating a section of pod wall at 4 DAF.

Fig.11: Scanning electron micrograph showing the distribution of the pod wall tissues with seed coat attached to funicle.

Fig.12: Scanning electron micrograph illustrating the development of cotyledon seed at 13 DAF.

Fig.13: Scanning electron micrograph illustrating the different layers of seed pod.

Fig.14: Light micrograph showing some differentiation of the outer epidermis.

Fig.15: Scanning electron micrograph illustrating cell layers of the seed pod.

Fig.16: Scanning electron micrograph showing the inner surface of the pod wall.

Figs.17,18: Scanning electron micrographs showing a breakdown of the innermost layer of the inner epidermis.

Figs.19-21: Light micrographs showing the different layers of the seed coat cells.

Figs.23-26: Light micrographs showing a series of sections through the seed coat of pea at 4 DAF.

Fig.27: Diagram showing the position of sections in Figs. 23-26.

Figs.28,29: Light micrograph showing the structural changes of the seed coat.

Fig.30: Light micrograph illustrating vascular bundles.

Fig.31: Light micrograph showing the embryo sac.

Fig.32,33: Light micrograph of vertical sections through part of the seed at 6 DAF.

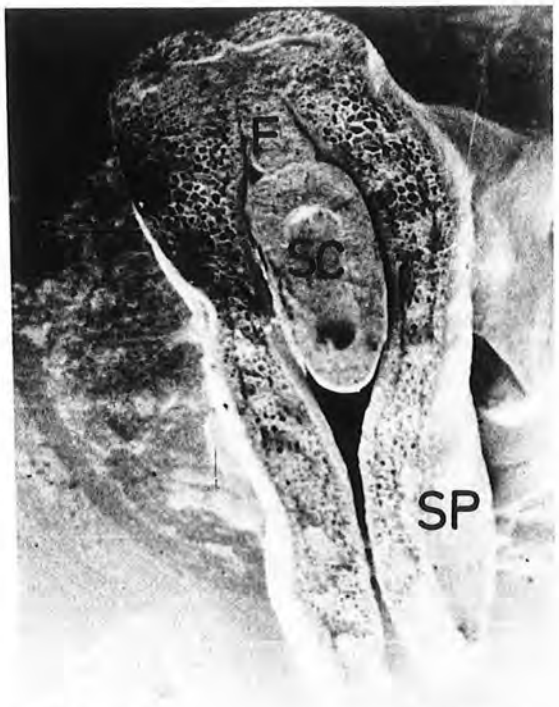


FIG.11 400µm

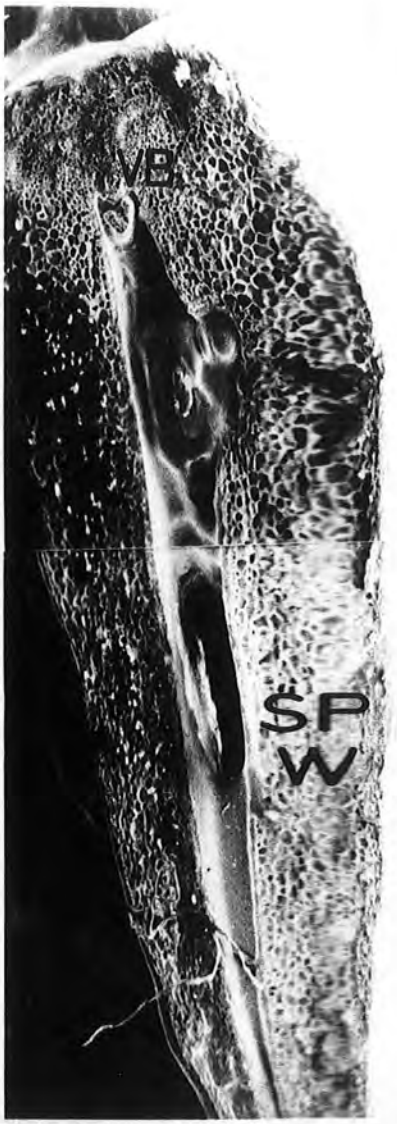


FIG.10 500µm

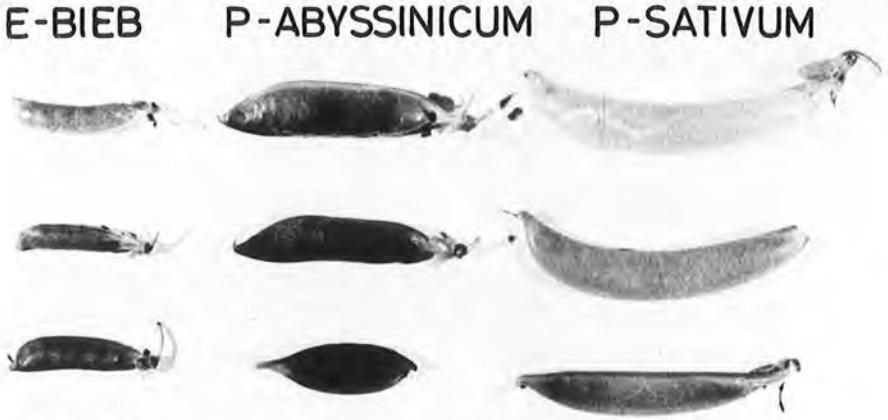


FIG.9 5cm

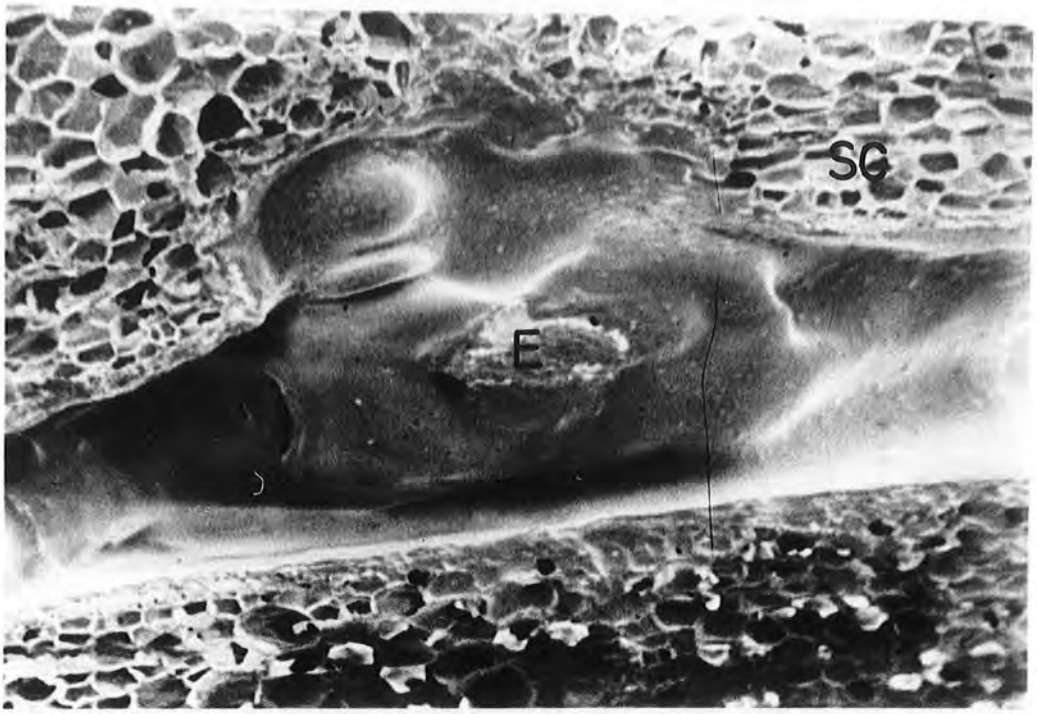


FIG.16

200 µm

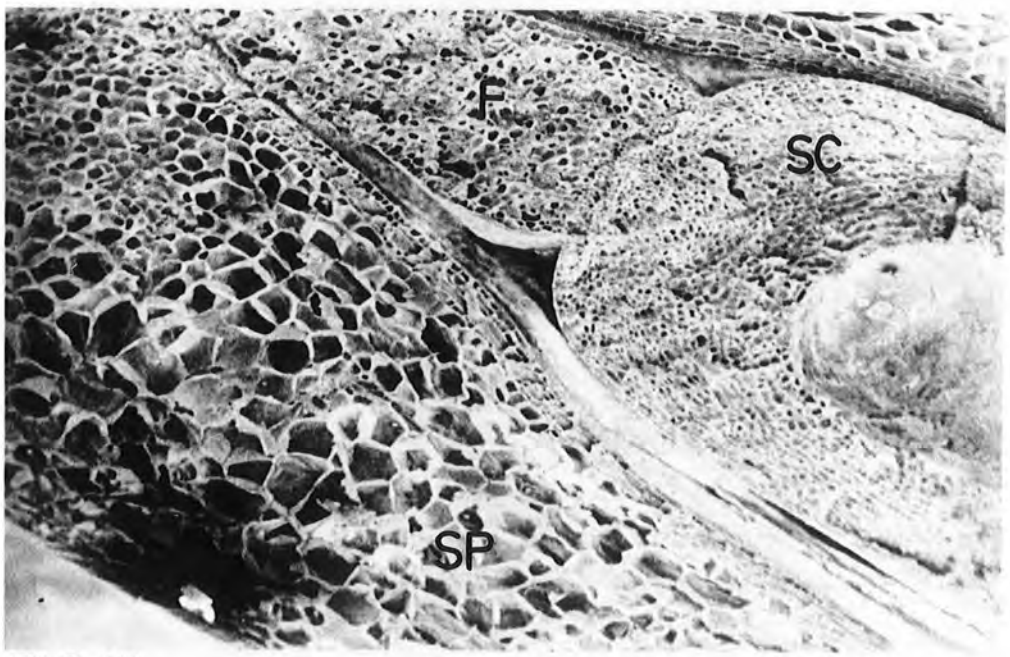


FIG.15

200 µm

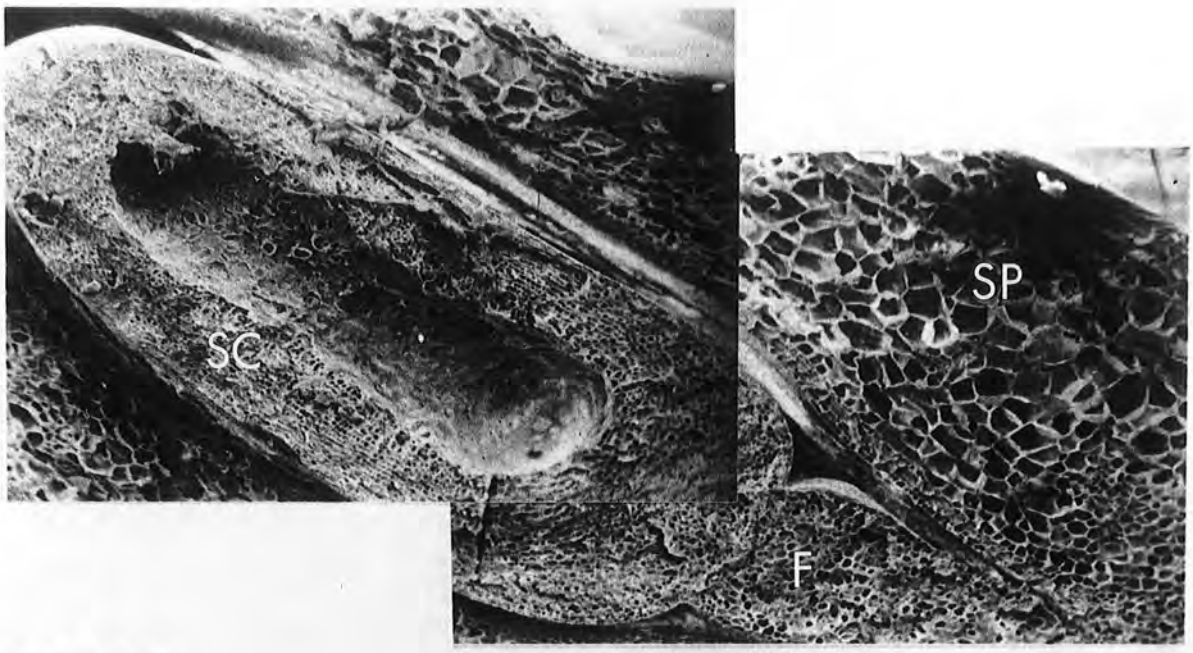


FIG.17

200 µm

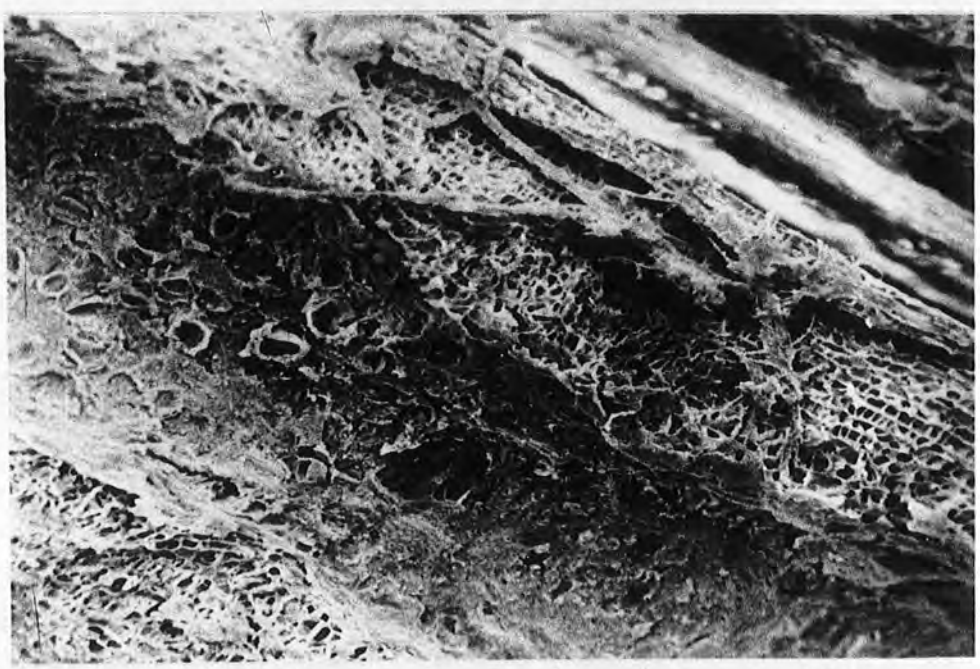


FIG.18

100 µm

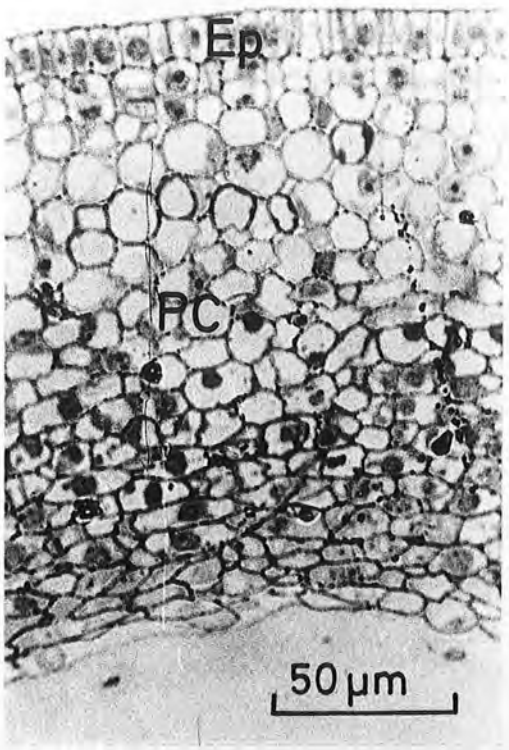


FIG.19

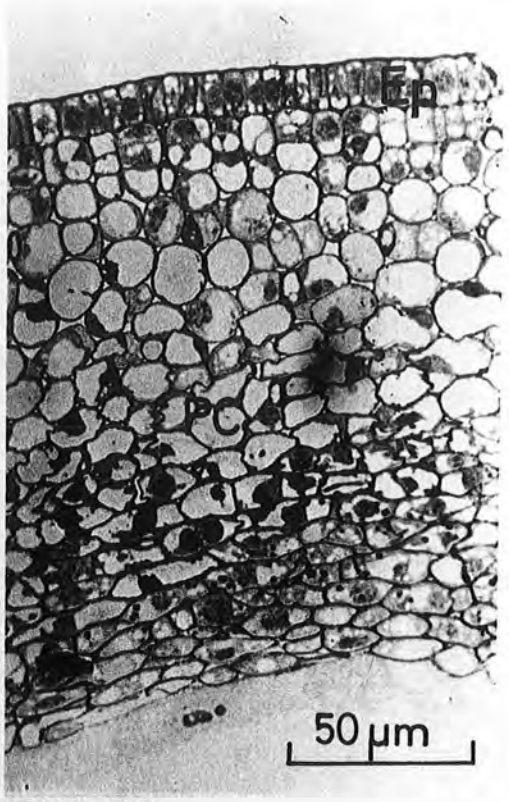


FIG.20

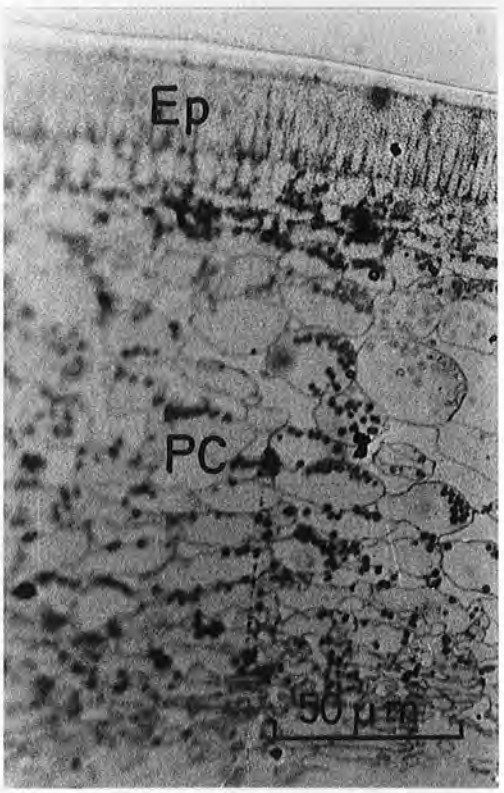


FIG.21

FIG.19 - Seed coat at 9 days

FIG.20 - " " " 16 "

FIG.21 - " " " 20 "

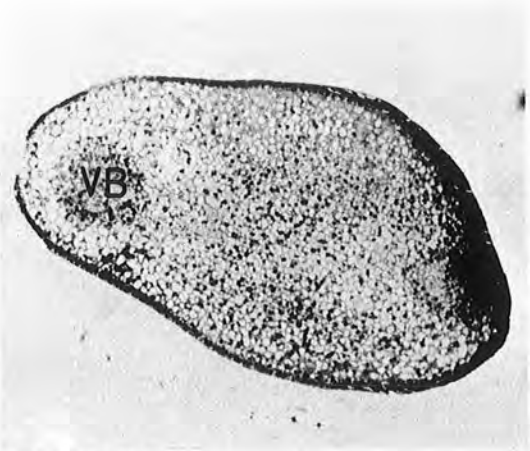


FIG.23

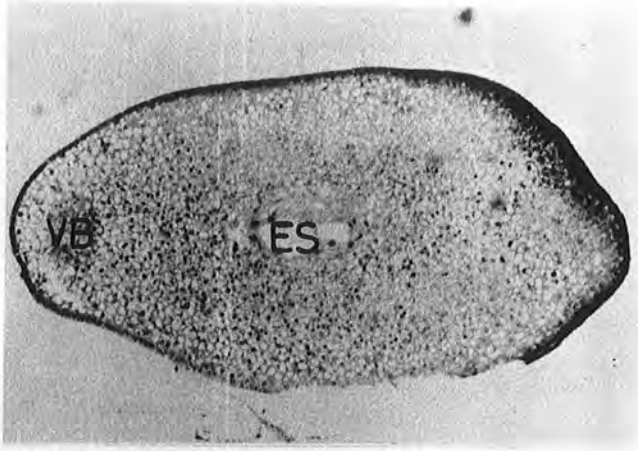


FIG.24

ALL 100μm

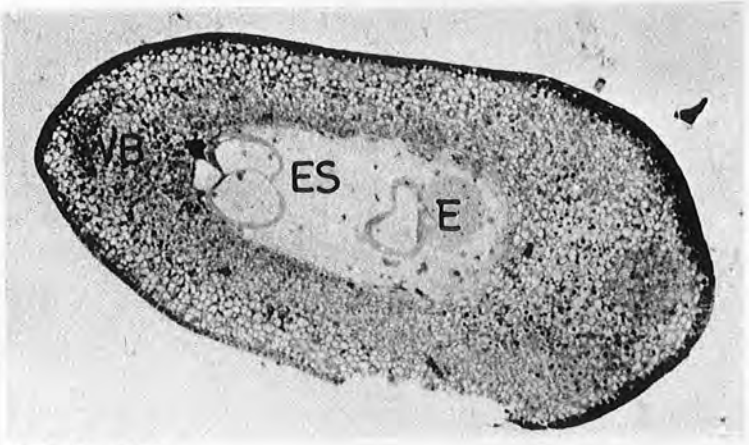


FIG.25

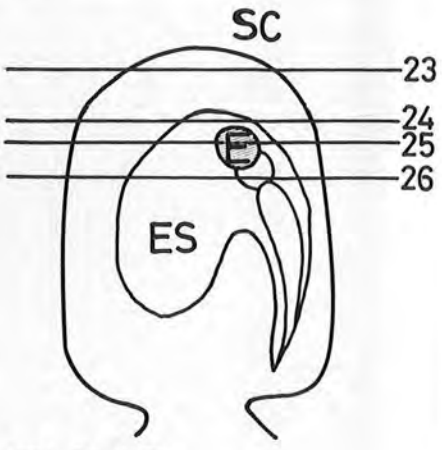


FIG.27

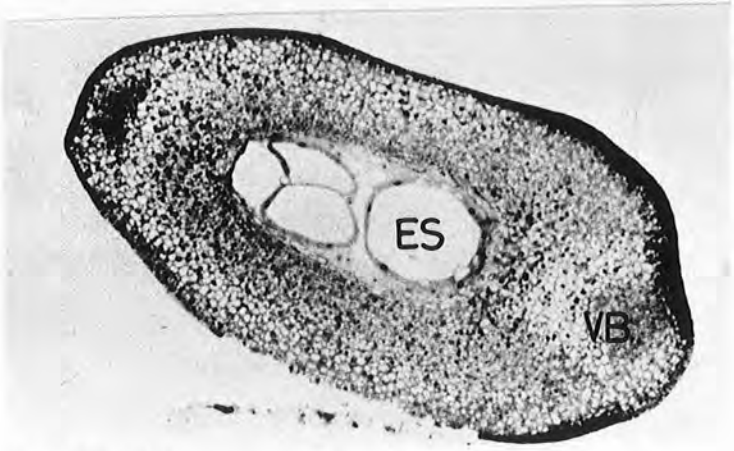


FIG.26

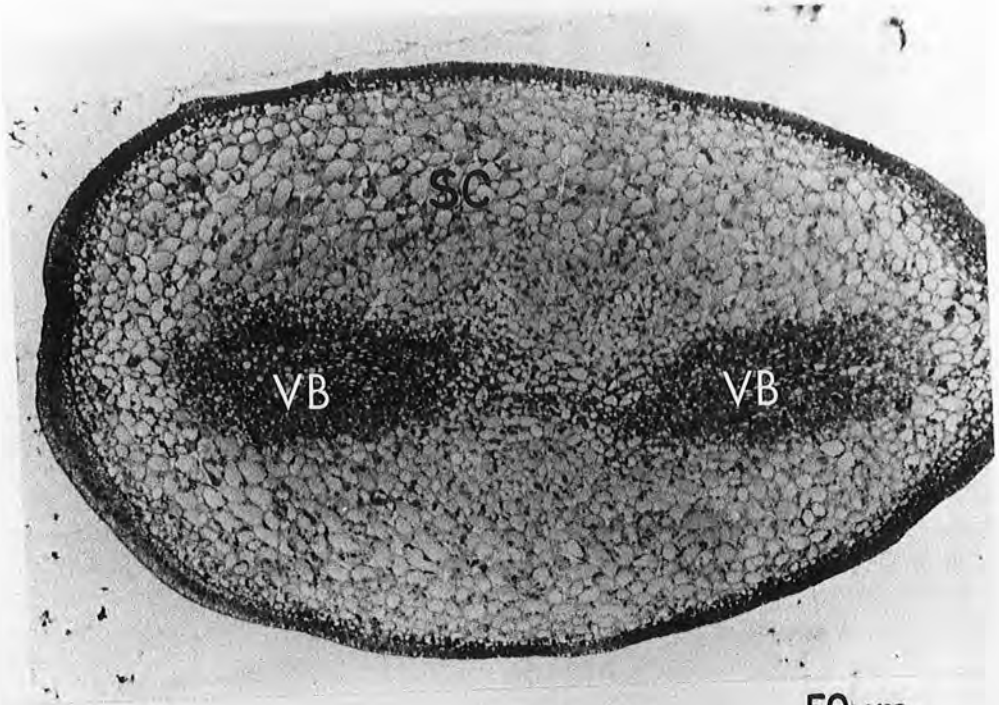


FIG. 28

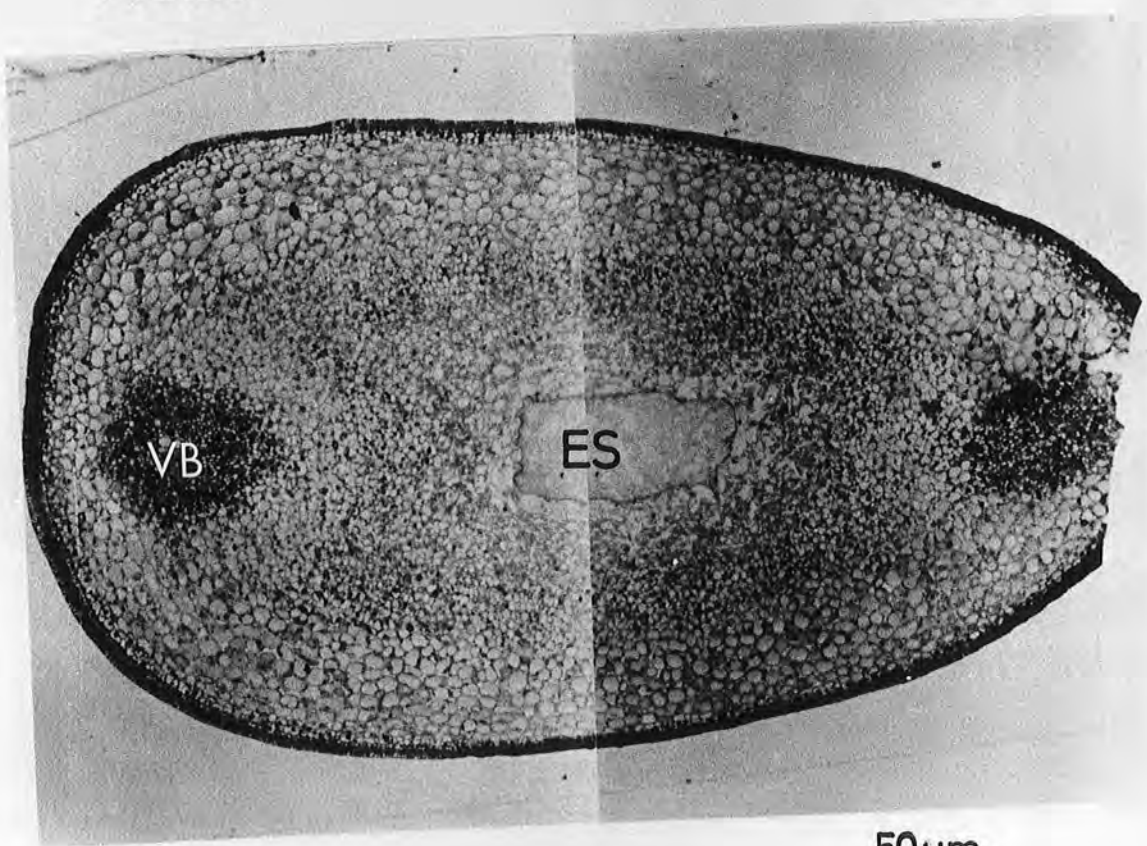


FIG. 29

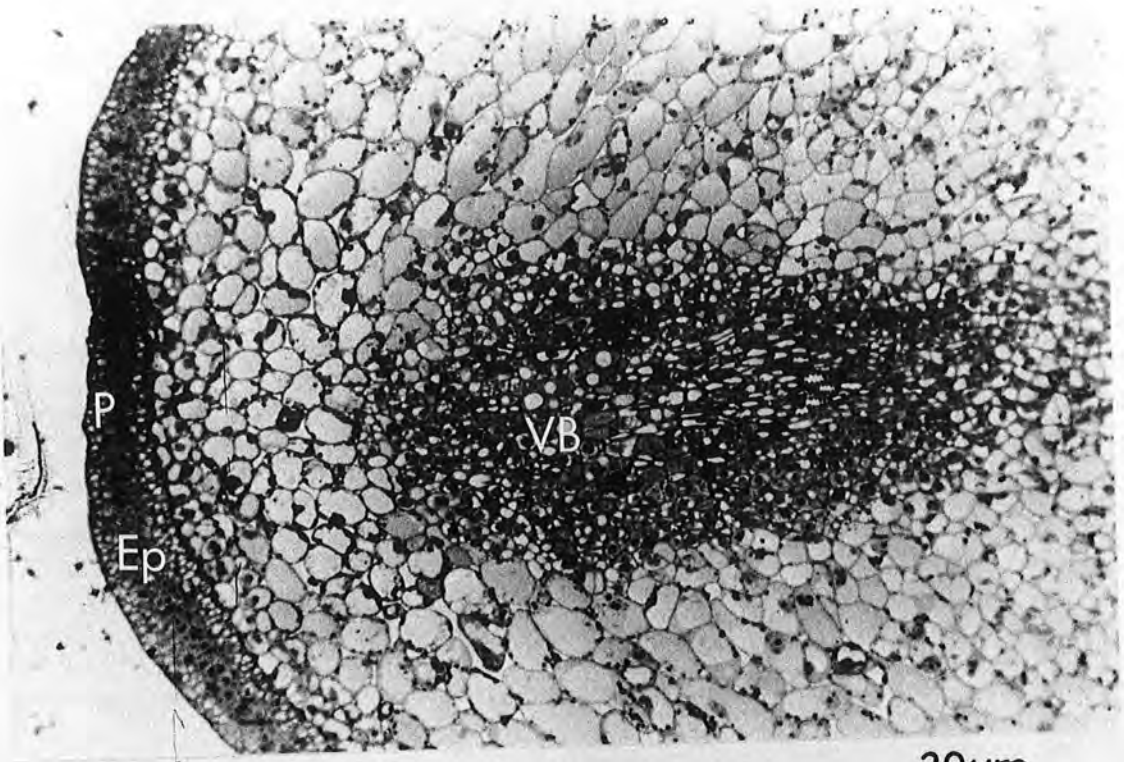


FIG. 30

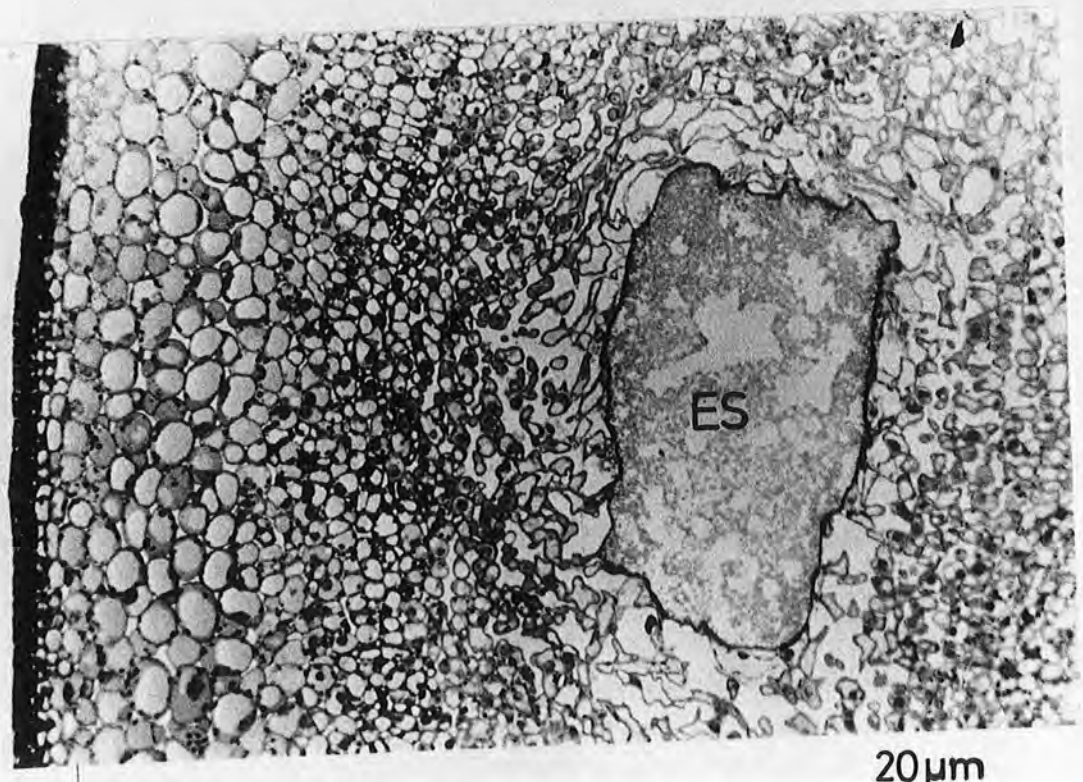


FIG. 31

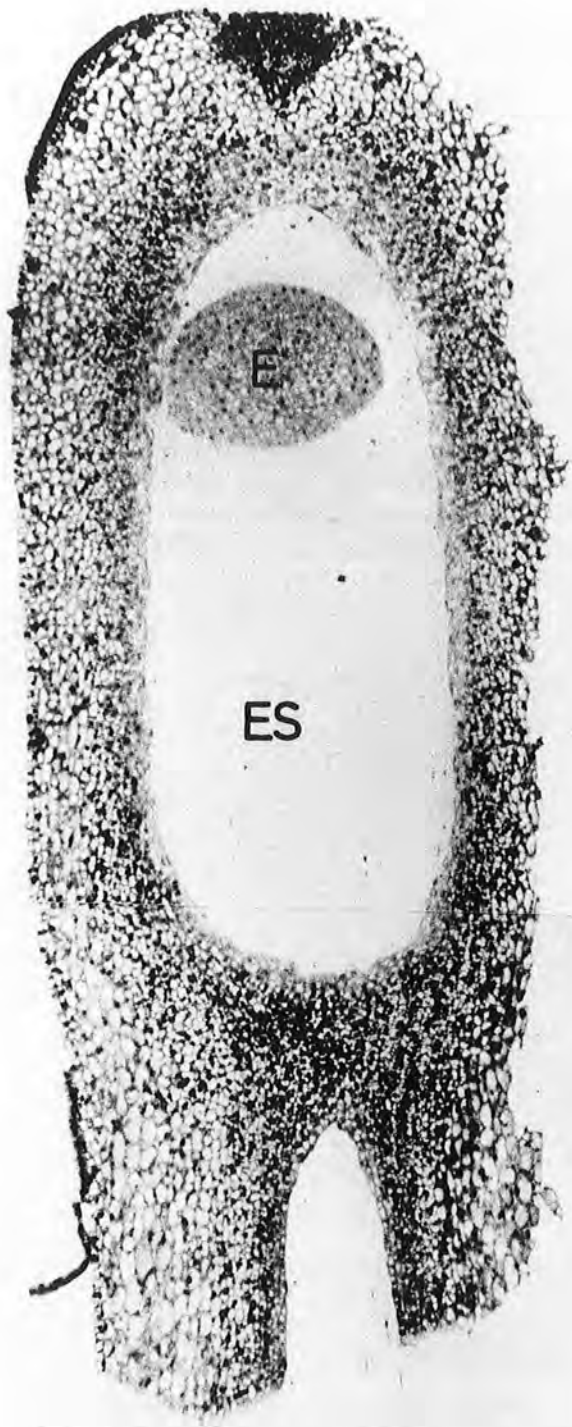


FIG. 32 50µm

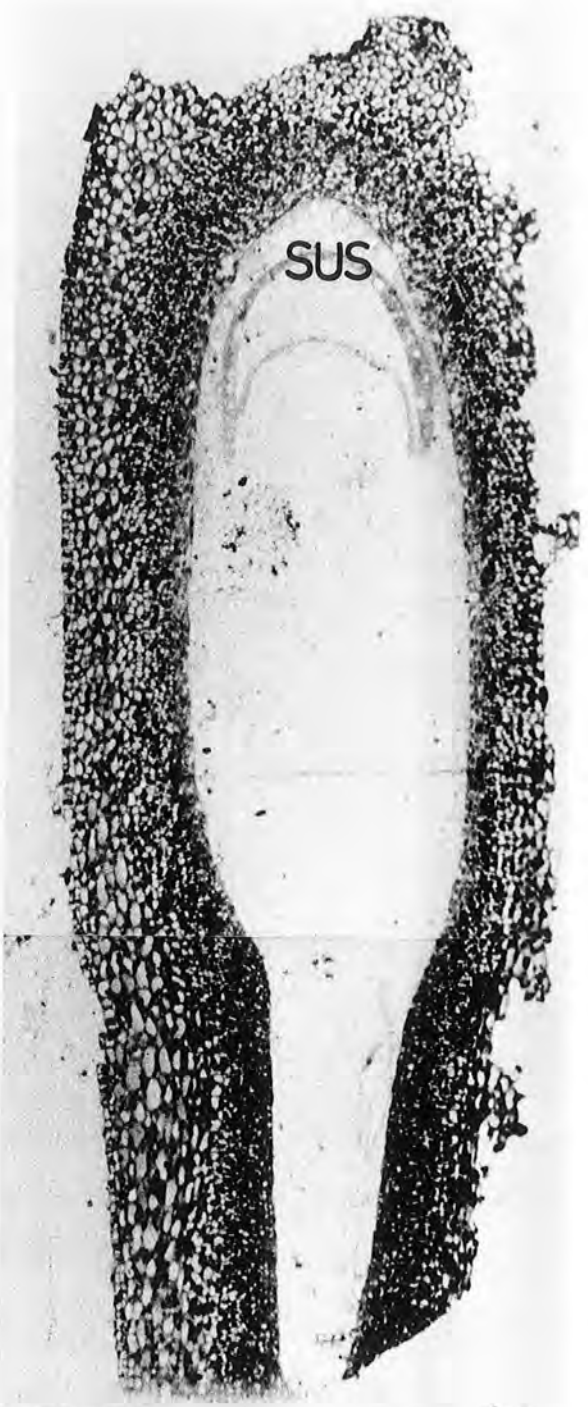


FIG. 33 50µm

1.3 Transmission electron microscopy of early embryo development.

The structural relationship of the pea embryo to its environment within the seed coat has been studied by electron microscopy. The general pattern of development as shown by our light microscope studies and previously published results (Marinos, 1970, Hardham, 1976) is presented diagrammatically in Fig. 34. Following fertilization the suspensor elongates and the embryo is pushed from the micropylar end of the embryo sac. During this time the embryo sac enlarges and the endosperm persists as a lining around the embryo sac boundary wall. Light microscopy indicates that the embryo is anchored by the development of cross boundary walls between an extra embryonic wall and the embryo sac boundary wall at the top of the embryo sac (Marinos, 1970; Hardham, 1976). The embryo then enlarges firstly to a globular form and then a 'heart-shaped' embryo which, while still attached to the suspensor, enlarges to form an embryo with radicle, plumule and two cotyledons attached at the hypocotyl. During this development the suspensor is crushed as the radicle enlarges and enters a radicle pocket of the embryo sac boundary wall and the endosperm is lost as the cotyledons enlarge and fill the majority of the embryo sac.

Transmission electron microscopy of these tissues illustrated numerous features which are of significance regarding the structural development and, in particular, the nutrition of the developing embryo.

Figures 35 - 38 show the ovular tissue which is adjacent to the embryo sac boundary wall at 6 DAF. During early embryo development this layer of cells develops a high proportion of intercellular spaces

with the cell wall junctions containing few plasmodesmata. The innermost layer of cells is attached to the embryo-sac boundary wall although no evidence of plasmodesmata through these junctions was found. The walls of this layer of seed coat cells do not show any development of the transfer cell form, in marked contrast to the embryo sac wall. The long, thin-walled cells of the inner seed coat layer do however, have a high cytoplasmic content and, as well as a nucleus, contain many mitochondria, plastids, dictyosomes and ER. A few small vacuoles are present although the cytoplasm does contain numerous small vesicles, many associated with the numerous dictyosomes. The plastids are generally of an immature, amoeboid form with a highly-developed peripheral reticulum. The plasmalemma is frequently seen with an irregular outline, suggestive of a high flux of the many vesicles which are in close proximity.

The micrographs shown in Figs 35 - 38 represent different regions of the embryo-sac boundary wall. The relative positions of these micrographs and also Figs. 40 - 48 show in the diagram of Fig. 39.

Comparison of the boundary-sac wall in the micrographs indicates that there are different degrees of wall modification at the different regions of the embryo-sac. The extent of development of wall is far greater near the apex of the embryo-sac (Fig. 35 and 36) and around the radicle pocket (Fig. 38, 40 and 41) than at the wall on the opposite side to the radicle pocket (Fig. 37).

The endosperm, seen in Figs. 35 - 38, lining the embryo-sac boundary wall, contains many organelles and small vacuoles. Many mitochondria are seen in the endosperm cytoplasm particularly adjacent to the protrusions of the boundary wall. The greatest densities of

mitochondria are found near the boundary wall below the main seed coat vascular bundle (Fig. 35, 36) and around the radicle pocket region (Fig. 38). Between the numerous small vacuoles the cytoplasm contains many ribosomes and polyribosomes. Immature plastids of an amoeboid form, with a well developed peripheral reticulum, are found scattered throughout the endosperm cytoplasm (Figs. 37, 38). Cisternal and tubular ER (Figs. 36, 37, 38) is present as are a few dictyosomes (Figs. 36, 37). The outer boundary of the endosperm cytoplasm is a membrane which follows closely the many complex protrusions of the embryo-sac boundary wall. An inner membrane lies adjacent to the developing embryo. At 6 DAF there is found no ultrastructural evidence of the formation of cell walls either to sub-divide the endosperm cytoplasm or to act as future anchors for the positioning of the embryo.

Figure 40 is an electron micrograph of a lower part of the suspensor with endosperm and embryo sac wall. The suspensor contains dense cytoplasm with ribosomes and also mitochondria with prominent nucleoids and an unusual form of plastid, without developed thylakoids and grana or lipid globules. The endosperm cytoplasm contains many small vacuoles and vesicles and mitochondria with prominent cisternae although little apparent ER.

Fig. 40 also shows the embryo sac boundary wall attached to the seed coat cells (inner ovular cells). Protrusions of the boundary wall are surrounded by endosperm cytoplasm with a high proportion of mitochondria-classical transfer cells.

The proximity of the suspensor to the embryo sac boundary wall which is forming the radicle pocket is shown in Fig. 41 (see relative

position in Fig. 39). This micrograph shows the suspensor cell cytoplasm with many mitochondria adjacent to the structurally modified wall.

Those 'vesicles' not associated with Golgi often have attached ribosomes and many, in fact, represent sections of tubular ER, unusual in that it is 'rough'. The suspensor wall has a systematic thickening of a transfer cell type with the protruberances only on the suspensor cytoplasmic side. Many mitochondria with prominent cisternae line the wall. The plasmalemma is apparently smooth and there are few vesicles in close proximity indicating trans-membrane transport rather than pinocytotic movement of metabolites. The endosperm shows very dense cytoplasm with many ribosomes and mitochondria with large and closely packed cisternae.

Fig. 42 is an electron micrograph of central regions of the suspensor cytoplasm showing the typical form of the many suspensor cell nuclei. The amoeboid shape produces a very high surface area to volume ratio and has great contact with material surrounding the nucleus. The nucleus contains much heterochromatin, some of which is attached to the nuclear envelope. The central cytoplasm of the suspensor contains numerous organelles. The unusual plastid is present as are some mitochondria and many vacuoles. In marked contrast to the peripheral cytoplasm of the suspensor cells there is much ER of both cisternal and tubular form. The cisternal ER has associated ribosomes and occasionally dilates to form small vacuoles. There are differences between the cytoplasmic material at the centre of the suspensor and at the edge. Near the centre the cytoplasm contains few plastids, many more vacuoles but few mitochondria. There is much

more ER and ribosomes at the centre of suspensor cytoplasm.

The boundary beween the suspensor and cells of the embryo is shown in Fig. 43. The suspensor with its characteristic plastids is seen in the lower part of the micrograph. A few suspensor cell mitochondria lie adjacent to the endosperm (Fig. 40, 41). Unlike the suspensor wall adjacent to the endosperm the dividing wall to the embryo has no transfer-type protrusions, although there are a few plasmodesmata with cytoplasmic bridges from the suspensor to the wall. The separating wall has two distinct layers, the thicker of the suspensor cell and a thinner portion of the cells of the embryo (at this stage still in a globular form).

The cells of the developing embryo are typically meristematic in their ultrastructural appearance. There are few vacuoles and a high proportion of cytoplasm is filled with numerous organelles and ribosomes. The cell walls are very thin. The mitochondria of the embryo are smaller than those of the suspensor cell and contain conspicuous nucleoid regions between the well-developed cristae. The plastids show differentiation of a thylakoid system although granal stacking is limited to only a few discs. Constriction division of the plastids may be shown in Fig. 43. Development of a few cisternal ER profiles is evident although the vast majority of ribosomes and poly-ribosomes are in the cytoplasm. Dictyosomes of the Golgi apparatus are present although in the cells adjacent to the suspensor the contents of dictyosome vesicles do not stain. The embryo-cell nucleus shown in Fig. 43 has reticulate heterochromatin and a very prominent nucleolus. Examination of the nucleolus reveals both fibrillar and granular regions, indicative of ribosomal RNA synthesis and packaging.

Variation in the range of cellular development in the 6 day embryo is shown by comparison of Figs. 44 and 45. Both are of epidermal cells although in the former the cell has begun to develop a population of vacuoles whereas the cell shown in Fig. 45 is 'younger' and without any vacuoles. At this period the embryo is undergoing a phase of cell division although it is evident from these micrographs and the cells shown in Fig. 49 that the division is not synchronised.

All epidermal cells (Fig. 44, 45) contain a nucleus with prominent nucleolus, with well-defined fibrillar and granular regions. In some cases (e.g. Fig. 45) there is a nucleolar 'vacuole'. The epidermal cells also contain rough cisternal ER profiles and numerous dictyosomes. A few of the dictyosome vesicles contain densely staining material. Various stages of vacuole ontogeny are illustrated in Figs. 45 and 46, which illustrate 'clearing' of cytoplasmic regions (starred) and ER dilation (arrowed). The plastids of the epidermal cells show developing thylakoids with small stacks of grana and a peripheral reticulum more obvious than in plastids of neighbouring inner cells (Fig. 46: detail of Fig. 45). Some plastids contain phytoferritin deposits. The outer embryo wall is thicker than the walls within the embryo, however, it shows no evidence of development of irregular thickening at this stage. The inner membrane of the endosperm lies close to the embryo cell wall although the intervening space contains may fine tubular extensions of the membrane (Fig. 45 small arrows). These are shown in greater detail in Fig. 47. The tubules sometimes contain ribosome-rich cytoplasm and many are seen closely associated with the outer fibres of the embryo wall. The inner surface of the embryo wall is rather diffuse and apparently

granular in appearance (Fig. 47). The 'granularity' results from cross-sectioning of the inner wall fibrils.

Fig. 47 shows the embryo epidermis of the side of the embryo towards the centre of the embryo sac. The embryo wall and its association with the endosperm membrane is similar all around the embryo.

One of the many cell division figures found within the embryo at this stage is shown in Fig. 49. The metaphase pairing of the 7 chromosomes is evident as are the characteristic patterns of chromatin staining. An incomplete boundary of nuclear envelope/ER delineates a region at the centre of the cell where most organelles are excluded from the central 'nuclear cytoplasm', although this has been penetrated by some ER.

The very thin dividing wall between the embryo cells is little more than a middle lamella at this stage although there are numerous plasmodesmata bridges.

Figures 50 and 51 are electron micrographs of embryos of pea at 6 DAF showing the next stage of development of cells in passing from the developmental phase i) to ii) and contain the embryo cells at different stages of differentiation. Only a few cells are dividing (e.g Fig. 51) and most cells are starting to vacuolate and expand.

Fig 50 shows embryo cell with highly dense cytoplasm containing numerous vacuoles, mitochondria, ER, ribosomes and plastids. The cell wall is more substantial than that seen at the earlier stage (e.g Fig. 49). Fig. 50 also shows that the majority of the embryo cells at this time have nuclei with 2 or more nucleoli. The nucleoli are frequently

hollow with a so-called 'nucleolar vacuole'. As well as an increasing amount of rough cisternal ER in a cytoplasm still very dense with free ribosomes, the numerous dictyosomes are now more evident in the micrographs as many of them have vesicles containing electron dense material. The plastids have not developed very much further than at e.g. Fig. 46, although there is no longer evidence of constriction division of plastids as was found earlier. Fig. 51 illustrates one of the few cell divisions found in the embryo cells at 6 DAF. As in Fig. 49 a broken nuclear envelope/ER boundary excludes most organelles from the nuclear volume which contains 7 pairs of chromosomes at metaphase. The clusters of small electron dense vesicles are always associated with dictyosomes, although these are not always seen because of the plane of the sectioning.

Fig.34: Diagram of the development and enlargement of the embryo within the embryo sac.

Figs.35-38: Electron micrographs showing the ovular tissue which is adjacent to the embryo sac boundary wall at 6 DAF. X6536, X7400, X9675, X6966.

Fig.39: Diagram showing the relative positions of Figs.35-38.

Fig.40: Electron micrograph of a lower part of the suspensor, X6525.

Fig.41: Electron micrograph showing the proximity of the suspensor to the embryo sac wall, X19250.

Fig.42: Electron micrograph of the central regions of the suspensor cytoplasm.

Fig.43: Electron micrograph illustrating the boundary between the suspensor and cells of the embryo.

Figs.44.45: Electron micrographs illustrating the variations in the range of cellular development in the 6 DAF embryo, X24750, X34200.

Fig.46: Electron micrograph showing the plastids of the epidermal cells, X30600.

Fig.47: Electron micrograph showing the inner surface of the embryo wall.

Fig.48: Electron micrograph showing cross sectioning of the inner wall, X20250.

Fig.49: Electron micrograph showing cell division.

Figs.50,51: Electron micrographs of the embryo of pea at 6 DAF, X6534, X10890.

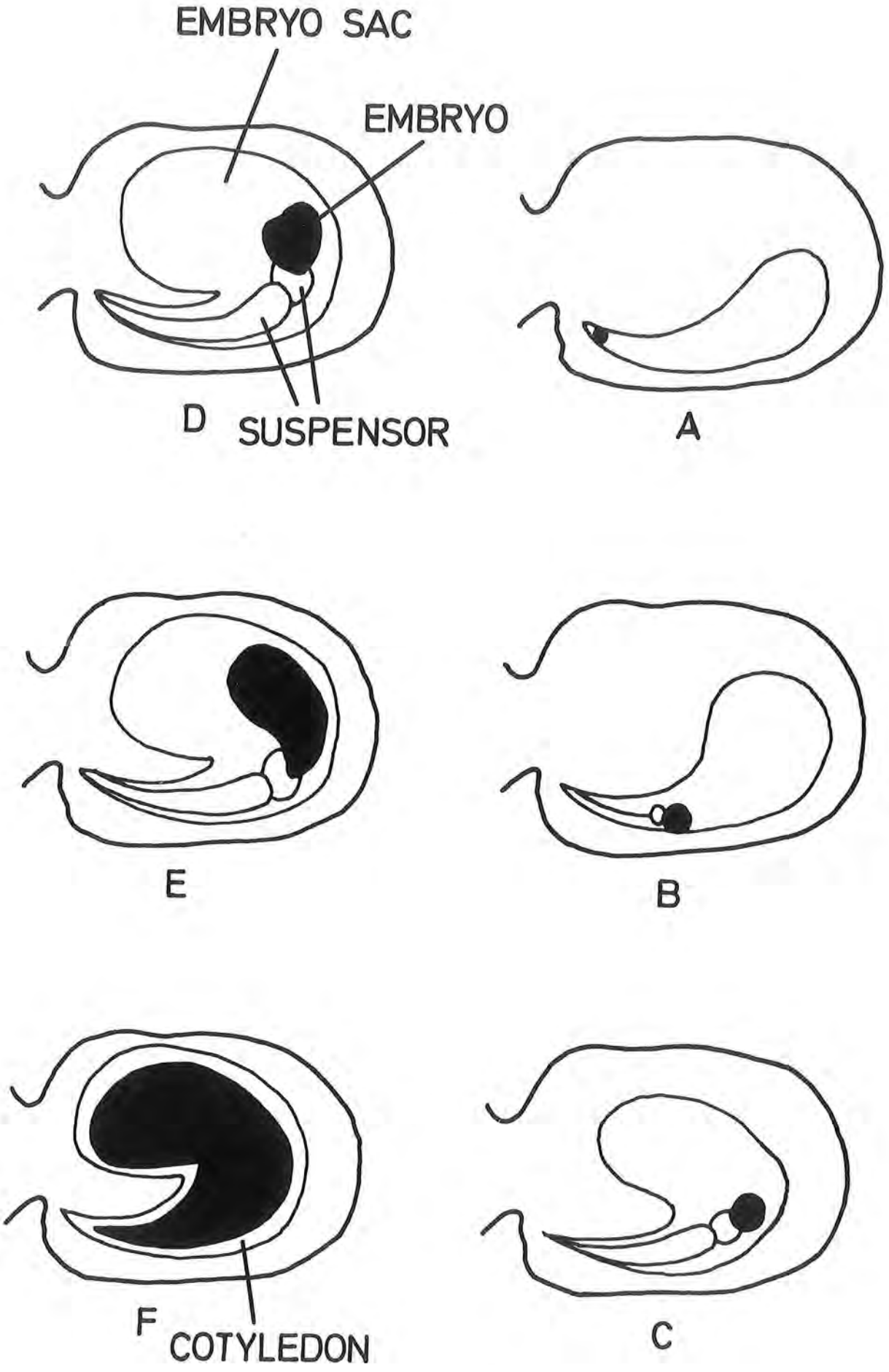


FIG.34

NOT TO SCALE

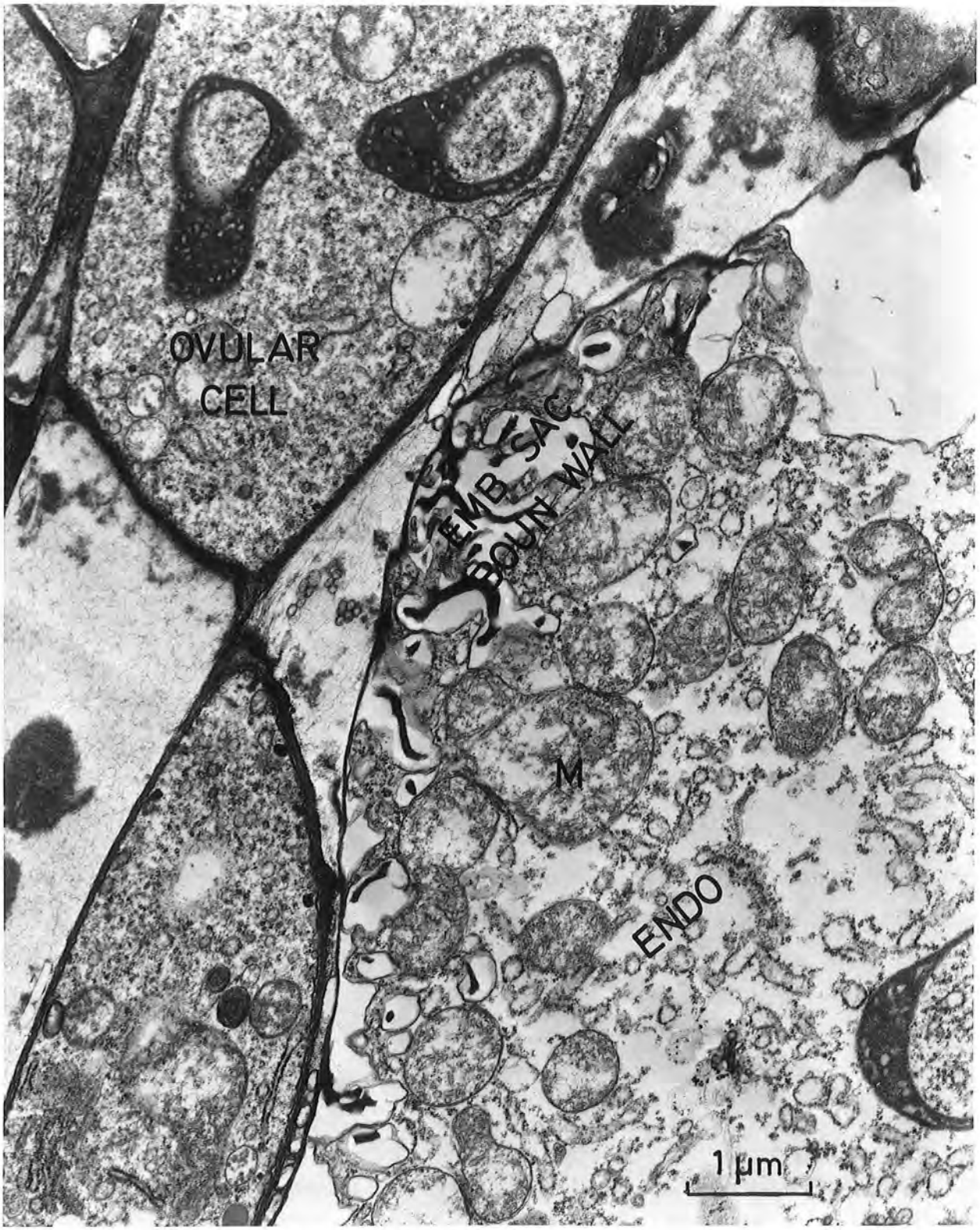


FIG. 36

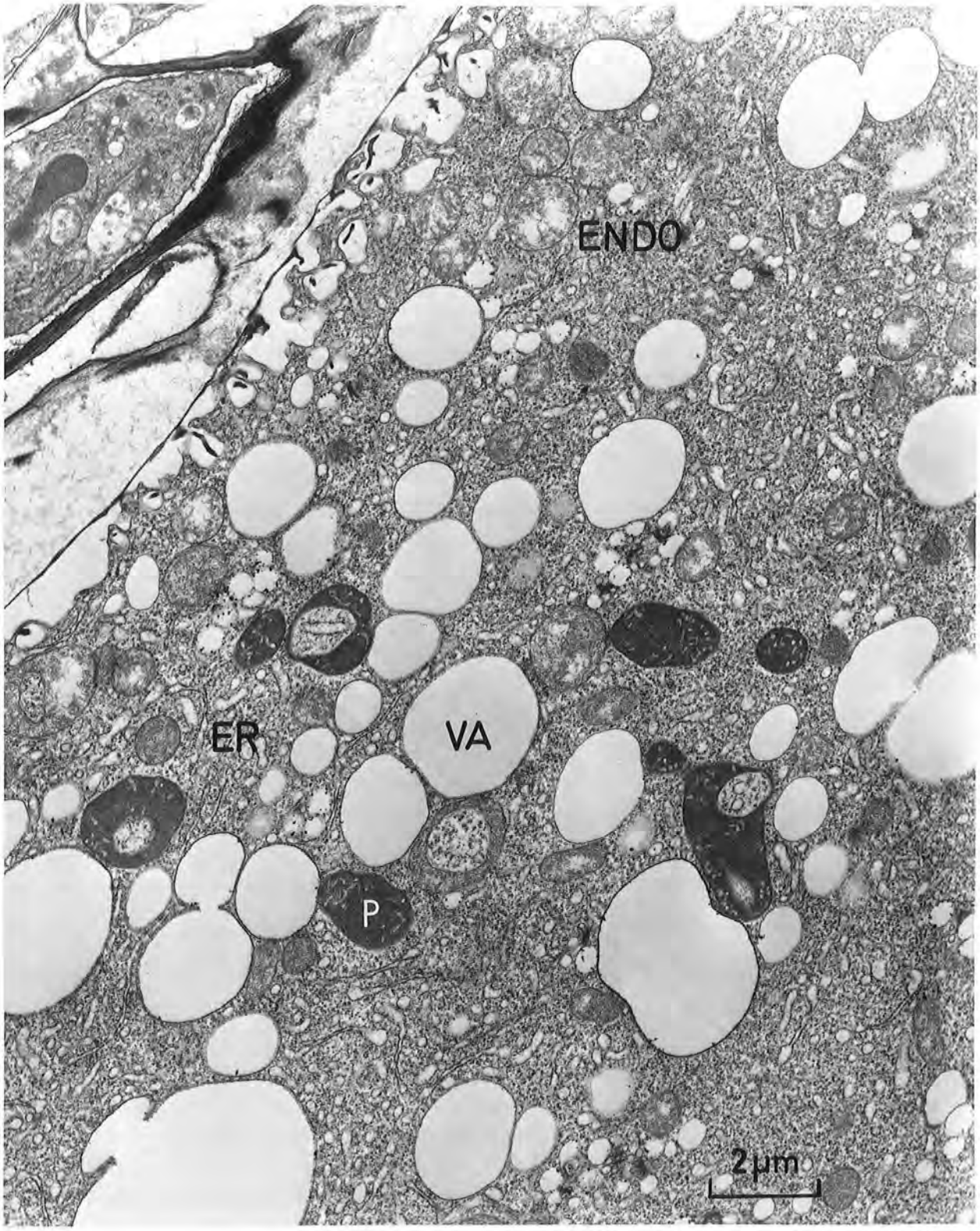


FIG. 37

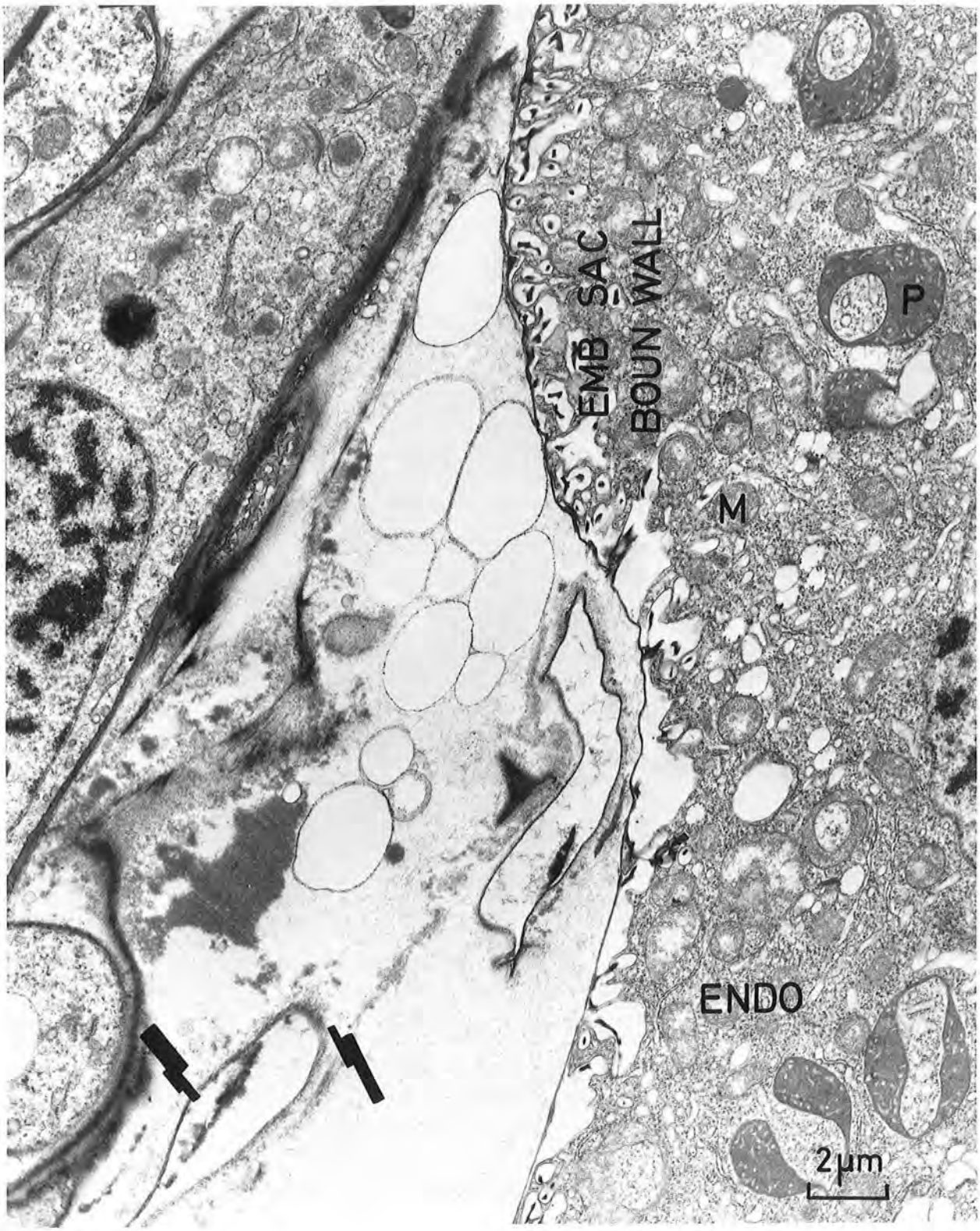


FIG. 38

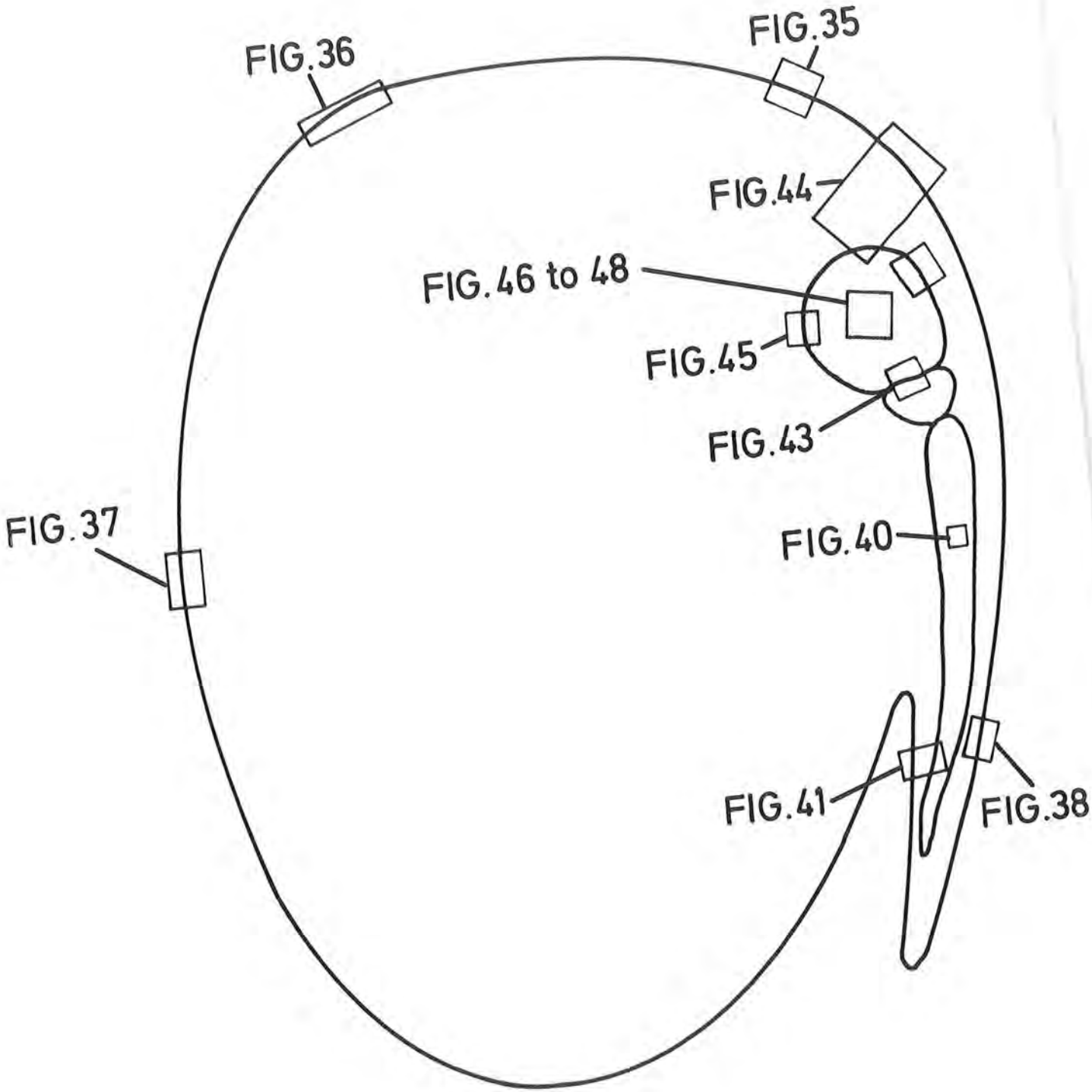


FIG.36
FIG.35
FIG.44
FIG.46 to 48
FIG.45
FIG.43
FIG.37
FIG.40
FIG.41
FIG.38

FIG.39

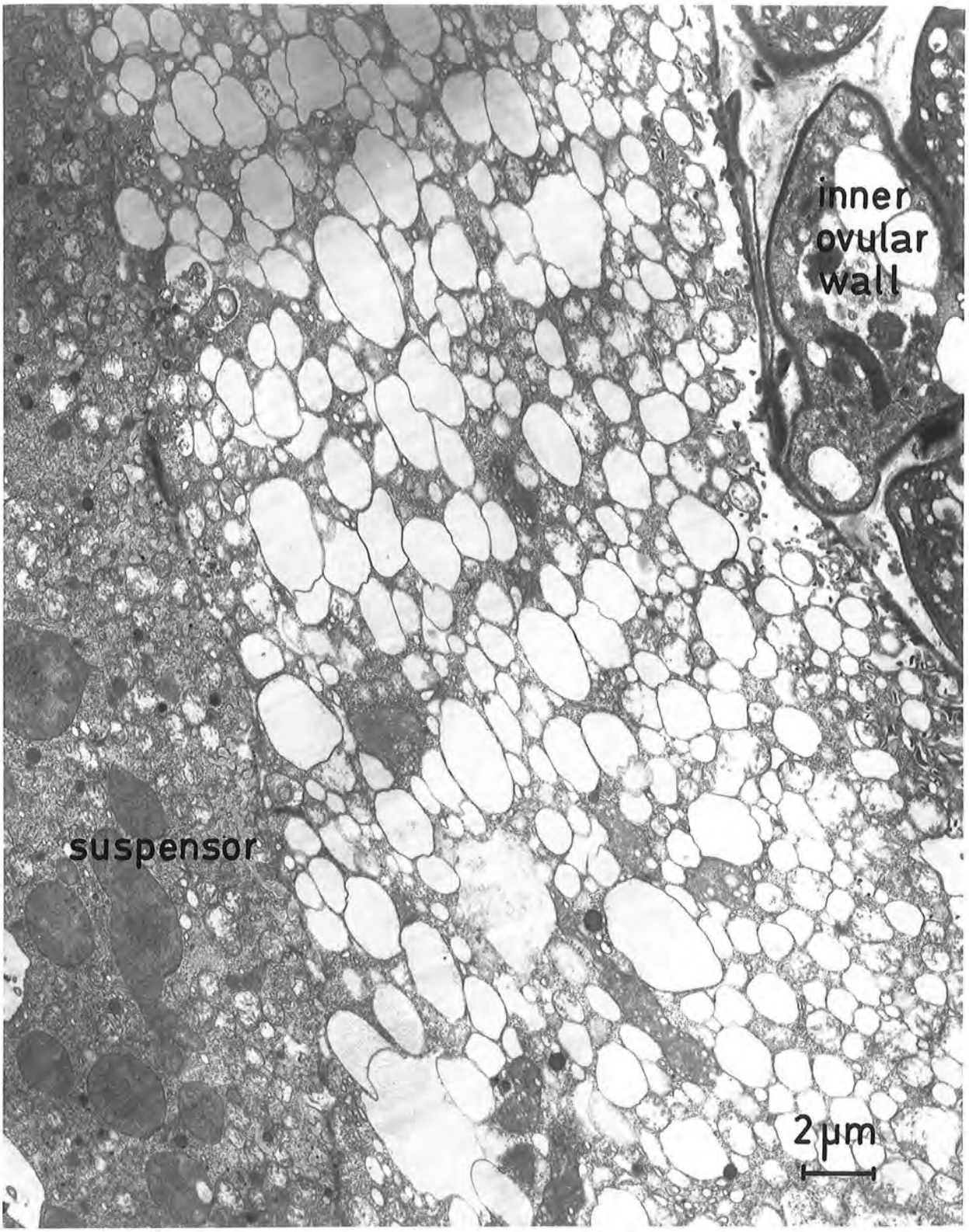


FIG. 40

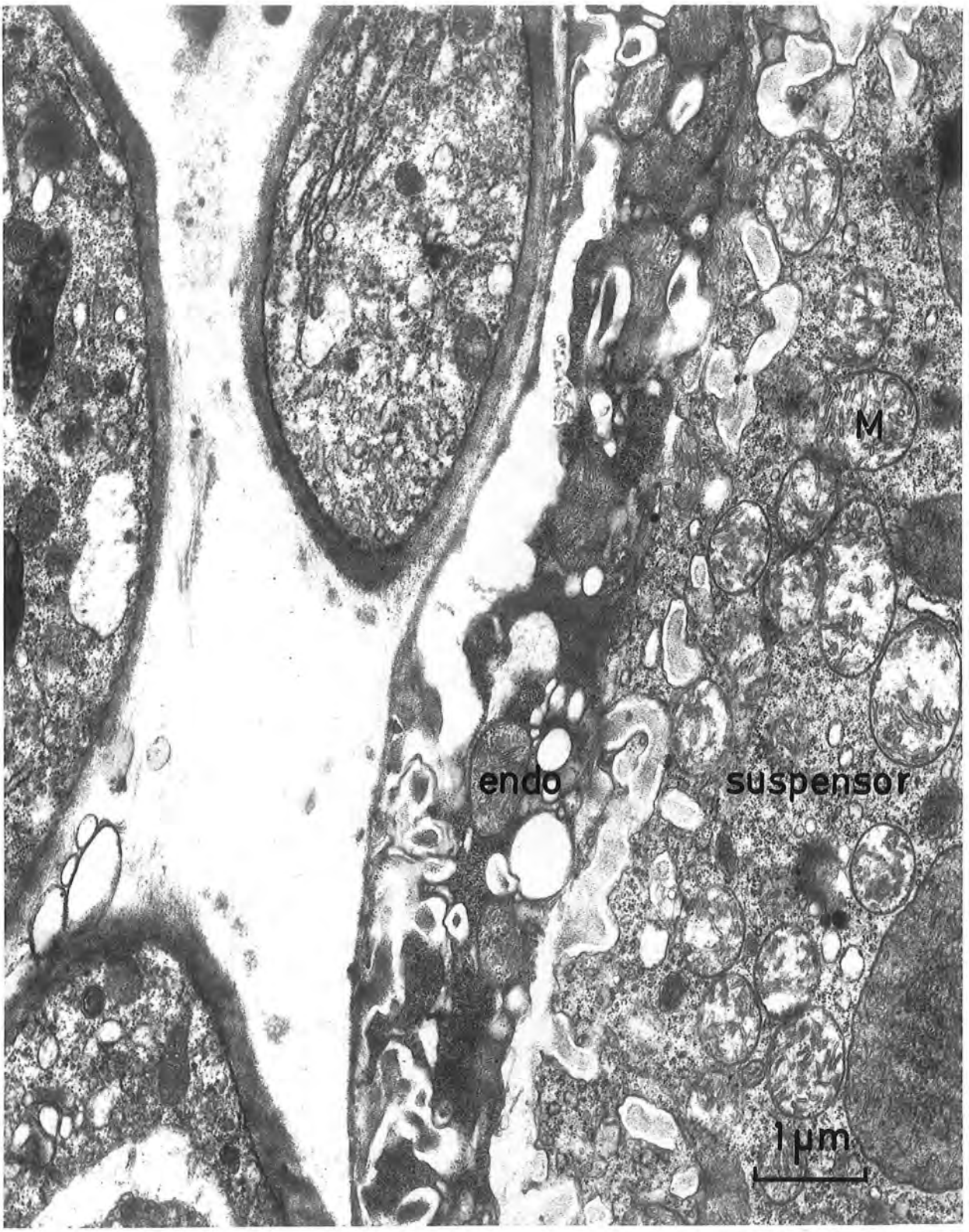


FIG. 41

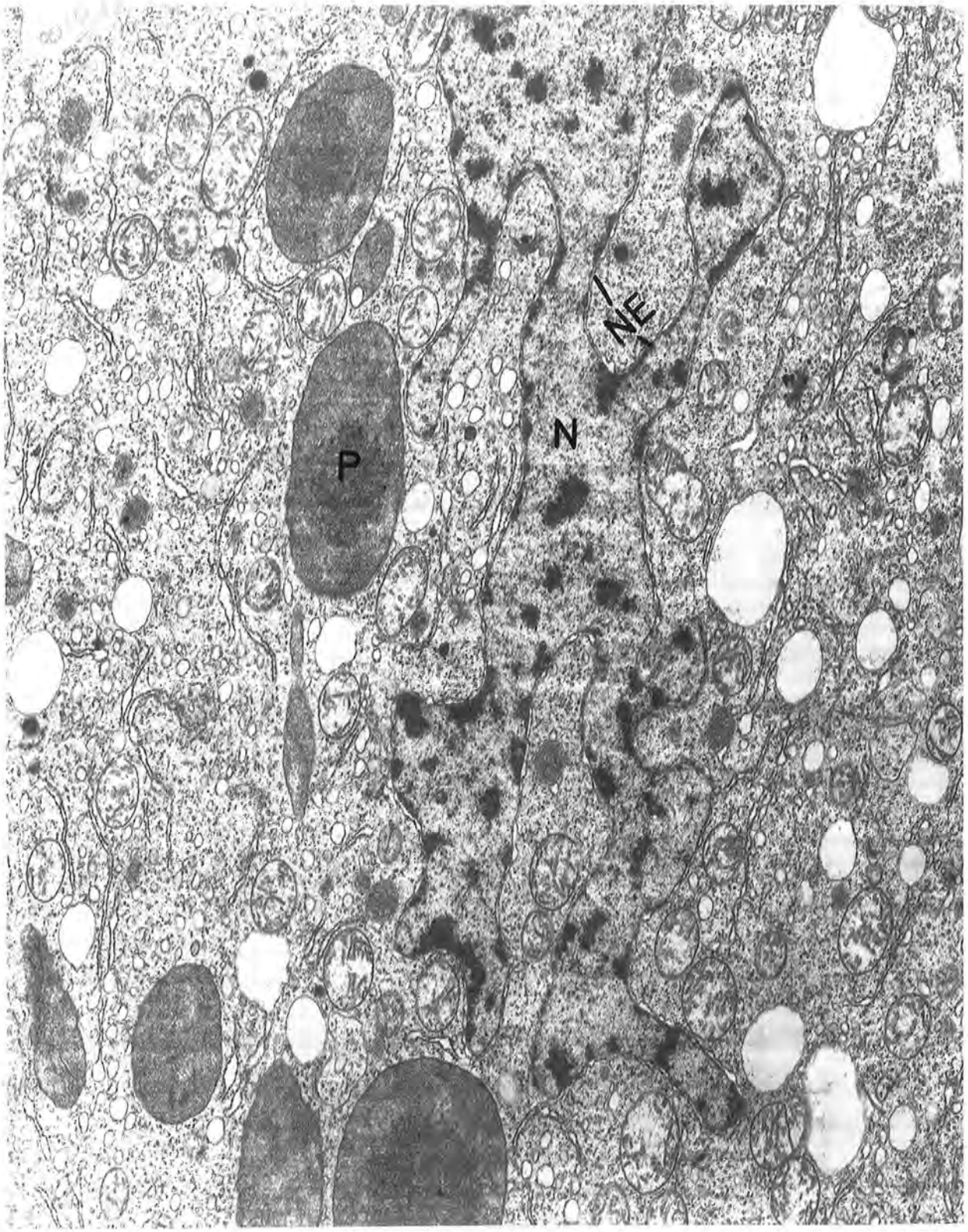


FIG. 42

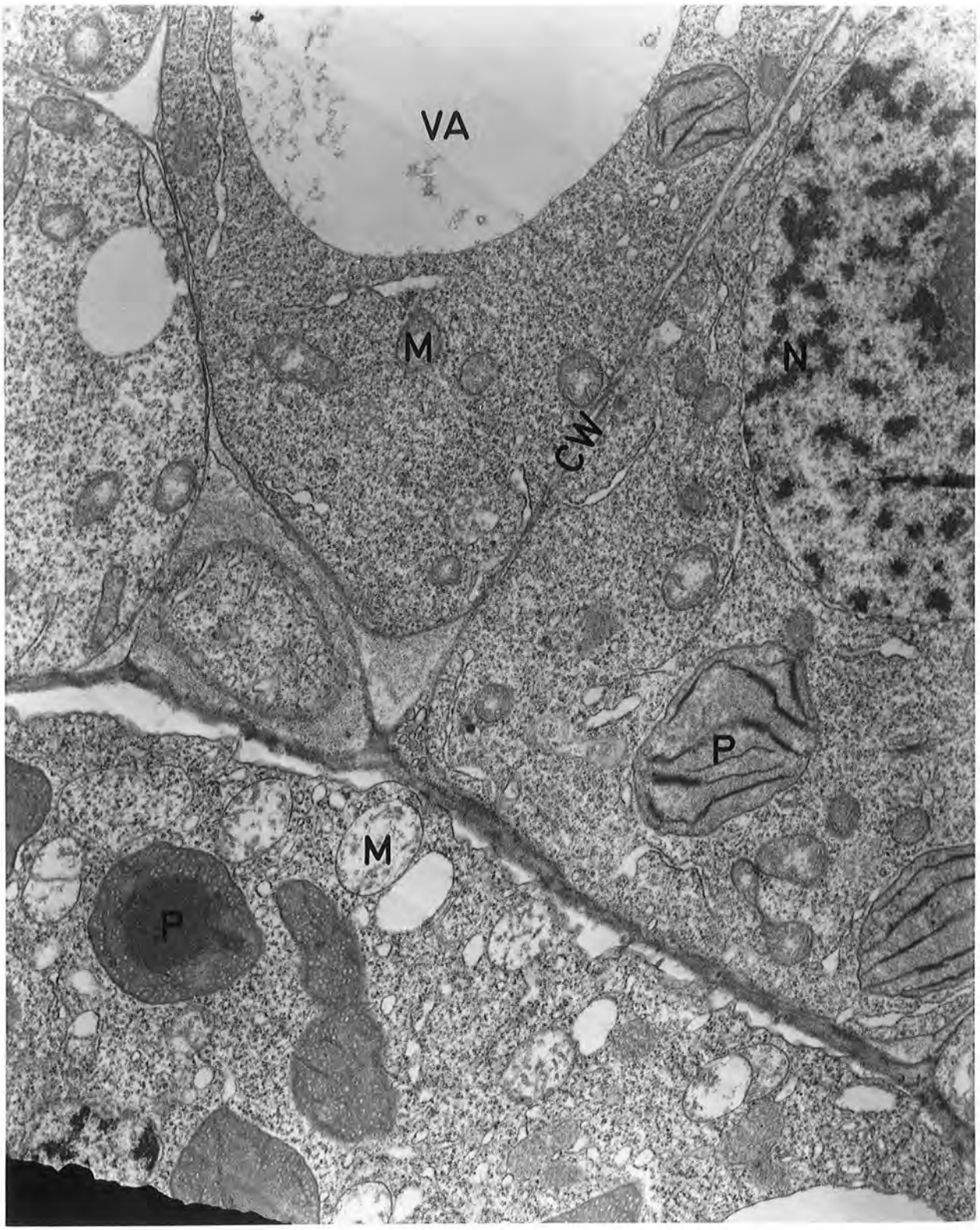


FIG.43

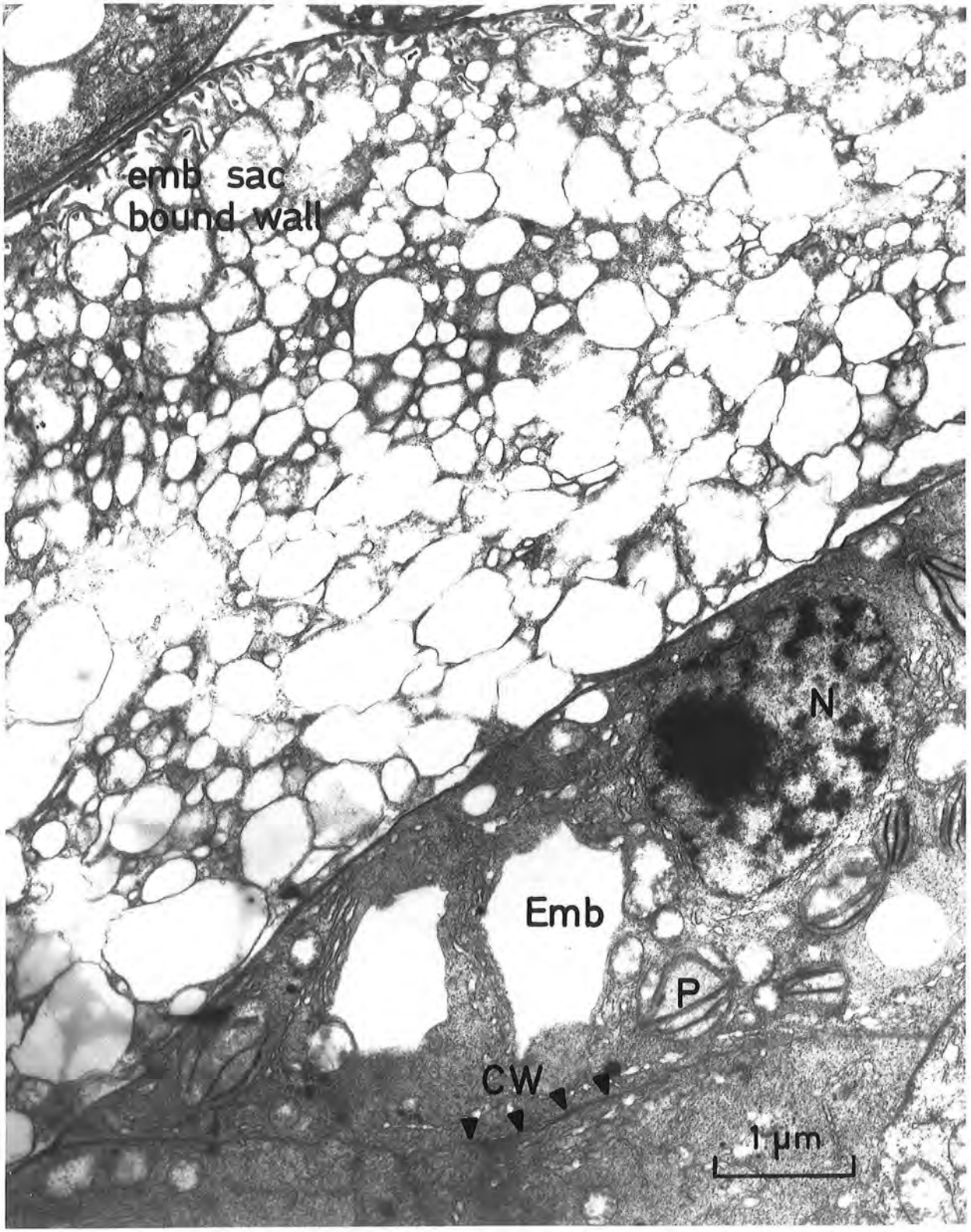


FIG. 44

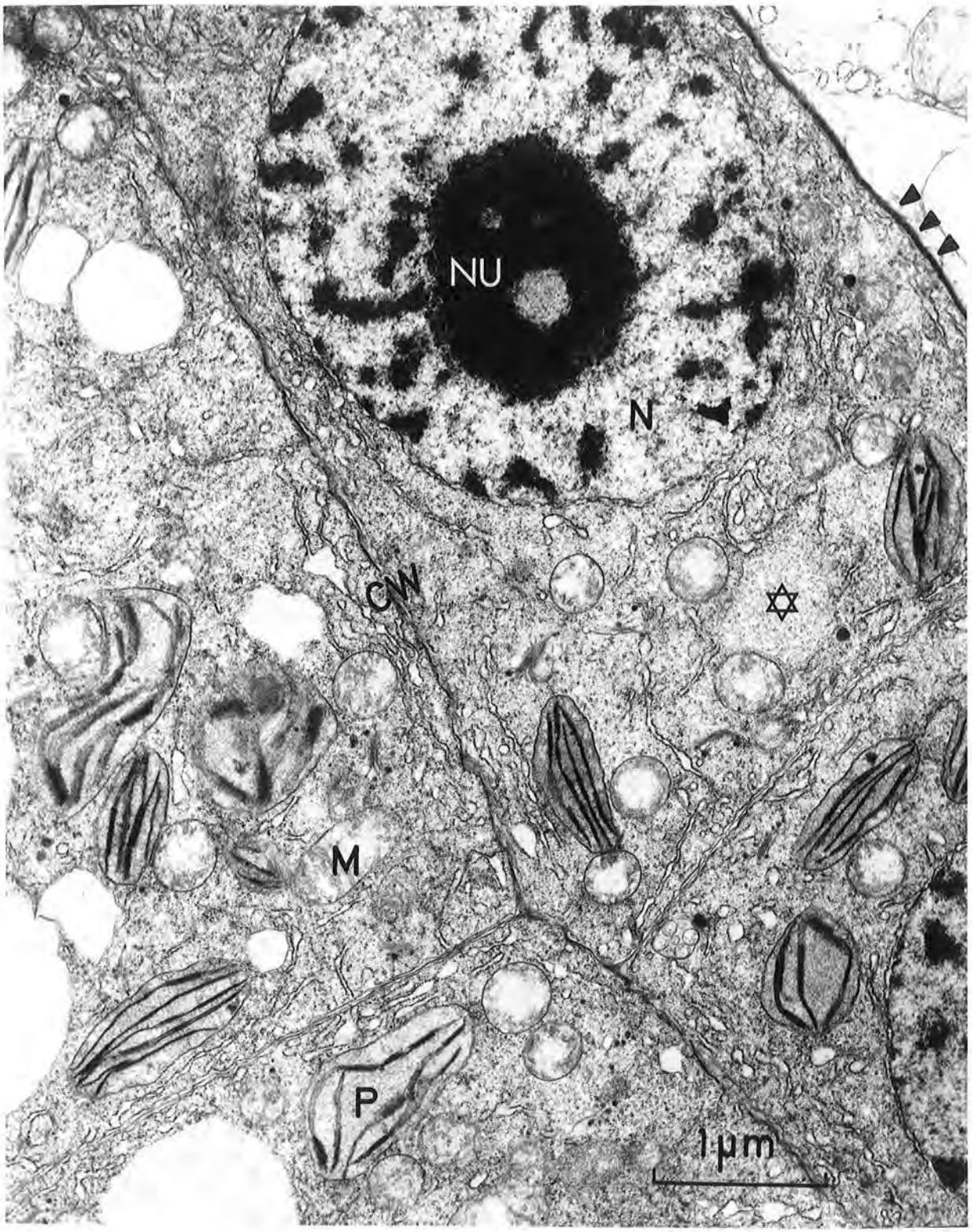


FIG. 45

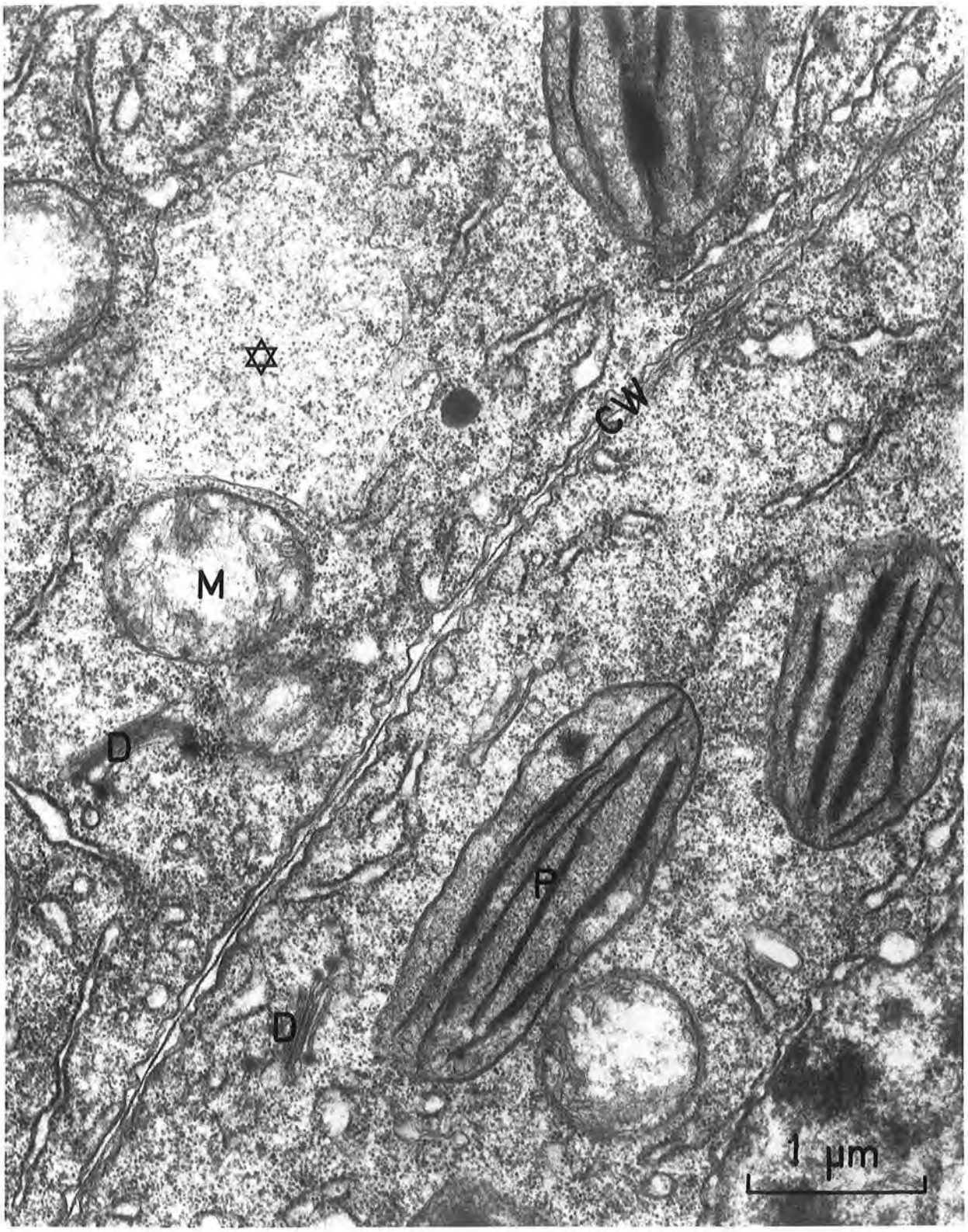


FIG. 46

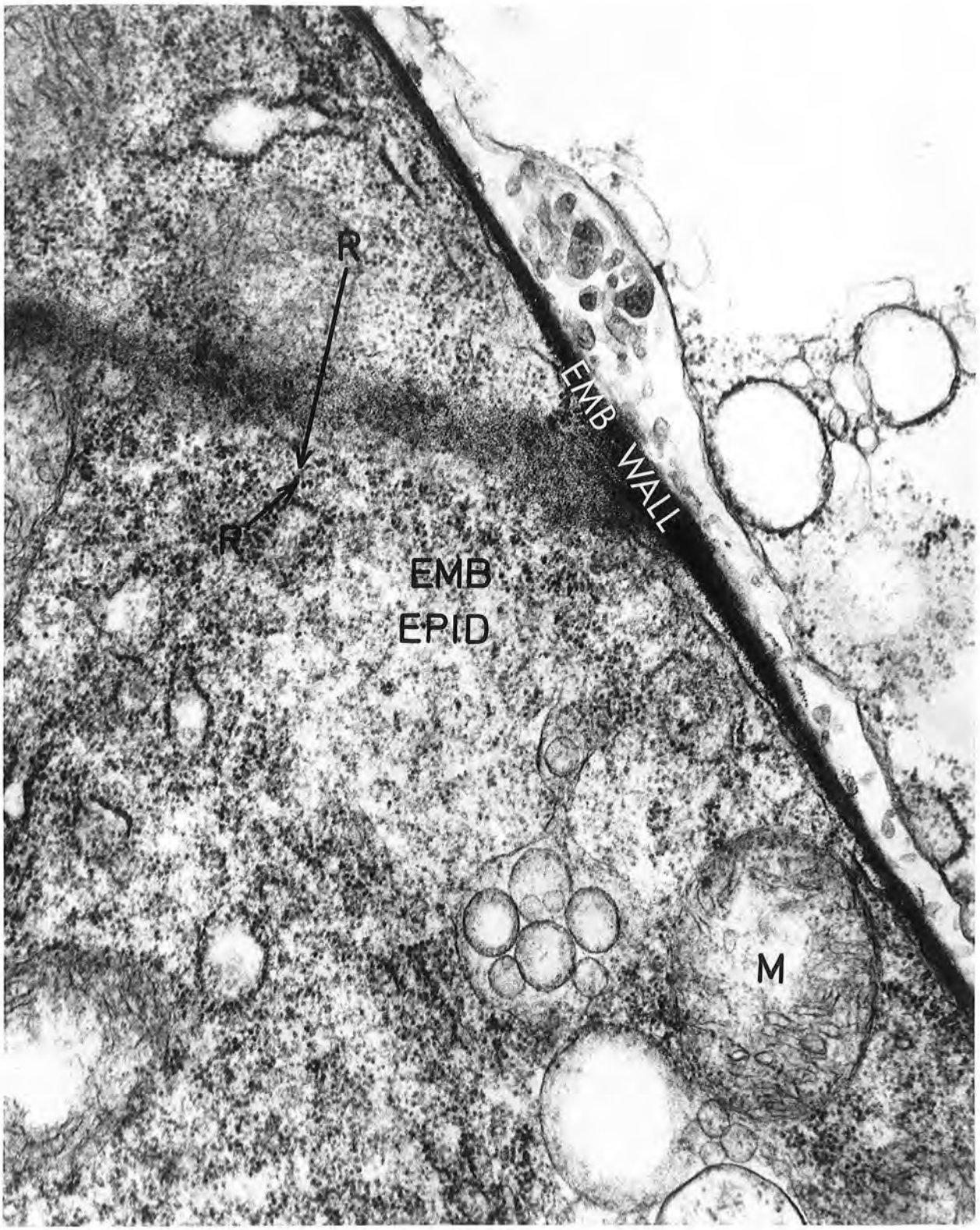


FIG. 47

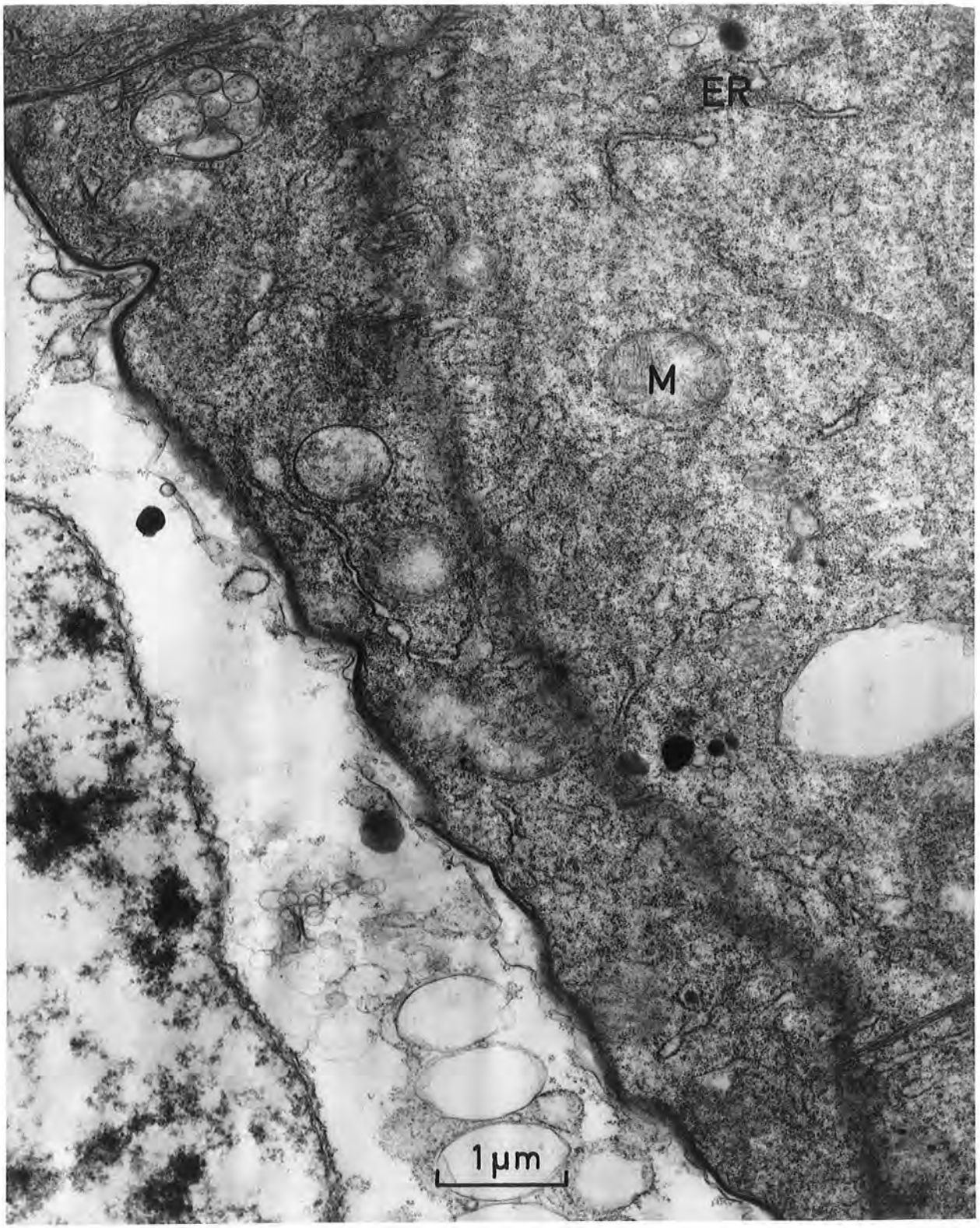


FIG. 48

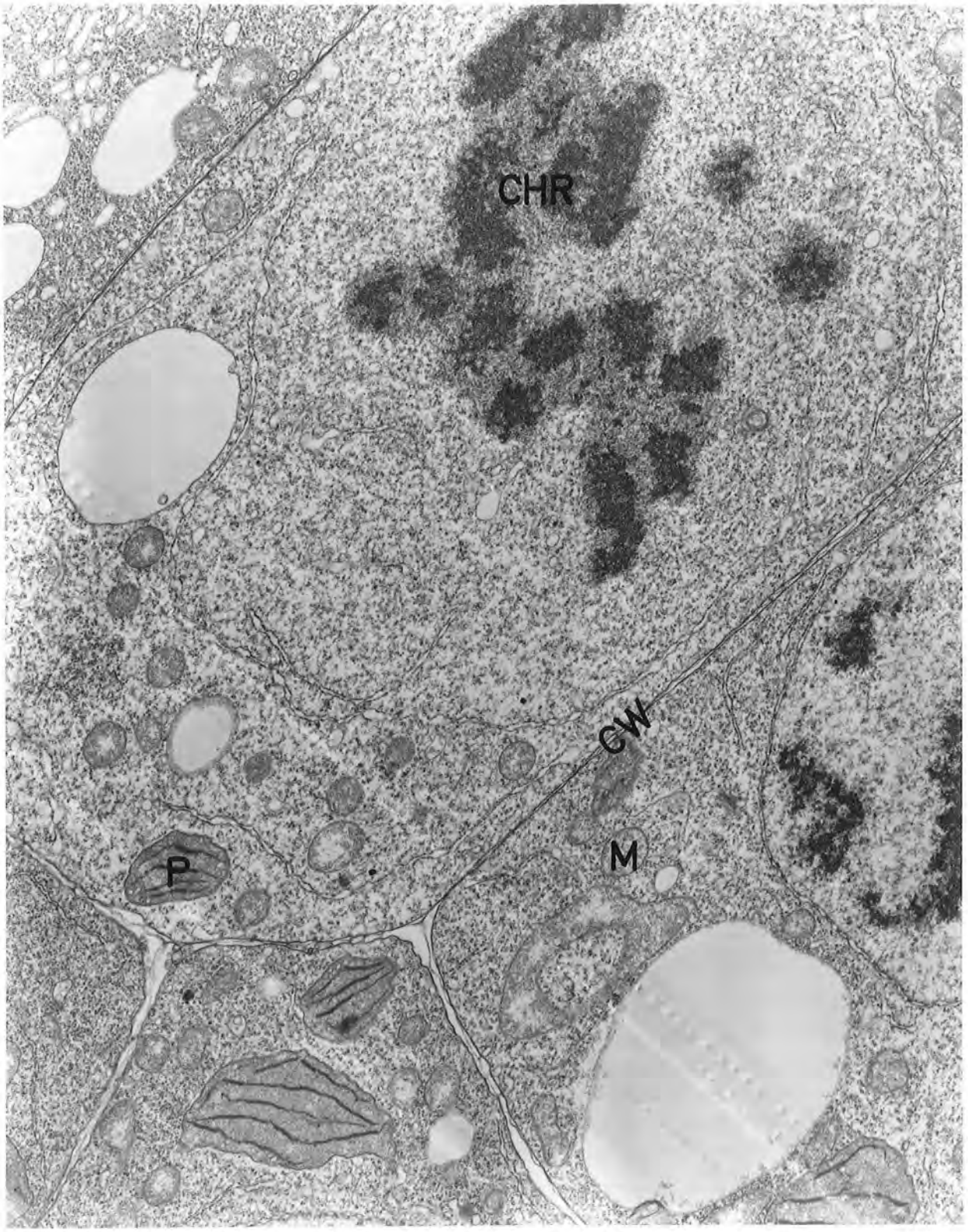


FIG. 49

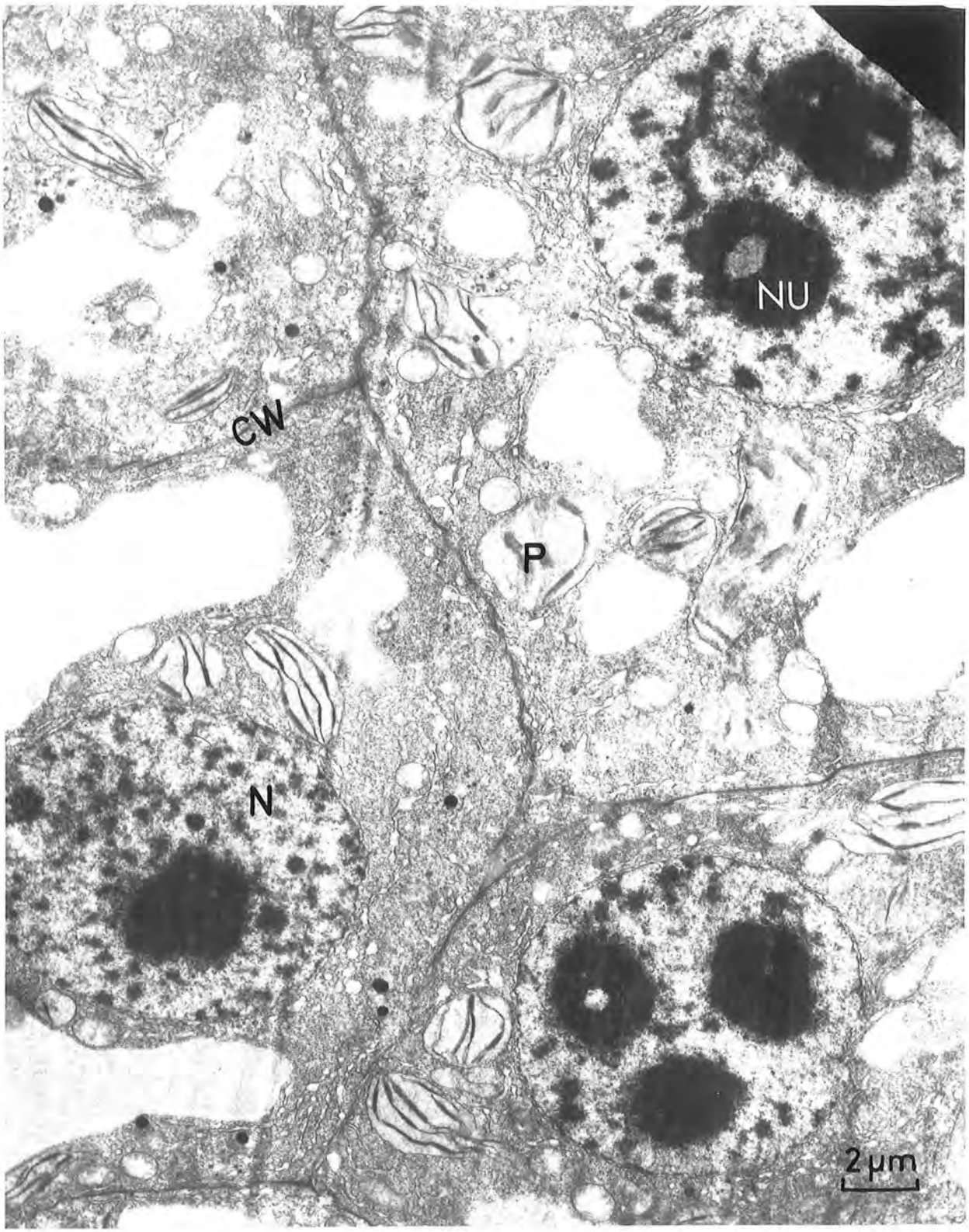


FIG. 50

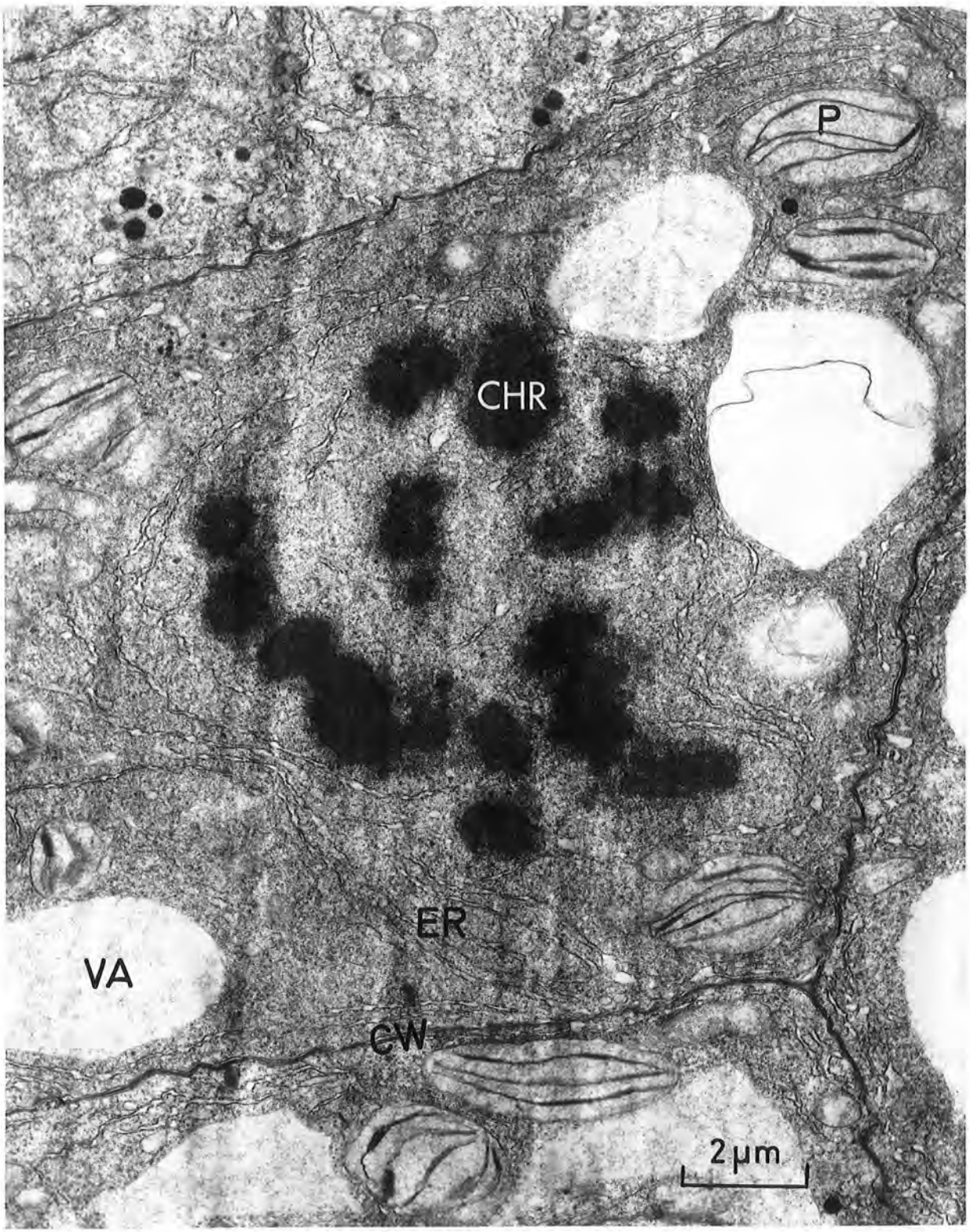


FIG. 51

1.4 Light microscopy of cotyledon development.

The structure of the early embryo has been described above. The globular embryo changes to a heart-shape, still with attached suspensor, and as this enlarges the embryo develops two recognisable cotyledons and an emergent radicle and shoot apex. The cotyleons act as the major site of storage deposition. This part examines the changes in cotyledon structure during storage deposition as shown by light microscopy.

Examination of an embryo of 12 DAF pea indicates a number of significant features at the early stage of storage deposition. Figure 52 shows several 1μm sections cut through a pea embryo and also a section of seed coat. The main vascular traces from hypocotyl to cotyledons are seen in section 7 (arrows) and the transversely sectioned minor vascular bundles running throughout the cotyledons are shown in other sections in an arc. The outer (abaxial) side of the cotyledon is apparently darker between the epidermis and the vascular bundles with a more transluscent parenchyma between the bundles and the inner (adaxial) epidermis. The major cell layers of the seed coat and outer cotyledon layers are presented diagrammatically in Fig. 53, 54. The palisade and parenchyma layers of the seed coat are illustrated, also the residual endosperm and outer cotyledon layers.

It is evident that whilst there is an apparent difference in the development of cells through the cotyledon from abaxial to adaxial epidermis in any one section there is no difference in the development between the tissues shown in the series of sections cut along the cotyledon.

In order to examine this apparent difference through the cotyledon

samples were fixed and embedded and sectioned for examination by light microscopy after staining with toluidine blue. Seeds at increasing stages of maturity were examined in this manner to determine the pattern of storage depositions.

The sequence of protein body formation in cotyledonary cells of developing pea seeds has been followed in this way. Results here will be presented for three ages of tissue, 12, 16, and 20 days after flowering.

At 12 DAF, in the middle of the period of cell expansion, there is a large increase in the size of the cotyledon parenchyma cells but little deposition of storage protein, although starch has been laid down within the plastids. The vacuolar system has differentiated and expanded from a single large vacuole so that each cell now has several large vacuoles. Some protein material has accumulated on the inner surface of the membrane of the cytoplasmic vacuoles and vesicles. Figs. 55 and 56 show cells just under the abaxial epidermis with Fig. 55, 3 - 5 cells from the epidermis and Fig. 56, 6 - 10 cells from the surface. Figs. 58 and 57 show tissue at similar positions relative to the adaxial epidermis. In comparing the typical representative cells of the four layers shown in Figs. 55 - 58 it is evident that although there is a general similarity in the cytology, there is a progressive increase in cell size through Figs. 55 to 57 , but with a slight decrease in cell size in Fig. 58 relative to those in Fig. 57. There is also a change in the relative proportion of intercellular space and there are more developed starch grains per cell towards the adaxial side. In each cell one vacuole is distinguishable by its more densely stained limiting membrane. Serial sectioning indicated that

this vacuole and others were in fact isolated from each other.

By 16 DAF the cotyledon parenchyma cells contain many small vacuoles with amorphous material shown to be protein by histochemical staining and hence the organelles will be termed protein bodies. Figs. 59 and 60 show tissue from similar portions in the cotyledon to those shown in Figs. 55 and 58. The density of staining is not the same in different protein bodies within a cell and there is a difference in the extent of filling of protein bodies in adjacent cells. The small sub-epidermal cells shown in the corner of Fig. 59 contain less densely filled protein bodies and similarly the cells close to the adaxial surface of the cotyledon (Fig. 60) are similarly stained, with protein only adhering to the limiting membrane. The central cotyledon parenchyma cells contain protein bodies each with an even distribution of protein although with some quantitative variations between protein bodies. Gradually each cell contains a small number of larger, more densely stained protein bodies and a far greater number of smaller, less intensely stained protein bodies. The starch reserves have also increased by 16 DAF with a similar number of starch grains per cell throughout the cotyledon, with the exception of the epidermal and sub-epidermal cells.

At 20 DAF there are extensive deposits of storage protein with mostly spherical shape. Of the protein bodies, Figs. 61 and 62 are of the abaxial and adaxial regions respectively and show the increase in parenchyma cell walls from 16 DAF. Light microscopy shows that there are no significant changes in the cytology of pea cotyledon cells at different regions (abaxial and adaxial) at the same age.

The finding that storage deposition was relatively even throughout

the cotyledons from abaxial to adaxial epidermis was a little surprising as the 1 µm tissue sections had indicated a 'darker' outer region, closer to the source of nutrients moving from embryo sac boundary wall to outer cotyledon epidermis. The light microscopy however indicated some differences in cell size and extent of intercellular spaces and these paremeters were quantified.

Two aspects were examined, the proportion of cell as percentage of tissue at each of the regions through the cotyledon and also the extent of cell packing as indicated by the ratio of cell wall contact with the other cells as opposed to intercellular space (a cell to cell/cell to space ratio) Table 1A.

The comparison of different parts of the cotyledon was made using sections taken from 4 regions: 1) close (within 3 to 5 cells) to the abaxial epidermis, 2) 6 to 10 cells from the abaxial epidermis, 3) 6 to 10 cells from the adaxial epidermis, 4) close to the adaxial epidermis. The sections were examined using stereological techniques and the results are shown in Table 1B. This gives the proportion of cell volume, including walls, as a percentage of tissue volume for the different regions of the cotyledon at 12, 16 and 20 DAF. There is a decrease in the % volume of cells from abaxial to adaxial side of the cotyledon. The intercellular space is approximately doubled in the adaxial regions and this could account for the apparent difference (i.e. the 'darker' outer region) seen in cotyledon slices and 1 µm sections. During the maturation phase (20 DAF) there is a decrease in % volume of cotyledon cells as the tissues dehydrate.

The quantitation of tissues can be very useful as it can show the relative importance of the various features and may be used as a

1.5 Transmission electron microscopy of cotyledon development.

The results from light microscopy examination of developing pea cotyledon indicated that there is a uniformity in the rate of development of storage reserves throughout the cotyledon. The detail of cell form was examined by transmission electron microscopy and the changes in fine structure of the developing storage parenchyma cells are shown in this section.

The fine structure of cotyledon storage parenchyma cells at 7 DAF is shown in Figs. 63 to 69. At this time the cotyledon cells are completing the phase of cell division and commencing a phase during which there is considerable cell expansion. One of the few cell division figures is shown with chromosomes at early metaphase (Fig.63, top right). The interphase nuclei are characterised by conspicuous nucleoli usually of a hollow sphere form (Fig. 64) with distinct fibrillar and granular regions. The nuclei also contain highly stained dispersed heterochromatin throughout the nucleoplasm. Fig. 66 also shows the cells are producing the vacuoles at the centre of the cells. The size of cotyledon at this stage of development is approximately 0·5mm; apart from the presence of the central vacuoles, in structure the cells are very similar to each other. The presence of several plastids with few internal membranes, and some small dictosomes can be seen. It is also possible to observe several mitochondria and ER in Fig. 63. Details of the plastids is shown in Fig. 65 and 66. A few thylakoids with shallow granal stacks lie appromimately parallel to each other although separated by regions of stroma. The peripheral reticulum is not highly developed. Fig. 63 reveals the presence of several dark bodies approximately 0·25 µm

diameter and clusters of smaller, similarly stained vesicles. The numerous electron dense deposits are associated with dictyosomes (Fig. 66) and membrane bound (Fig. 67). Work with similar deposits in Vicia faba and cowpea has shown that the vesicle contents are pronase-digestible.

The cytoplasm of the cells illustrated in Figs. 63, 64 contains a high proportion of cytoplasmic polyribosomes as well as an increasing amount of cisternal ER with associated (membrane-bound) polyribosomes (cf. parenchyma cell cytoplasm at early embryo stage).

The cell walls of the parenchyma cells are primary with cellulose fibrils loosely woven (Fig. 68) and with some parallel orientation (Fig. 69). Figure 69 also illustrates some of the numerous plasmodesmata connections between the parenchyma cells. The plasmodesmata frequently occur in pairs.

The onset of the rapid phase of cell expansion is illustrated by the extent of vacuolation in the parenchyma cells (Fig. 70). This occurs within 24h of the development shown in Fig. 70 (i.e. 8 DAF). The cells develop a few large vacuoles, with the cytoplasm now mostly confined to a layer lining the walls, containing a population of cytoplasmic vesicles. The nuclei are generally found in the middle of the cells, surrounded by the large vacuoles, but with a few cytoplasmic bridges to the peripheral layer. The plastids, lying close to the cell walls, have not developed any significant starch reserves although areas of plastid stroma appear 'clear'.

By 10 DAF the ultrastructure of the storage parenchyma cells has changed markedly. (Fig. 71). The cells no longer contain a few large vacuoles but a larger population of smaller vacuoles and vesicles.

These contain small, densely-stained deposits, often associated with the vesicle membranes (tonoplast). The deposits were identified histochemically as protein. The plastids now contain starch reserves.

Details of the nucleus at this stage (fig. 72) illustrates the many nuclear envelope pores, most of which contain a central granule. The dispersed heterochromation is mostly granular with some peripheral fibrillar regions. The cytoplasm contains much rough ER. There are now few free cytoplasmic polyribosomes, although the cisternae are small. Numerous dictyosomes are present throughout the cytoplasm, characterised by clusters of electron dense vesicles (Figs. 73, 74).

By 12 DAF the storage parenchyma cells have undergone further change in their ultrastructural appearance. The vacuoles and vesicles now contain dipersed electron dense material (Fig. 75) rather than the dense peripheral deposits present in e.g. Fig. 71. Many of the nuclei have become amoeboid in shape with numerous projections and invaginations of the nuclear envelope, and in some cases cells have become multi-vacuolate (e.g. Fig. 75). An increase in the size of the ER cisternae occurs during this period so that by 13 - 14 DAF, when the vacuoles have accumulated more material, the cytoplasm contains large amounts of rough cisternal ER with many profiles of large surface area (these appear 'long' in this section) (Fig. 76, 77). The cytoplasm contains numerous dictysomes each with characteristic dense vesicles.

The increase in wall thickness during this stage of cotyledon development is illustrated in Fig. 76, (although exaggerated by a semi-tangential section). The increase results from the addition of loosely packed fibrils in regions of the wall free of plasmodesmata.

Synthesis of storage proteins and their deposition into the vacuolar protein bodies occurs for a few more days (15 - 19 DAF). During this period protein bodies fill with protein so that each has a uniform amorphous appearance (Figs. 78, 79). There are no globoids or crystalline deposits present. Slight variations in staining intensity occur both between adjacent protein bodies within a cell and between protein bodies of adjacent cells. The structure of the endoplasmic reticulum changes with a breakdown of some of the large cisternae into smaller pieces (Fig. 79) and the cytoplasm contains fewer dictyosomes at this stage. From 20 DAF the seed undergoes a phase of maturation with cessation of biosynthetic activity and a reduction in fresh weight. The protein bodies become more uniformal y spherical and the cytoplasm contains only a small amount of ER, although numerous polyribrosomes are still evident (Fig. 80).

The sequence of protein body formation in the cotyledon cells of developing wild pea seed was also examined by electron microscopy to find out the sequence of ultrastructural changes in the cell, and deposition of protein material to compare development with modern agricultural pea varieties.

The result indicated a similar pattern of development, both of the seed in general and ultrastructural aspects of storage deposition. To illustrate the similarity we have chosen only one stage of the developing wild pea for comparison with the result above; a storage equivalent to 10 DAF (Fig. 81), although in the wild pea this stage takes longer to reach because of the slower rate of maturity.

Fig. 81 shows cells with this cell wall and vacuoles, with protein material deposited inside the tonoplast. Starch grains are

also present at this stage. The cytoplasm contains ER, some mitochondria and few dictyosomes. Fig. 82 shows a nucleus with nuclear envelope and nucleus with surrounding cytoplasm containing ER, mitochondria and dictyosomes. Fig. 84 shows a change similar to that seen in P. sativum, with more and aligned, cisternal ER, associated with the transition of storage protein from granular to more fibrous within the vacuoles. Comparison of the wall at intercellular spaces shows that in wild pea cells, wall corner thickenings stain more densely (Fig. 83). Figures 83 and 84 show the configuration of the plasmalemma at the stage of storage deposition. There are numerous small invaginations and corrugations suggestive of either pinocytosis, vesicle deposition, or an increased surface area mechanism . As there is no apparent increase in vesicle density in the cytoplasm adjacent to the plasmalemma extracellular vesicle deposition seems unlikely.

Fig.52: Light micrograph showing several 1 mm sections cut through a pea embryo and seed coat.

Figs.53,54: Showing diagrammatically the major cell layers of seed coat and outer cotyledon layers.

Figs.55,56: Light micrographs showing the abaxial epidermis of cotyledon cells of pea at 12 DAF.

Figs.57,58: Light micrographs showing the adaxial epidermis of cotyledon cells of pea at 12 DAF.

Figs.59,60: Light micrographs showing the abaxial and adaxial epidermis cotyledon cells of pea at 16 DAF.

Figs.61,62: Light micrographs showing the abaxial and adaxial epidermis cotydedon cells of pea at 20 DAF.

Table 1A: Showing the ratio of cell wall contact with the other cells as opposed to intercellular space.

Table 1B: Showing the volume of cell as total proportion.

Figs.63-69: Electron micrographs illustrating the structure of cotyledon storage parenchyma cells at 7 DAF, X7251, X15800, X13210, X44600, X41500, X45000.

Fig.70: Electron micrograph showing the structure of cotyledon cells at 8 DAF, X9777.

Fig.71: Electron micrograph illustrating storage parenchyma cells at 10 DAF, X5500.

Fig.72: Electron micrograph showing the nucleus, X21060.

Figs.73,74: Electron micrograph showing highly dense cytoplasmic material, X9040, X5280.

Fig.75: Electron micrograph illustrating vacuoles and vesicles now containing electron dense material, X6820.

Figs.76,77: Electron micrographs showing the cytoplasm containing large amounts of RER, X10530, X13770.

Figs.78,79: Electron micrographs illustrating protein filling the protein bodies, X6050, X7310.

Fig.80: Electron micrograph showing the protein bodies more uniformally, and cytoplasm containing only a small amount of ER, X7700.

Figs.81-84: Electron micrographs illustrating protein body formation in the cotyledon cells of developing wild pea, X8750, X6900, X7700, X8110.

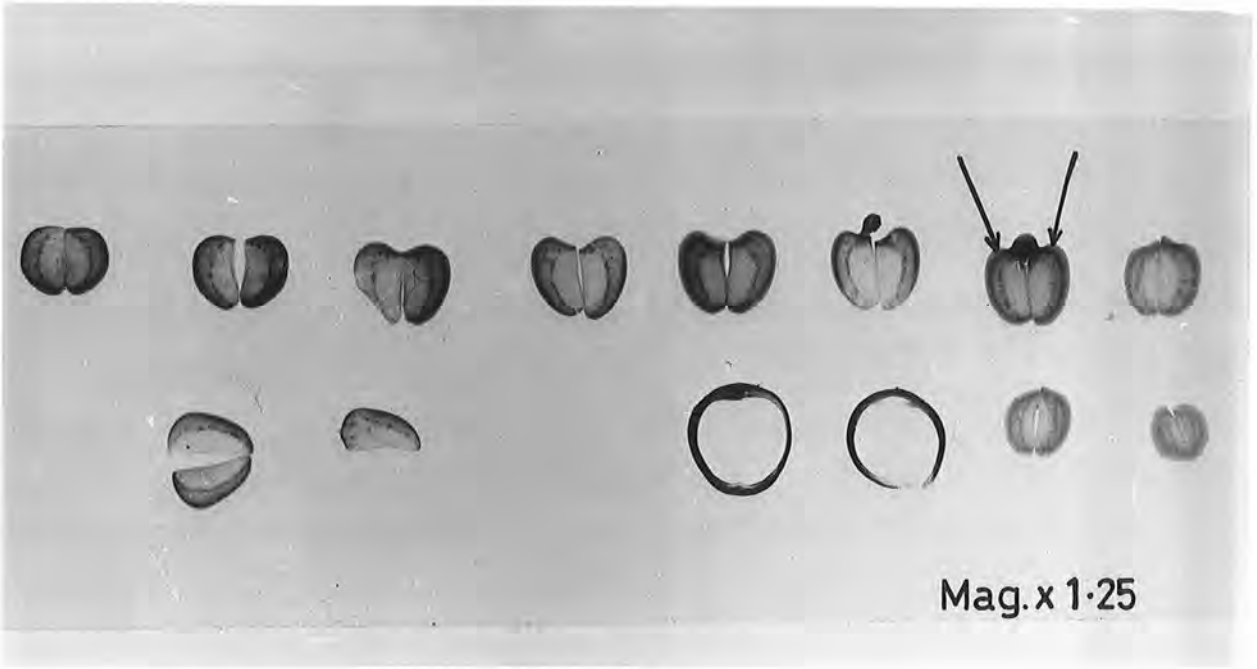


FIG. 52

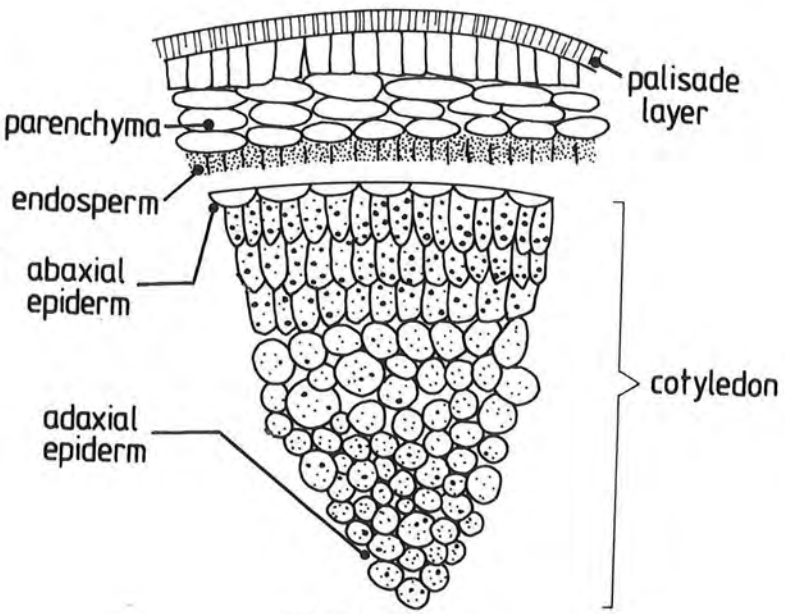


FIG. 53

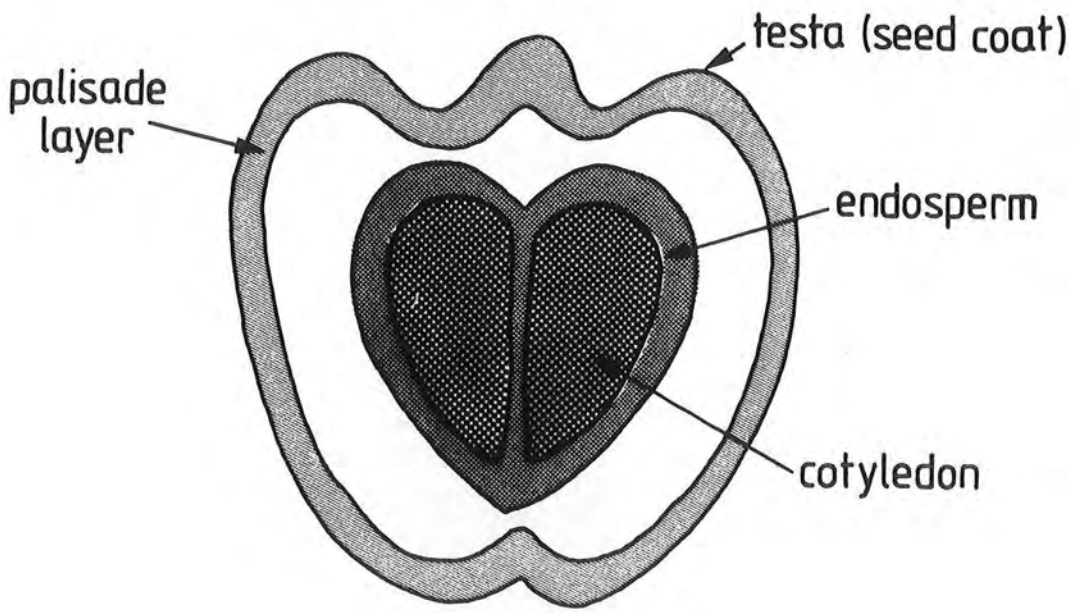


FIG. 54

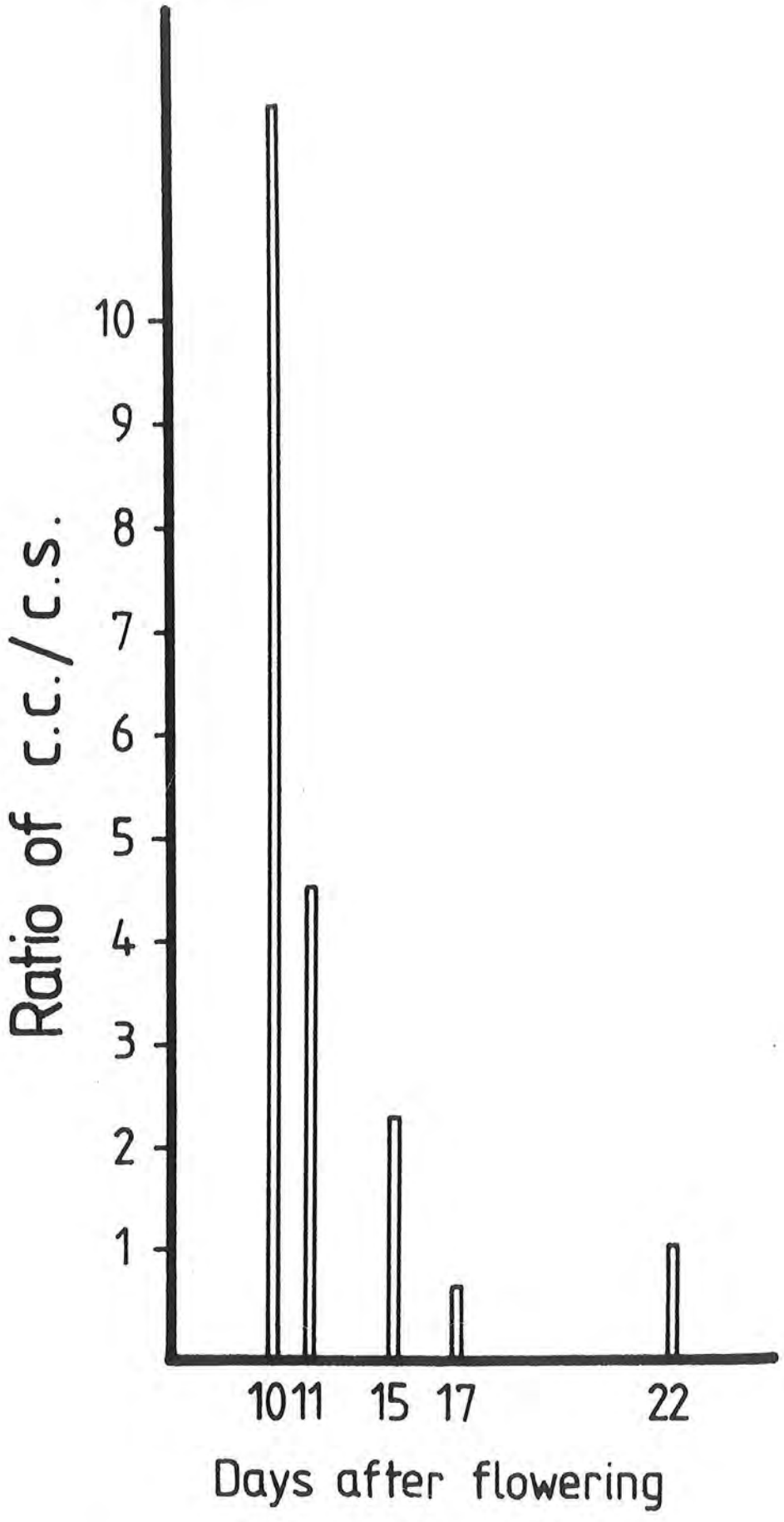

Ratio of c.c./c.s.
10
9
8
7
6
5
4
3
2
1
10 11 15 17 22
Days after flowering

TABLE A

	Abaxial I	Abaxial II	Abaxial II	Abaxial I
12	91 , 92 , 94 , 97 , 101 95 = $\bar{X}$ 95 ± 1·82	89 , 90 , 94 , 98 , 104 95 = $\bar{X}$ 95 ± 2·76	85 , 87 , 87 , 91 , 95 89 = $\bar{X}$ 89 ± 1·79	86 , 86 , 88 , 92 , 93 89 = $\bar{X}$ 89 ± 1·48
16	93 , 94 , 95 , 95 , 98 95 ± 0·84 $\bar{95} = \bar{X}$	91 , 94 , 94 , 96 , 100 $\bar{95} = \bar{X}$ 95 ± 1·48	85 , 88 , 90 , 92 , 95 95 = $\bar{X}$ 90 ± 1·7	86 , 88 , 89 , 90 , 92 89 = $\bar{X}$ 89 ± 1
20	88 , 90 , 93 , 95 , 99 93 = $\bar{X}$ 93 ± 1·92	84 , 90 , 91 , 95 , 95 91 = $\bar{X}$ 91 ± 2·02	89 , 91 , 93 , 95 , 97 93 = $\bar{X}$ 93 ± 1·41	78 , 80 , 84 , 87 , 91 84 = $\bar{X}$ 84 ± 2·35

TABLE 1B. Percentage cell of total volume at different regions of cotyledon ; (I) 3-5 cells from epidermis. (II) 6-10 cells from epidermis.

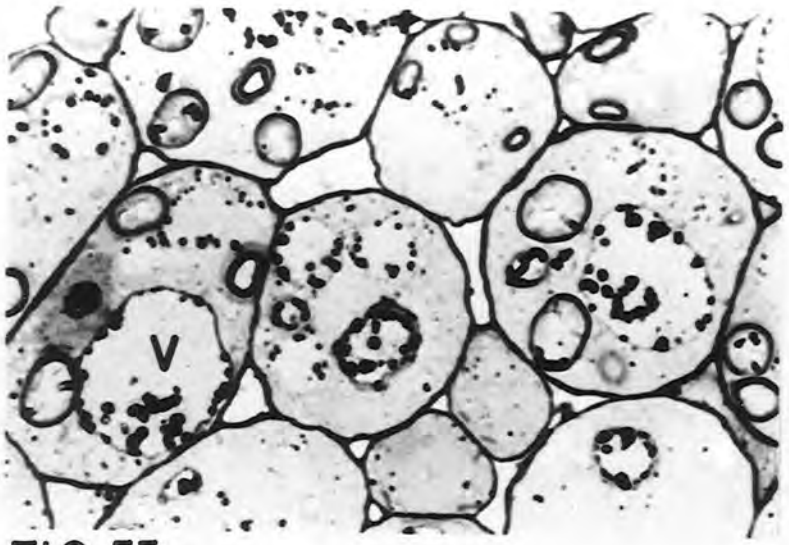


FIG.55

ALL 50μm

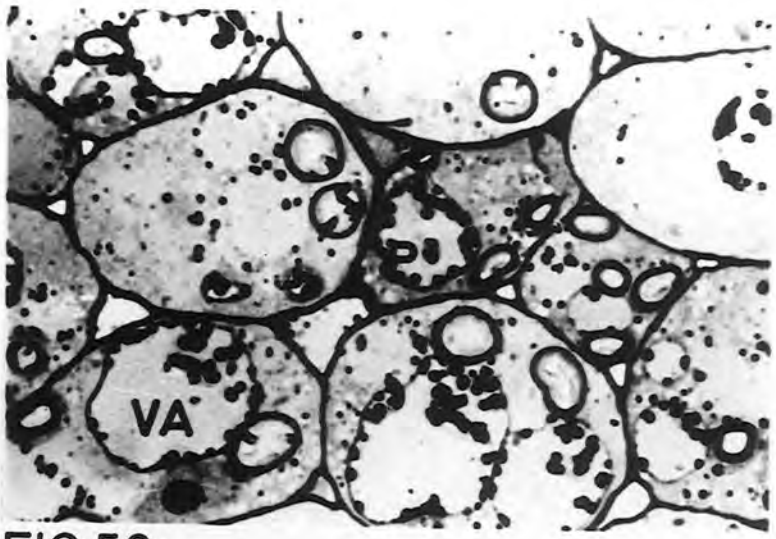


FIG.56

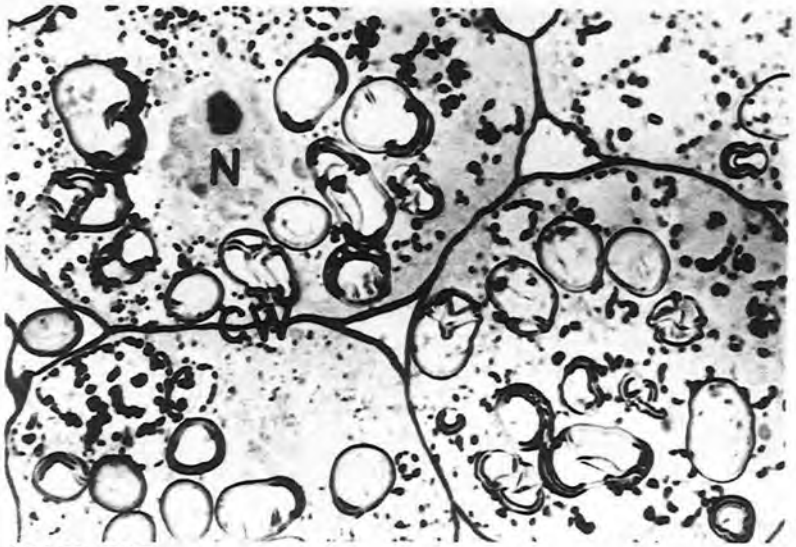


FIG.57

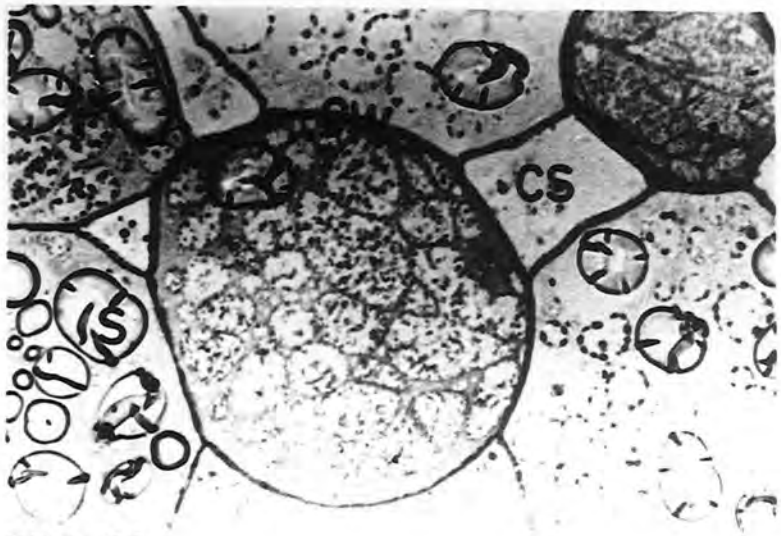


FIG.58

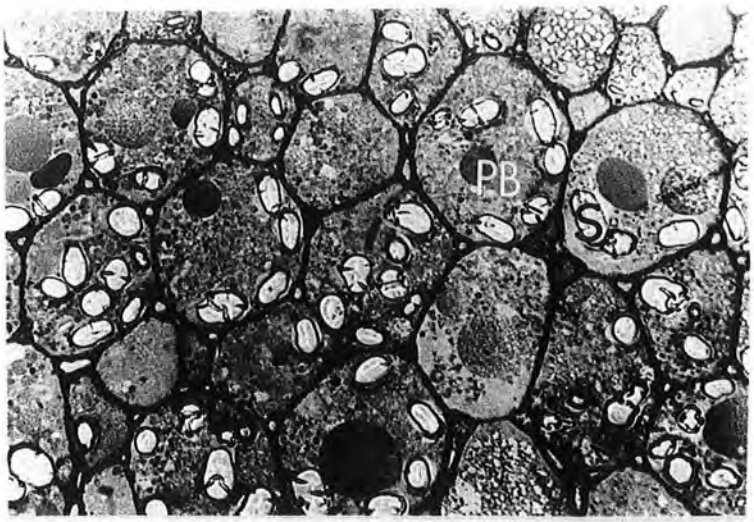


FIG.59

50μm

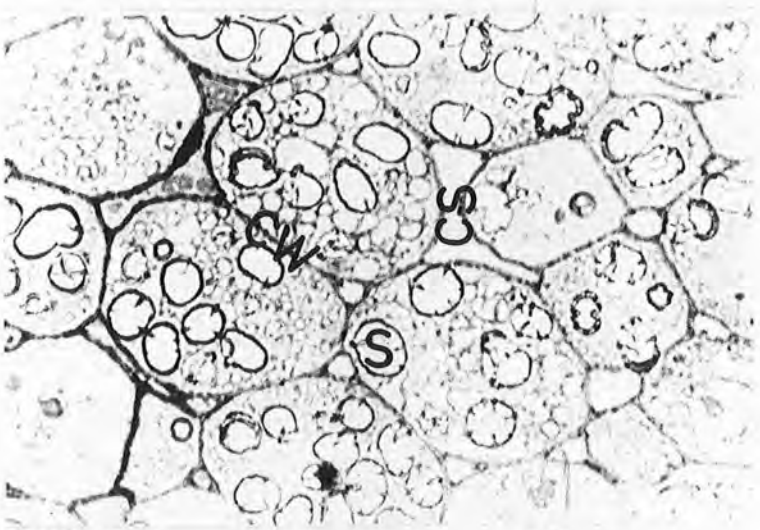


FIG.60

50μm

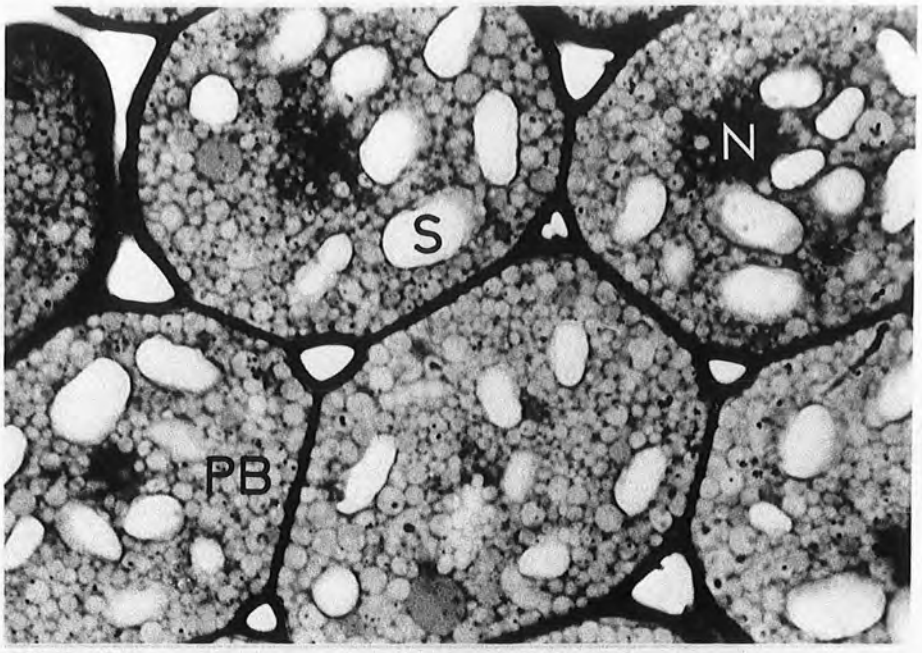


FIG.61

50μm

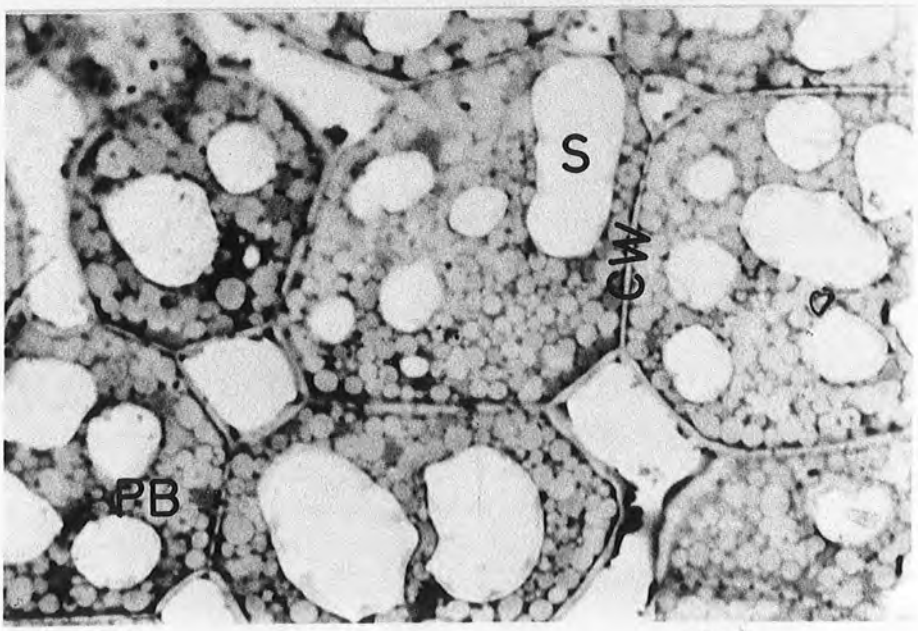


FIG.62

50μm

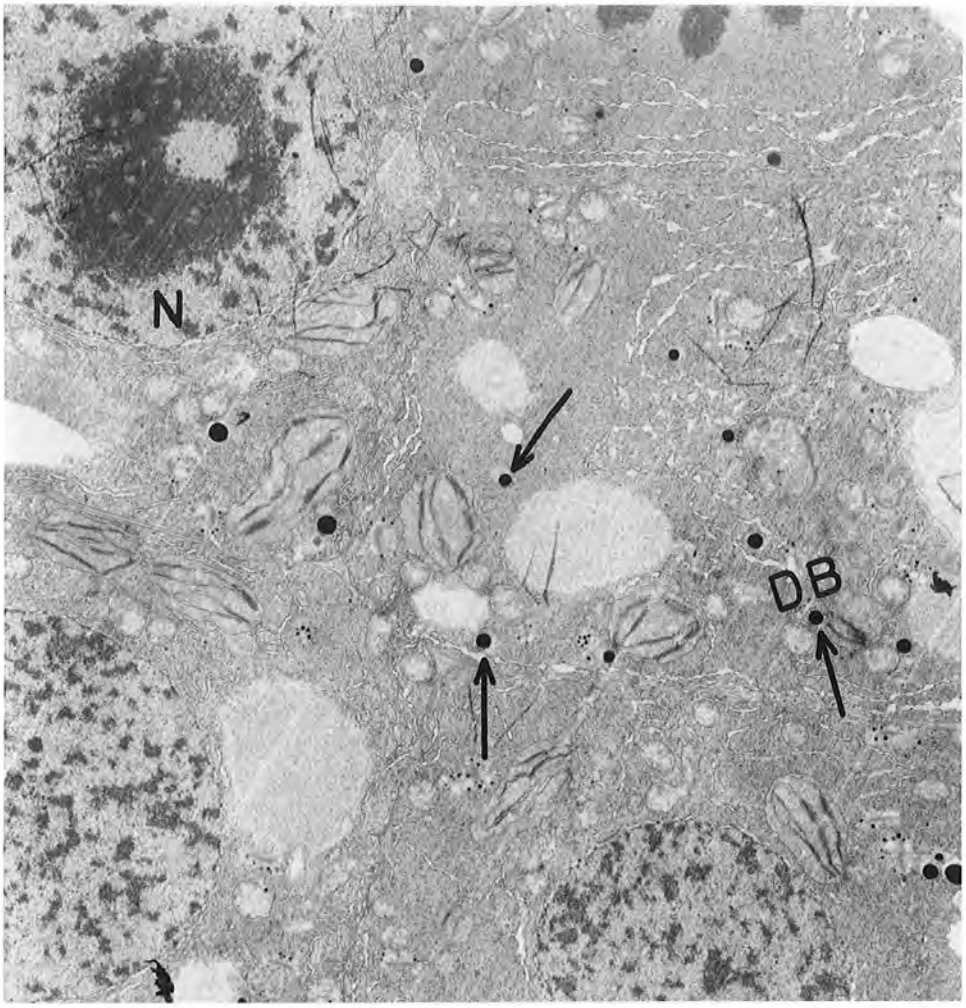
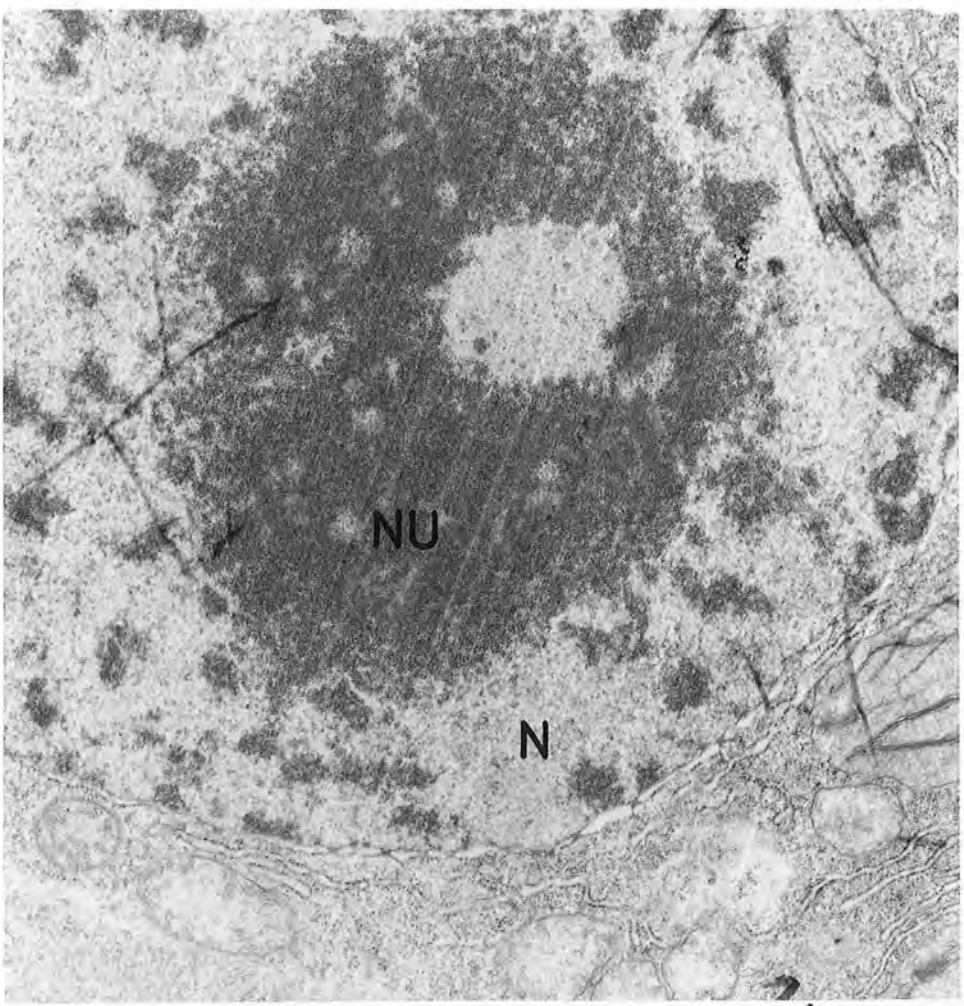


FIG. 63

2μm

FIG. 64



1μm

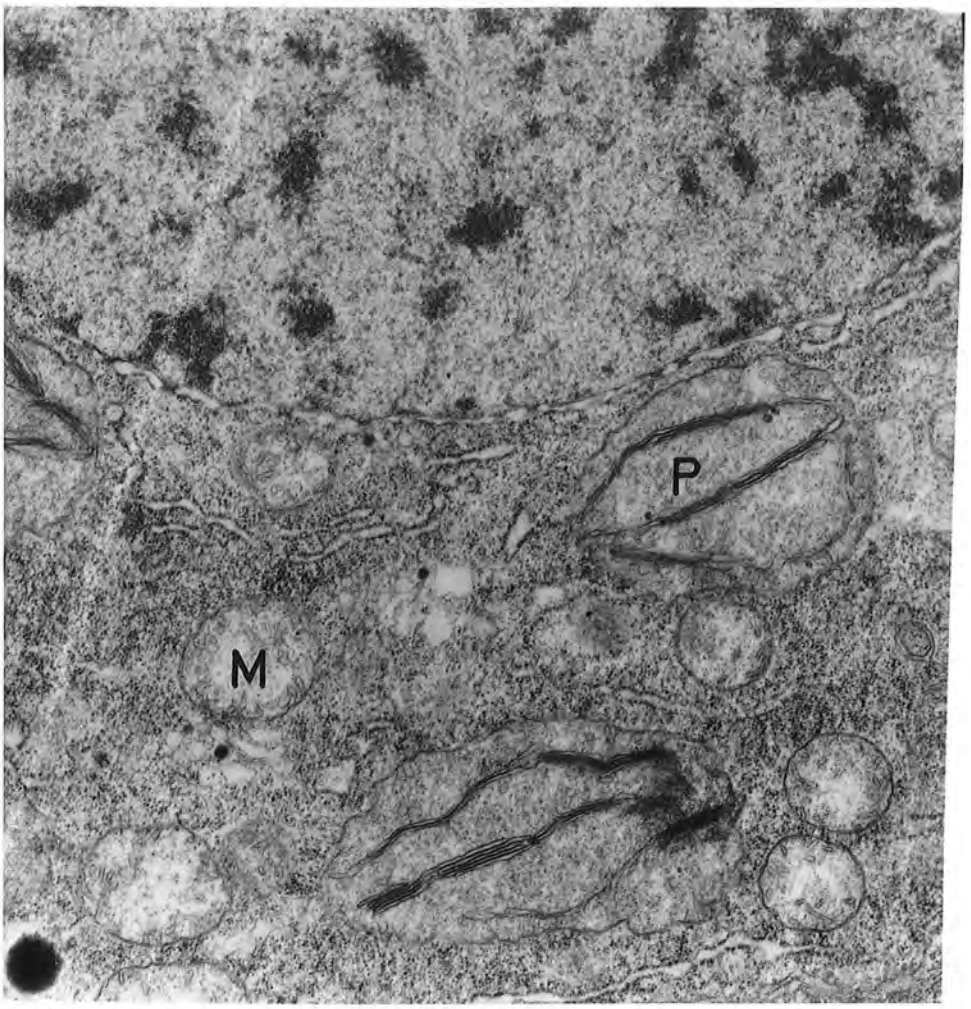
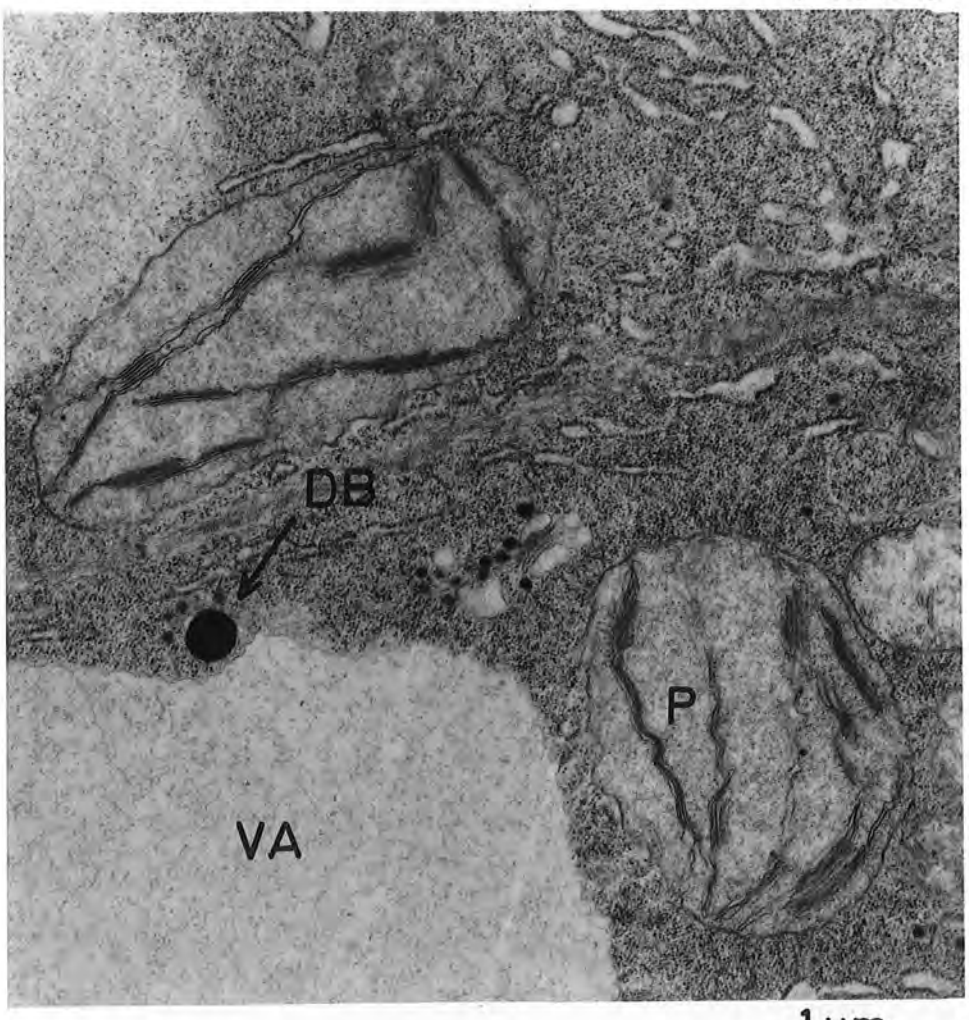


FIG.65

1μm

FIG.66



1μm

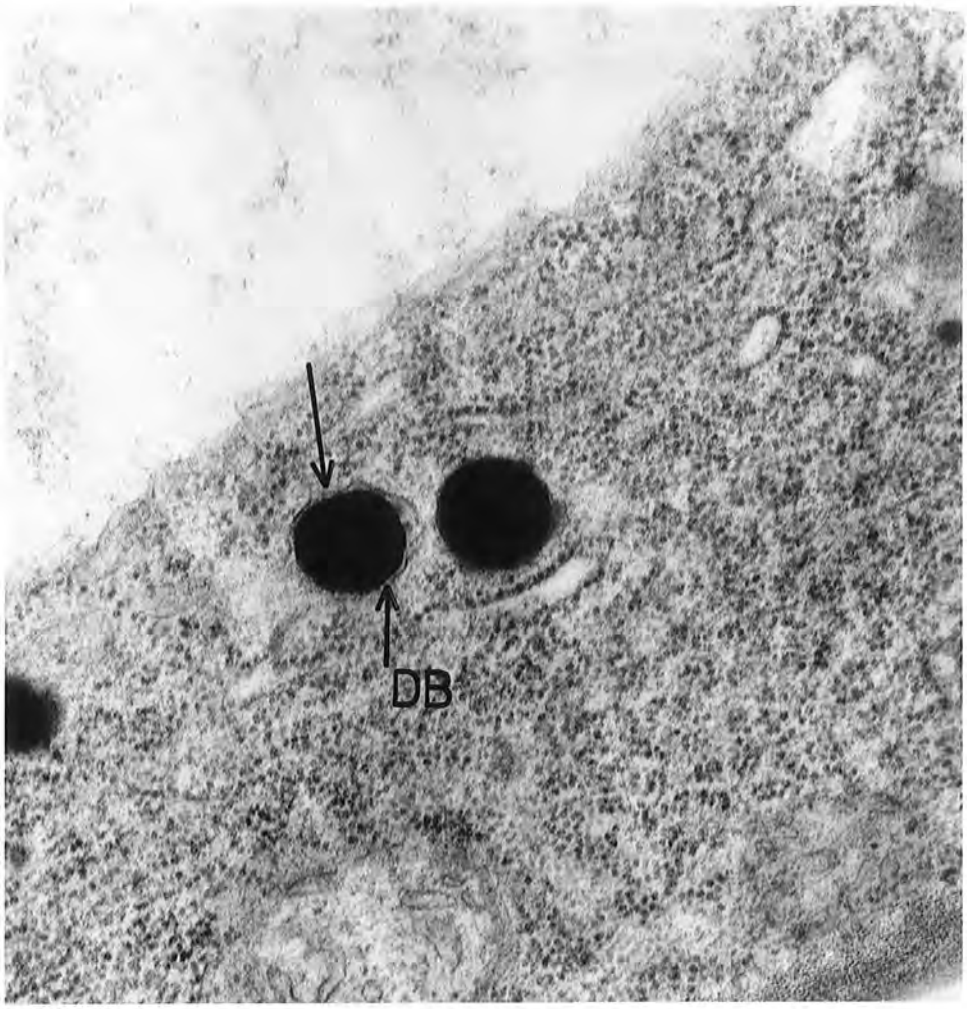
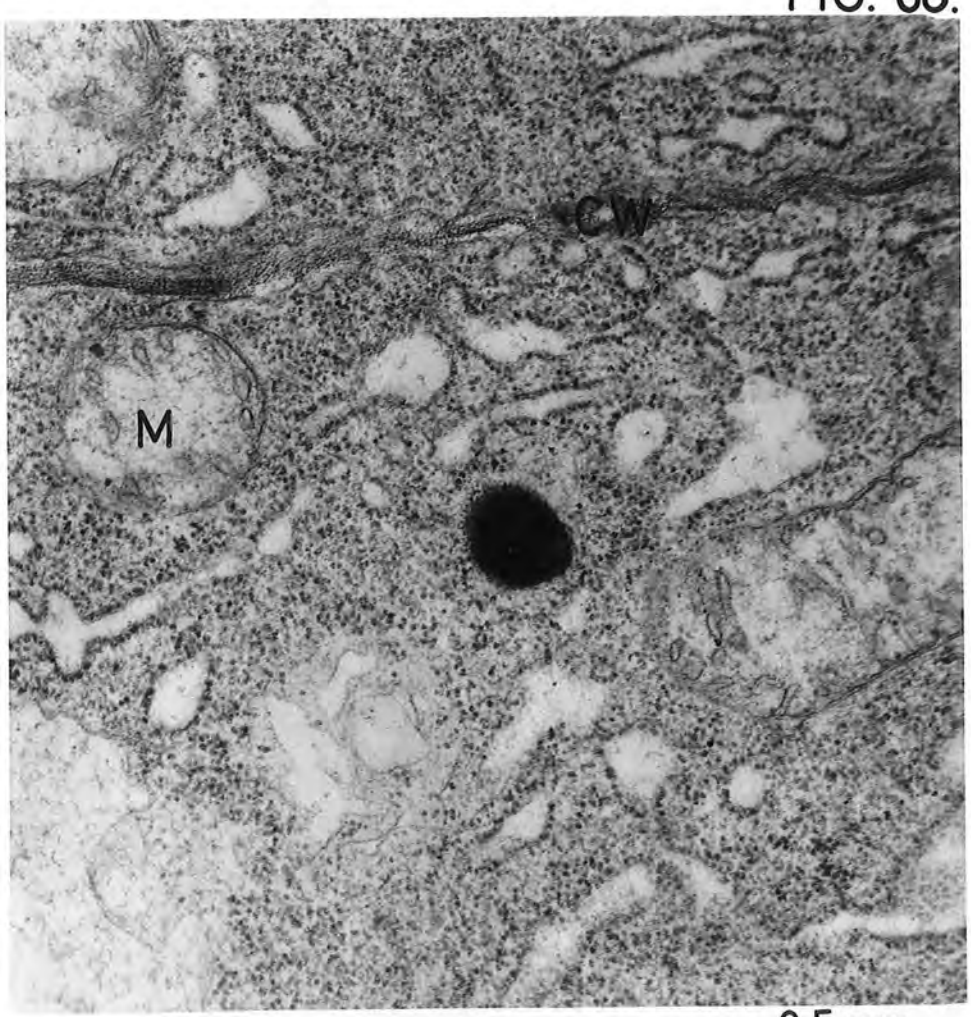


FIG. 67.

0·5 μm

FIG. 68.



0·5 μm

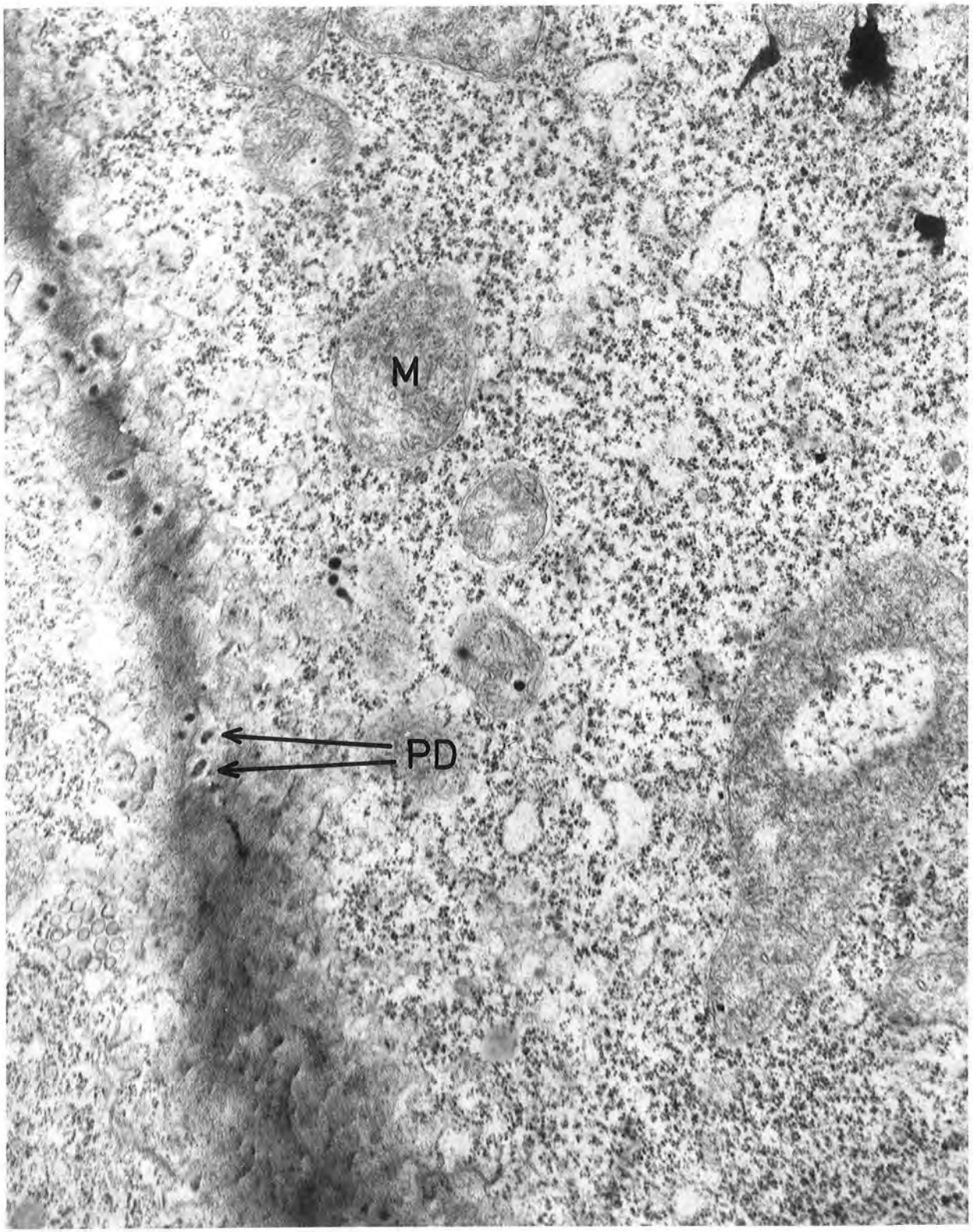


FIG. 69.

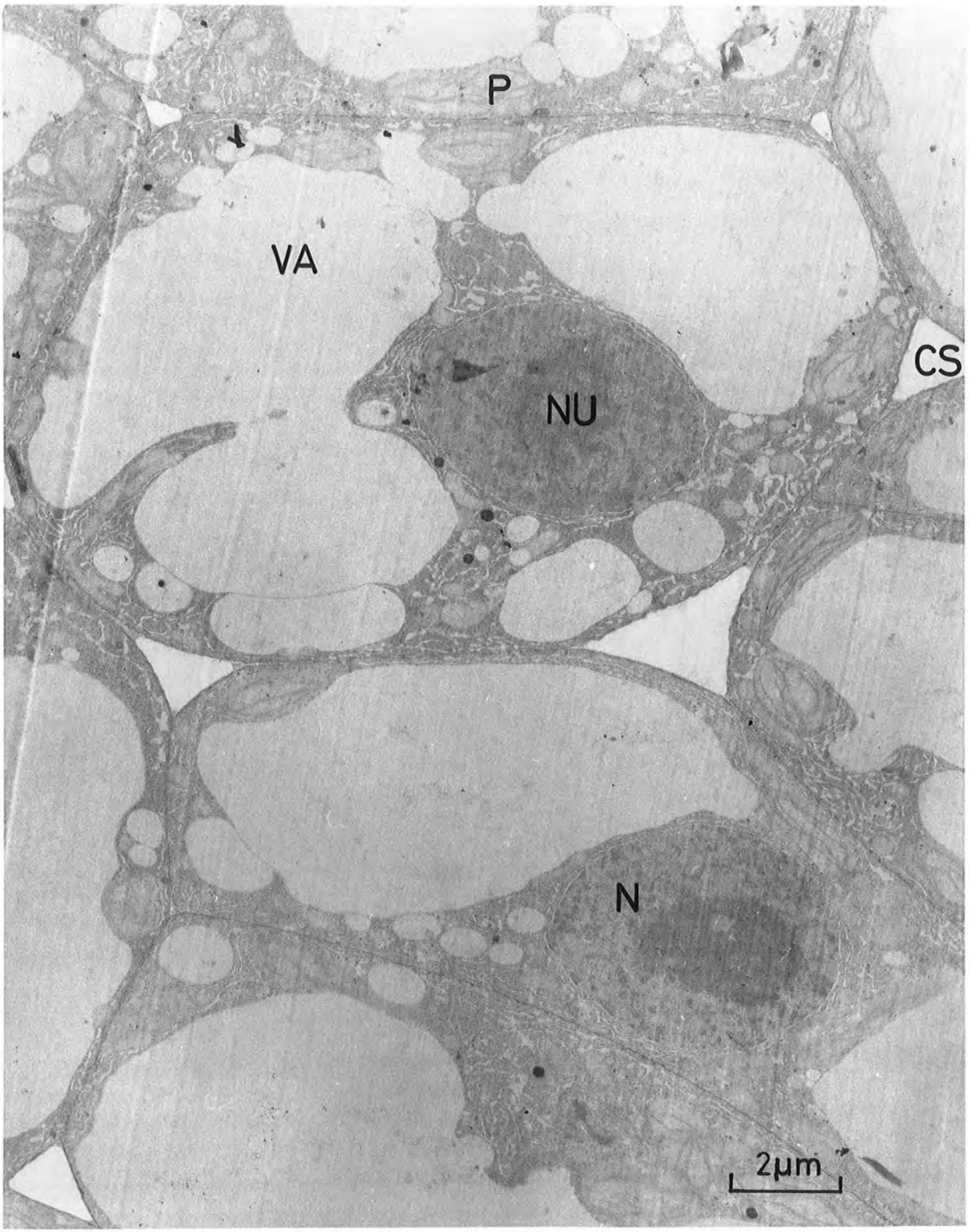


FIG. 70.

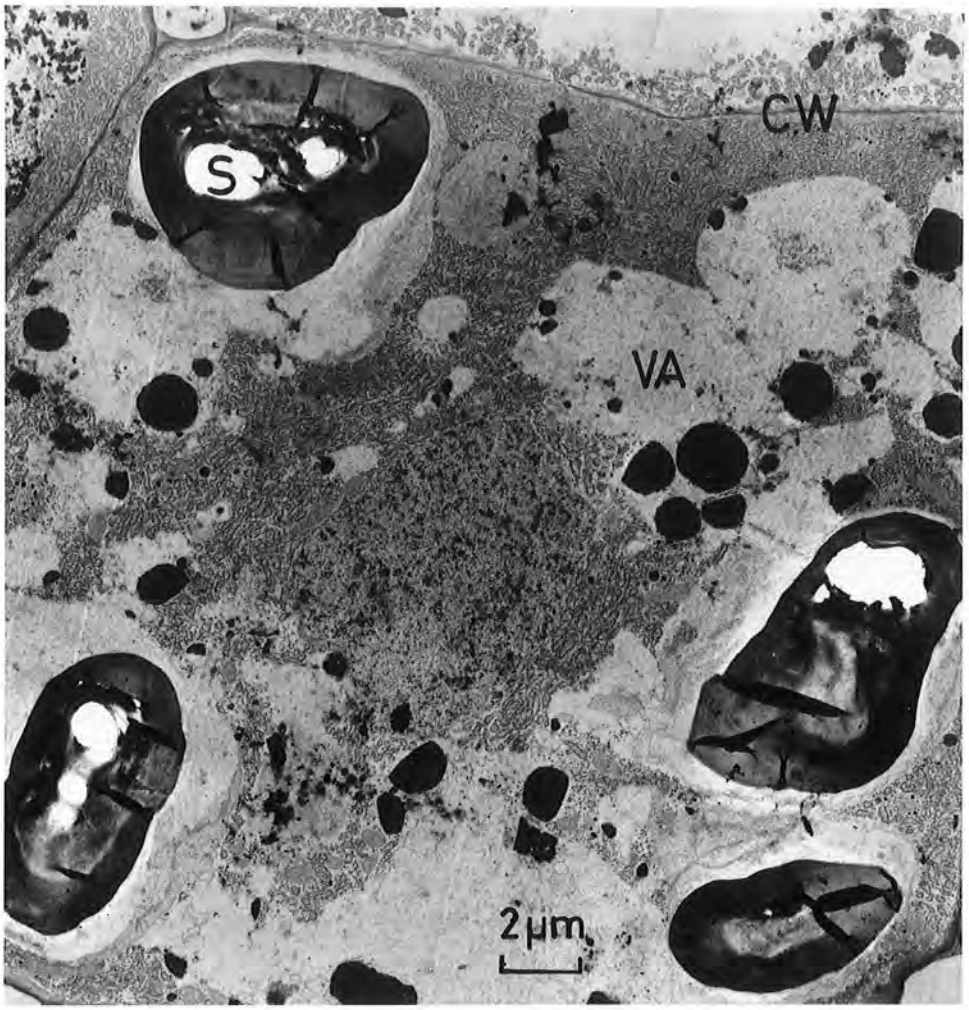
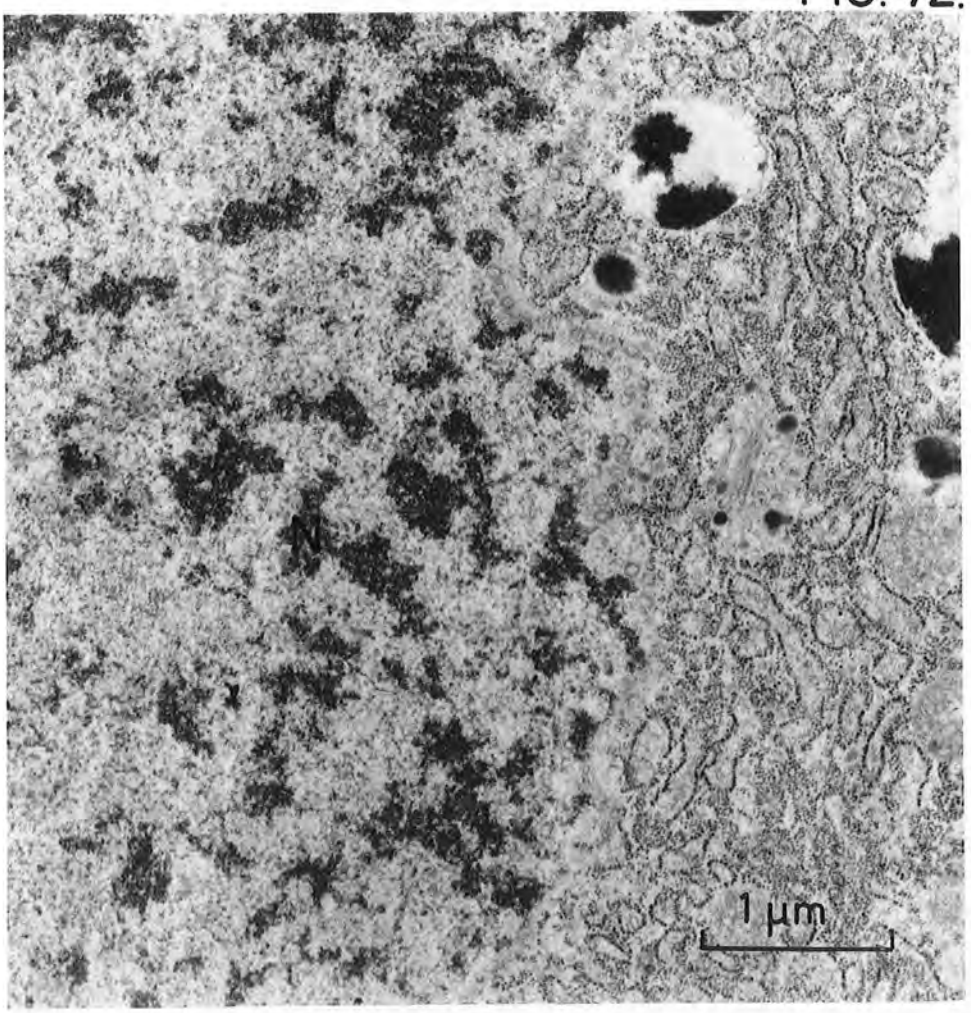


FIG. 71.

FIG. 72.



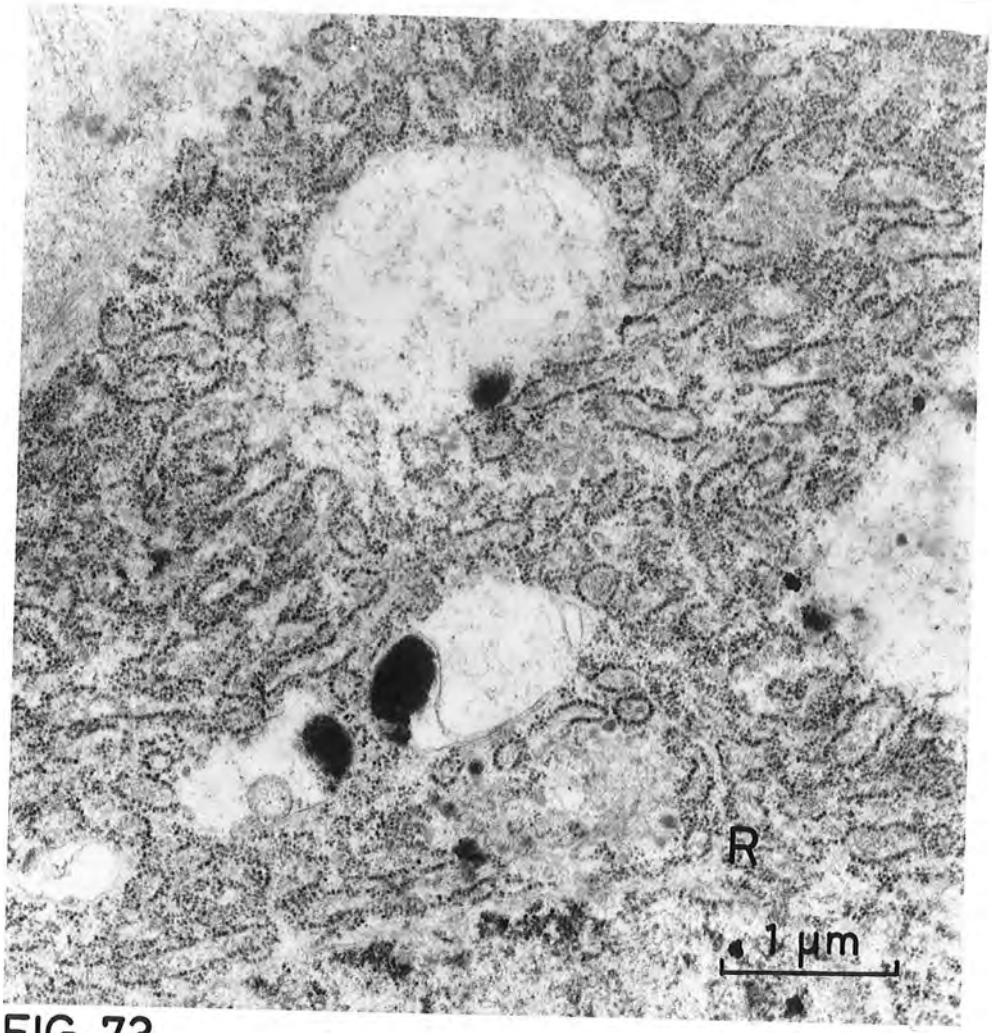
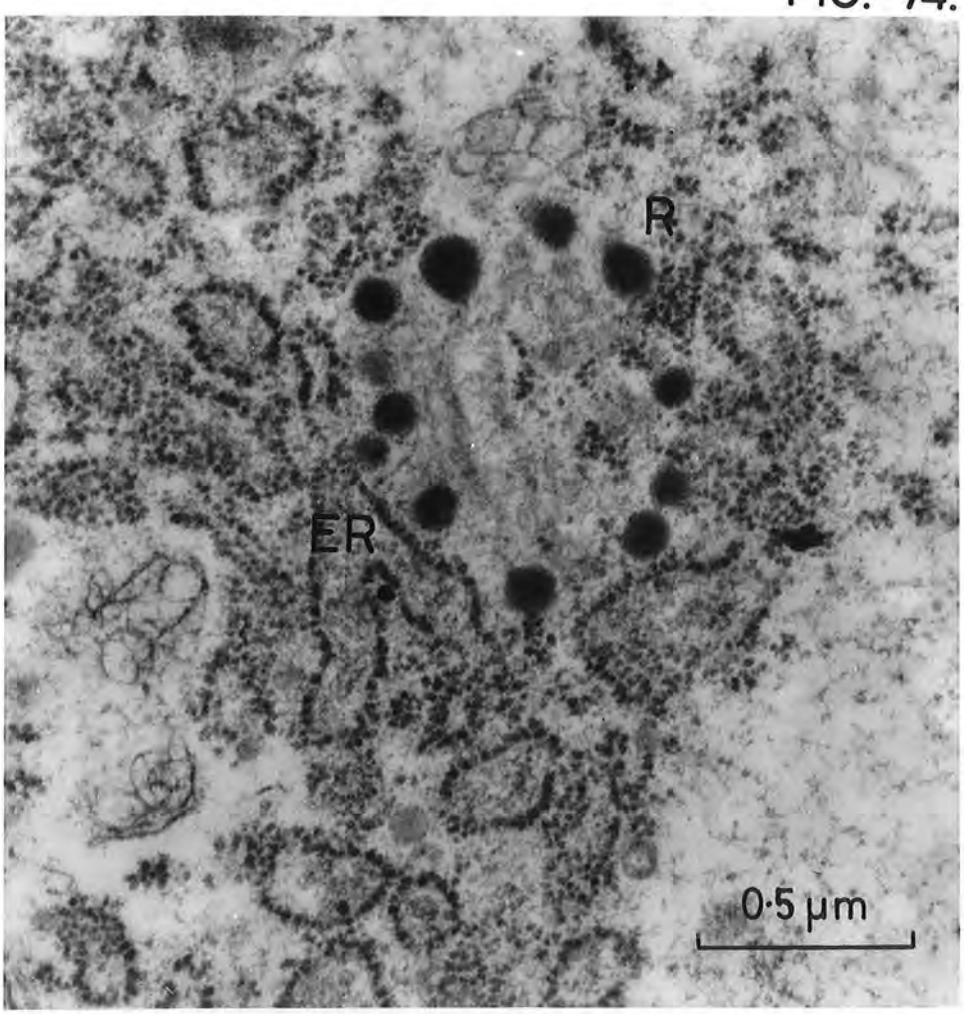


FIG. 73.

FIG. 74.



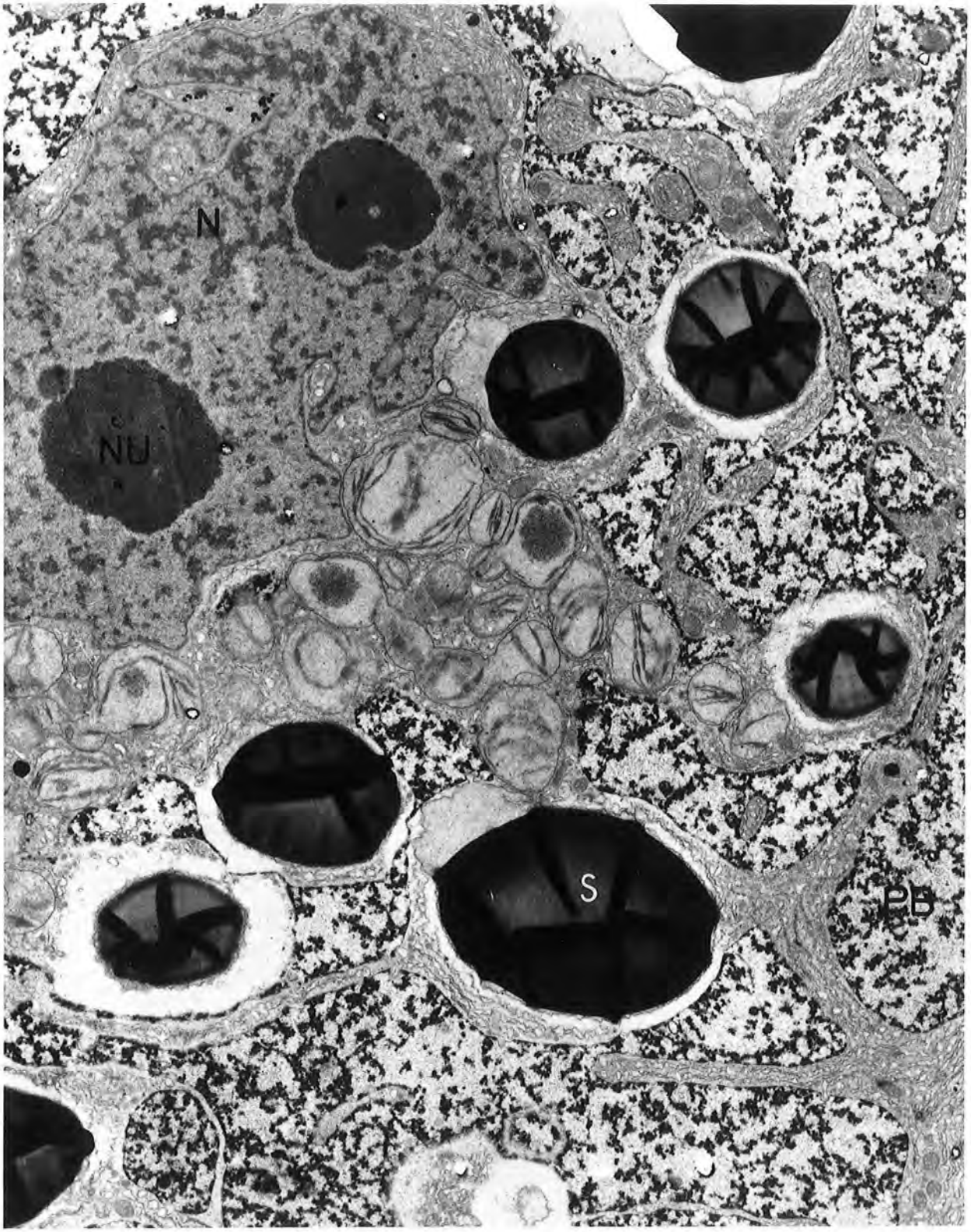


FIG. 75

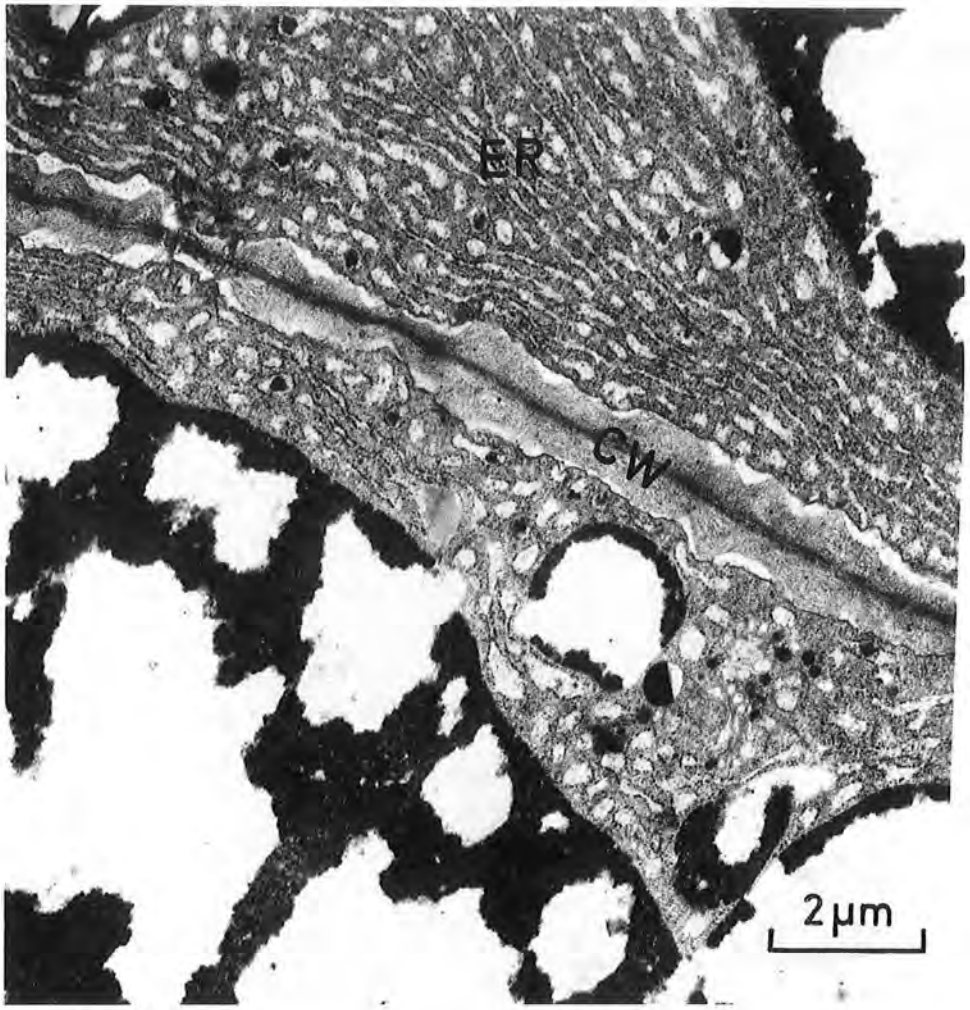
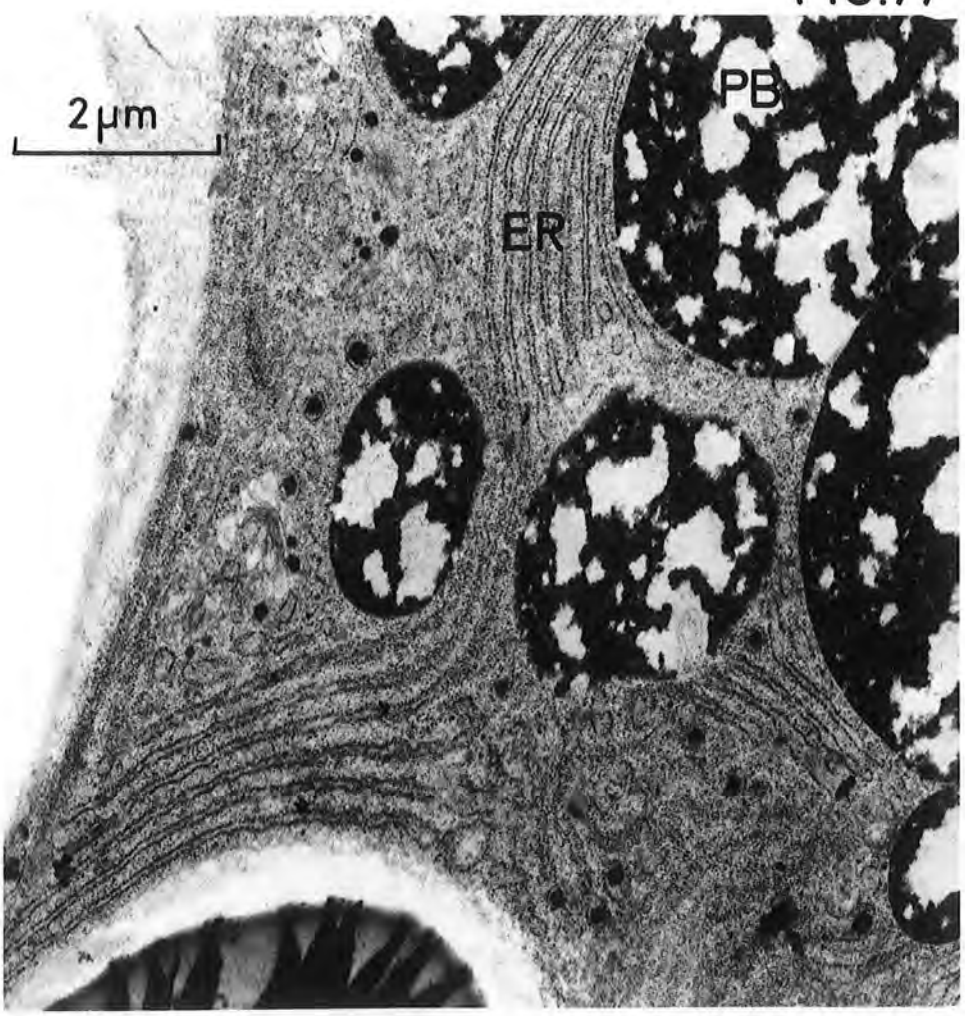


FIG.76

FIG.77



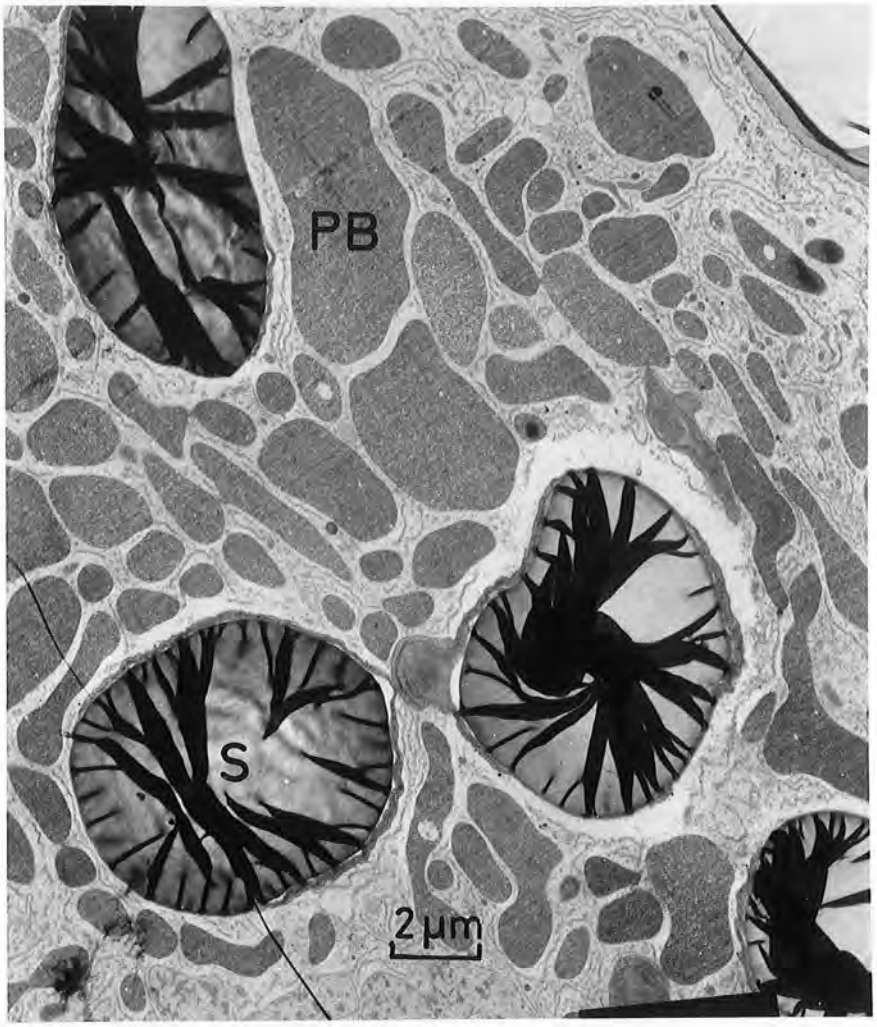
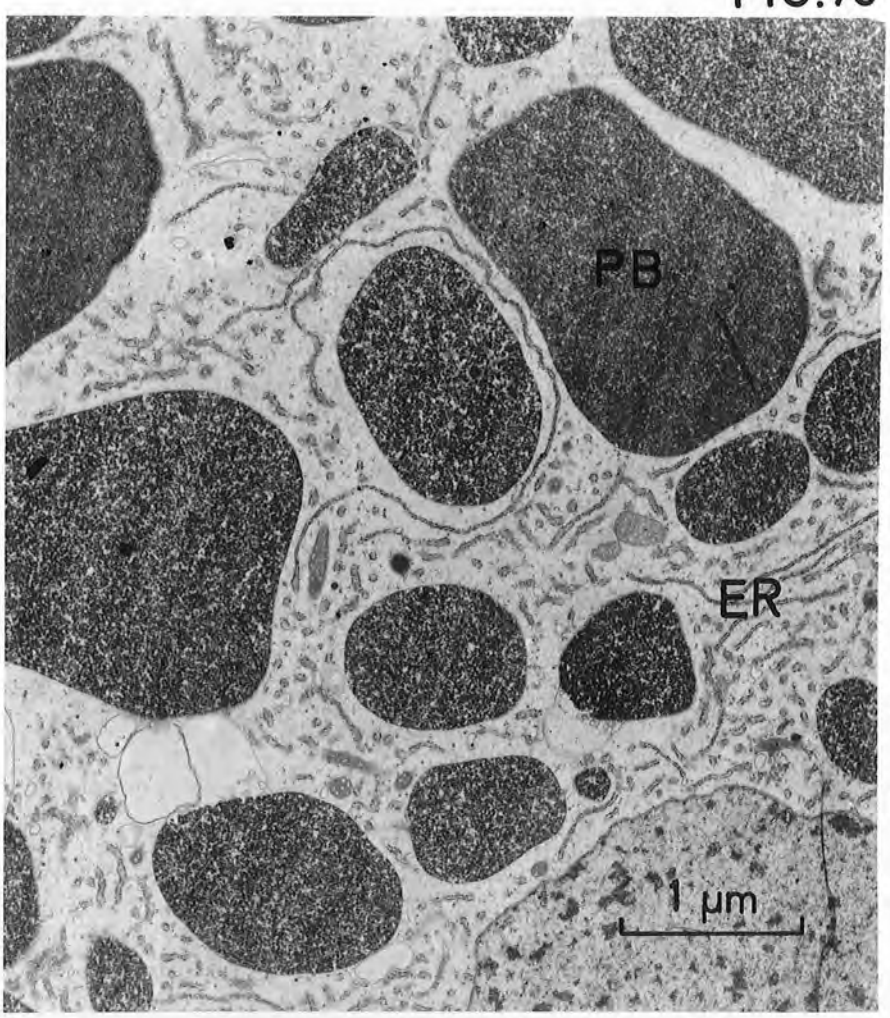


FIG. 78

FIG. 79



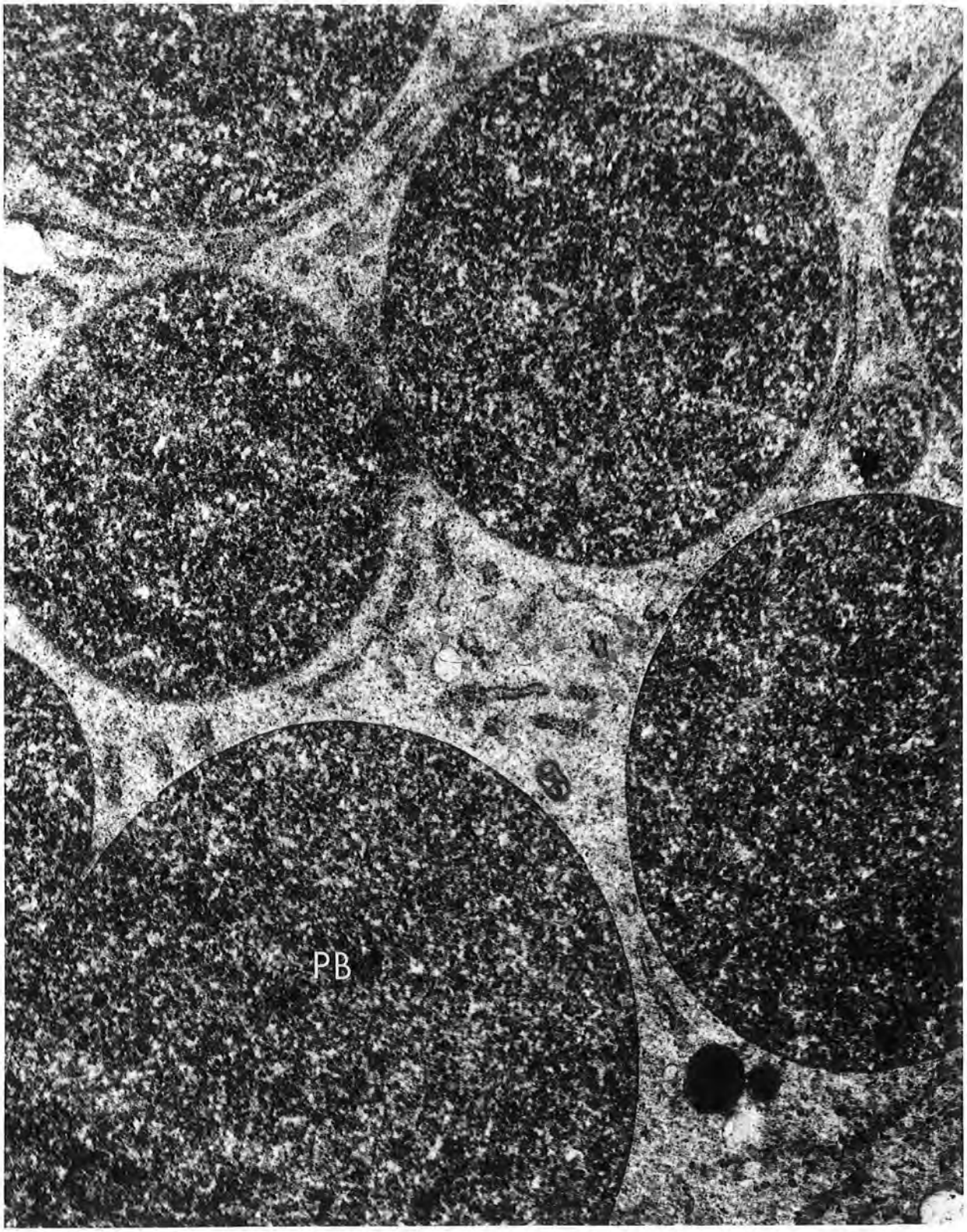


FIG. 80

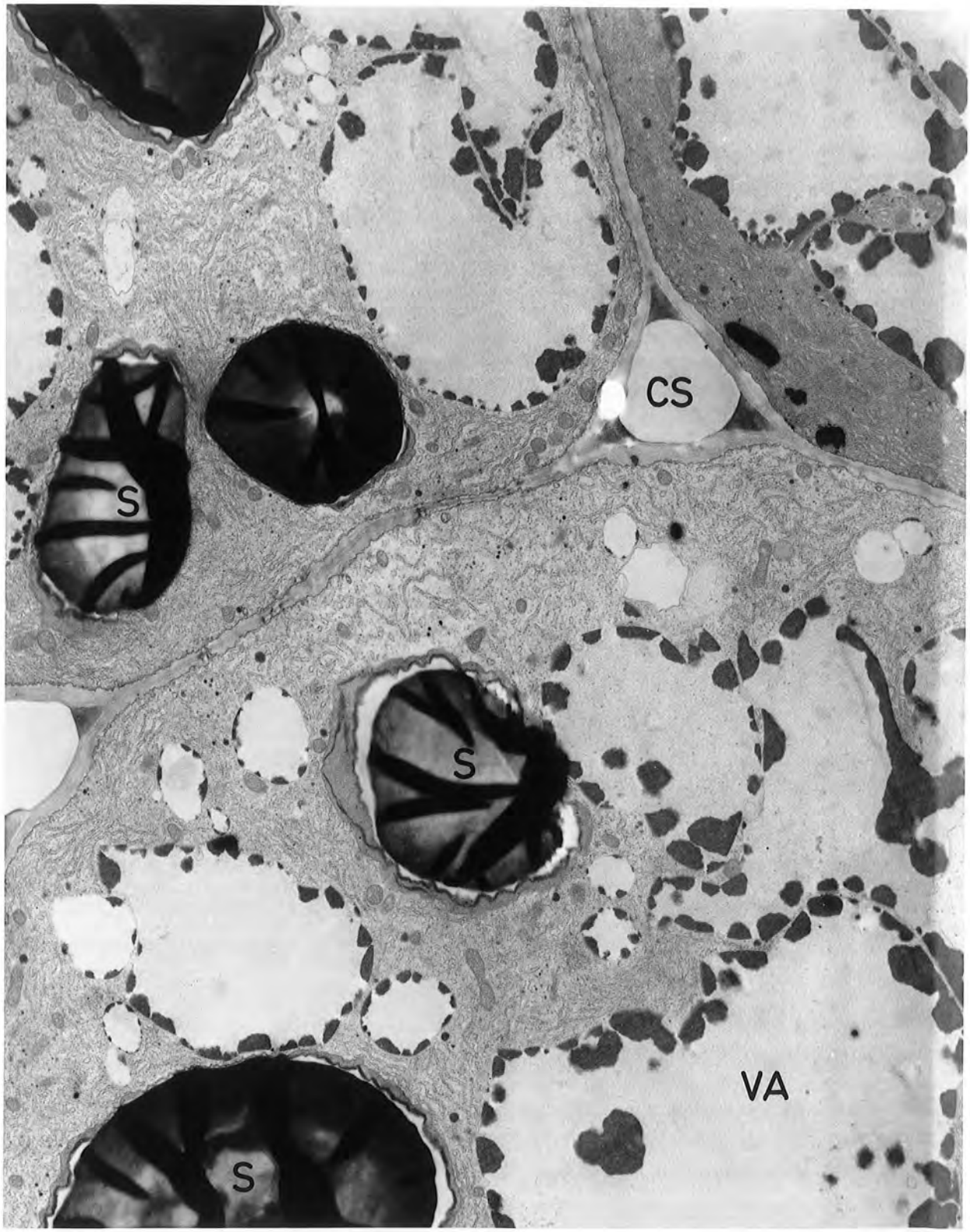


FIG. 81

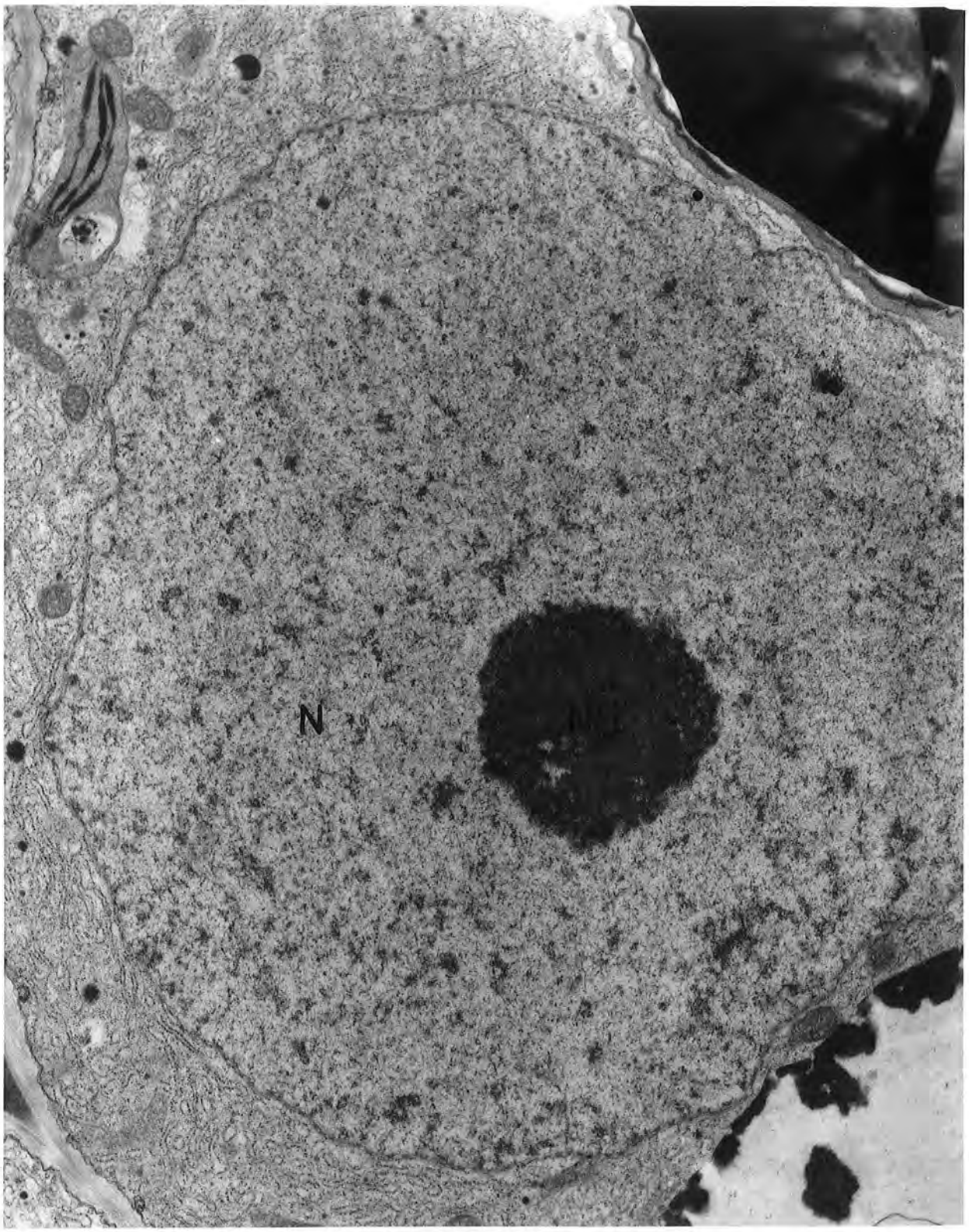


FIG. 82

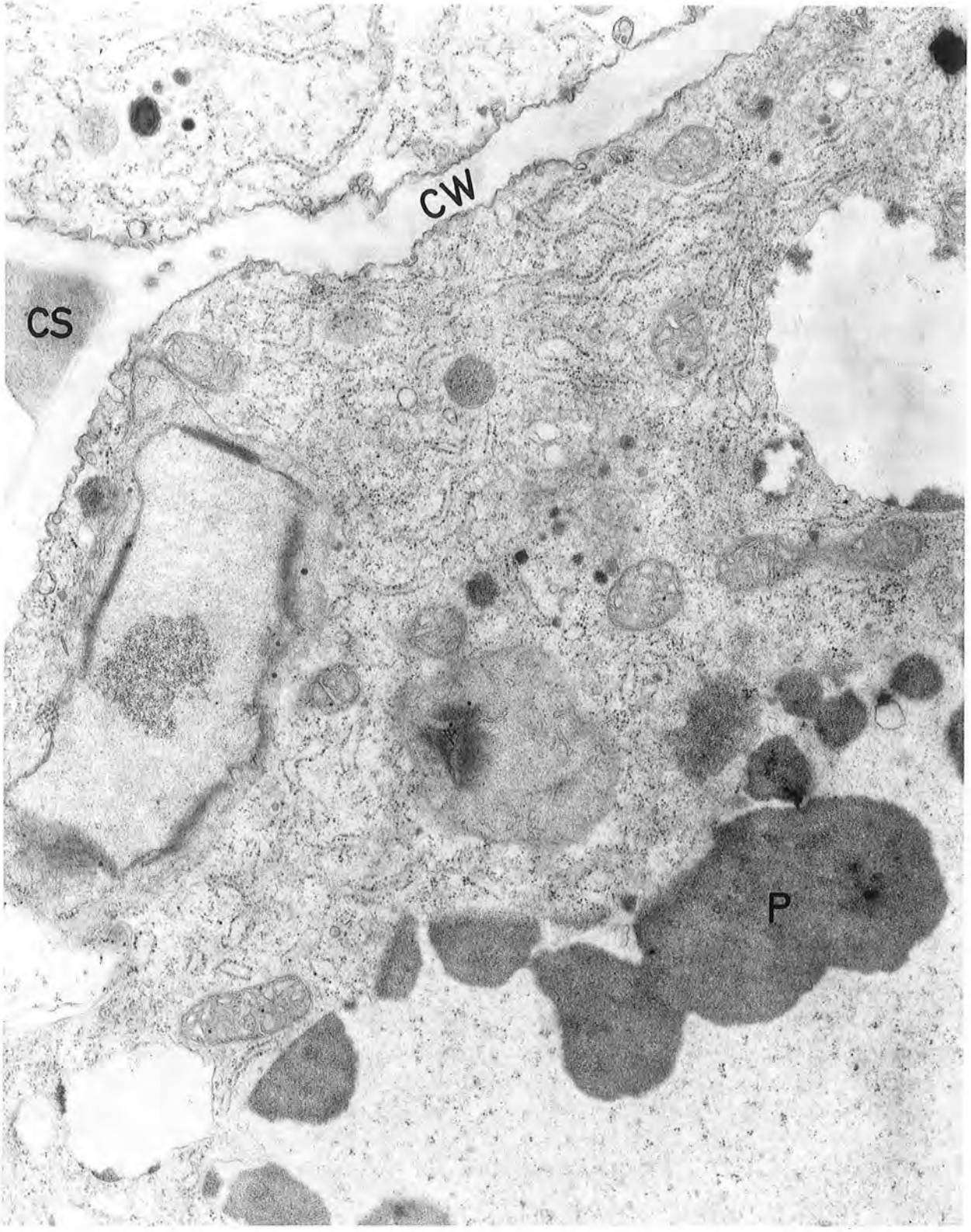


FIG. 83

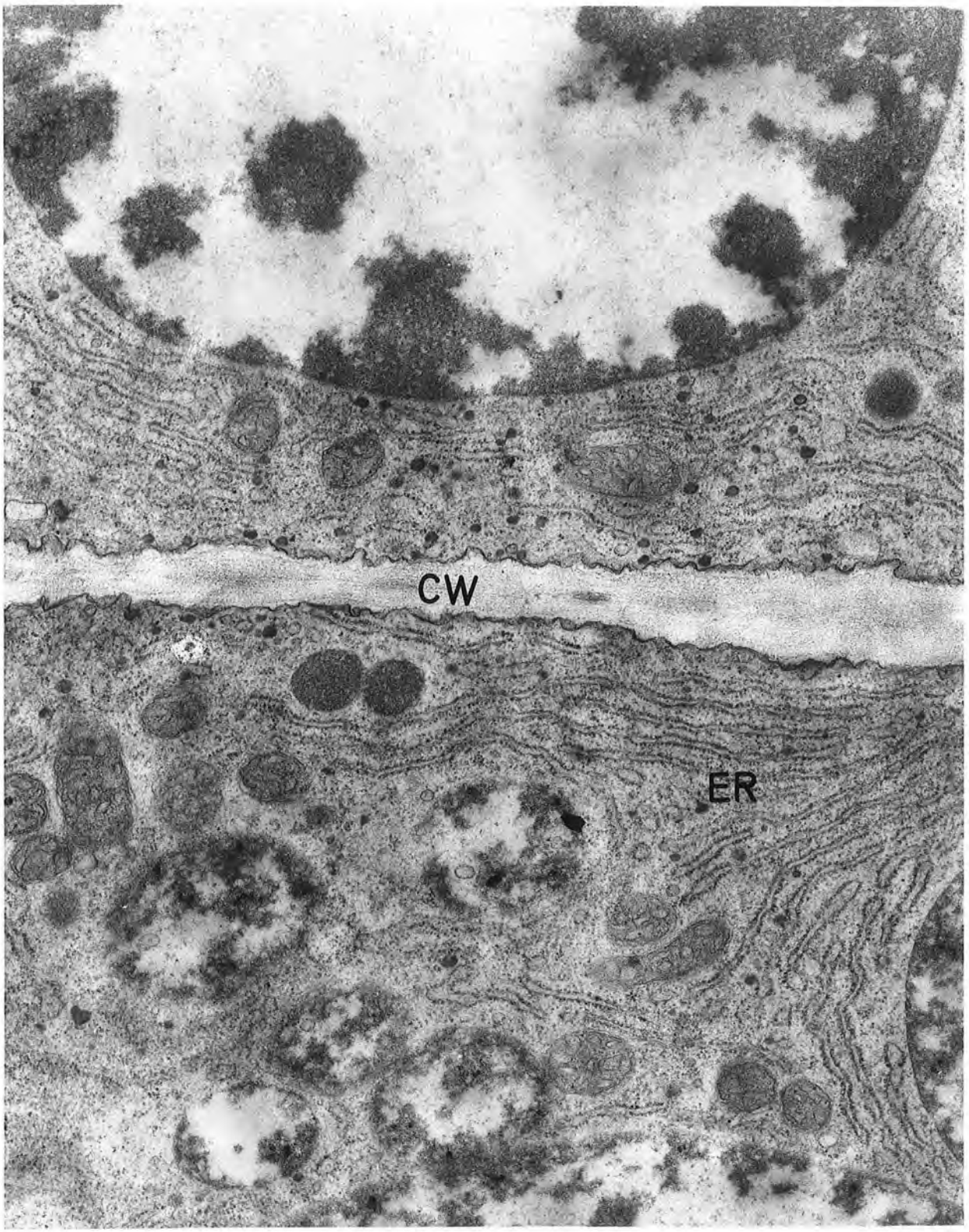


FIG. 84

1.6 Movement of fluorescent tracers into developing tissues.

The fluorescent tracers ANS and fluorescein were used to examine the possible routes, both through the apoplast and symplast, that might be available for the movement of nutrients and metabolites in the developing pea seed.

Seeds at different stages of development were isolated with a small portion of pod wall tissue which was placed in either 0.1% fluorescein or 0.01% ANS (Gates & Oparka, (1982)). Care was taken to prevent contact between the seed coat, including funicle, hilium, and tracer solution. After periods up to 4.5 hours the seeds were removed from tracer solution and after sectioning the tissues were examined using a Nikon Diaphot fluorescent microscope with TMD-ER fluorescent attachment lamp.

The uptake of the apoplast tracer ANS for 1.5 hrs by a 6 DAF seed resulted in movement through the xylem of the funicle (Fig.85) and up the main seed coat vascular bundle (Fig. 86) Fluorescence was found at the embryo sac boundary wall at the top region of the sac but there was no ANS fluorescence at the radicle pocket region (Fig. 87), although chloroplast autofluorescence is evident.
Counterstaining with the fluorescent cellulosic-binding calcofluor indicates the distribution of tissues around the radicle pocket (Fig. 88).

Occasional specimens were loaded with the funicle in direct contact with the fluorochrome. In these cases the funicle parenchyma accumulated considerable quantities of fluorochromes but these were not passed through the columnar epithelial layers of hilium; fluorochromes only moved via the main vascular bundle connection.

After 2.5 hr., continued uptake of ANS had resulted in an

increased accumulation of fluorochrome in the region close to the top of the embryo cavity, within the loose parenchyma layers lining the embryo sac boundary wall. Fluorescence is most intense in the region adjacent to the developing embryo (Fig.89). Figure 90 is a calcofluor counterstained image of Fig. 89 to indicate distribution of tissues. Comparison of Fig. 89 with the flourescent micrograph resulting from uptake of the symplastic tracer fluorescein for a similar period (Fig. 91) indicates movement of tracer in phloem, xylem and vascular parenchyma and in the parenchyma adjacent to the embryo sac boundary wall. There is no spread of fluorescence through the seed coat to the radicle pocket with either fluorochrome, even after 4 hrs loading of young seeds. (Fig. 92 - 6 DAF seed, ANS), although with more developed seeds (10 - 15 DAF) the fluorochromes did accumulate at the radicle pocket lining after 6 hrs incubation.

Both ANS and fluorescein were found to pass to the embryo cotyledons after 2.5 - 4 hrs (Fig. 93: 8 DAF, ANS) although in both cases the distribution of fluorescence was rather 'hazy' indicating an apoplastic rather than symplastic movement within the cotyledons. The calcofluor counterstaining of the cotyledon of Fig. 93 indicates the stages of early differentiation of a vascular bundle (Fig. 94).

The results show that both apoplastic and symplastic routes are available for transport of nutrient from the pod to the seed coat with accumulation at the loose parenchyma layer lining the embryo sac boundary wall only in the region of the embryo. Unloading of the symplastic and apoplastic transport to the developing embryo is also indicated.

Figs.85,86: Light micrographs showing the uptake of ANS in seed at 6 DAF.

Figs.87,88: Light micrographs showing ANS movement in seed at 6 DAF.

Figs.89,90: Light micrographs illustrating that ANS fluorescence is most intense in the region adjacent to the developing embryo.

Fig.91: Light micrograph showing the movement of tracers in the phloem, xylem and vascular bundles.

Fig.92: Light micrograph illustrating ANS in a 6 DAF seed.

Fig.93: Movement of ANS from seed coat to cotyledon epidermis (CE)

Fig.94: Calcofluor counterstaining of cotyledon tissue showing differentiating vascular bundle. No uptake of either flourescein or ANS was found in these bundles.

2.0 Seed development in Vigna unguiculata (cowpea).

The results above indicate a fairly uniform rate of deposition of storage reserves in the cotyledon parenchyma tissues of developing pea. It has been shown that the mobilisation of reserves during seed germination follows different patterns in different legumes. In pea there is a general mobilisation throughout the cotyledons with a progressive wave in high catablic activity, whereas in cowpea the pattern and development of both protein and starch mobilisation is closely related to the distribution of vascular tissues in the cotyledon leaves. To determine whether such a variation in pattern might occur during seed development, and be related to the mobilisation patterns of seed germination, a range of legumes has been examined. This section of the results deals with the development of cowpea seeds and in particular the deposition of protein and starch reserves in the storage parenchyma tissues.

2.1 Scanning electron and light microscopy of cowpea pod and seed coat.

The distribution of tissues in pod and seed coat of a fruit, just prior to the stage of storage deposition in the seed, is shown in Fig. 95. The pod epidermal layer encloses a band of large, layered chlorenchyma cells which contain many plastids. The inner parenchyma layers are of small cells without any obvious layering. The parenchyma tissues of the pod are separated by a central layer of large, thickened, structurally supportive sclerenchyma cells. The vascular strand is seen passing through the funicle to the seed coat. In marked contrast to the pea results above, the inner layer of the seed coat lining the embryo sac is composed of large columnar cells which form a smooth embryo sac boundary wall. Detail of the seed

coat is shown in Fig. 96 which also shows the development of the radicle pocket with an inner projecting wall much thinner than the boundary layer seed coat cells. The long cells attached to the embryo sac boundary wall are shown in Fig. 97.

A 1μm toluidine-stained section of seed coat at 6 - 8 DAF is shown in Fig. 98. The section shows the young seed coat attached to the funicle. There are two large vascular bundles running through the seed coat, serial sections showed that these had branched from a single vascular strand passing through the seed coat/funicle junction (Fig. 98). The columnar epithelium lining the embryo sac is clearly illustrated in Fig. 98 with a layer of looser parenchyma at the base of the columnar epithelium. The packing of the loose cells is shown in Fig. 99 and also in a horizontal section through the seed coat near its apex (Fig. 100).

2.2 Light microscopy of cotyledon parenchyma development.

The development and changes in the cotyledon cells of cowpea at a cytological level were examined by study of 1μm sections of fixed, resin embedded samples stained with toluidine blue. Particular attention was paid to changes in cell vacuoles and the rate and distribution of storage protein in parenchyma cells. The cotyledon parenchyma structure was studied between 10 and 30 DAF during which period storage deposition of both starch and protein occured.

Figures 101 and 102 show cotyledon cells at 15 DAF. The cells of the cotyledon were highly vacuolate with very thin cell walls. The cytoplasm is present as a thin layer around the cell with a few transcellular strands. The cells' nuclei are seen either at the cell centre supported by transcellular cytoplasmic strands, or lying close

to the cell wall. In some cells the nucleolus is particularly conspicuous, often with a central clear 'vacuolar' region.

The cell dimensions were measured on light micrographs and changes in cotyledon cell diameter quantified from 10 to 30 DAF. At 10 DAF the cotyledon parenchyma cells were 50 μm in diameter and increased in size to 200μm by 30 DAF.

At 15 DAF the cotyledon parenchyma cells were characterised by the presence of one to five large vacuoles (Figs. 101,102). There was no evidence in light micrographs of protein deposition at the tonoplast or in the cytoplasm. The cotyledons of 15 DAF seeds were examined both close to the abaxial epidermis (Fig. 101) and close to the adaxial epidermis (Fig. 102). At this time the cells are at a stage of expansion (phase 2). Comparison of Figs.101 and 102 shows no size differences between the cells of the two parts of the cotyledon. The vascular bundles in cowpea cotyledons are distributed throughout the leaf, not as an arc as in pea cotyledons. The vascular bundle in Fig. 102 shows division of the vascular parenchyma although at this time there is no obvious differentiation of phloem or xylem with secondary wall deposition.

At 25 DAF three layers of cotyledon cells were examined of the now enlarged seeds (see diagram with Figs. 103,104,105, and 106). The cells of the outer (abaxial) region of the cotyledon are smaller in size than in either of the other two parts examined. The distribution of intercellular spaces is notably different to that shown for 15 DAF parenchyma, with the now thicker-walled cells in less direct contact and an increase in the relative volume of apoplast. At 25 DAF there are extensive deposits of both starch and storage protein in the

cotyledon parenchyma. The shape and protein content of the vacuolar protein bodies varied. The cells of the abaxial region contained spherical protein bodies with generally uniform amorphous contents (Fig. 103). The cells in the middle region were more variable, particularly close to any vascular bundles where protein bodies at the 'earlier' stage with coagulated, heavy-stained deposits at the tonoplast are seen. However adjacent cells are similar in appearance and maturity to the abaxial region. The cells of the adaxial region were similar in size to those of the central region, however the protein body population was uniform with evenly stained amorphous deposits throughout the cells (Fig. 105). The vascular bundle in Fig. 104 illustrated the extent of vascular differentiation at this stage of maturity. Apart from a few major vascular bundles in each cotyledon there is little differentiation of xylem with secondary thickening, however the majority of vascular bundles now contain differentiated phloem.

Starch distribution was found to be even throughout the cotyledon at 25 DAF with numerous large starch grains in cell storage parenchyma cells.

The last stage of development examined by light microscopy was 30 DAF, by which time the cotyledons had reached maximum fresh weight and development (Fig. 107). The cotyledon cells are filled with protein bodies and starch reserves and have much thicker cell walls than the earlier stages examined. The seeds cells are at the maturity (stage 4) phase of development.

2.3 Transmission electron microscopy of cowpea inner seed coat.

The light microscopy of the seed coat of the young developing

cowpea showed a substantially different structure for the seed coat layers adjoining the embryo sac than the structures reported here and elsewhere (Marinos, 1970) for developing pea. The inner columnar epithelial layer of the cowpea seed coat has been examined by electron microscopy as part of an investigation of the structural relationship of the cowpea embryo to its immediate growth environment.

The ultrastructural features of the columnar epithelial cells are illustrated in Figs. 108 to 116.

Figure 108 shows the general form of the cells of the inner seed coat layer with their dense cytoplasmic contents. The cells contain interphase nucleus, numerous plastids, often of an immature amoeboid form, dictyosomes, ER and numerous small vacuoles/vesicles rather than one or a few large vacuoles. The numerous dictyosomes contain up to 12 cisternae with elaborate peripheral reticulum and many vesicles (Fig. 108). The dictyosome vesicles do not contain any stained material although some are 'coated' suggesting transport to or from the plasmalemma. The cell walls are penetrated by large numbers of plasmodesmata connecting neighbouring columnar cells (Fig. 110). The symplastic interconnection is not, however, maintained at a high level between the columnar cells and the cell layer at their base. The base layer cells are thick-walled and many are in various stages of senescence (e.g. Fig. 111) but there are no apparent plasmodesmatal connections between the two layers of cells either in vertical section (Fig. 111) or a glancing section (Fig. 112) which enhances the number of plasmodesmata seen between adjacent columnar cells.

The cell wall at the apex of the columnar cells is a two layered structure with loosely woven fibrils (Fig. 113,114). Close to the cell

apices lies the embryo sac boundary wall with the space between containing loose fabrillar material with some electron dense aggregates (Figs. 113,114). On the inner side of the embryo sac boundary wall is a thin lining of endosperm cytoplasm. The embryo sac boundary wall does not contain any wall processes of the typical transfer cell type, however, there are occasional electron dense thickenings which are usually associated with pockets of plasmalemma into the endosperm cytoplasm (Fig. 114).

The multinucleate endosperm cytoplasm contains numerous mitochondria, polyribosomes - both free and bound to ER - and some membrane bound amorphous organelles similar in appearance to peroxisomes (Fig. 115).

As the embryo develops the endosperm contents degenerate to a granular mass. There is no development of cell wall processes during this time either on the columnar apices, the embryo sac boundary wall or the embryo epidermal cells (Fig. 116). The cells of the developing embryo are characterised by the presence of plastids with developing thylakoid and small granal stacks (Fig. 116).

2.4 Transmission electron microscopy of cowpea cotyledon storage parenchyma.

The sequence of protein body formation in cotyledonary cells of developing cowpea seed was examined by electron microscopy. The sequence of ultrastructural changes described was based on the date after flowering (DAF). The time period for comparison of materials in cotyledon cells was between 5 and 30 DAF. At the earliest stage examined (15 DAF) cells of the cotyledons were highly vacuolate with a thin lining of cytoplasm to the cell wall and few transcellular

strands or membranes. No further evidence of cell division was seen and chloroplasts with limited internal membrane system were present and occasionally contained small stack of grana. The cytoplasm contained some free ribosomes, ER and occasional small dictyosomes and mitochondria (Fig. 117). Figure 118 illustrates that division of the main vacuoles was apparent in the cotyledon cells of 18 DAF seeds with some showing cytoplasmic strands penetrating vacuoles. Also at this stage numerous cytoplasmic vesicles were present near the nucleus and close to the cell walls (Fig. 119). By this time cotyledon parenchyma cells were characterised by few smaller vacuoles in contrast with earlier stages and contained stainable aggregate generally appressed to the tonoplast (Fig. 118). This material was observed in light and electron microscopy and identified using toluidine blue to stain protein deposits. These deposits will be referred to as protein deposits and in later stages of development as protein body. The cells contain a nucleus with prominent nucleolus. The nucleolar staining showed a characteristic reticulate granular pattern. Many plastids with starch grains were located either at the cell centre or around the cell wall, and ER was found distributed throughout the cytoplasm. The development of cotyledon cells at 25 DAF is indicated in Fig. 120. Protein deposition was apparent in cotyledon cells of 25 DAF. with electron-dense precipitation both in cytoplasmic vesicles and vacuoles, which are termed protein bodies from this point. The cytoplasm now contained conspicuous rough endoplasmic reticulum and Golgi bodies with vesicles containing electron-dense material (Fig. 121). The Golgi were found throughout the cytoplasm and not just adjacent to the cell walls. The nucleoli have retained the staining

pattern shown at 18 DAF although some have taken a highly amoeboid form with numerous projections and invaginations of the nuclear envelope (Fig. 122).

The last stage of development examined was 28 - 30 DAF by which time the cotyledons had reached near maximum fresh weight (end of phase 3). Development at this stage showed a fairly uniform population of protein bodies (Fig. 123). The apparent differences in size of protein bodies shown in Fig. 123 is due to sectioning at various levels through spherical protein bodies. A few dictyosomes and a small amount of ER remain. The frequency of starch grains/cell in the late stage and early stage was the same as if no plastids division had occurred during maturation and increase in total starch was due to increase in grain size rather than number.

The change in cell walls is another conspicuous feature accompanying maturity of cotyledon parenchyma cells. The parenchyma cell walls show increased thickening during development. Fig. 124 shows the cell wall at 15 DAF which is thin with little cellulose added to the middle lamella. At this stage plasmodesmata are readily visible. Some ribosomes and ER are visible around the cell wall. The structure of the cell wall at 18-20 DAF is shown in Fig. 125. At this stage the cell wall is thicker and middle lamella is quite visible, with some plasmodesmata. There is an increase in the extent of intercellular space. The amount of mitocondria and ribosomes and ER has also increased.

The next stage which is at 28 - 30 DAF is shown in Fig. 126. The cell wall is much thicker. The primary wall and middle lamella are also thickened but the amount of plasmodesmata is the same as previous

stage. Fewer ribosomes and dictyosomes are seen in the cytoplasm around the cell wall and there are some protein bodies also indicated close to the cell wall.

3.0 Development of Vicia faba.

Structural changes in cotyledon cells of Vicia faba (Broad Bean) during the development were studied using varieties of Vicia faba (Maris Bead and Sudanese Triple White), which are known to differ in the rate of growth and seed development. Maris Bead was used as a variety which has slow growth and Sudanese Triple White used as a variety of fast growth and both were grown in the same greenhouse under similar conditions.

The tissues from Vicia faba (Maris Bead and Sudanese Triple White) were taken from cotyledon leaves of seed at different stages of maturity (15,20,25,35, and 40 DAF). The cotyledon cells were examined by light and electron microscopy and the four developmental phases, cell formation, cell expansion, synthesis of storage reserves, and maturation and dormancy were recognized in the development of the embryo.

3.1 Light microscopy of cotyledon parenchyma development in Sudanese Triple White.

Sections were taken from different parts of cotyledon, close to the adaxial surface and abaxial surface. The 1μm sections were cut from resin embedded tissue and placed onto glass slides and stained with toluidine blue for 20 seconds.

Figure 127 shows cotyledon parenchyma cells of tissue taken from the abaxial surface epidermal part 15 DAF The parenchyma cells usually contain one or two large vacuoles, although in some cells a number of small vacuoles were present. The nucleus and nucleolus were prominent after staining with toluidine blue, although the cell wall is thin and intercellular spaces are not very big. Smaller cells immediately

under the epidermis are seen at top right. Fig. 128 shows the typical structure of parenchyma cells in the mid-region of the cotyledons at 15 DAF. The cell cytoplasm and vacuole are similar to those described in the abaxial layers in terms of vacuole distribution, nucleus, cell walls and the relationship between cells. Fig. 129 shows the adaxial parenchyma cells of a cotyledon at 15 DAF. Again there is great similarity in the structure of the cells, cell wall and cytoplasmic material, nucleus and vacuoles.

Figures 130, 131 and 132 show the parenchyma of abaxial, mid and adaxial region cotyledon cells at 20 DAF. There are now more vacuoles per cell than at 15 DAF and they are smaller, although early deposition of protein into their vacuoles is apparent in the form of densely-stained granules lying close to the tonoplasts. Similar deposits are also seen in the cytoplasm. The extent of protein deposition, and also starch grain development, is similar throughout the cotyledon parenchyma including those cells close to vascular bundles (eg. Fig. 131). Progressively the small vacuoles and vesicles filled with amorphous protein deposits from 25 DAF until filled protein bodies were evenly distributed throughout the cotyledon parenchyma tissues by 40 DAF.

3.2 Light microscopy of cotyledon parenchyma development - Maris Bead.

Figures 133 and 134 are light micrographs of toluidine blue-stained sections of parenchyma cells of 20 DAF cotyledon showing the extent of vacuolation at this early stage of seed development when cell expansion was taking place with most cells containing one or two big vacuoles. At this stage the cell walls are thin with a cytoplasmic layer lining the walls. The only prominent organelle is

the nucleus and, as found in other legume seeds at this stage, the nucleolus is a heavily staining spherical form. Fig. 133 is from the abaxial part of parenchyma cell cotyledon and Fig. 134 part of the mid region of the cotyledon. There is little stuctural change between these parts, as regards vacuole size and cell wall and cytoplasmic structure, and similar form was found in the adaxial region. The vascular bundle in Fig. 134 shows no differentiation of secondary xylem or phloem. The cytoplasmic structure of day 20 of Maris Bead, a slow growing variety, has got the same structure as Sudanese Triple White at 15 DAF. Examination of 25 DAF cotyledons from Maris Bead showed no discernible differences in cytological detail from those shown in Figures 133 - 134 for 20 DAF material. By 35 DAF, however, the appearance of the cotyledon cells had changed dramatically (Fig. 135 and 136). The abaxial epidermis contained cells with thick outer walls highly modified in a transfer-cell form (Fig. 135) and the parenchyma cells beneath contain numerous vacuoles and vesicles with storage protein deposited as an even finer granular/fibrillar form. The parenchyma cells at the mid (Fig. 136) and adaxial regions (Fig. 137) of the cotyledon however show vacuoles at an apparently earlier stage with the protein deposits appearing as dense coagulates close to the tonoplast membrane. The parenchyma cells of the abaxial region are generally smaller and contain fewer starch grains than in the other part of the cotyledon.

By 45 DAF the protein deposition is similar throughout the cotyledon (Fig. 138, abaxial region; Fig. 139, mid region; Fig. 140, adaxial region) with a fairly uniform distribution of intensely stained spherical protein bodies. The cells close to vascular bundles

(eg. Fig. 139) contain a similar distribution of stored protein as those immediately below the epidermal layer of transfer cells (Fig. 138) and other parenchyma cells (eg.Fig. 140). The starch grains of the abaxial region cells are generally smaller than those in the other parts of the cotyledon. The vascular bundle (Fig. 139) shows differentiation of both xylem and phloem.

3.3 Transmission electron microscopy of cotyledon parenchyma development in Sudanese Triple White and Maris Bead.

The ultrastructure of the storage parenchyma cells was examined and particular attention was paid to the pattern of protein deposition within the cotyledons of developing Vicia faba. The fast developer, Sudanese Triple White is to be compared with the slower growing Maris Bead variety.

As the main features of the ultrastructural differentiation of storage parenchyma cells of both varieties of Vicia faba are similar to the sequence described for Pisum and Vigna, only a few representative micrographs are presented here.

Following the phase of cell expansion to produce highly vacuolate cells with a thin lining of cytoplasm adjacent to the cell walls, the first indication of differentiation of a storage parenchyma cell is the increase in proportion of cytoplasm (Fig. 141; Sudanese Triple White, 15 DAF). The cytoplasm contains numerous free ribosomes and some bound to ER as well as plastids with developing thylakoids and grana, but no starch, and dictyosomes with characteristic electron dense vesicles (Fig. 142: detail of Fig. 141). The ultrastructural features of 20 DAF Sudanese Triple White are illustrated in Fig. 143. The dense protein deposits are seen close to the tonoplast of the

larger vacuoles and also located within smaller membrane-bound cytoplasmic vesicles. The ER is now formed of large cisternae with numerous attached ribosomes; free cytoplasmic ribosomes are far fewer in number than at 15 DAF. A similar but slower pattern of ultrastructural change was observed for slower maturing Maris Bead.

A notable feature illustrated by the results from light microscopy was the differential maturity of the abaxial and other parenchyma regions of the 36 DAF Maris Bead. The ultrastructural detail of this is illustrated in Fig. 145 where the cells of the adaxial region are to the right of the micrograph. Comparison of the ultrastructural detail of the cells containing the different types of protein body indicated that there are no other apparent differences in the form or distribution of the other organelles. The form of the abaxial epidermal layer transfer cells is shown in Fig. 144 (Sudanese Triple White, 20 DAF).

The subsequent maturation of the storage parenchyma cells follows the pattern described previously for other legumes, with a progressive filling of the protein bodies to an amorphous, uniformly-staining material and a reduction in the extent of cytoplasmic organelles such as mitochondria and ER prior to seed maturation.

4.0 Development of Phaseolus vulgaris.

The development of the storage parenchyma tissues of cotyledons of Phaseolus vulgaris was followed by light and electron microscopy. Samples were taken from green-house grown plants at 15, 25, 33 and 40 DAF. After fixation and embedding sections were cut either at 1μm and stained with toluidine blue for light microscopy or at 80 - 100nm and stained with uranyl acetate and subsequently lead citrate for electron microscopic examination.

The sequence of development was generally as described above for Pisum, Vigna and Vicia with phases of cell division, cell expansion, deposition of storage deposits and maturation. Examination of tissues from the different regions of the cotyledon showed a uniform sequence of stages throughout the storage tissues with first storage deposition following the phase of cell expansion, and then storage protein deposition. Unlike Pisum and Vicia, however, where protein is initially as dense deposits associated with the tonoplast membranes of large vacuoles, in Phaseolus sub-division of the large vacuole occurred before protein deposition. The dense deposits were then followed by a more uniform amorphous protein within the protein bodies. Deposition of the storage reserves was accompanied by a change in the inter-relationships of the storage parenchyma cells with an increasing proportion of intercellular spaces and less cell to cell contact. These features are illustrated in Figures 146 - 153.

The light micrograph, Fig. 146 illustrates the formation of numerous smaller vacuoles and vesicles replacing the few large vacuoles at a time prior to the first deposition of protein. The nuclei are frequently seen near the centre of the parenchyma cells,

often surrounded by developing starch grains. The nucleoli do not contain a large 'vacuolar' region as is the case in Pisum and Vicia at this stage of vacuole development; instead there are a few small clear regions in otherwise densely stained nucleoli. Protein deposition within the vacuoles is initially in the form of dense granular deposits frequently associated with the tonoplast membranes In (Fig. 147), however, these are replaced by more uniform amorphous protein bodies which frequently have a similar staining as the cytoplasm when toluidine blue is used. Thus the mature seed shows storage parenchyma in which individual protein bodies are difficult to distinguish by light microscopy (Fig. 148). Figure 148 does illustrate a considerable cell wall thickening that occurs late in seed development, with thickened regions of the cell wall being associated with the intercellular spaces. The columnar cells of the abaxial epidermis with transfer cell type thickening on their outer wall are seen in top left of Fig. 148; they contain little protein reserves.

The ultrastructural detail of the unusual features of storage parenchyma development in P. vulgaris is shown in Figs. 149 - 153. The early deposition of starch at a stage when the cytoplasmic volume is increasing, with presence of numerous free ribosomes but relatively little rough ER, is shown in Fig. 149. The formation of cytoplasmic vesicles and smaller vacuoles prior to protein deposition in the large vacuoles is illustrated in Fig. 150. Conspicuous rough ER is seen in the cytoplasm as well as dictyosomes and electron dense deposits of a form and size previously identified as protein filled dictyosome vesicles (Fig. 151). The even filling of protein bodies with storage protein and the development of uneven wall thickenings late in seed

development is shown in the electron micrograph of tissue from 40 DAF seed (Fig. 152). The extra wall material is laid down on wall adjacent to the intercellular spaces, which during the course of seed development have increased significantly. Plasmodesmatal connections between developing storage parenchyma cells are restricted to pit field regions where even the limited increase in cell wall thickening seen on other wall to wall regions, does not occur. The extra wall thickening is also always found on parenchyma cell wall adjacent to vascular phloem tissue (Fig. 153) but not always adjacent to differentiated xylem.

Fig.95: Scanning electron micrograph showing the distribution of tissues in pod and seed coat of cowpea.

Fig.96: Scanning electron micrograph showing details of the seed coat.

Fig.97: Scanning electron micrograph showing the long cells attached to the embryo sac boundary wall.

Fig.98: Light micrograph illustrating the seed coat at 6 - 8 DAF.

Figs.99,100: Light micrograph showing the loose packing of the cells.

Figs.101,102: Light micrograph showing cotyledon cells of cowpea at 15 DAF.

Figs.103-105: Light micrographs illustrating different parts of the cotyledon at 25 DAF.

Fig.106: Diagram showing the position of Figs. 101 - 105.

Fig.107: Light micrograph showing cotyledon cells of cowpea at 30 DAF.

Fig.108: Electron micrographs illustrating the ultrastructure of columnar cells, X4500.

Figs.109,110: Electron micrographs showing dictyosomes, vesicles, ER and plasmodesmata, X31000, X25000.

Figs.111,112: Electron micrographs illustrating the thick cell wall between the columnar cells and the cell layer at their base, X4800, X5500.

Figs.113,114: Electron micrographs illustrating two-layered columnar cell wall structure and the embryo sac boundary wall, X9200, X45500.

Fig.115: Electron micrograph showing multinucleate endosperm cytoplasm, X11200.

Fig.116: Electron micrograph showing the cells of the developing embryo, X6300.

Fig.117: Electron micrograph of cotyledon cells at 15 DAF, X18800.

Figs.118,119: Electron micrograph of cotyledon cells at 18 DAF, X3800, X4500.

Fig.120: Electron micrograph showing cotyledon cells at 25 DAF, X8650.

Fig.121: Electron micrograph illustrating RER, and Golgi bodies with vesicles, X13400.

Fig.122: Electron micrograph showing nucleoli and nuclear envelope, X11024.

Fig.123: Electron miccrograph showing a uniform population of protein bodies, X8250.

Figs.124-126: Electron micrographs illustrating the increase of cell wall during the development of the cotyledon cells.

Fig.127: Light micrograph showing cotyledon parenchyma cells from the abaxial epidermis of Sudanese Triple White at 15 DAF.

Fig.128: Light micrograph showing the typical structure of the parenchyma cells of Sudanese Triple White at 15 DAF.

Fig.129: Light micrograph showing the adaxial parenchyma cells of Sudanese Triple White at 15 DAF.

Figs.130-132: Light micrographs illustrating the parenchyma cells of the abaxial, mid and adaxial regions of the cotyledon cells of Sudanese Triple White at 20 DAF.

Figs.133,134: Light micrographs illustrating the cells of the cotyledon of Maris Bead at 20 DAF.

Figs.135-137: Light micrographs showing the cotyledon cells of Maris Bead at 35 DAF.

Figs.138-140: Light micrographs illustrating the abaxial, mid and adaxial regions of Maris Bead at 45 DAF.

Figs.141,142: Electron micrographs showing Sudanese Triple White at 15 DAF, X15400, X39000.

Fig.143: Illustrating Sudanese Triple White at 20 DAF, X6300.

Fig.144: Electron micrograph showing the parenchyma regions of Maris Bead at 36 DAF, X15200.

Fig. 145: Illustrating the adaxial epidermal layer of Sudanese Triple White at 20 DAF, X4180.

Figs.146-148: Light micrographs illustrating the deposition of protein within the protein bodies and a considerable cell wall thickening occuring late in seed development.

Figs.149-153: Electron micrographs illustrating the ultrastructural detail of the unusual features of storage parenchyma development in P. Vulgaris, X5940, X11200, X624, X12100.

FIG.85

FIG.86

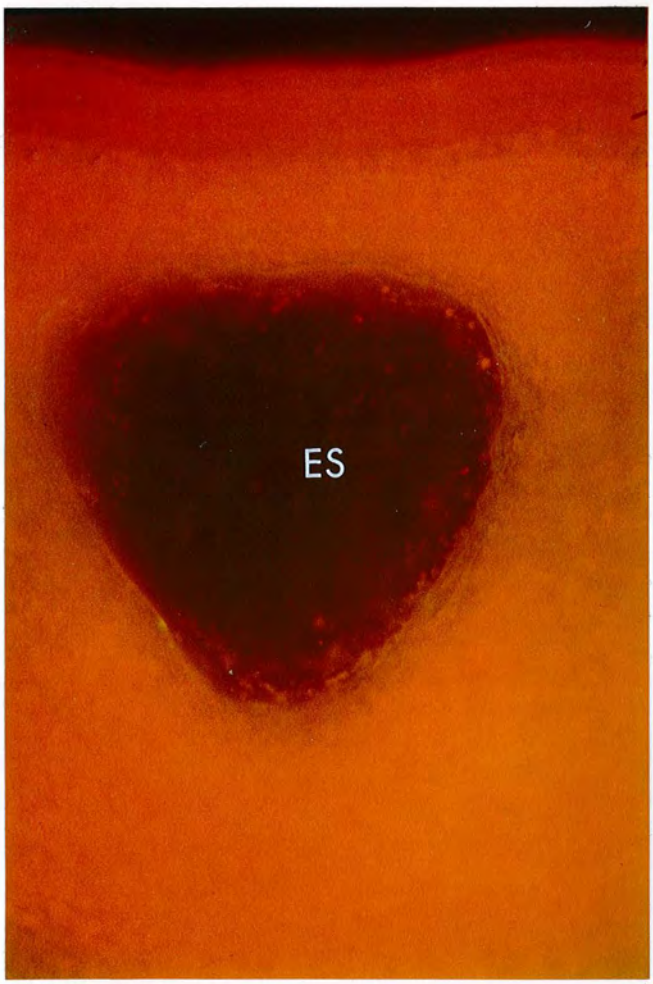


FIG.87

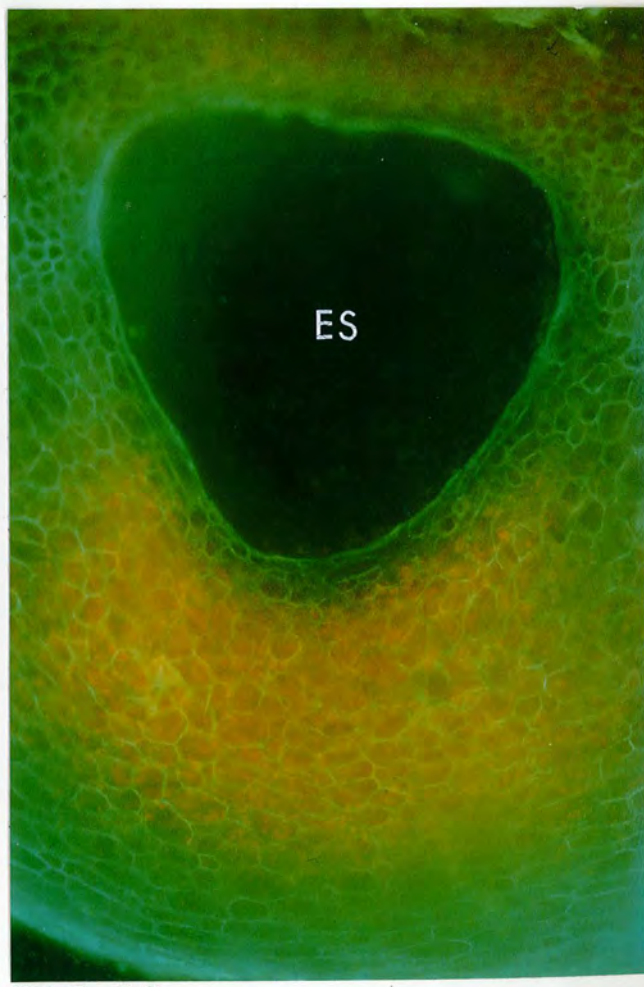


FIG.88

ALL 200μm

FIG.89

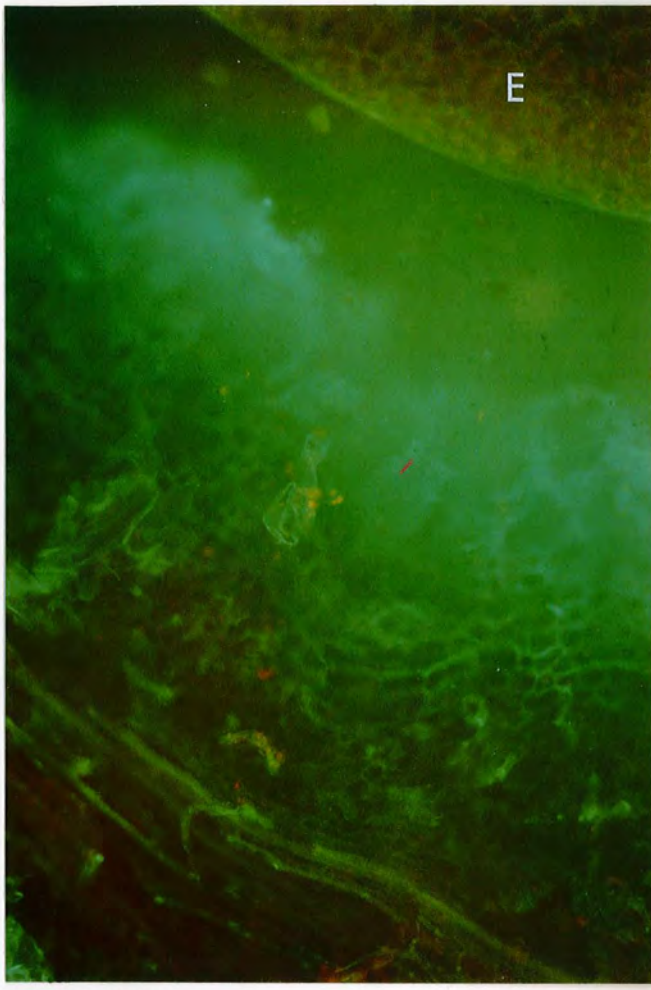


FIG.90

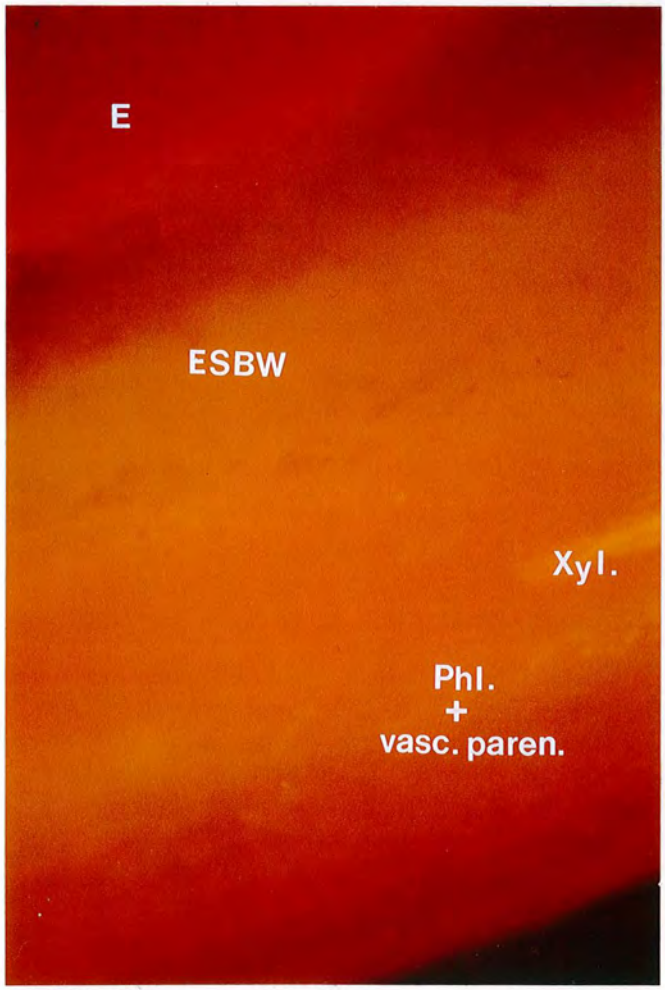


FIG.91

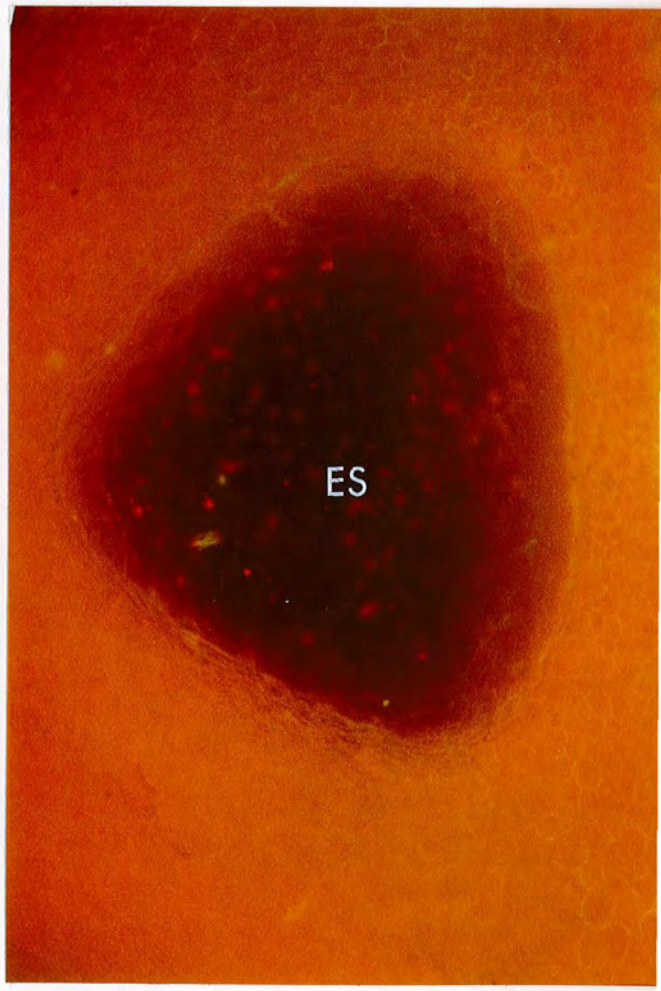


FIG.92

ALL 200 µm

FIG. 93

200 µm

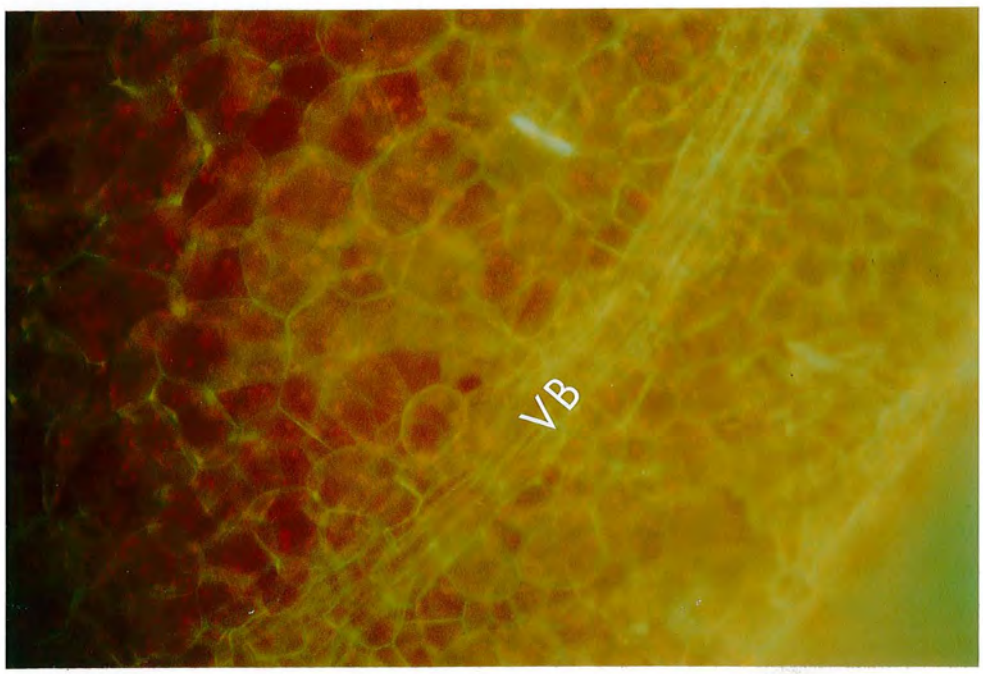


FIG. 94

100 µm

200μ

FIG. 95

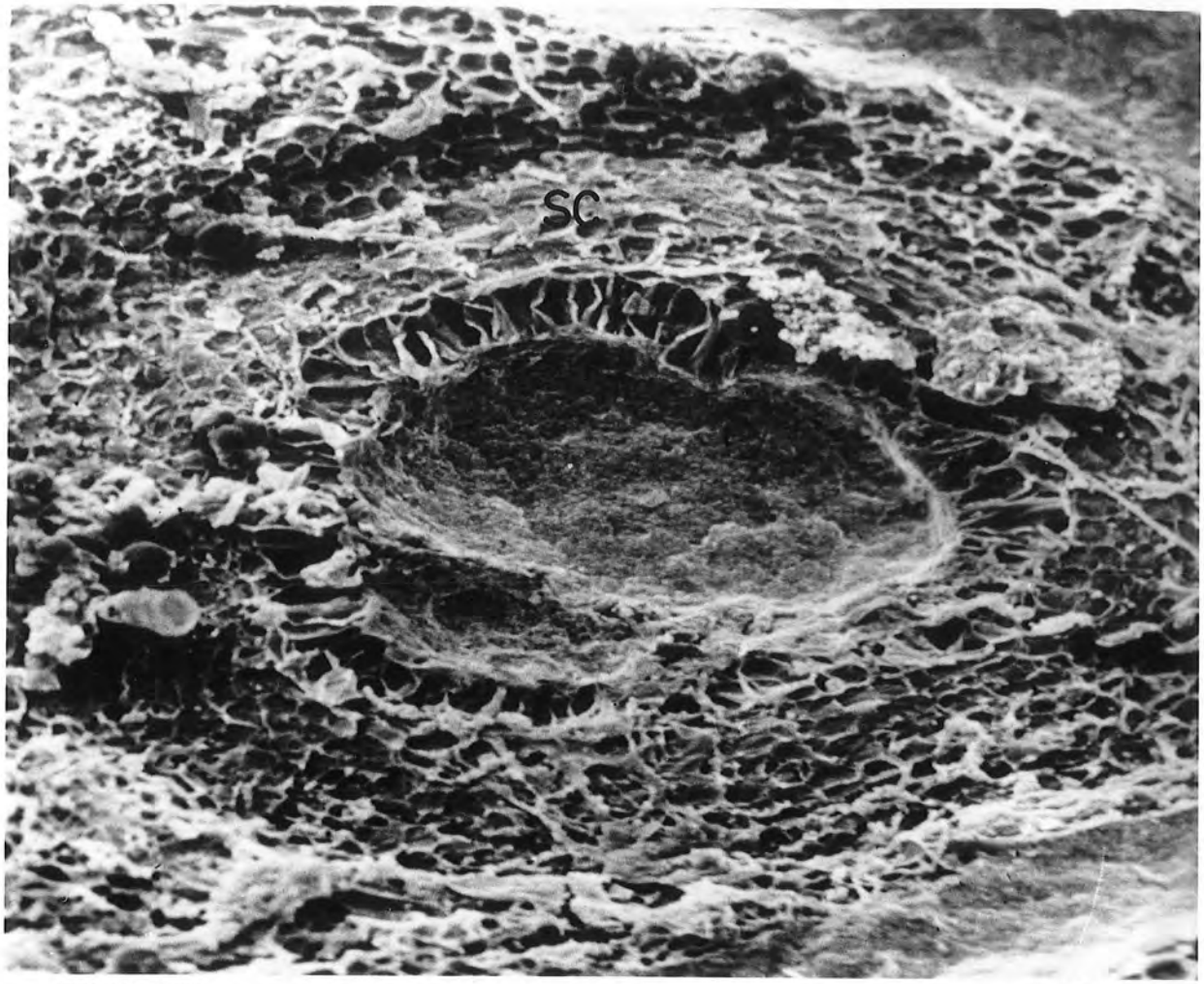
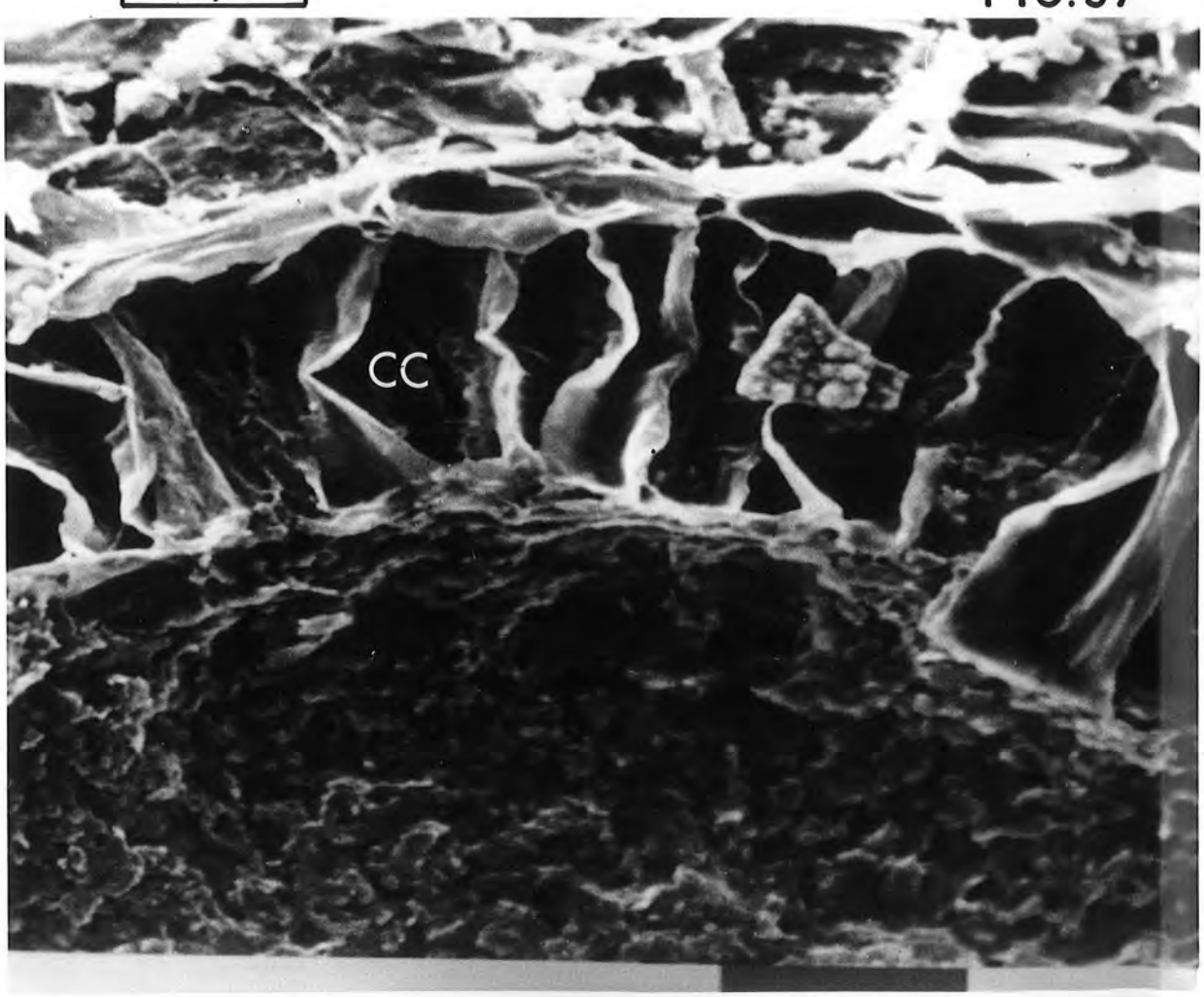


FIG.96

100 µm

20 µm

FIG.97



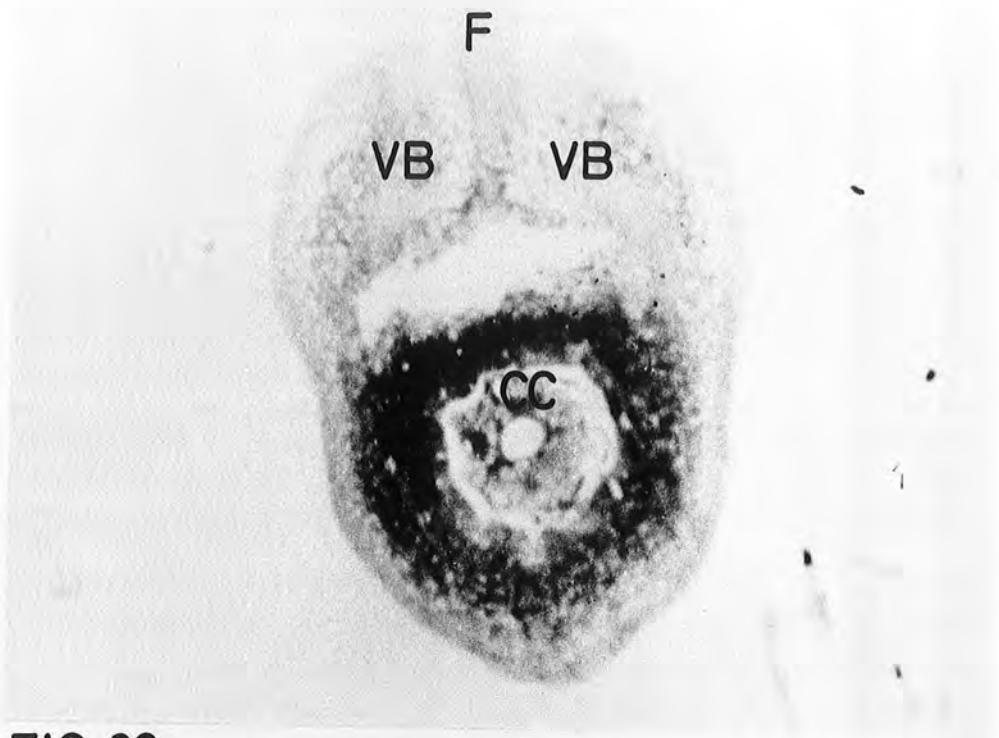


FIG. 98

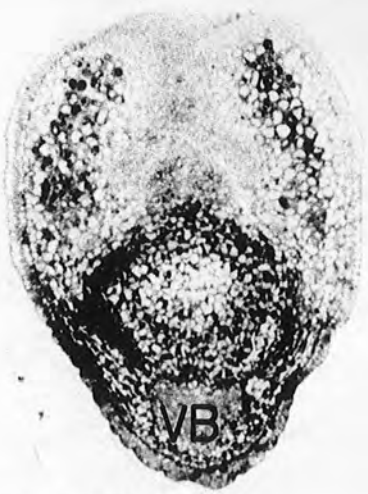


FIG. 100

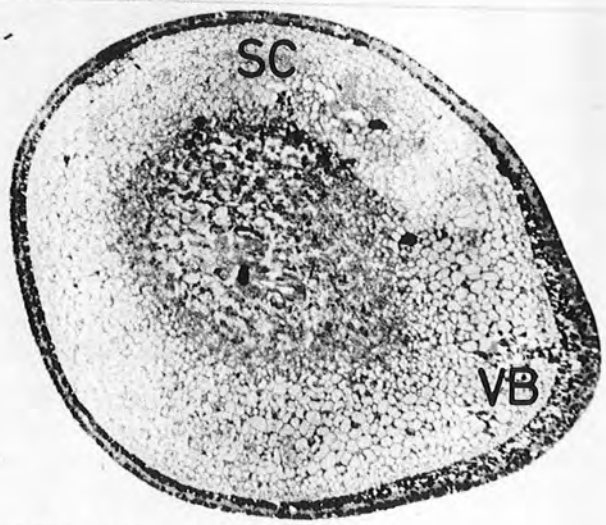


FIG. 99

ALL 100μm

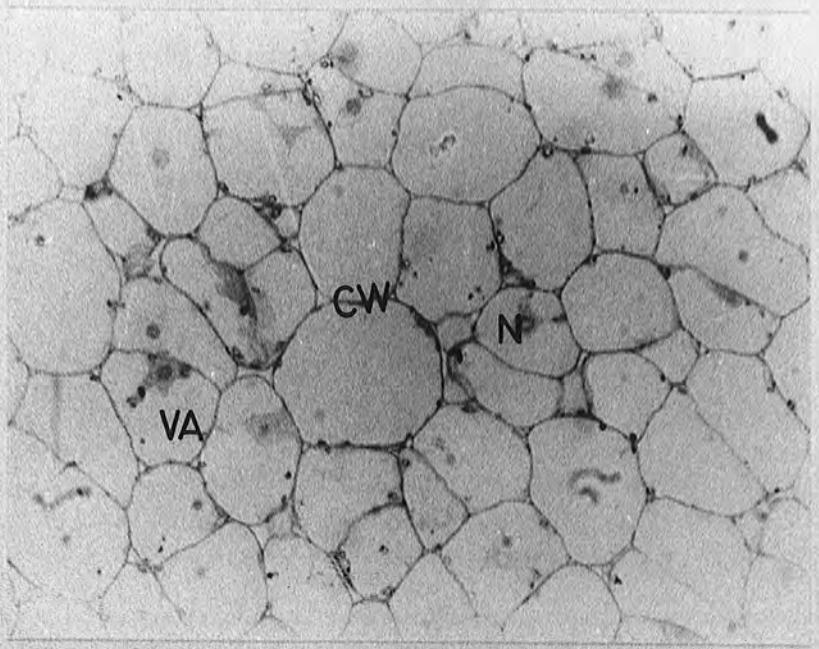


FIG.101

50 µm

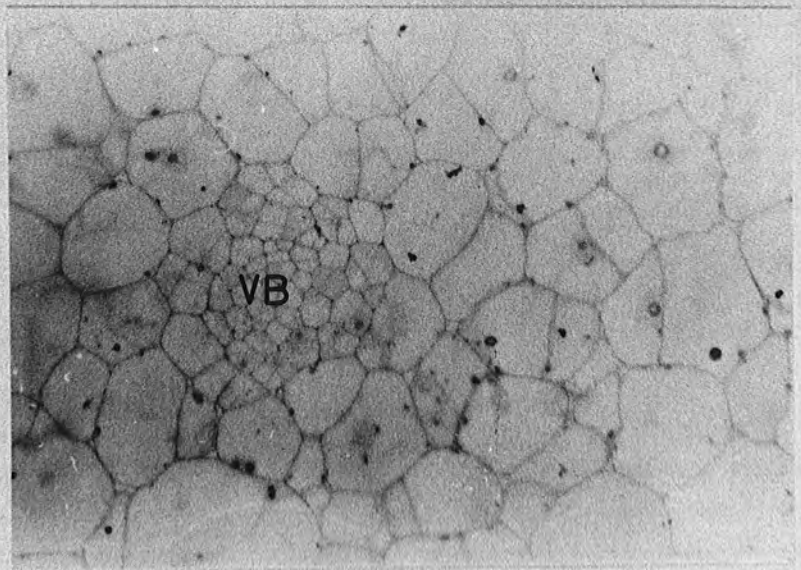


FIG.102

50 µm

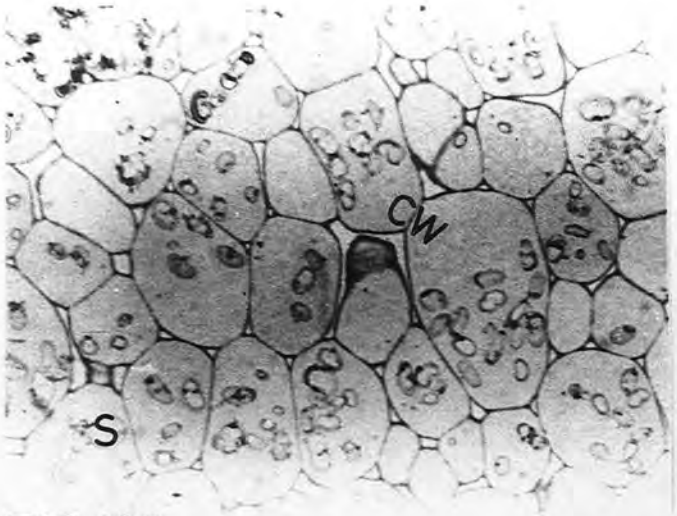


FIG.103

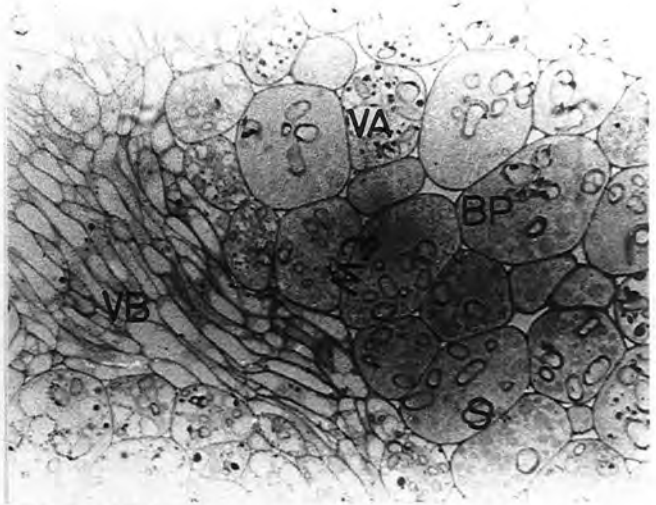


FIG.104

ALL 50μm

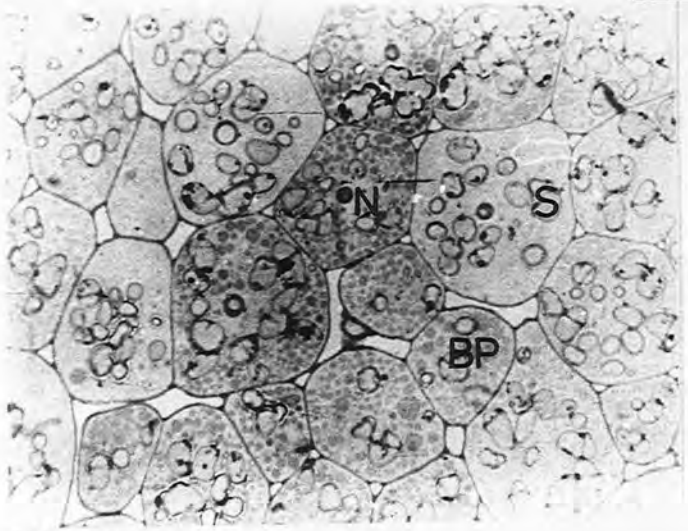
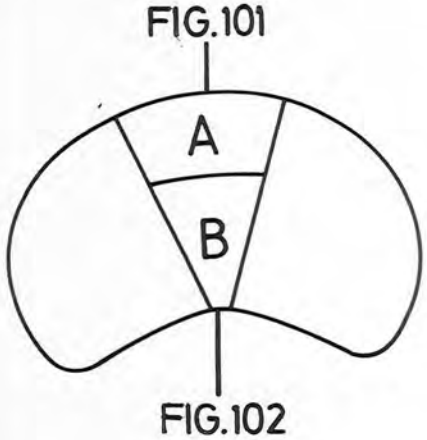


FIG.105



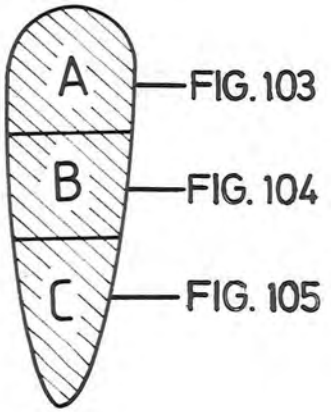


FIG.106

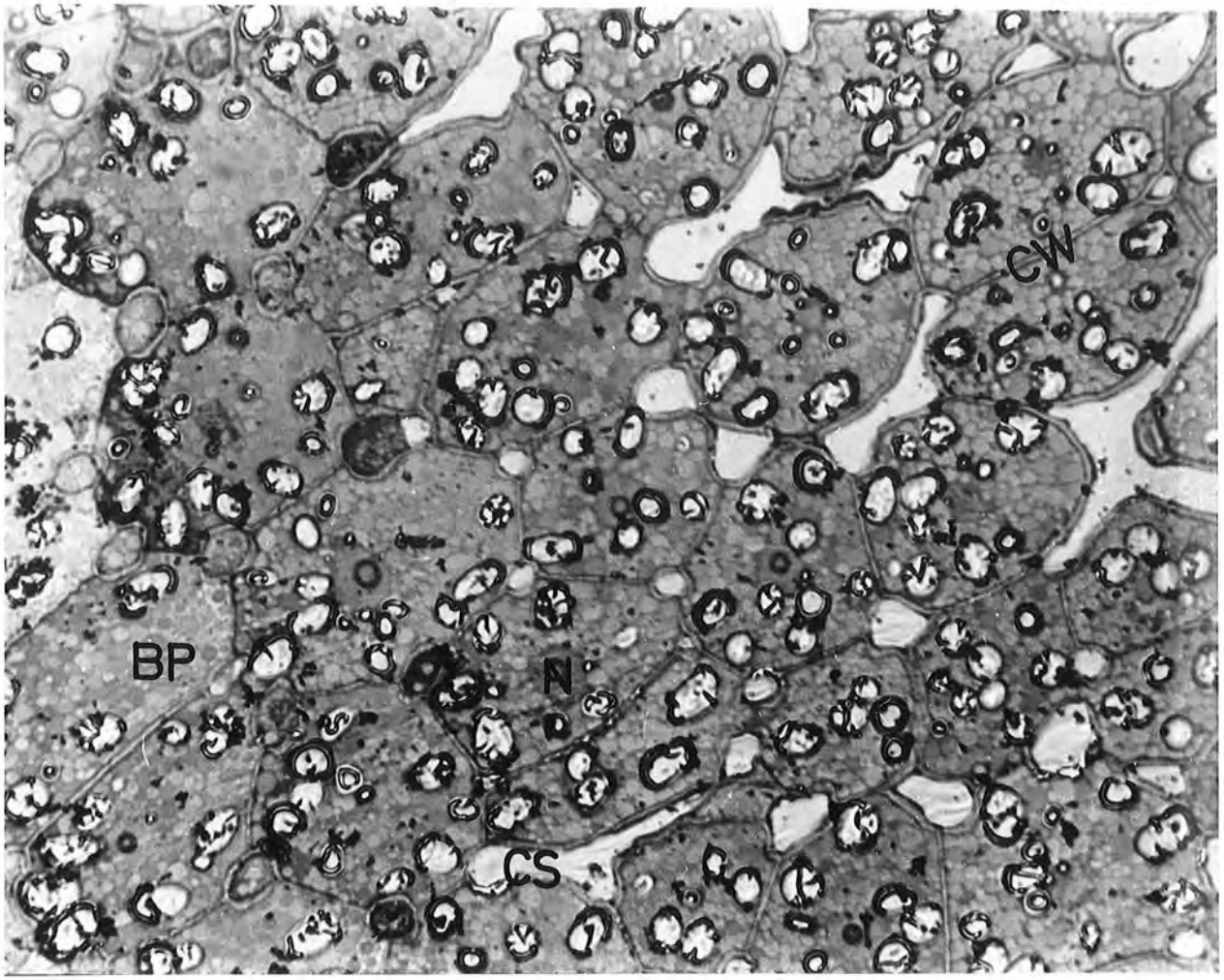


FIG.107

50 µm

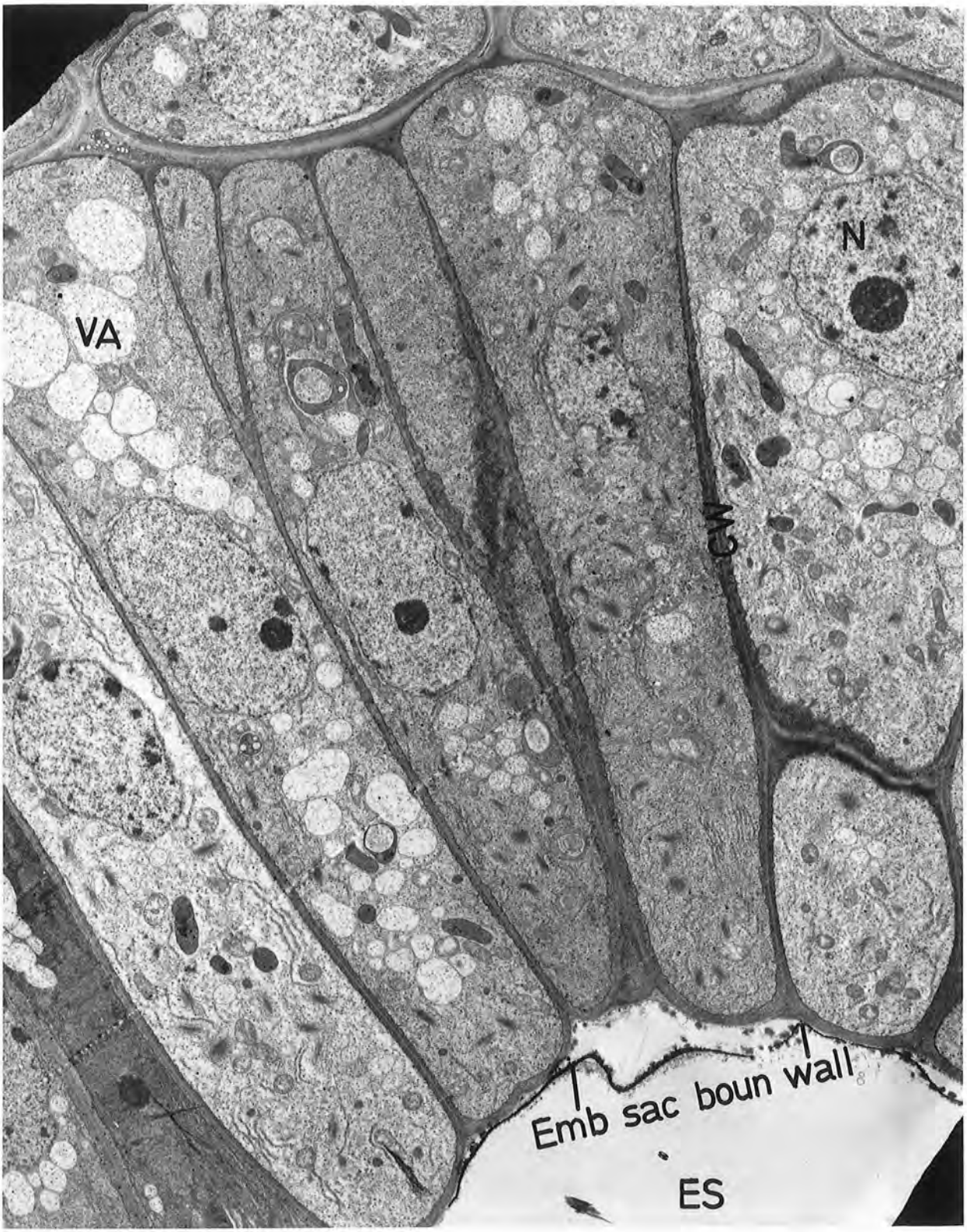


FIG. 108

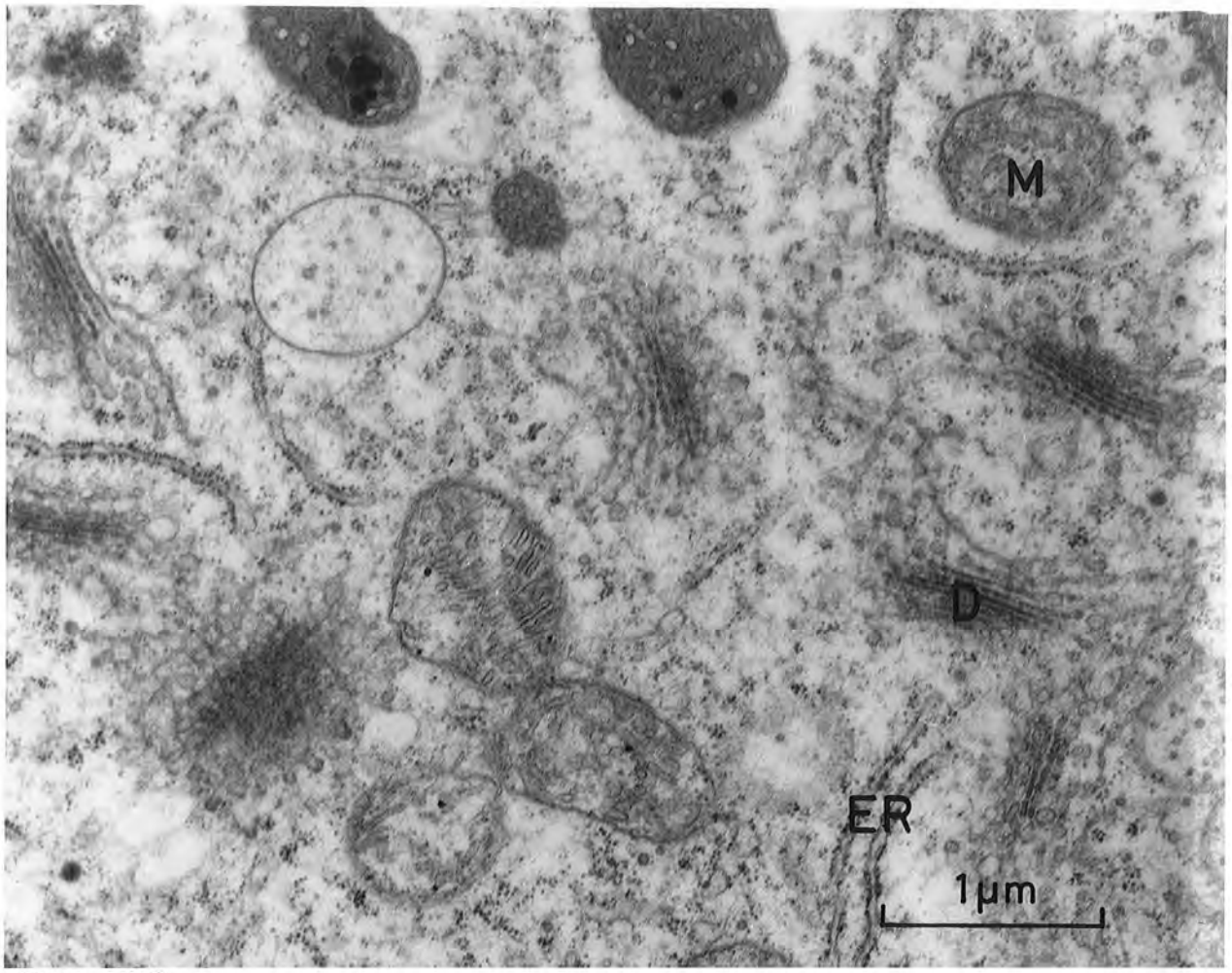
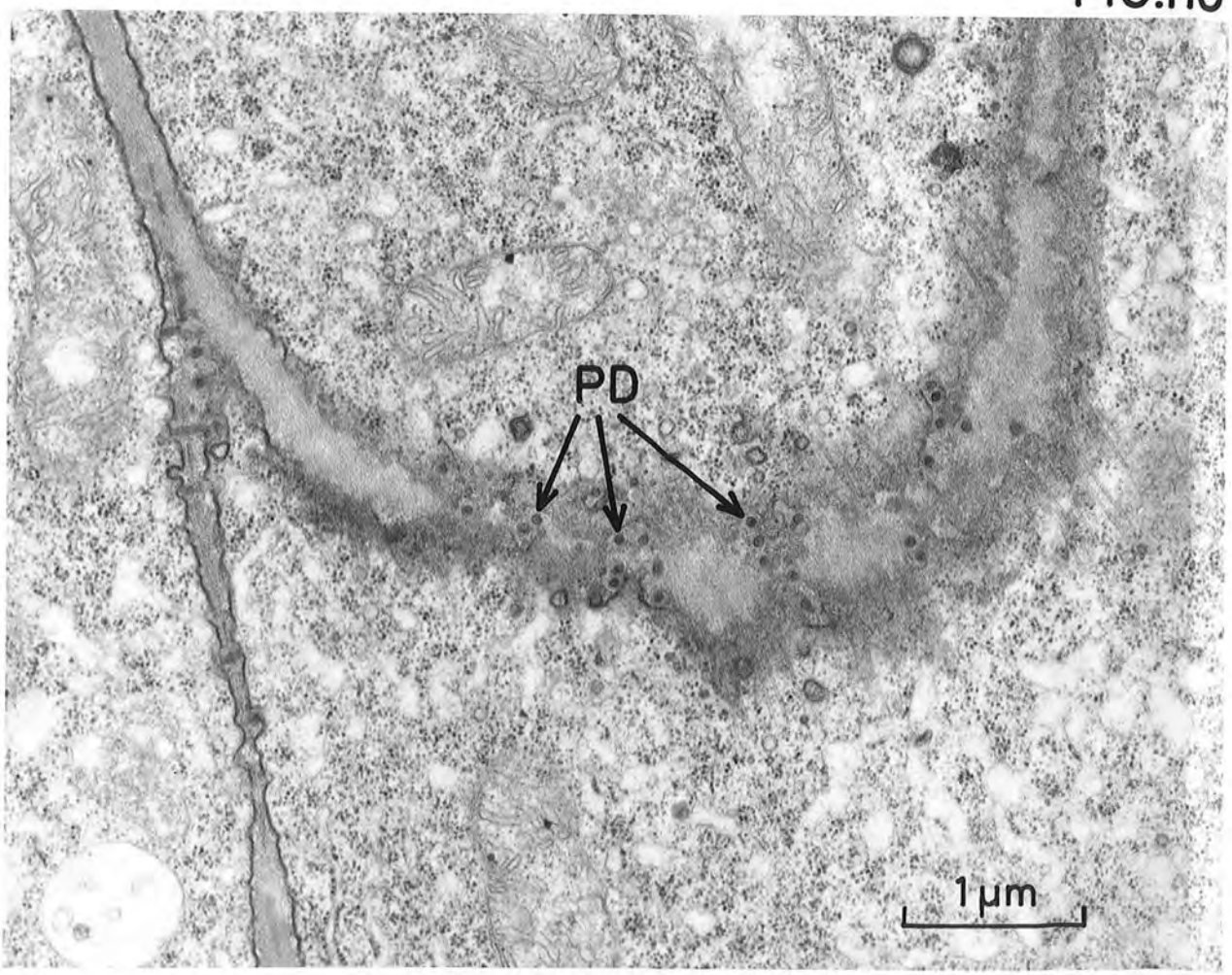


FIG.109

FIG.110



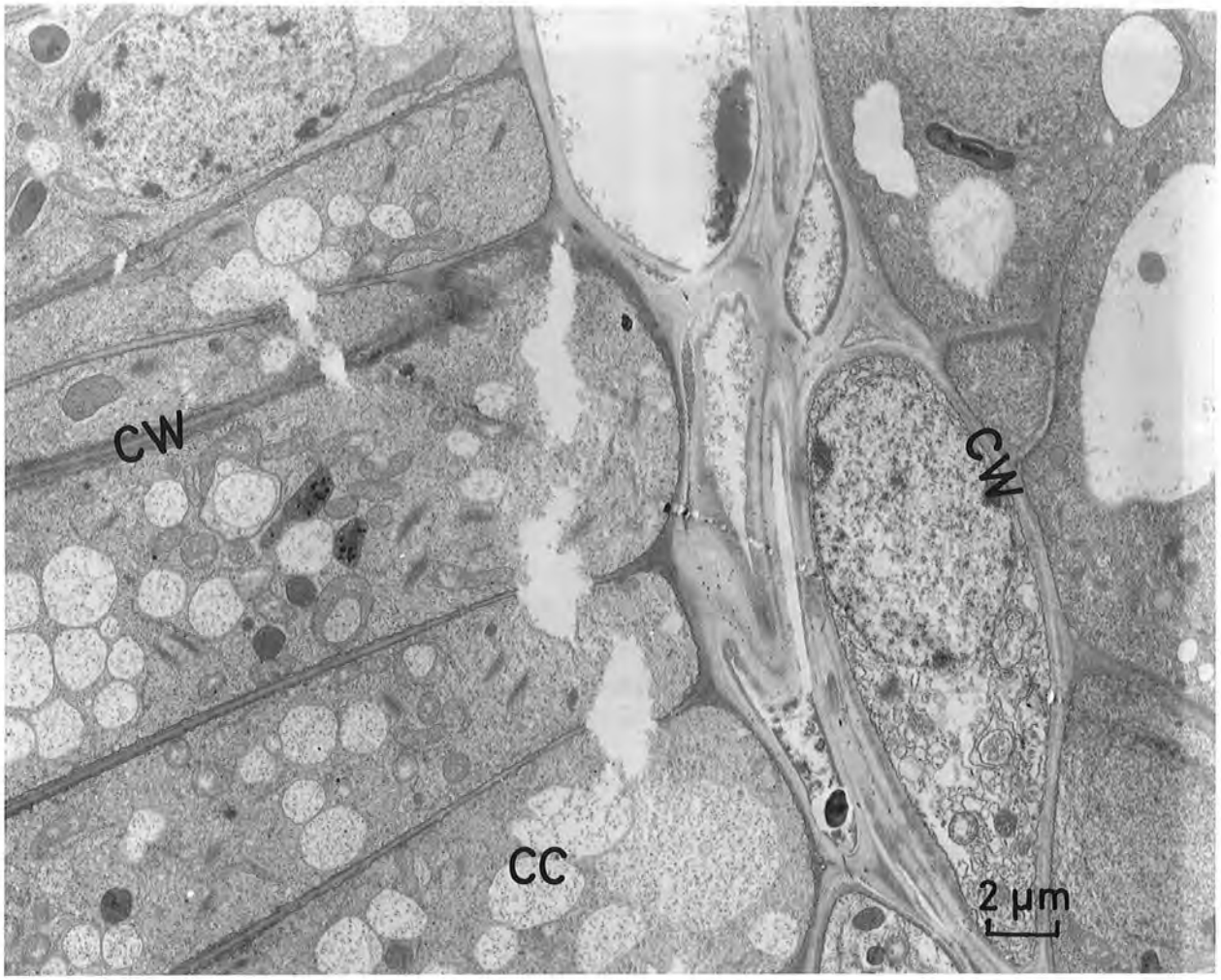
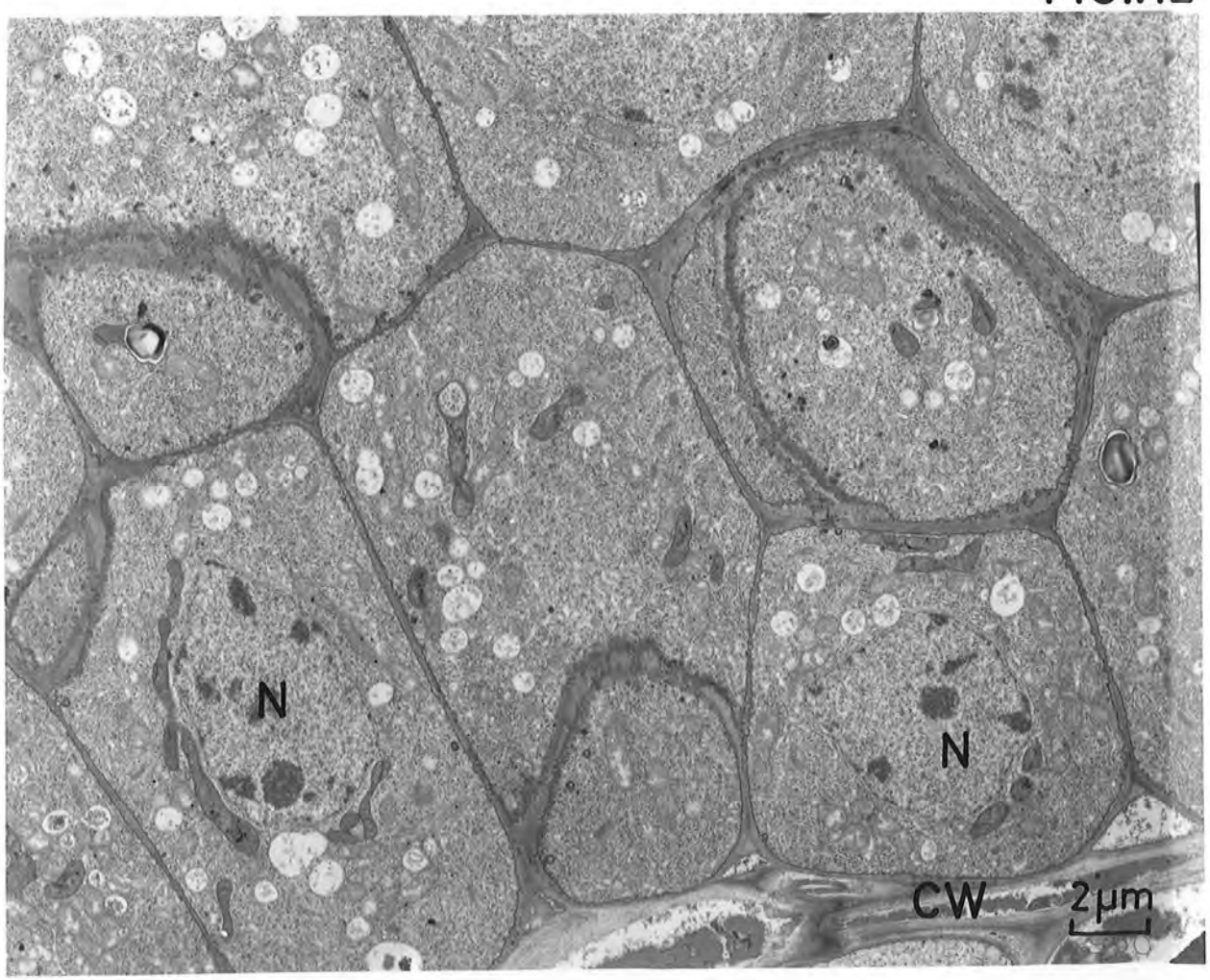


FIG.111

FIG.112



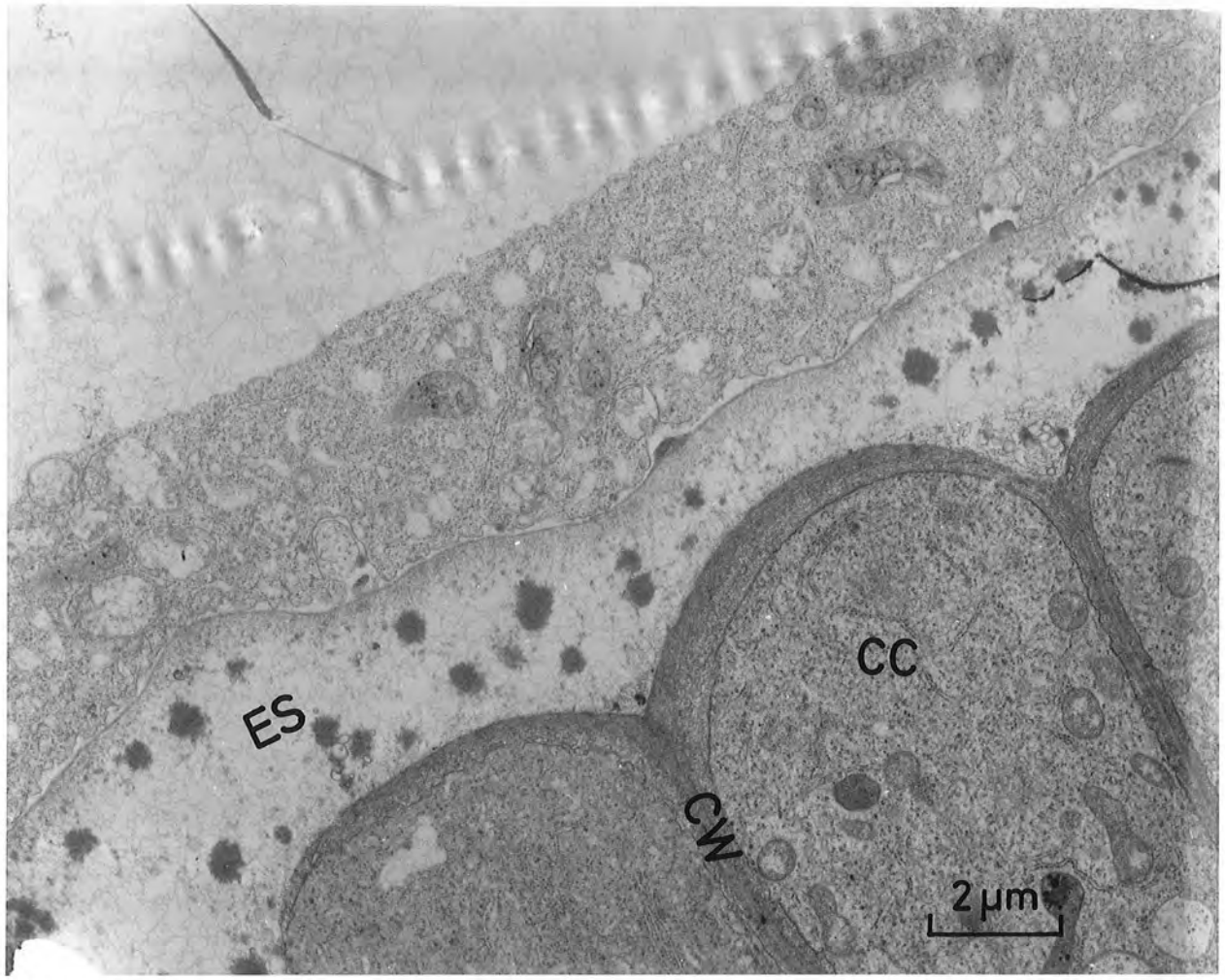
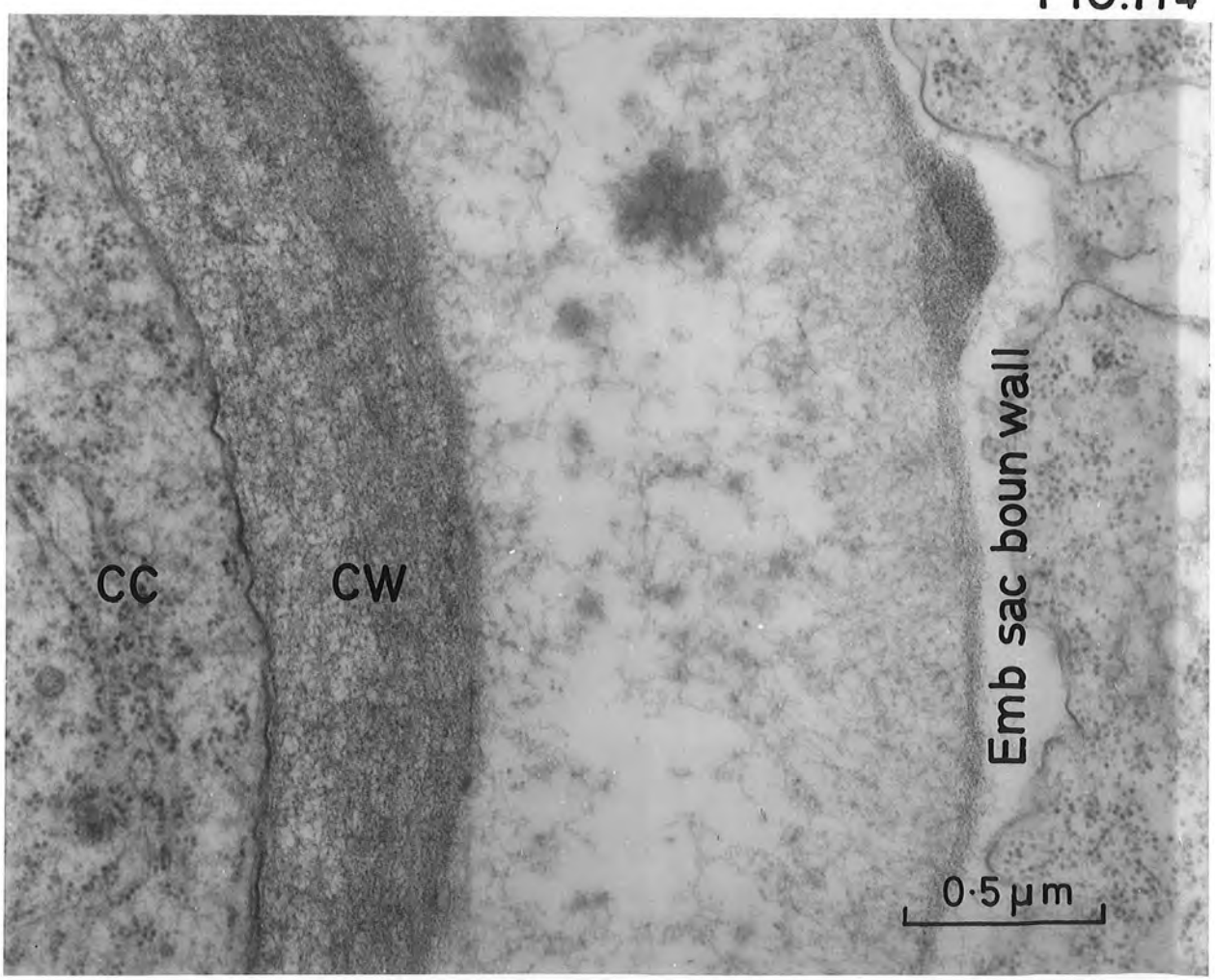


FIG.113

FIG.114



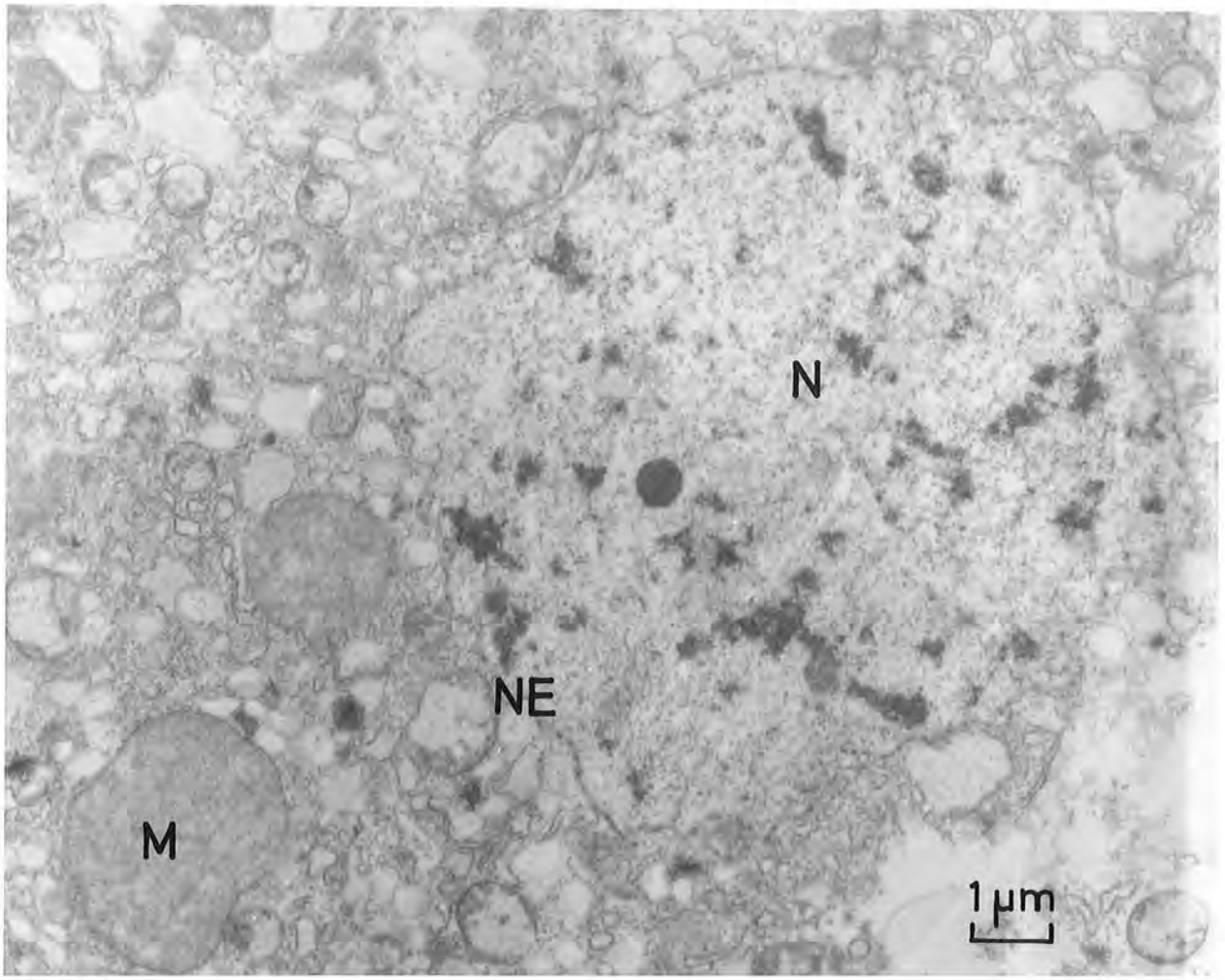
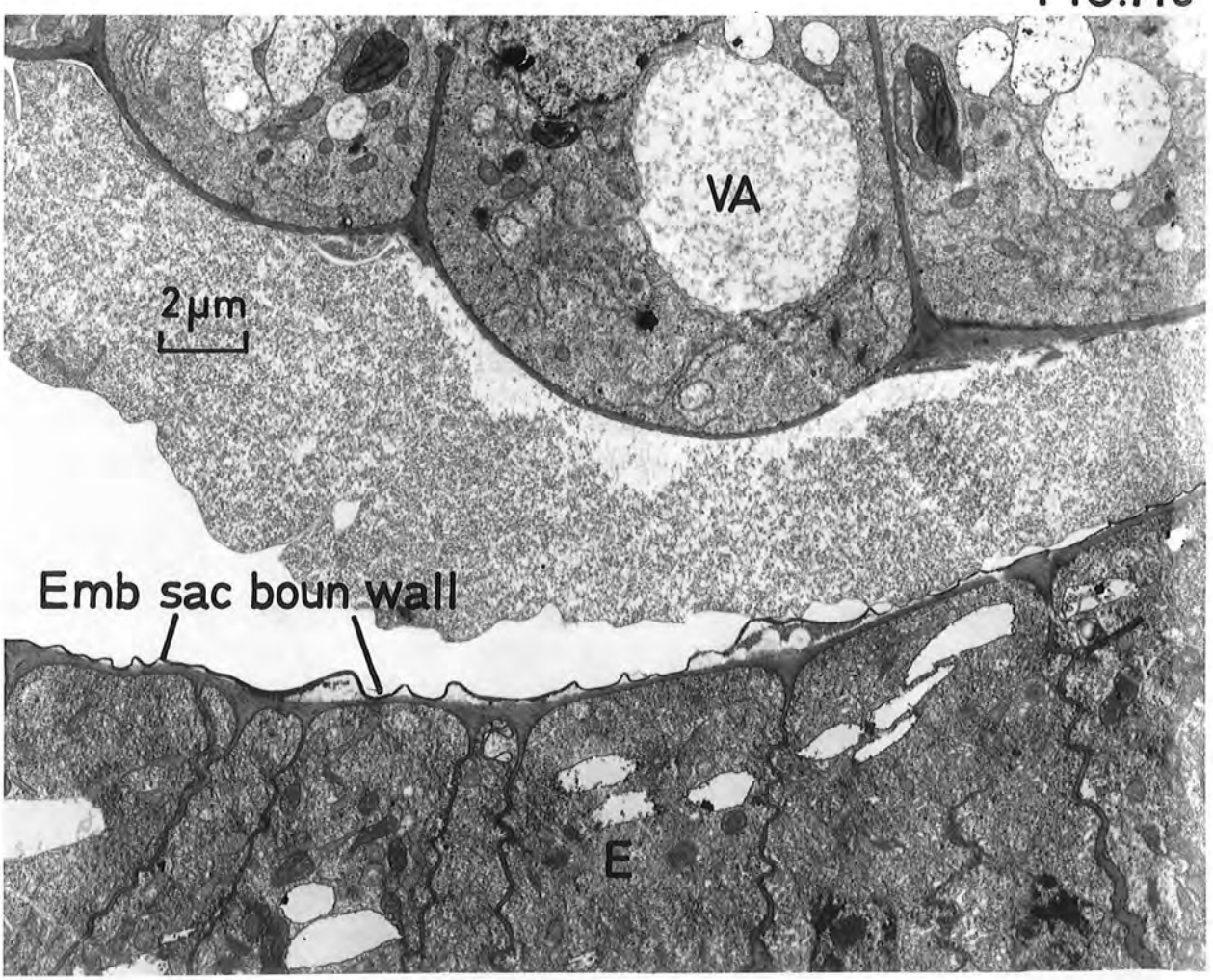


FIG.115

FIG.116



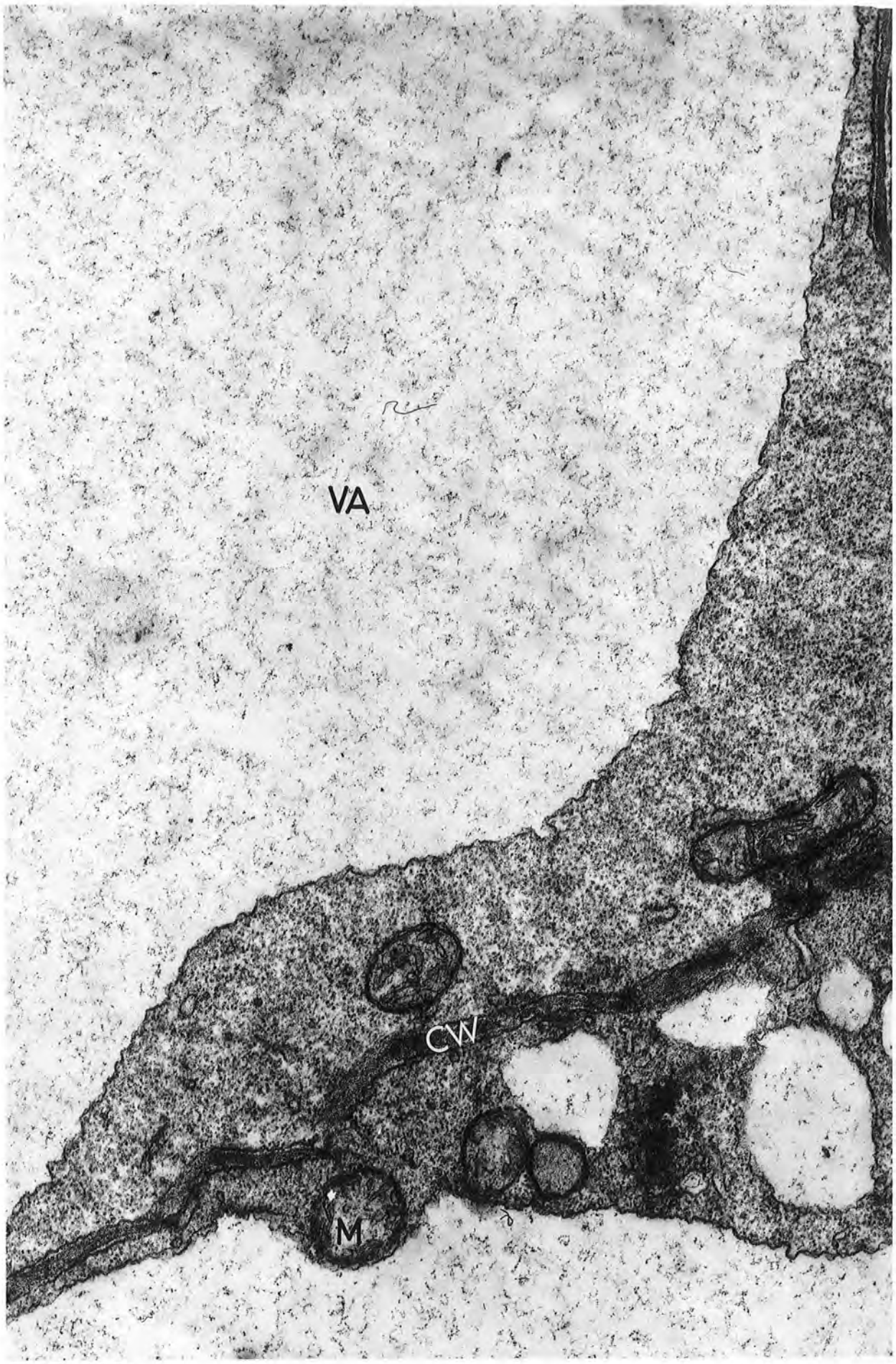


FIG. 117

1 µm

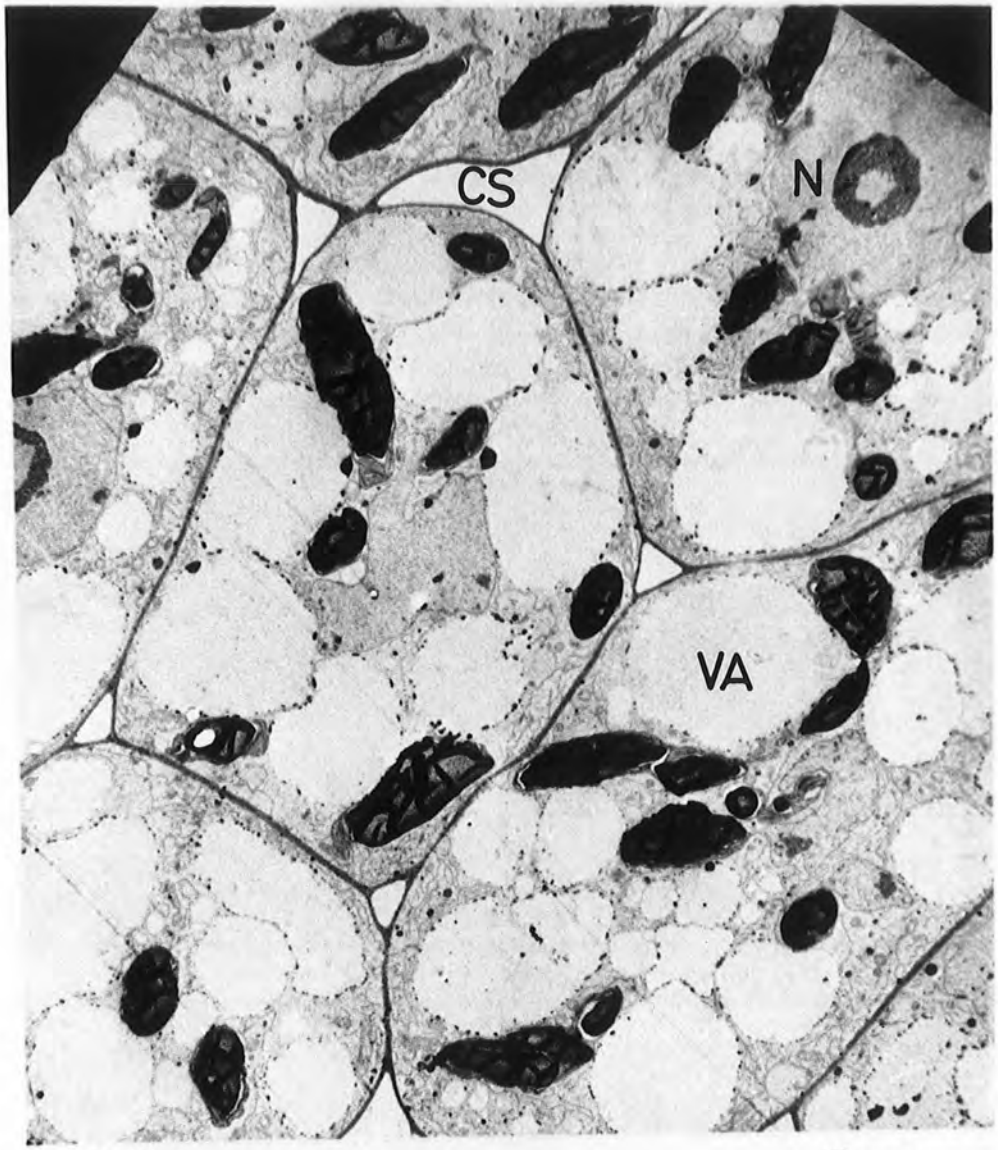


FIG.118

5μm

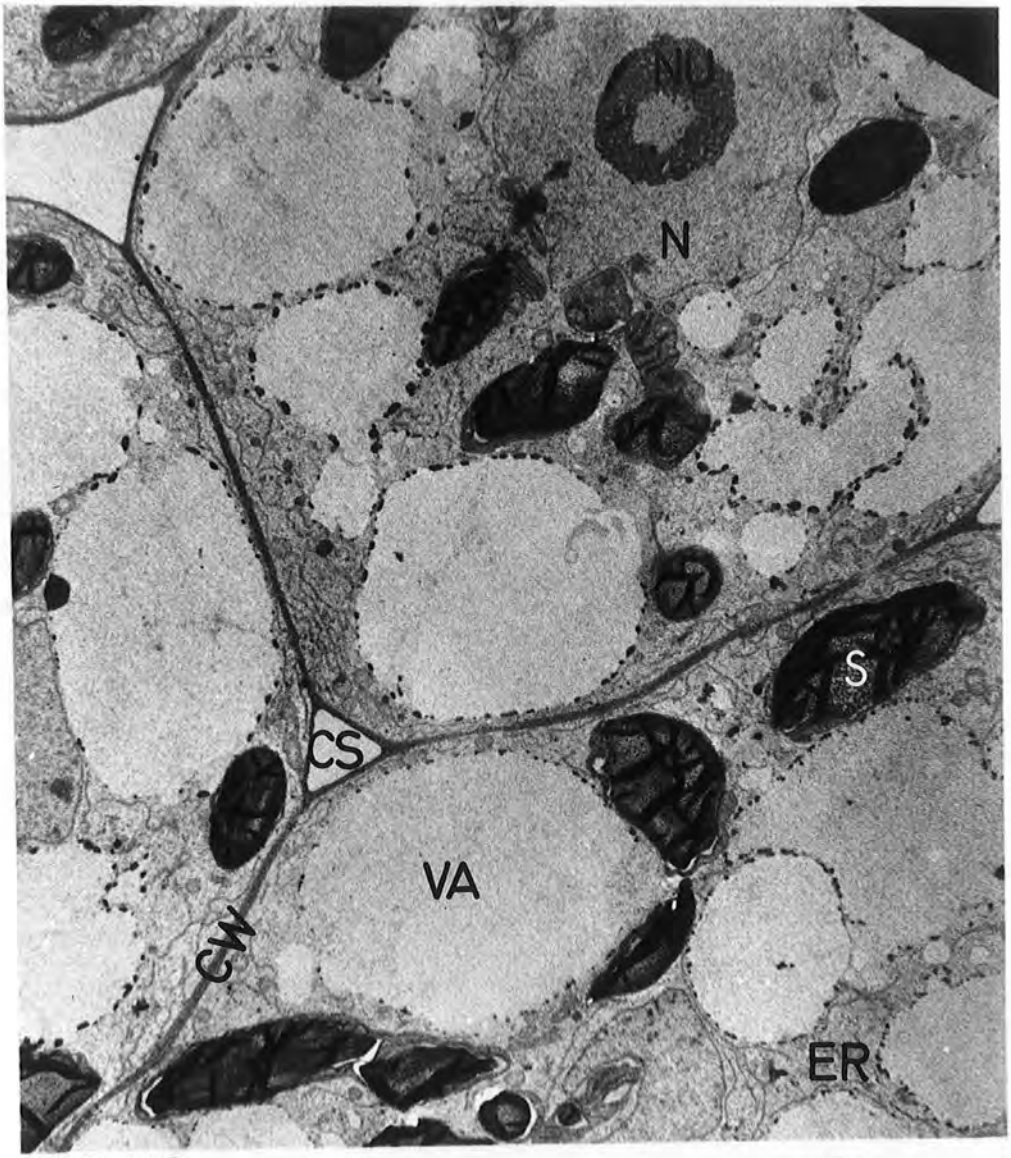


FIG.119

5 µm

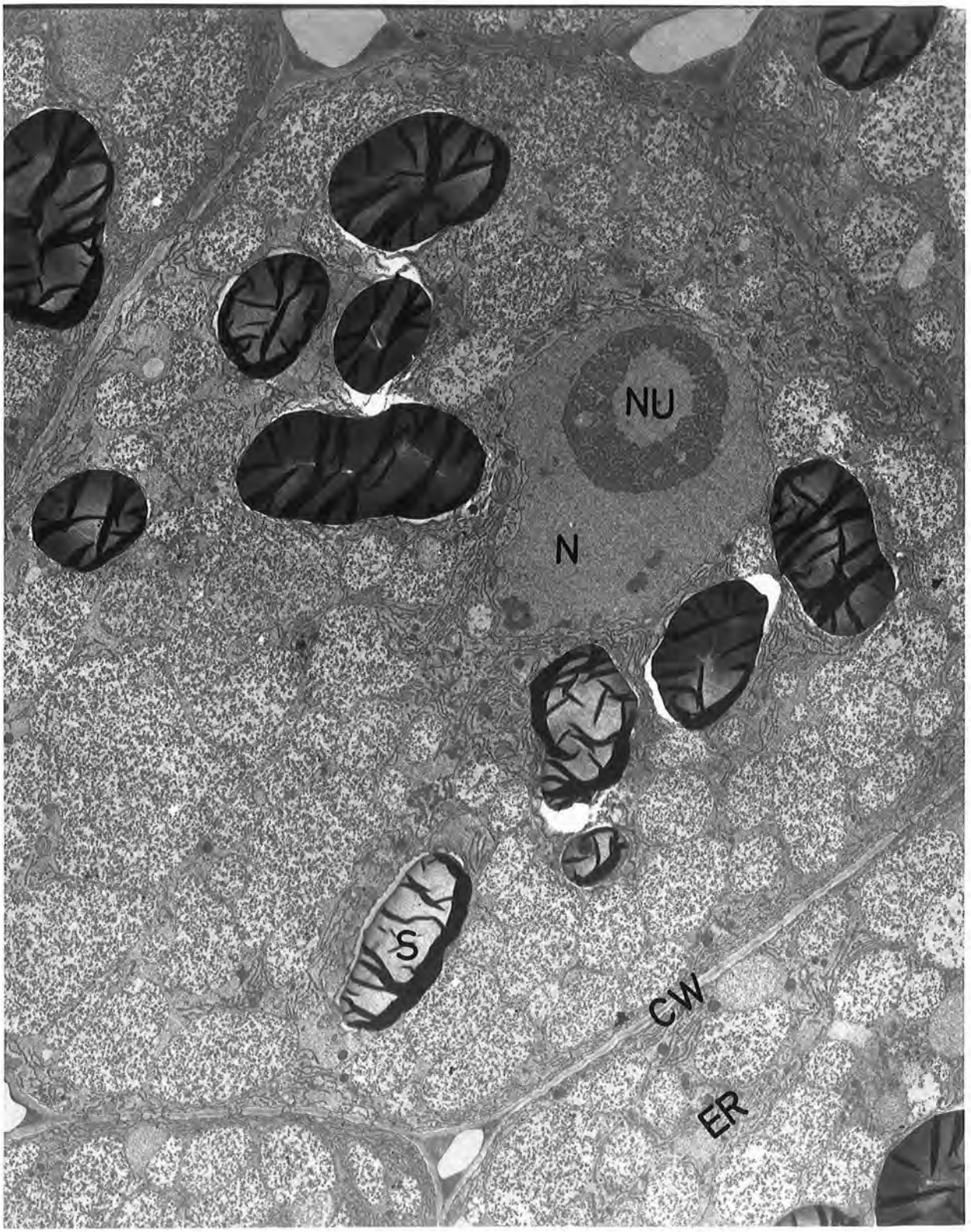


FIG. 120

2 µm

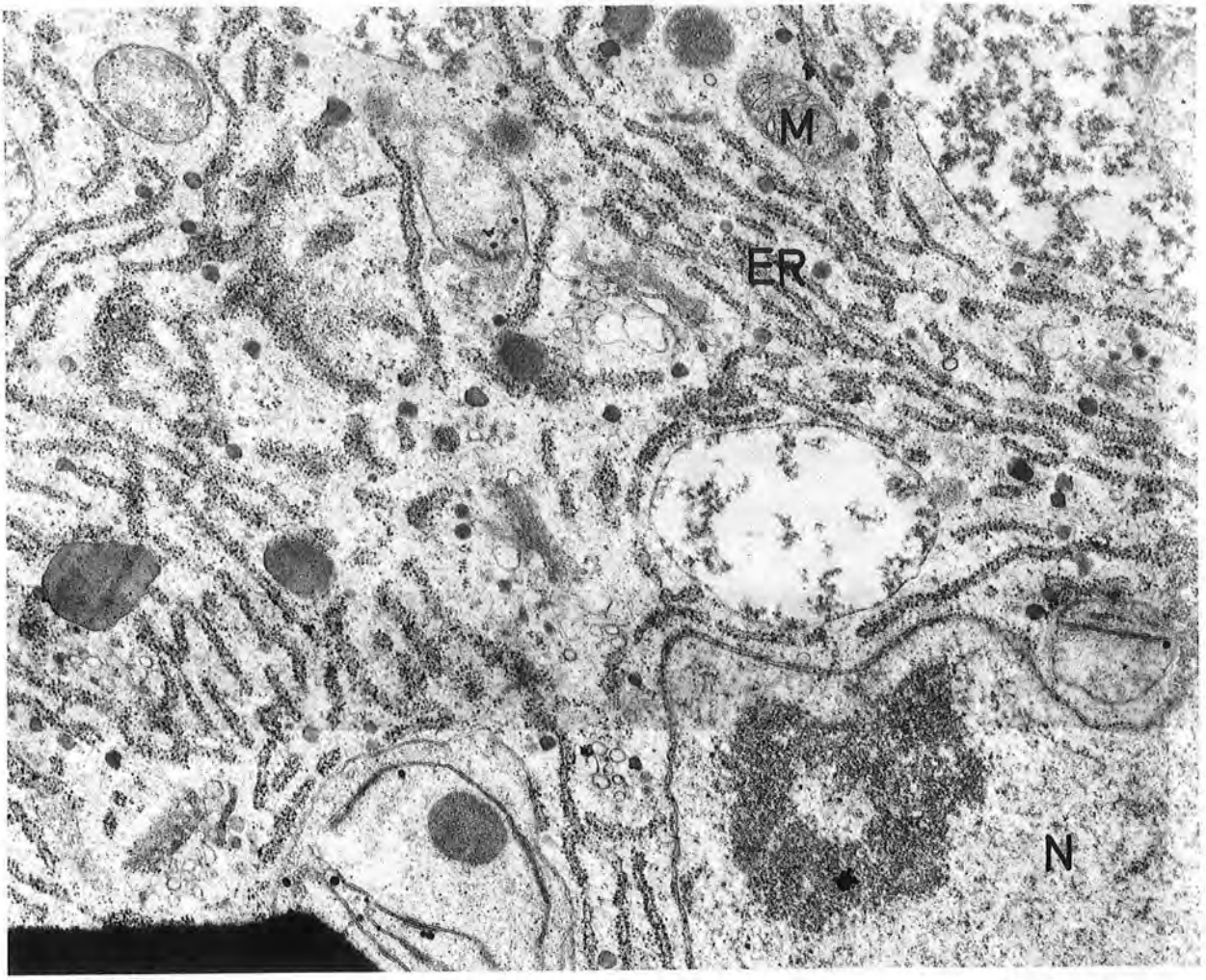
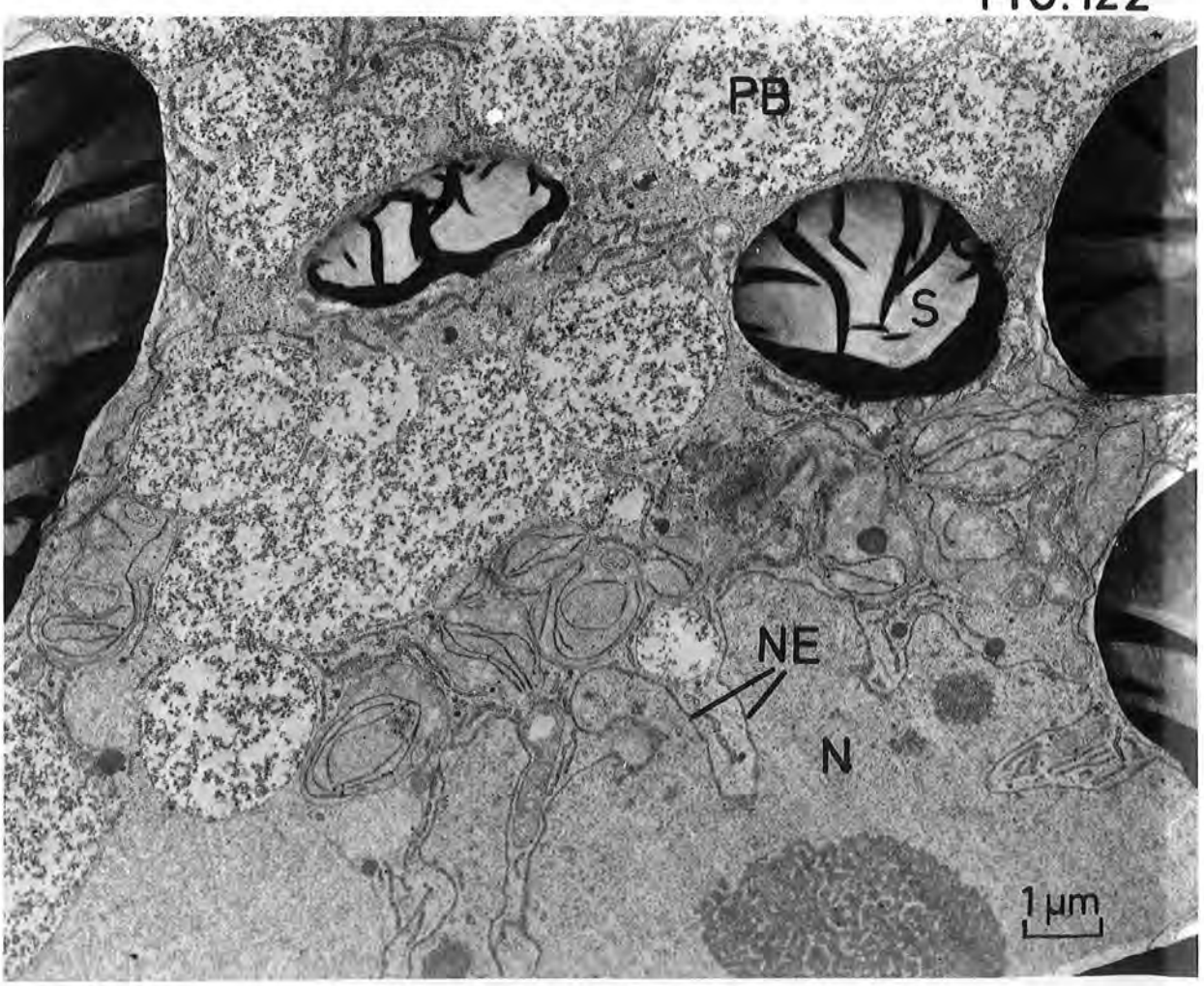


FIG.121

0·5 µm

FIG.122



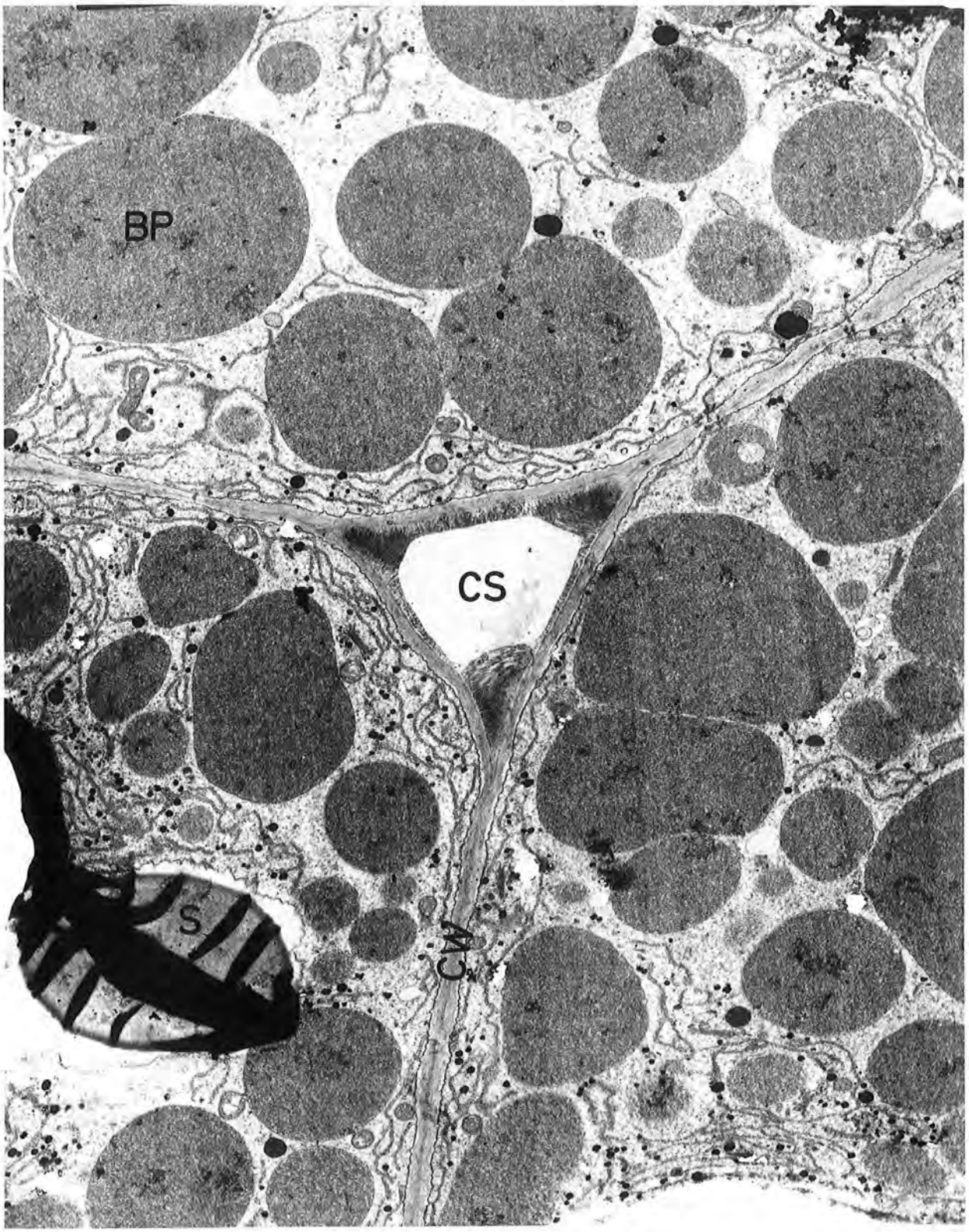


FIG.123

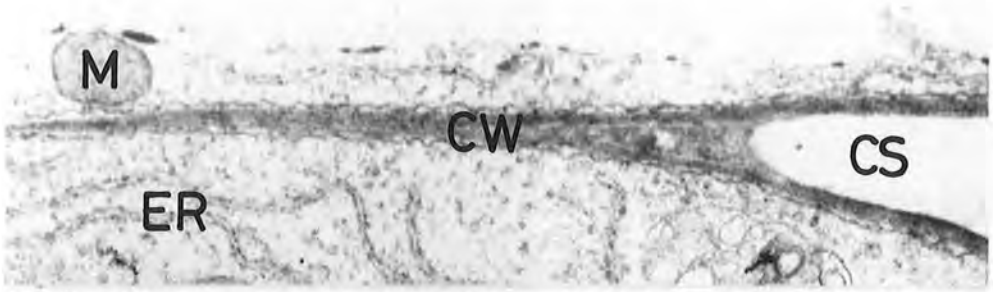


FIG.124

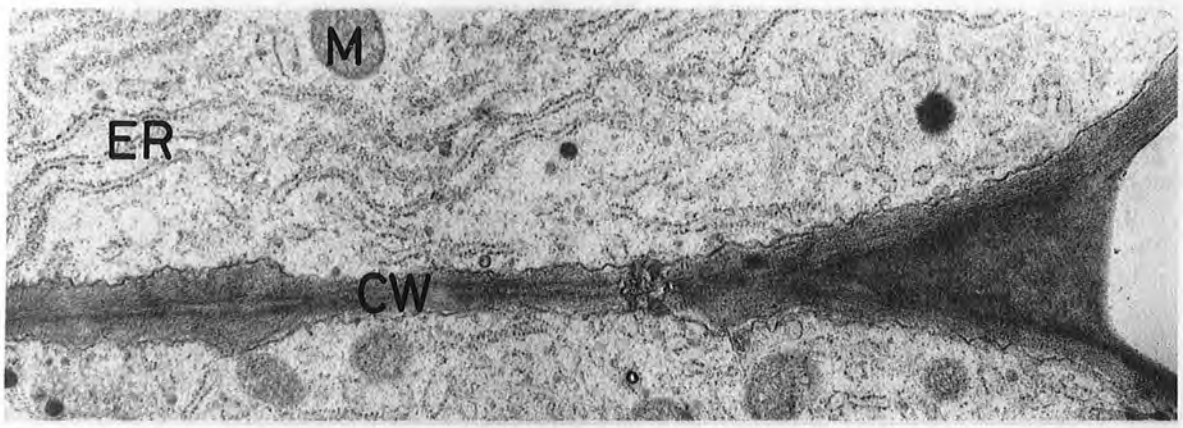


FIG.125

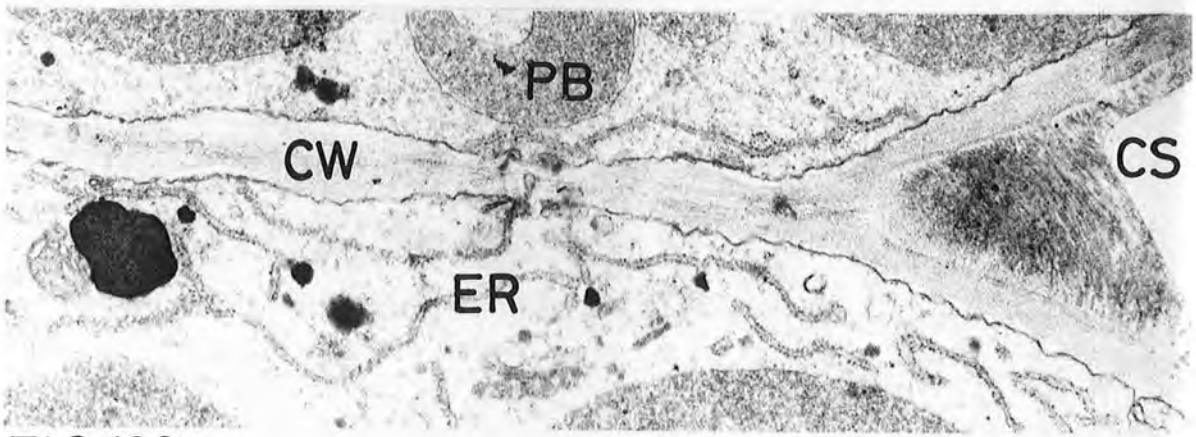


FIG.126

ALL 0·5 µm

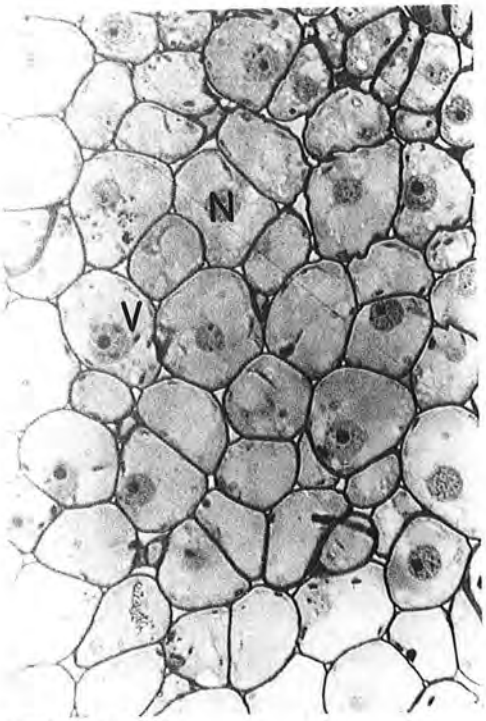


FIG.127

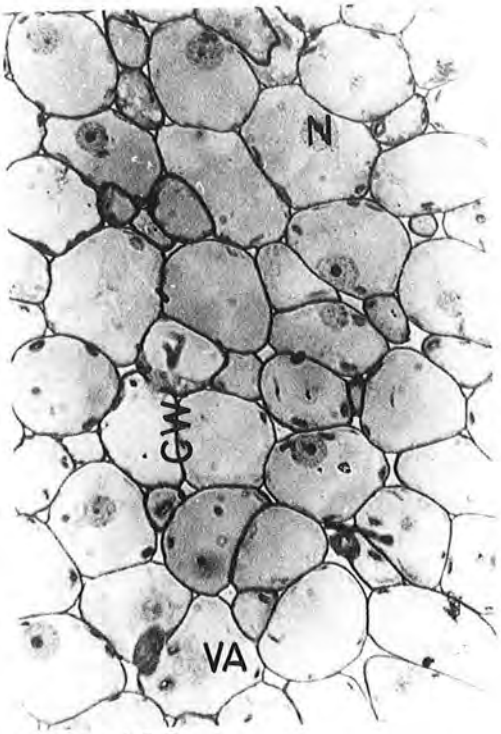


FIG.128

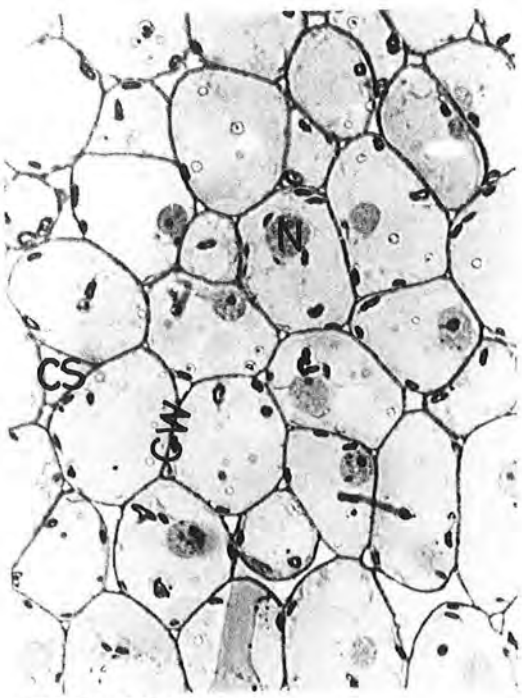


FIG.129

ALL

50 µm

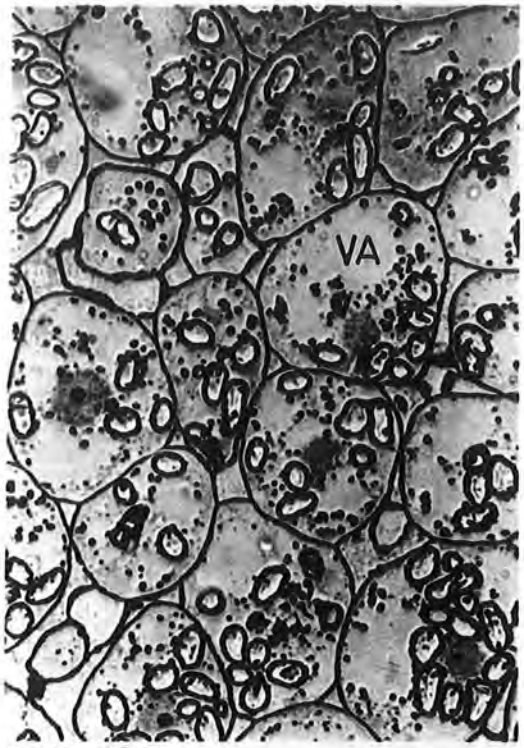


FIG.130

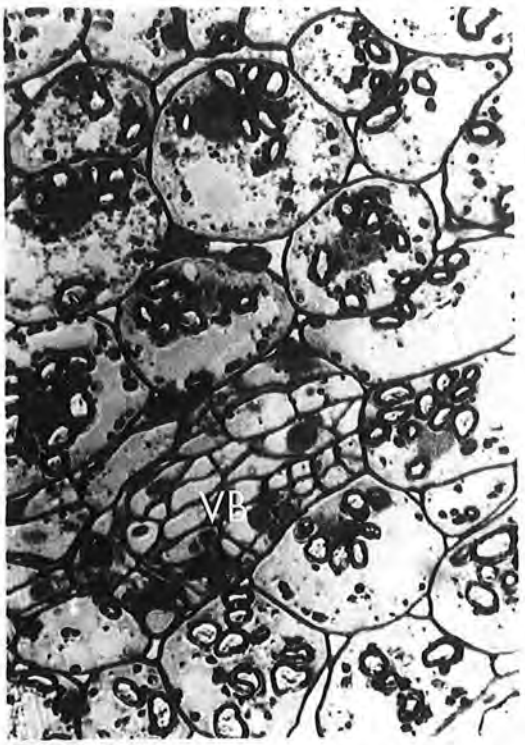


FIG.131

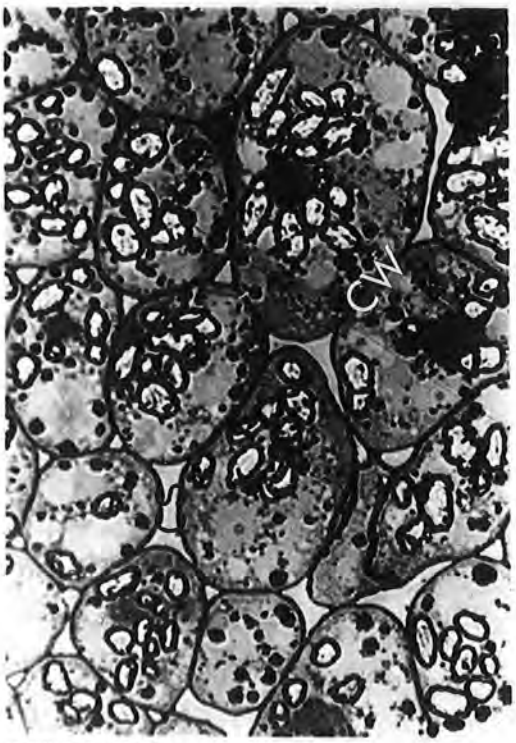


FIG.132

ALL

50 µm

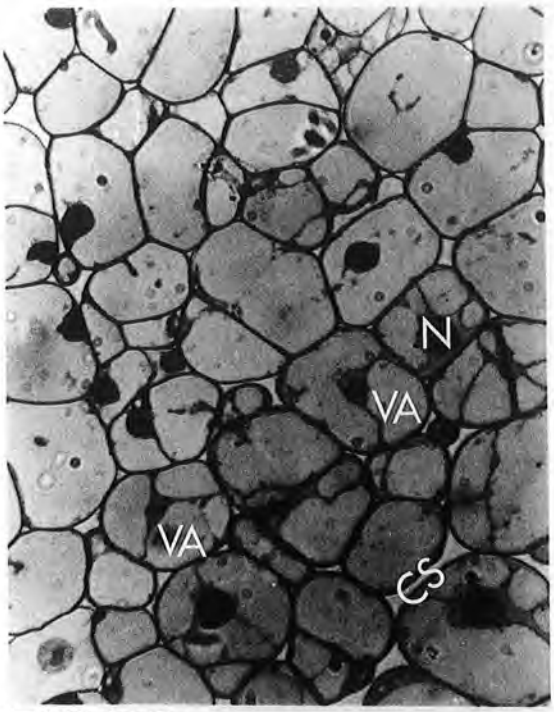


FIG.133

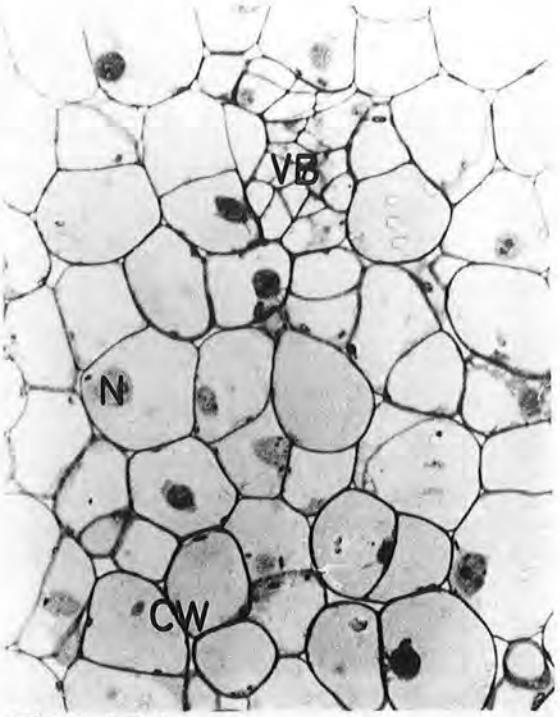


FIG.134

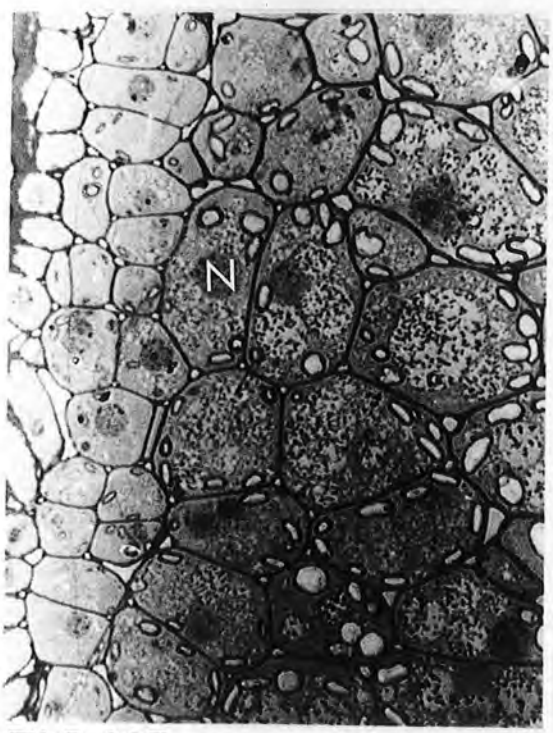


FIG.135

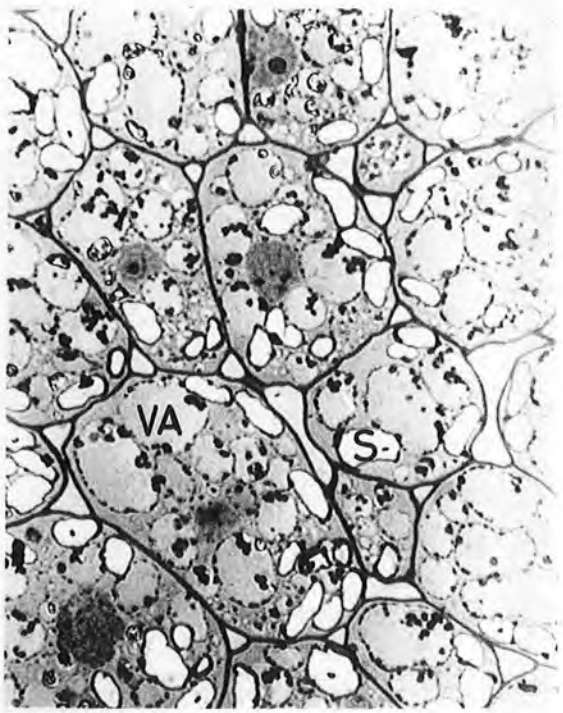


FIG.136

ALL 50 µm

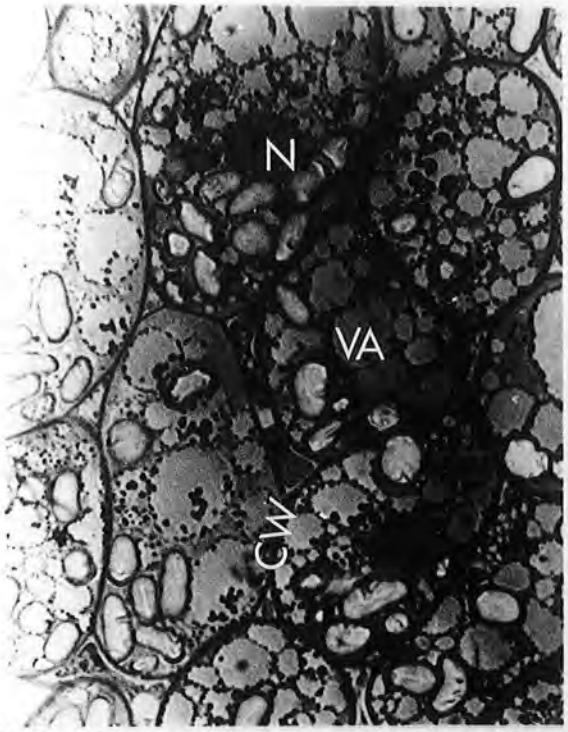


FIG.137

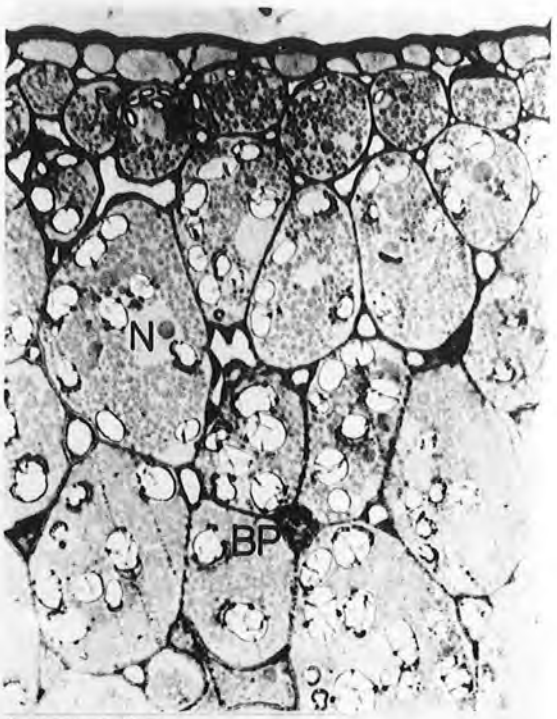


FIG.138

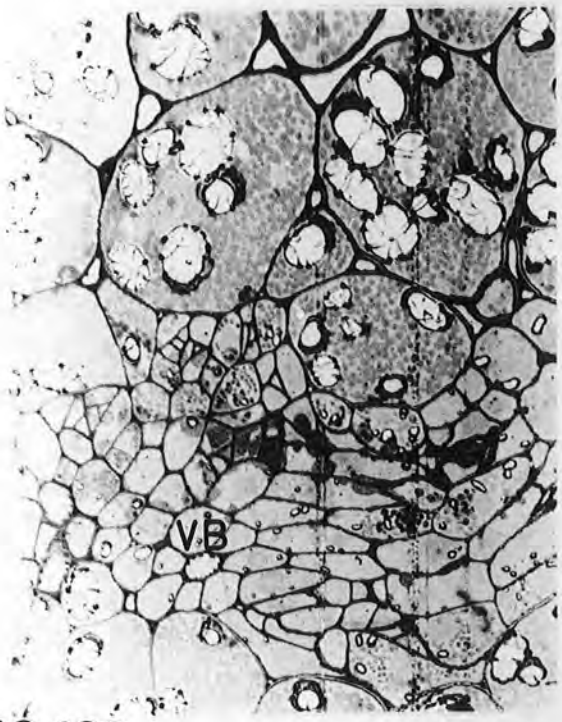


FIG.139

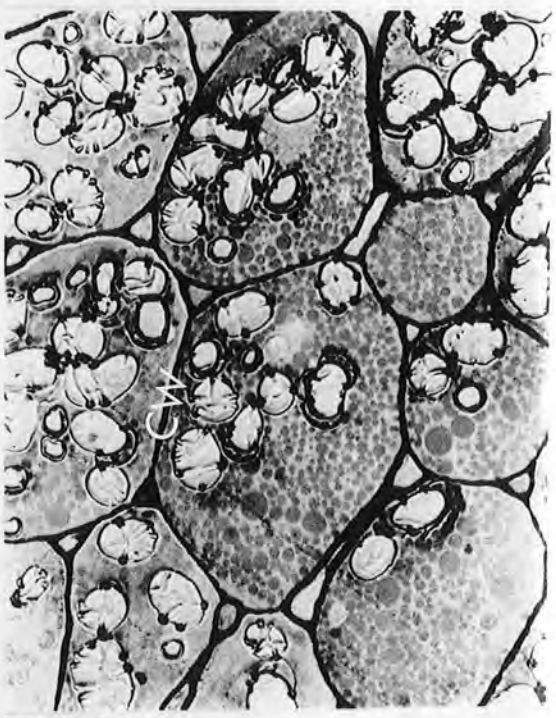


FIG.140

ALL 50 µm

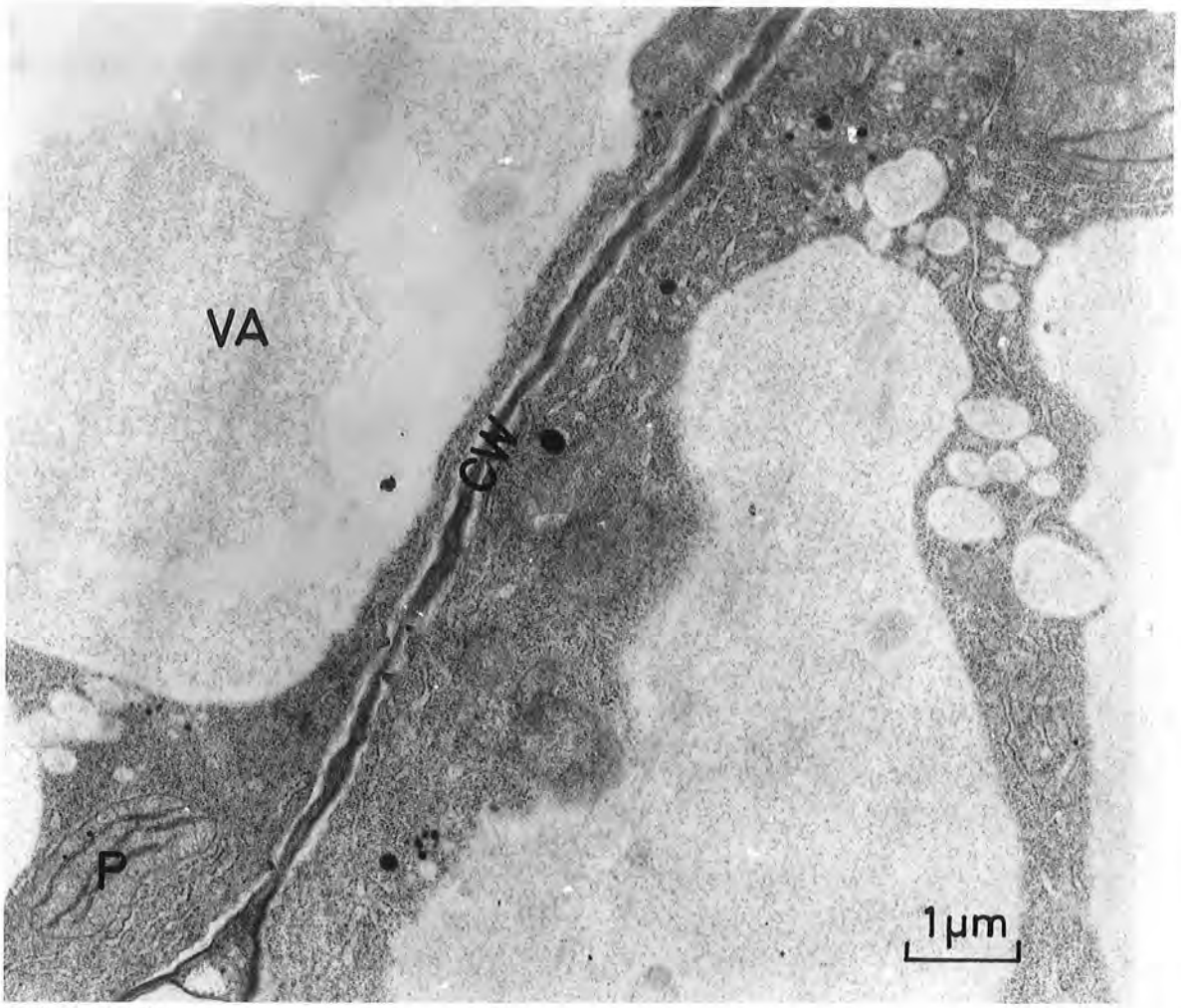
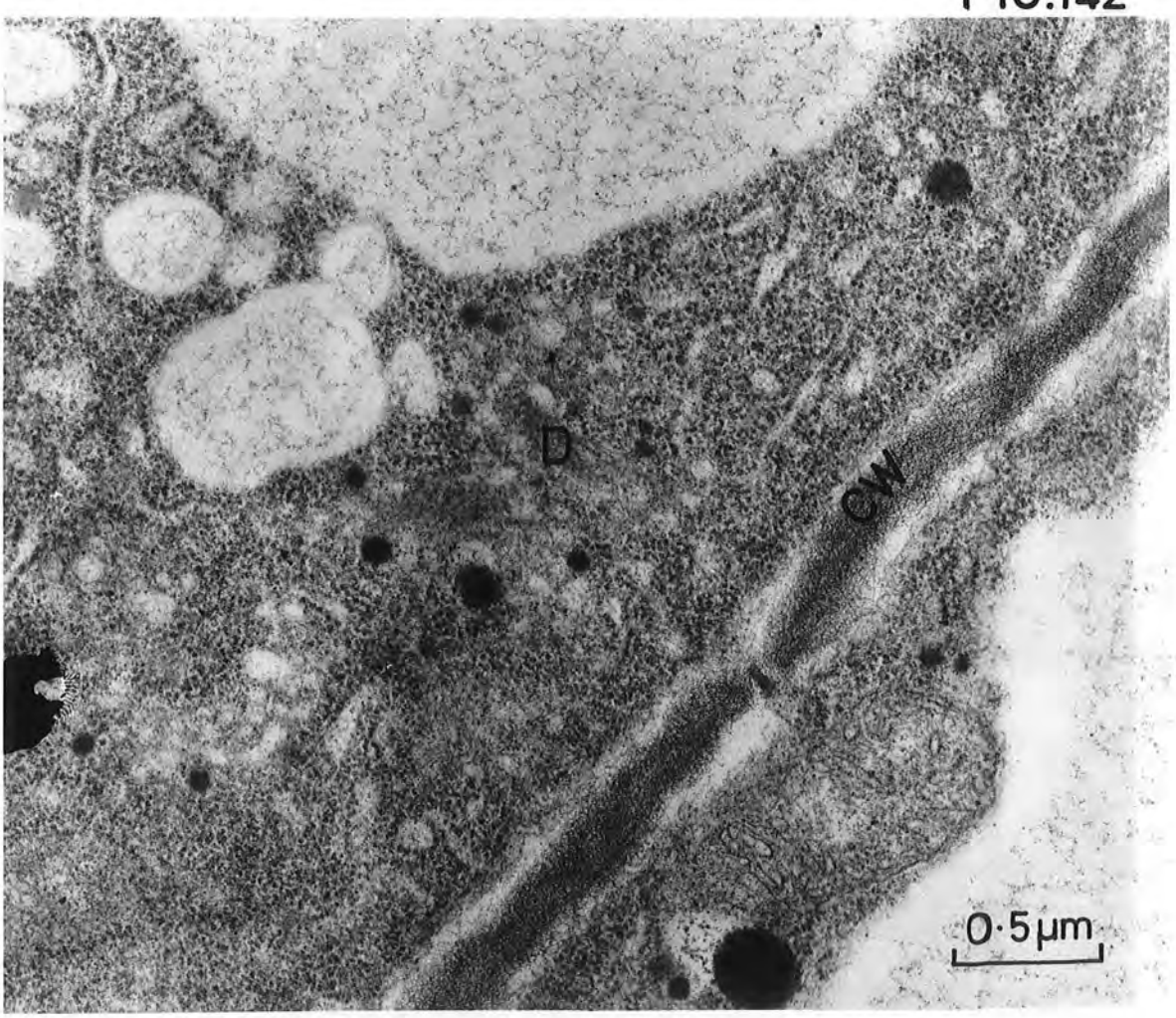


FIG.141

FIG.142



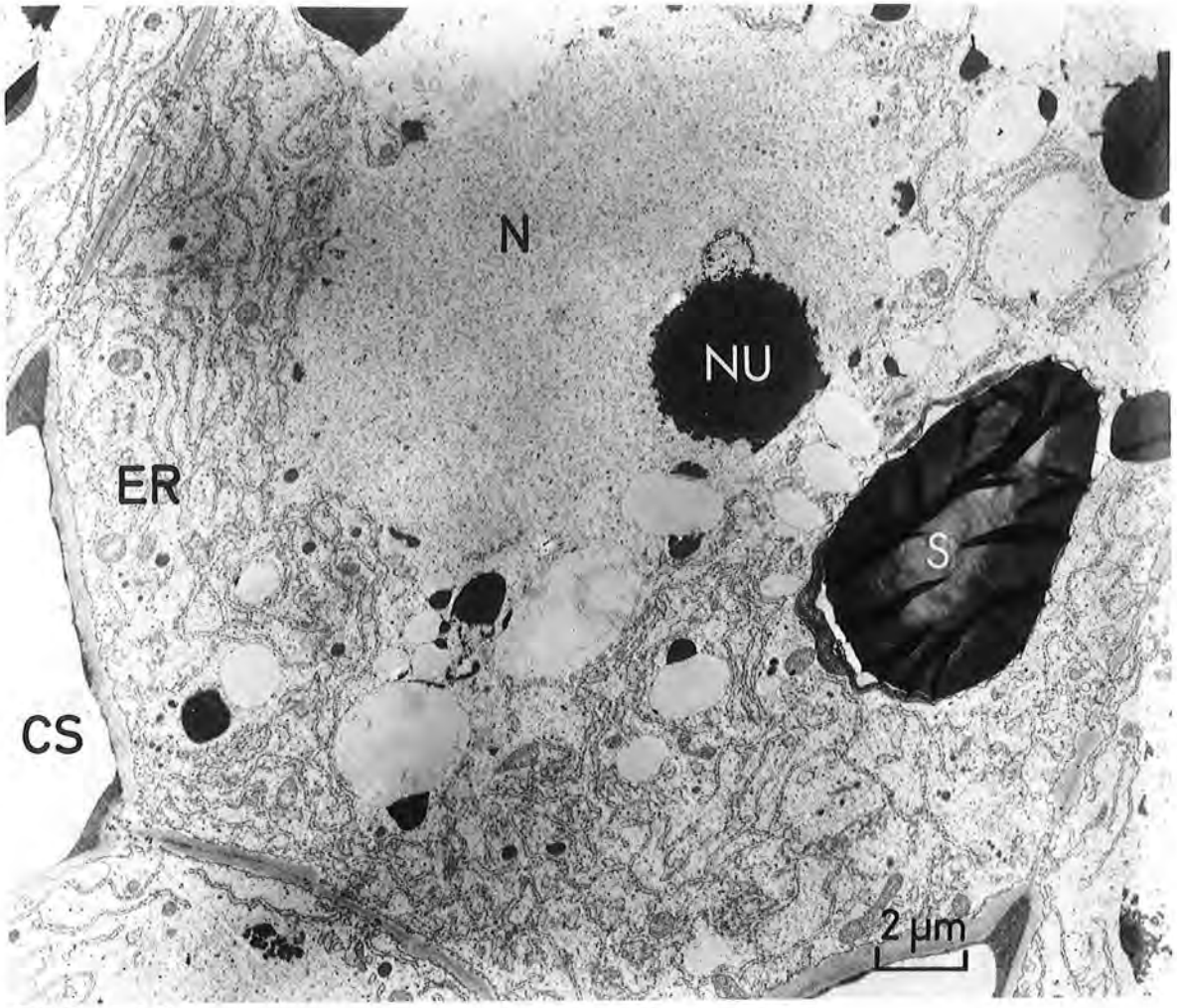
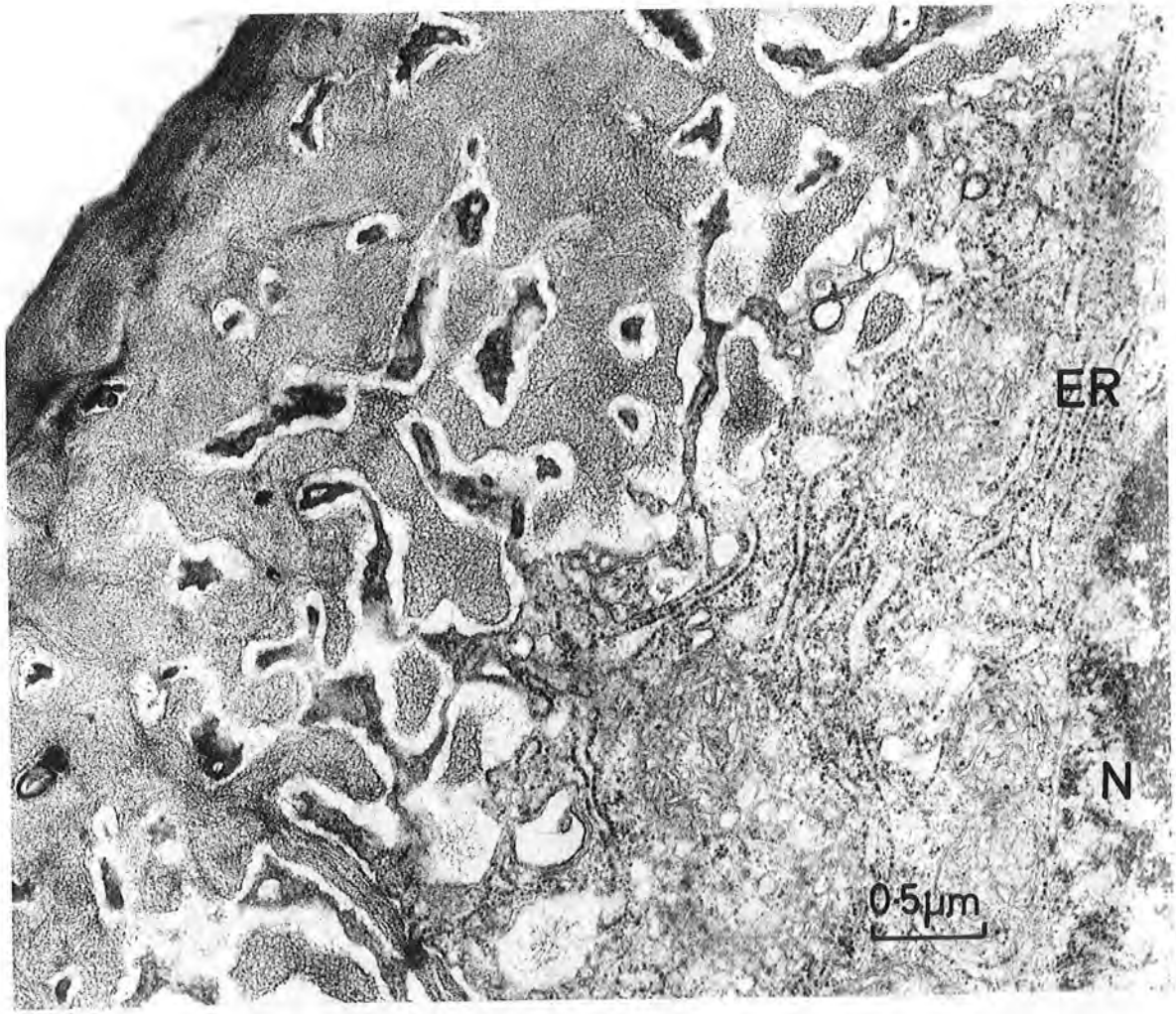


FIG.143

FIG.144



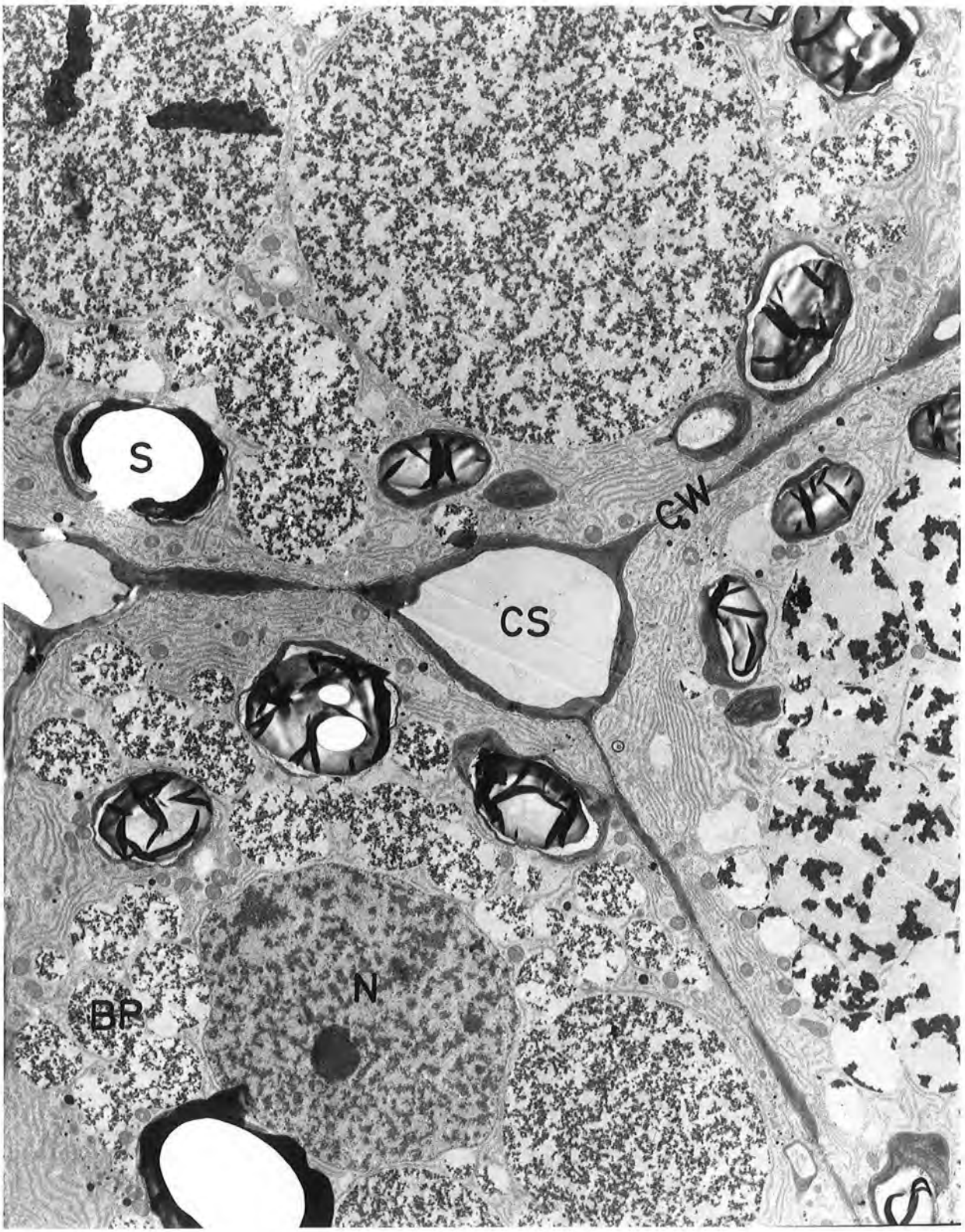


FIG.145

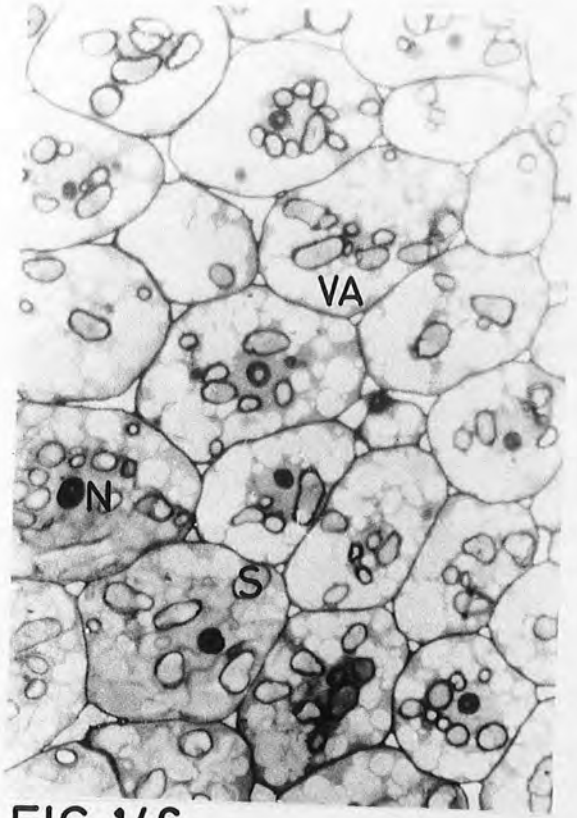


FIG. 146

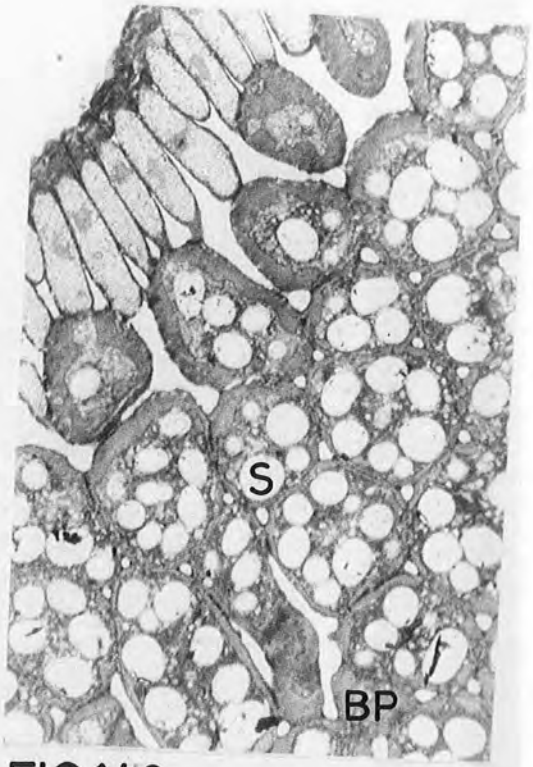


FIG. 148

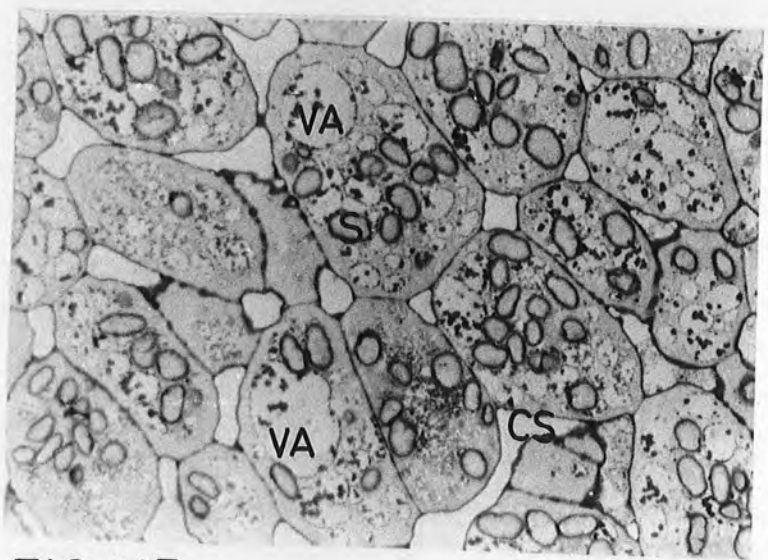


FIG. 147

ALL 50 µm

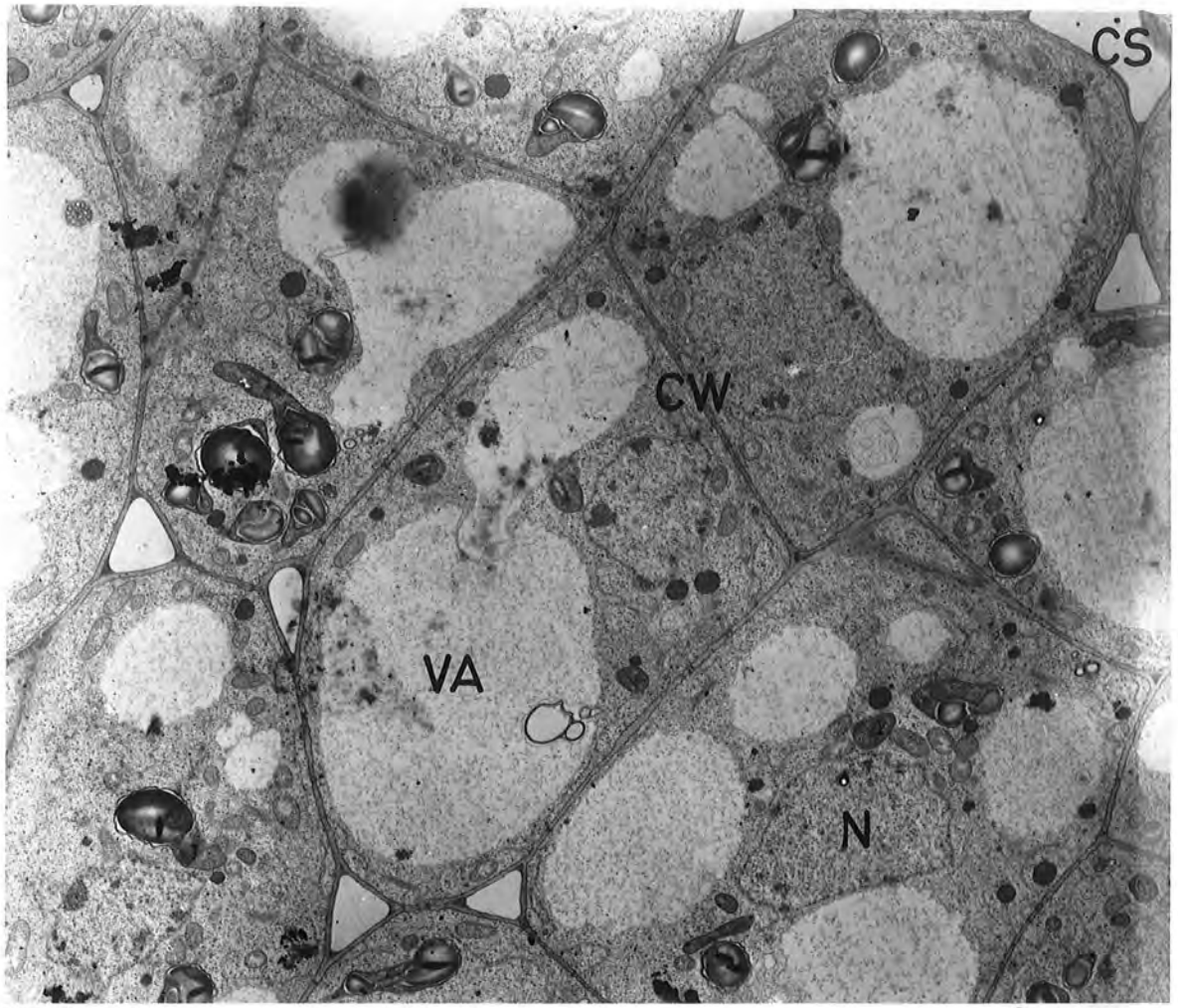
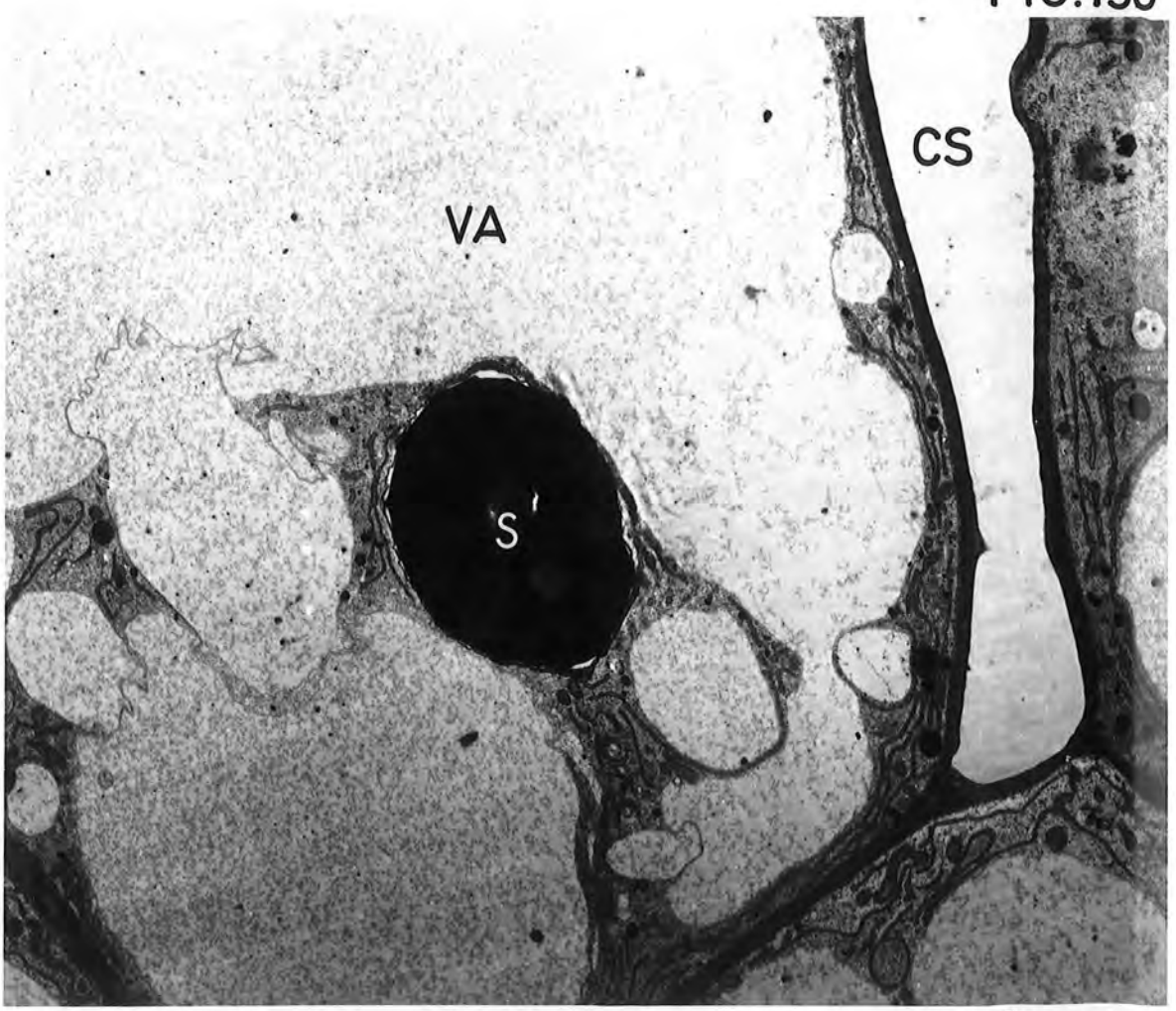


FIG. 149

2µm

FIG. 150



1µm

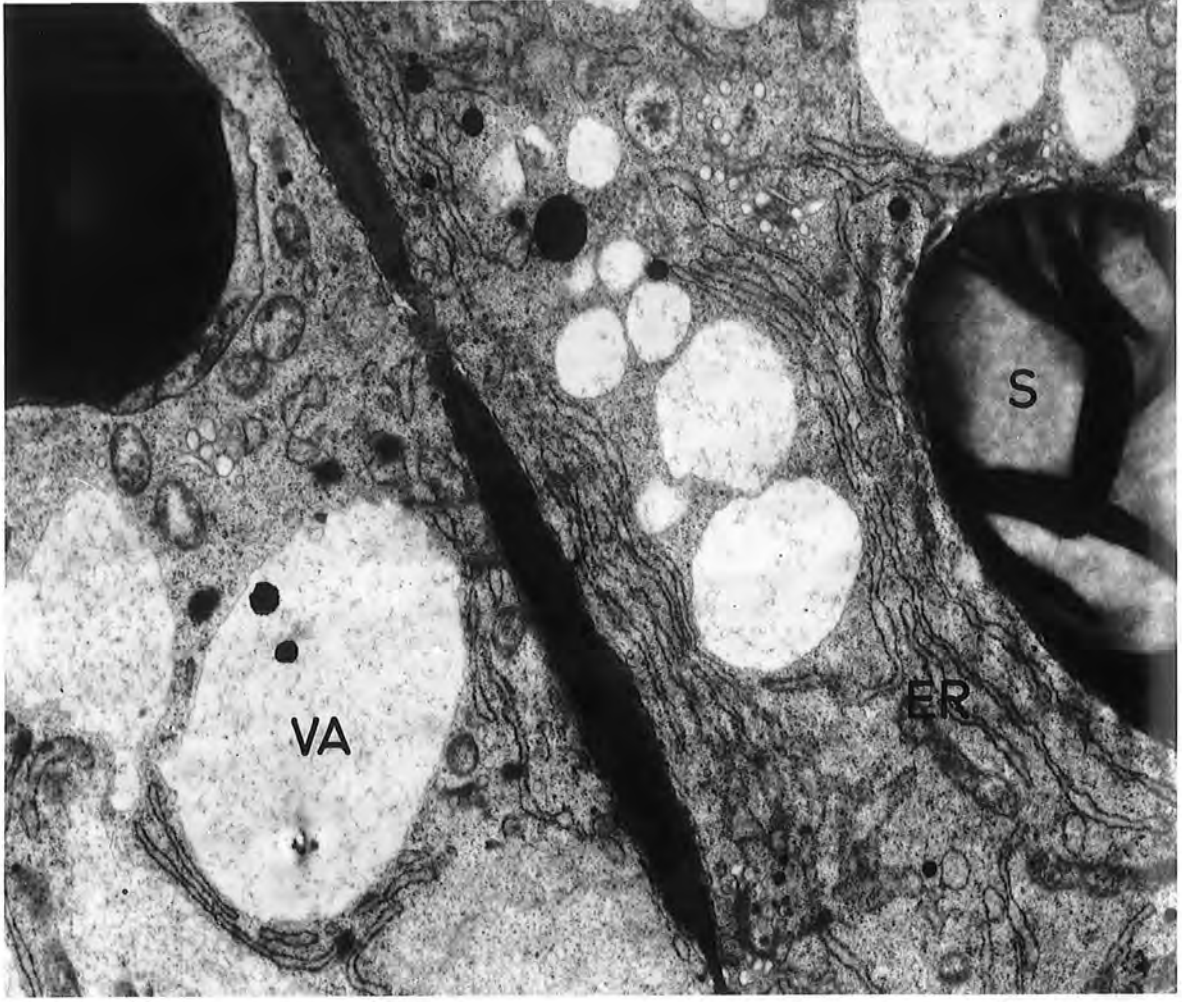
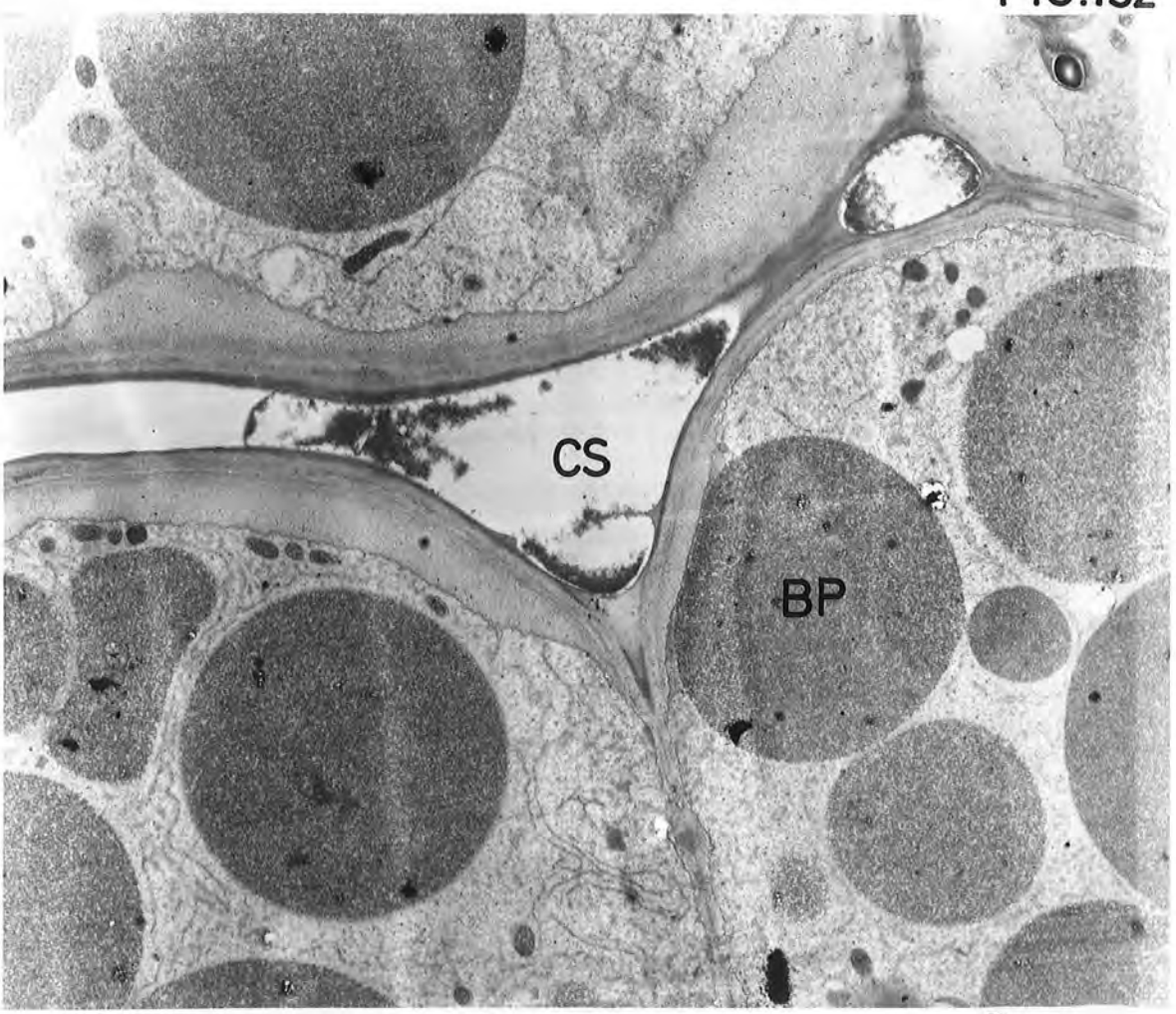


FIG. 151

1 µm

FIG.152



2 µm

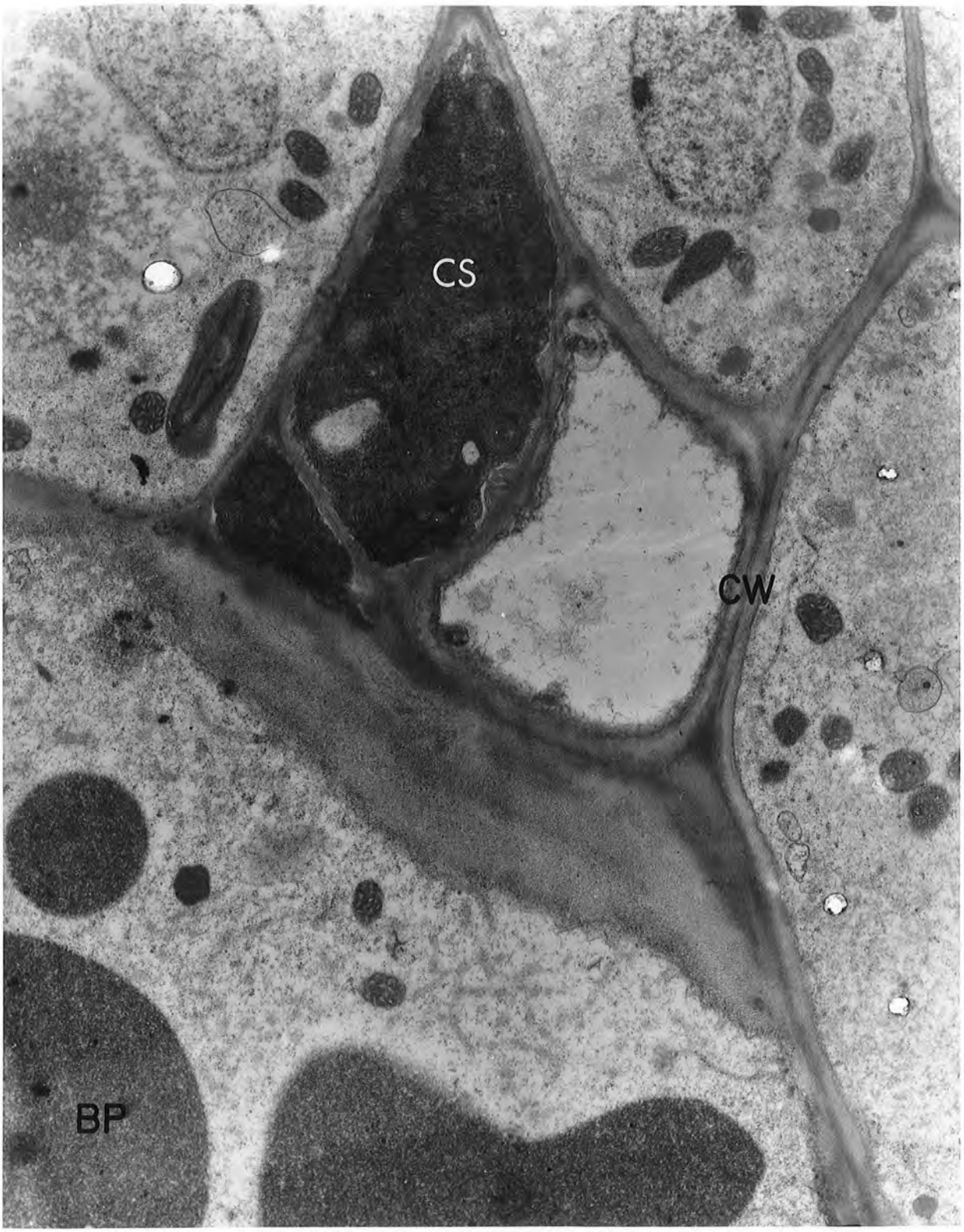


FIG.153

DISCUSSION

Legumes have been grown and used as a food source by man for more than 10,000 years, and are one of his most ancient food crops. Legumes are important agriculturally mainly for the production of seeds which are rich in protein, in some cases up to 50% on a dry weight basis. These seeds are used for human consumption in both the tropics and temperate regions and also for animal feed in the temperate zones. In some cases the whole plant of certain legume species is utilized as a fodder crop. Legumes also play an important role in improving soil fertility by replenishing nitrogen through root nodule associated fixation and have long been used as as free source of nitrogen input for mixed rotation forms of subsistence agriculture. The extent of protein malnutrition is a major and widespread problem in many developing countries. Deficiency of dietary protein is particularly harmful for children and has serious consequences in increased susceptibility to numerous diseases. In the 1960's fear was expressed that a world shortage of seed protein would increase and as part of the response biologists took an increasing interest in the process of synthesis and deposition of seed proteins which account for some 70% of the edible protein produced in the world. Reassessment of the minimum dietary protein requirements particularly in relation to carbohydrate intake, have reduced fear of a massive shortage of edible protein, however the research into seed proteins has continued in an effort to improve quality and productivity for both human and animal nutrition, particularly with regard to regions where seed proteins constitute the major protein food source. Information on their characteristics, their synthesis

and deposition are required along with studies of the controls operating at the genetic, biochemical and physiological levels that regulate the size of the storage protein fraction and the proportion of the individual proteins in this fraction. This knowledge will help to define the limits within which seed proteins can be modified and could indicate new selection criteria for plant breeding programmes relating to seed protein improvement.

The development of legume cotyledons has been described (Bain & Mercer (1966), Pisum sativum; Briarty et al. (1969) Vicia faba) as consisting of four phases (i) cell division; (ii) cell expansion; (iii) reserve biosynthesis; (iv) seed maturation and dormancy. These authors and others (Briarty (1973), Phaseolus vulgaris; Harris & Boulter (1976), Vigna unguiculata; and Opik (1968) P. vulgaris) have reported changes in vacuolar size and number during the development of legume cotyledon. Changes in the vacuolar system and cytoplasm of cotyledon parenchyma cells during the period of storage protein deposition in P. sativum were reported by Craig, Goodchild and Hardham (1979). In this study we have also found evidence of these four phases of development in all the legumes we examined under LM and EM, i.e. Pisum sativum, Vicia faba, Vigna unguiculata, and Phaseolus vulgaris. In addition we have shown that in these legumes deposition of storage deposits shows a uniform sequence of stages throughout the different regions of the storage tissue of the cotyledons with the first stage of deposition, following the phase of cell expansion, involving the synthesis of starch and then storage protein deposition.

The formation of protein bodies during the development of legumes has been followed by a number of workers in recent years (Bain &

Mercer (1966); Opik (1968); Briarty Coult & Boulter (1969)). It is generally agreed that protein fills vacuoles which are present from an early stage in seed development, forming the protein bodies of the mature seed. The proteins of the bodies have been widely investigated. In the case of the seeds of V. faba there is very strong evidence that the globulin proteins, vicilin and legumin, occur only in the protein bodies and consititute 90% of their total protein (Morris, 1969). The sites of synthesis and post-translational modifications of the protein remains uncertain. The suggestion of Morton and Raison (1964) that the protein bodies are equipped with their own protein synthesizing machinery has now been discounted (Wilson 1966). Payne & Boulter (1969) found very strong circumstantial evidence linking the formation of rough endoplasmic reticulum with the synthesis of the storage protein of V. faba ,as did Opik (1968), working with P. vulgaris. The origin of the protein bodies and their relationship to the large vacuoles of early development is uncertain (eg. Pernollet, 1978). There are two main hypotheses concerning the vacuolar changes. The first postulates that two distinct populations of cell vacuoles exist, the large empty vacuoles of the early stage of development being replaced by some small vacuoles as a result of sub-division and later protein absorption on the tonoplast of vacuoles, and are replaced by protein bodies at a later stage of development. It was thought that the protein synthesized on the ER and stored in the protein bodies was transferred between sites by vesicles arising from swellings at the ends of the ER. Recently Harris (1979) has shown that previously described small ER-derived vesicles are not in fact vesicles but profiles of large ER

tubules connected to the RER. Although many attempts have been made by investigators to account for protein transport and protein body formation the results were not very clear and they could not find a single and universal answer to this biochemical reaction.

Storage proteins are important storage components in legume seeds and of these proteins vicilin and legumin are particularly important. Each of these consists of a family of closely related sub-unit molecules. Two of the legumes in which the structural biochemistry of storage protein has been well studied are V. faba and P. sativum. We used these legumes as a model in our study. Many investigators have studied protein production and storage in different seeds and several attempts have been made to analyse the role of this substance in the seed and subsequently in plant development. The results of these detailed studies have revealed that not all the proteins produced in seeds are storage proteins, and sometimes it would be difficult to differentiate the non-storage from storage proteins. The study of biochemistry of protein synthesis and biochemical reactions in protein synthesis has established that the basic and main reactions are the same in all the systems when the protein is synthesized. This whole process of the synthesizing of the storage protein in legume seeds can be divided into three major systems, A) transcription, B) translation, and C) deposition of storage protein. In transcription several genes (in addition to those genes which are involved in the basic cell metabolism) play a main role. These genes are transcribed by RNA polymerase and then transported from the nucleus to the cytoplasm by RNA. At this stage they are associated with ribosomes. During translation the

association of aminoacyl RNA and ribosomes takes place and the information for the production of amino acids sequence is translated. Following these processes the proteins are produced. During his detailed study, Boulter (1981) showed the different sequences in this procedure. After the production of proteins they are deposited in the protein bodies. Many investigators have studied the exact place and method for this deposition and it has been suggested that these proteins are initially deposited in the rough endoplasmic reticulum (RER) (Bailey et al., 1970; Opik, 1968; Payne & Boulter, 1966). Then they are stored in the single membrane bound protein bodies. These organs have however no power in the synthesis of storage protein. The studies by immunofluorescent light microscopy have revealed that both vicilin and legumin-types of protein are stored in the same protein bodies, (Graham & Gunning (1970); Craig et al., 1979). These findings were supported by Harris during his study by EM (personal communication). After the protein has been stored in the special 'protein bodies' the main task in the seed development is its secretion into the system when it is needed (during germination). It is clear from many studies including our own that during the seed development a phase of cell division is followed by a stage in which the cells enlarge and develop a specific cytoplasm containing a few large empty vacuoles. During the later phase of storage synthesis these large vacuoles will be eliminated to give room to the much smaller vacuoles in which the protein will be stored (Harris & Boulter, 1976). We could show this phase in V. faba, and V. unguiculata. However, Neuman et al., (1978) in their studies on V. faba could not show any relationship and development continuing

between vacuoles and protein bodies.

It should be emphasised here that in this thesis, discussion of the origin of protein bodies refers to those in the cotyledonary leaves; other protein bodies, such as those in cereal development or in legume embryonic axis, might have completely different origins.

Protein bodies are membrane bound organelles, normally spherical, which are usually located in cotyledons. The size and shape of these organelles varies from one legume to another and are mostly about 1 - 10μm in diameter surrounded by a single membrane. Protein is not the only material which exists inside the protein bodies as they contain other substances such as phytin and different minerals which are usually magnesium or calcium salts of inositol hexaphosphoric acid. However, as most of the studies on the structure of protein bodies have been carried out qualitatively using the light or EM microscope the true nature of these substances and the purity of them must be considered cautiously.

The formation of protein bodies is a subject on which there are different views. Generally, however, during the cell development the large vacuoles are replaced by numerous protein bodies containing storage protein. This process may take place following several pathways. For instance, it is possible that the large vacuoles may be divided into organelles which then can receive protein from Golgi bodies. Another possibility is that after the large vacuoles have been dismantled, the protein bodies may be formed by merging of ER and Golgi vesicles. Some investigators believe that protein bodies can be formed directly from ER. However, whatever the procedure is, the protein bodies of legume cotyledons do not contain ribosomes on their

surface membrane. A dual origin of protein bodies was reported by Bain & Mercer (1960).

We have made a comparative examination of a range of legume seeds to determine common features of storage parenchyma development and also the points of difference which could give information on the possible differences in synthesis and deposition of the different storage proteins. The developing seeds have also been examined to determine whether different patterns of protein deposition occur which may be related to the different and specific patterns of protein mobilization which occur during seed germination. The structure of pod and seed coat have also been examined in some cases to illustrate possible routes for nutrient flow into the developing seeds.

Our results show changes in pod wall structure during seed development which indicate a significant role as a potential reservoir for nutrient supply for early embryo development, and secondly, as a barrier to dehydration of the developing seed. Dehydration is a considerable problem during the early and mid phases of the seed development but is an essential part of the later seed maturation. Development of the vascular supply from pod to developing seed in Pisum sativum has been examined in detail by Hardham (1976) and our work has built upon this. We examined the movement of tracers in the vacuolar supply network from pod to seed, and also the changing morphology of the developing seed coat particularly in relation to the embryo inside.

The structure of the seed coat was studied in pea and cowpea. The results show that the seed coat in these legumes has in general a similar pattern, however, there are significant

differences in the structure of the layer(s) adjacent to the embryo sac boundary wall. The results also show that in the domestic type studied the seeds were larger and with a different form of seed coat The study of pea and cow pea could not reveal the definite role of the testa regarding the control of growth. Ballard (1973) in his study on the legumes' seed coat, showed its major role in the size and growth of the seeds. We also examined the seed coat to find features of importance in controlling the exchange of water and nutrient between plant and seed during the development of the seed embryo. The surrounding seed coat undergoes considerable change during the course of seed development.

We also studied the structure of the pea embryo and its relationship to its environment. The general pattern of development is shown by LM and EM. Following fertilization the embryo suspensor elongates with the embryo being pushed from the micropyl ar end of the embryo sac. During this time the embryo sac enlarges and endosperm persists as a lining around the embryo sac boundary wall. The embryo then enlarges to firstly a globular form and then a heart-shaped embryo which, while still attached to the suspensor, enlarges to form an embryo with radicle, plumule, and two cotyledons attached at the hypocotyl. During this development the suspensor is crushed as the radicle enlarges and enters a radicle pocket of the embryo sac boundary wall. The endosperm is lost as the cotyledons enlarge and fill the majority of the embryo sac. Comparison of the boundary-sac wall in the micrographs indicates that there are different degrees of wall and modification at different regions of the embryo sac. The extent of the development of the wall is far greater near the apex of

the embryo sac and around the radicle pocket than at the wall on the opposite side to the embryo pocket. Marinos (1970) and Hardham (1976) also studied the early development of pea embryo. They suggested the formation of extra boundary wall but our results did not show any such extra boundary wall.

As the result of examination of the early embryo of pea we could show that the main seed coat layers and embryo sac boundary wall have an important role in nutrient pathway during the first period of early development. The endosperm seen lining the embryo sac boundary wall contains many organelles and small vacuoles; many mitochondria are seen in the endosperm cytoplasm particularly adjacent to the protrusions of the boundary wall. The greatest densities of mitochondria are found near the boundary wall below the main seed coat vascular bundle and around the radicle pocket region. Between the numerous small vacuoles the cytoplasm contains many ribosomes and polyribosomes. In the suspensor, immature plastids with a well developed peripheral reticulum are found scattered throughout the cytoplasm. The micrographs also showed evidence of plasmalemma function. It is apparently smooth and there are few vesicles in close proximity indicating trans-membrane transport rather than pino cytotic movement of metabolites. The suspensor has very dense cytoplasm, with many ribosomes and mitochondria with large and closely packed c ristae. These ultrastructural features emphasise the role of certain regions of embryo sac boundary wall and also the suspensor in the nutrient transportation during the development of the early embryo. The circumstantial evidence from electron microscopy is supported by the results we obtained from the fluorescent tracers ANS

and fluorescein used to examine the possible routes of the nutrients and metabolites into the developing seed. The results all indicate movement both through the apoplast and symplast. We found with these tracers that both apoplastic and symplastic routes are available for transport of nutrient from the pod to the seed coat with the accumulation at the loose parenchyma layer lining the embryo sac boundary wall only in the region of the embryo. Unloading of the symplastic route with apoplastic transport to the developing embryo is also indicated.

The cells of the developing embryo a few days after fertilization are typically meristematic in their ultrastructural appearance. There are few vacuoles and the high proportion of cytoplasm is filled with numerous organelles and ribosomes. The cell walls are very thin, the mitochondria of the embryo are smaller than those of the suspensor cell and contain conspicuous nucleoid regions between well developed c ristae. The plastids show differentiation of a thylakoid system although granal stacking is limited to only a few discs. Development of a few cisternal ER profiles is evident although the vast majority of ribosomes and polyribosomes are free in the cytoplasm. Dictyosomes of the Golgi's apparatus are present although in the cell adjacent to the suspensor the contents of dictyosome vesicles do not stain. The globular embryo develops to a heart-shaped embryo. The cotyledon leaves then develop to form the important food storage organs of the developing seed.

The results from light microscopy examination of developing pea cotyledons indicated that there is a uniformity in the rate of devlopment of storage reserves throughout the cotyledon. A comparison

of different parts of cotyledon was made with cells tken from regions close to both adaxial and abaxial epidermis and also from the central region. Our results indicate there is some difference in cell size and the extent of intercellular spaces. To qualify this result we used stereological techniques to examine the percentage of cell and space at each region throughout the cotyledons. We also examined the extent of the cell packing as indicated by the ratio of cell wall contact with other cells as opposed to cell wall to intercellular space. We found that the cells between the abaxial epidermis and vascular bundle are more densely packed with smaller volume of intercellular space than the cells between the vascular bundle and adaxial part of the cotyledon.

The ultrastructural detail of cell form was examined by transmission electron microscopy. The results show that the cotyledon cells at 7 DAF are completing the phase of cell division and commencing a phase during which there is considerable cell expansion. At this stage nucleoli are usually characterised by a hollow sphere form with distinct fibrillar and granular regions indicative of high rates of formation of pre-ribosomal particles. It is also possible to observe several mitochondria and some ER at this stage. Plastids are present in all cells and contain developing thylakoids although the peripheral reticulum is not highly developed. At this early stage we also found several dark bodies and clusters of smaller similarly stained vesicles; these electron dense deposits are always associated with dictyosomes and are membrane bound. Work with similar deposit in V. faba and cowpea has shown that the vesicle contents are pronase digestible. At 7 DAF we could see high proportions of cytoplasmic

polyribosomes. After the initial cell expansion (9 DAF) the extent of vacuolation in the parenchyma cells is increased with the development of a few large vacuoles which restrict the cytoplasm to a layer lining the wall. Nuclei are generally found in the middle of the cells, separated by a few transvacuolar strands and surrounded by the large vacuoles.

An increase in RER is apparent between 1 - 10 DAF and an increase in tubular ER with some attached ribosomes to mostly cisternal ER. At 10 DAF the storage parenchyma cells have changed markedly and the cells no longer contain a few large vacuoles but a larger population of smaller vacuoles and vesicles. All cells contain small densely-stained membrane bound deposits in the cytoplasm and similar material in all of the vacuoles and vesicles often associated with the vesicle membrane (tonoplast). These deposits are the early stage of protein deposition, histochemically identified by toluidine blue staining at light microscopy. At this stage the cytoplasm contains much more RER, and there are now few cytoplasmic polyribosomes. Numerous dictyosomes are present throughout the cytoplasm and they are characterised by clusters of electron dense vesicles.

By 12 DAF light microscopy shows that most cells contain protein bodies filled with less densely stained material. The storage parenchyma cells have changed in ultrastructural appearance with the vacuoles and vesicles now containing dispersed electron dense material rather than the dense peripheral deposits. Many of the nuclei have become amoeboid in shape with numerous projections and invaginations of the nuclear envelope and in some cases have become multi-nucleolate.

Synthesis of storage proteins and their deposition into the vacuolar protein bodies occurs for a few more days and during this period protein bodies fill with protein so that each has a uniform amorphous appearance.

By 20 DAF most cells are filled with protein, the cell walls are thicker than at the earlier stage and there is more intercellular space at the adaxial part of the cotyledon. From 20 DAF the seed undergoes a phase of maturation with cessation of biosynthetic activity and a reduction in fresh weight. The protein bodies become more uniformly spherical and the cytoplasm contains only a small amount of ER, although numerous polyribosomes are still evident. The biochemical changes, as well as formation of RER or SER are not very clear at the later stage of the cotyledon development because the study of these tissues is much more difficult. Our study of different legumes agrees with the results of other investigators in that the amount of RER is decreased at the maturity stage.

During seed development a small number of large apparently empty vacuoles in storage parenchyma cells are replaced by a large number of small bodies densely packed with protinaceous material. The mechanism of this transition is not clear. There may be two distinct populations of cell vacuoles, one consisting of large fluid vacuoles that are degraded and disappear during development and are replaced by a second population of ER or dictyosome-derived vesicles or vacuoles containing protein synthesized on the ER cisternae, as proposed by Harris & Boulter (1976), Khoo & Wolf (1970), and Savelberg & Van Parijis (1971). A second possible interpretation is that the protein

bodies fragment to form a small number of small protein-filled bodies as proposed by Bain & Mercer (1966), Opik (1968), Briarty (1969), and Rest & Vaughan (1972). In both interpretations, however, the large vacuoles that are initially present are lost during development and much additional tonoplast is produced. Craig, Goodchild and Miller (1979), describe the three-dimensional morphology of the vacuoles and protein bodies at various stages of pea cotyledon development and a possible relationship between these structures, and conclude that protein bodies arose by sub-division of the large vacuoles. They found multi-armed vacuoles which they claimed cut off the numerous protein bodies. Work in our laboratory indicates that such multi-armed vacuoles can be produced as fixation artefacts even at later stages of seed maturation if the wrong molarity is used with fixation (Harris, unpublished). Thus the ontogeny of protein bodies remains unresolved in a range of legumes despite recent papers (eg. Adler & Muntz, 1983).

Evidence that the storage proteins are synthesized at the RER comes from previous work which first illustrated the incorporation of labelled amino acids into protein bodies (Bailey, Cobb & Boulter (1970)), and subseqently the incorportion of labelled amino acids into storage protein sub-units (Baumgarther, Tokayasu & Chrispeels (1980)). The mechanism of protein transport from RER to protein bodies was assumed to be by vesicular transport directly from the RER. In thin section electon micrographs occasional swellings are seen at the ends of the RER and it was thought that these then separated and acted as carrier intermediates. Few such vesicles are seen free in the cytoplasm in micrographs of material at the potein synthesizing phase

but this could be explained by fixation time being longer than the transport/fusion to tonoplast period.

Comparative studies using thin and thick EM sections show the possibility of wrong interpretation about ER structure and the role of RER in protein synthesis and deposition absorption. In the thin sections pro-vesicles are found at the ends of RER. It is apparent that these 'vesicles' are simply due to the connections of tubular ER and cisternae which is very common especially during the protein formation (Harris, 1979). The thick section micrographs also show the extent of the tubular SER at this stage of development to be much greater than is apparent in thin sections. Harris (1979) suggested that this interconnection of the lumen of the cisternal and tubular ER may act as a route for the transport of different substances within the cell with the tubular ER being closely related to the dictyosomes which, because of their number, could also play a major role in this process. He describes the presence of a considerable number of dictyosome cisternae with electron dense vesicles, during the protein deposition phase of cell devlopment. The results presented above also indicate the roles of the ER and Golgi apparatus in storage protein synthesis and absorption. They indicate that in storage protein synthesis and deposition, protein body formation is by the development of cytoplasmic vesicles and vacuoles with the possibility of a minor component division of vacuoles. The ER and Golgi apparatus act in synthesis and translation of proteins to make the protein bodies.

In animal cells, it is now well established that the secretory proteins are synthesized on RER then moved to the Golgi apparatus from

where they enter condensing vacuoles for secretion from the cell. Crispeels, 1976, considers deposition of storage protein as a special case of protein secretion and concludes that the available evidence, although equivocal, supports the RER-Golgi apparatus sequence. In his recent work, Chrispeels (1983) indicates a direct role of the dictyosomes in post-translational modification of storage protein.

Cowpea: Vigna unguiculata

The development and changes in the cotyledon cells of cowpea were also examined at a cytological level and particular attention was paid to changes in cell vacuoles and rate and distribution of storage proteins in parenchyma cells. The four phases of development are similar to those of pea.

Light microscopy of the seed coat of the young developing cowpea, however, showed a substantially different structure to that of the pea seed coat. The inner seed coat layer was found to be a columnar epidermal layer in contrast to the loose epidermal layer of pea (Marinos, 1970; results above). The cowpea seed coat has been examined in greater detail by electron microscopy. The general form of the cells of the inner seed coat layer of cowpea was of columnar cells with dense cytoplasmic contents. The cells contain interphase nucleus, numerous plastids, often of an immature amoeboid form, dictyosomes, ER and numerous small vacuoles/vesicles rather than one or a few large vacuoles. The cell walls are penetrated by large numbers of plasmodesmata connecting neighbouring columnar cells. The cell wall at the apex of the columnar cells is a two layered structure with loosely woven fibrils. Close to the cell apices lies the embryo sac boundary wall with the space between containing loose fabrillar

material with some electron-dense aggregates. On the inner side of the embryo sac boundary wall is a thin lining of endosperm cytoplasm. The embryo sac boundary wall does not contain any wall processes of the typical transfer cell type, however there are occasional electron-dense thickenings which are usually associated with pockets of plasmalemma into the endosperm cytoplasm. The multinucleate endosperm contains numerous mitochondria and polyribosomes both free and bound to ER. As the embryo develops the endosperm contents degenerate into a granular mass. There is no development of cell wall processes during this time on the columnar apices or of the embryo sac boundary wall. The ultrastructural evidence suggests a quite different form of nutrient loading of the embryo sac to that which is seen in peas.

The pattern of protein deposition in cowpea is essentially similar to that described above for pea, and by other workers studying legume seed development. The development of cotyledon storage parenchyma cells consists of four phases, i) cell formation, ii) cell expansion, iii) synthesis, and iv) maturation and dormancy. Each phase was characterised by a distinctive sub-cellular organisation of the parenchyma cells. At the earliest stage examined the parenchyma cells of the cowpea cotyledon were highly vacuolate with a thin lining of cytoplasm to the cell wall and few transcellular strands or membranes. Later we observed that division of the main vacuoles was apparent in the cotyledon cells with some cells showing cytoplasmic strands penetrating the vacuole. The cytoplasm lining the cell wall contained some unbound cytoplasmic ribosomes, ER and occasionally small dictyosomes. Later at 18 DAF cotyledon parenchyma cells were characterised by more, smaller vacuoles which contained stainable

aggregate generally appressed to the tonoplast. At this time the nucleus is characterised by a reticulate granular pattern. At 25 DAF protein deposition was apparent in cotyledon cells. Cells showed electron-dense preciptiations both in cytoplasmic vesicles and vacuoles which are termed protein bodies from this point. More RER and Golgi bodies with vesicles containing electron-dense material were present throughout the cotyledons. The Golgi were found throughout each cell cytoplasm and not just adjacent to the cell wall. The evidence suggests an active role of RER and Golgi in development and transformation of protein to protein bodies. The nucleoli at 25 DAF have retained the staining pattern present earlier, although some nuclei have taken a highly amoeboid form with numerous projections and invaginations of the nuclear envelope. By 30 DAF each cell is filled almost entirely by a fairly uniform population of protein bodies with few dictyosomes and only small amounts of ER remaining. Cell wall changes occurred during the development of storage parenchyma cells. The parenchyma cell walls showed increased thickening during seed development and also an increase in intercellular space.

Vicia faba

Structural changes in cotyledon cells of Vicia faba during seed development were examined using varieties of V. faba. Two varieties were chosen for comparison because of their different rates of development; Sudanese Triple White was faster with regard to rate of flowering and maturity than Maris Bead. The main features of the ultrastructural differentiation of storage parenchyma cells of both varieties of V. faba are similar to the sequence described for Pisum

and Vigna. The sequence to maturity of Sudanese Triple White storage parenchyma cells follows the pattern described previously for other legumes, with a progressive filling of the protein bodies to an amorphous, uniformly staining material and reduction in the extent of cytoplasmic organelles such as mitochondria and ER just prior to seed maturation.

Prior to protein deposition cytoplasmic vesicles and small vacuoles formed within the cytoplasm. Rough ER was conspicuous and once protein deposition had commenced numerous dictyosomes with associated vesicles were also observed. Storage proteins were deposited within the forming vacuoles. Extra wall material is seen laid down on the wall adjacent to the intercellular space, which during the course of seed development has increased significantly. Plasmodesmatal connections between developing storage parenchyma cells are restricted to pit field regions and the limited increase in cell wall thickening is seen on the other wall to wall regions.

A similar but slower pattern of ultrastructural change was observed for slower maturing Maris Bead. Comparison of the ultrastructural detail of the cells containing the different types of protein body indicate that there are no other apparent differences in the form of distribution of the other organelles. The subsequent maturity of the storage parenchyma cell follows the pattern described previously for other legumes, with protein bodies progressively filling the cell. The only difference between the two varieties was that Maris Bead has a slower rate of maturity.

Phaseolus vulgaris

Study of the development of the storage parenchyma tissues of

cotyledons of P. vulgaris, showed that the sequence of development was generally as described above for Pisum, Vigna and V. faba with four phases of development. Examination of tissues from the different regions of the cotyledons showed a uniform sequence of stages throughout the storage tissue. The development of the storage parenchyma tissues of cotyledons of P. vulgaris was also studied. Generally the sequence of development was as described for Pisum, Vigna and Vicia, but unlike Pisum and Vicia, where protein is initially as dense deposits associated with the tonoplast membrane of large vacuoles, in Phaseolus sub-division of large vacuoles occurred before protein deposition. The dense deposits were then followed by a more uniform amorphous protein within the protein body. Also the nucleoli of storage parenchyma cell nuclei did not contain a large vacuolar region as is the case in Pisum and Vicia at this stage. Instead there are a few, small clear regions in otherwise densely stained nucleoli. Protein deposition within the vacuoles is initially in the form of dense granular deposits frequently associated with the tonoplast membranes as our results showed. There were some unusual features of storage parenchyma development in P. vulgaris; for example, the early deposition of starch at a stage when the cytoplasmic volume is increasing, and the presence of numerous free ribosomes but relatively little RER. Later, conspicuous RER is seen in the cytoplasm as well as dictyosomes although the replacement of the few large vacuoles by more smaller vesicles and vacuoles occurred as before, electron dense deposits of a form and size previously identified as protein were seen in the cells. Both LM and EM showed that extra wall material is laid down adjacent to the inter-

cellular space during the course of seed development. The extra wall thickening was also found on parenchyma cell walls adjacent to vacuolar phloem tissue, but not always adjacent to differentiated xylem.

The results of our study of a range of legume seeds show evidence that morphological and cytological changes during seed development follow a generally similar pattern.

Microscopical and stereological results indicate significant changes in the form of P. satvium storage parenchyma cell packing during cell development as well as differences between the abaxial and adaxial regions of the cotyledon. These latter differences explain the variation in appearance of cotyledon slices even though the cellular differentiation is relatively synchronised. The changes in cell contacts, with either other cells or intercellular spaces, indicates a substantial increase in the potential of an apoplastic pathway for movement of nutrients within the cotyledon, just prior to and during the early stages of protein deposition.

The development of transfer cells at the abaxial epidermis might imply a symplastic uptake of nutrients supplied from the seed coat, although the wall thickening may be related solely to protective functions (the epidermal cells contain little storage material even in the mature seed).

The anatomy of seed coat tissues and evidence from fluorescent tracers show that both apoplastic and symplastic routes are available for transport of nutrient from pod to seed coat. Unloading of the symplastic pathway and transport to the developing embryo is also indicated. In Pisum there is an accumulation of label at the loose

parenchyma layer lining the embryo sac boundary wall and the combined results imply a passive transfer from the seed coat tissues, mostly in the region of the embryo because of the vascular terminals. The development of 'transfer cell-type' wall processes to the inner side of the embryo sac boundary wall could imply active uptake into the embryo sac. In Vigna a different inner seed coat layer is present with a structure suggesting a different (i.e. actively secreted) form of nutrient transport to the embryo sac. In contrast to P. satvium, the embryo sac boundary wall does not develop localised thickenings. The role of the elongating suspensor cells in nutrient supply to the globular embryo situated at the top of the embryo sac is suggested. However as the embryo develops and expands to fill the embryo sac, nutrient can be taken directly from the endosperm through the symplastic regions to be evenly distributed to the storage parenchyma cells. The even deposition of storage protein throughout the cotyledons and role of various organelles in protein synthesis and transport have also been discussed.

These studies have shown a generally similar pattern of development in the storage tissues of a range of legume seeds. The cotyledon parenchyma cells within a seed are fairly synchronised with regard to formation of organelles and deposition of reserves. The pathways for nutrient supply to the developing seeds do, however, indicate significant and intriguing differences which may influence rates of seed development and affect the potential for increased crop exploitation.

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