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Electron microscopy of the cotton fibre: new observations on cell wall formation

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Abstract

The ultrastructure of the cotton fibre was examined after developing successful fixation methods. Fibre cells were fixed at different stages of development. In cells which were elongating and producing primary cell walls, the Golgi apparatus appeared to be directly involved in secretion and synthesis of primary wall components. In cells which were synthesizing thick secondary cell walls, evidence suggested a major role for the endoplasmic reticulum and plasma membrane in the synthesis and secretion of secondary wall materials. The possibility of a shift from a Golgi apparatus pathway for primary wall synthesis to an endoplasmic reticulum pathway for secondary wall synthesis is discussed. Plasma membrane micro-invaginations are present only during secondary wall synthesis and may represent sites of cellulose assembly. A model for primary wall biogenesis via the Golgi apparatus is presented, and the potential of the cotton fibre as a model system for studying cellulose biogenesis in higher plants is discussed.

Introduction

An estimated 10^{11} tons of cellulose are produced on earth each year (Hess, 1928), making it the most abundant macromolecule in the world (Hess, 1928; Ward, 1954). Despite the biological and commercial importance of cellulose, the cellular processes involved in its biosynthesis remain enigmatic. The mature cotton fibre is one of the few sources of virtually pure cellulose by dry wt, yet surprisingly little research has been directed toward understanding how the cotton fibre cell synthesizes cellulose and incorporates it into a cell wall.

Traditionally, fibre development has been divided into two phases: elongation and secondary wall thickening (Balls, 1915, 1928; Hawkins and Serviss, 1930; Anderson and Kerr, 1938; Schubert *et al.*, 1973). Following fibre differentiation from cells on the ovule epidermis, the elongation phase begins. During this phase lint fibres increase in length some 1,000 to 3,000 times their diameter. Fibre diameter is constant from the base to about 75% of the distance to the tip where the fibre tapers off. The elongation phase lasts from the day of anthesis to about 27 days post-anthesis, by which time the fibres have attained maximum length (Anderson and Kerr, 1938; Schubert *et al.*, 1973). It is during the elongation phase that the cell makes its primary (outermost) cell wall. Secondary wall production begins toward the end of the elongation phase, some 16 to 19 days post-anthesis, so that the secondary wall thickening phase actually overlaps the elongation phase to some extent (Schubert *et al.*, 1973). The cytoplasmic ultrastructure of the cotton fibre has been neglected except for research by Berlin and Ramsey (1970), Watson and Berlin (1973), Herth (1974) and the recent publication of Itoh (1974). In this report we shall present data on the ultrastructure of the cotton fibre during primary and secondary wall synthesis.

Materials and methods

Cotton plants *(Gossypium hirsutum* L. var. delta pine 16) were transplanted from the farm of Mr and Mrs Dillard Hopkins near Sanford, North Carolina, U.S.A., on 1 September

1973. After pruning they were potted and set in a greenhouse. Osmocote 14-14-14 was used as a fertilizer and Temik was applied as an insecticide. In December 1973 additional plants were grown from seeds harvested from the original plants. Cotton flowers were tagged on the day of anthesis, enabling a determination of the approximate age of the fibres on the ovules.

Unfertilized cotton ovules from greenhouse plants were grown in culture according to the method of Beasley and Ting (1973). Ovaries were removed from the plants 48 h after anthesis and surface sterilized for 20 min in a 20% aqueous clorox solution. Using a sterile technique, ovules were dissected from the ovaries and placed into sterile flasks or Petri dishes containing 50 ml of the Beasley and Ting liquid culture medium to which 5.0 μM gibberellic acid was added to stimulate fibre growth (Beasley and Ting, 1973). Since gibberellic acid is heat labile, it was added after the medium was autoclaved using a syringe fitted with an autoclaved Swinney attachment and a 0.45 μm Millipore filter. Cultures were incubated at 37°C in total darkness except for brief periods of inspection. Within 2 weeks, unfertilized ovules yielded about 75% of the cell wall

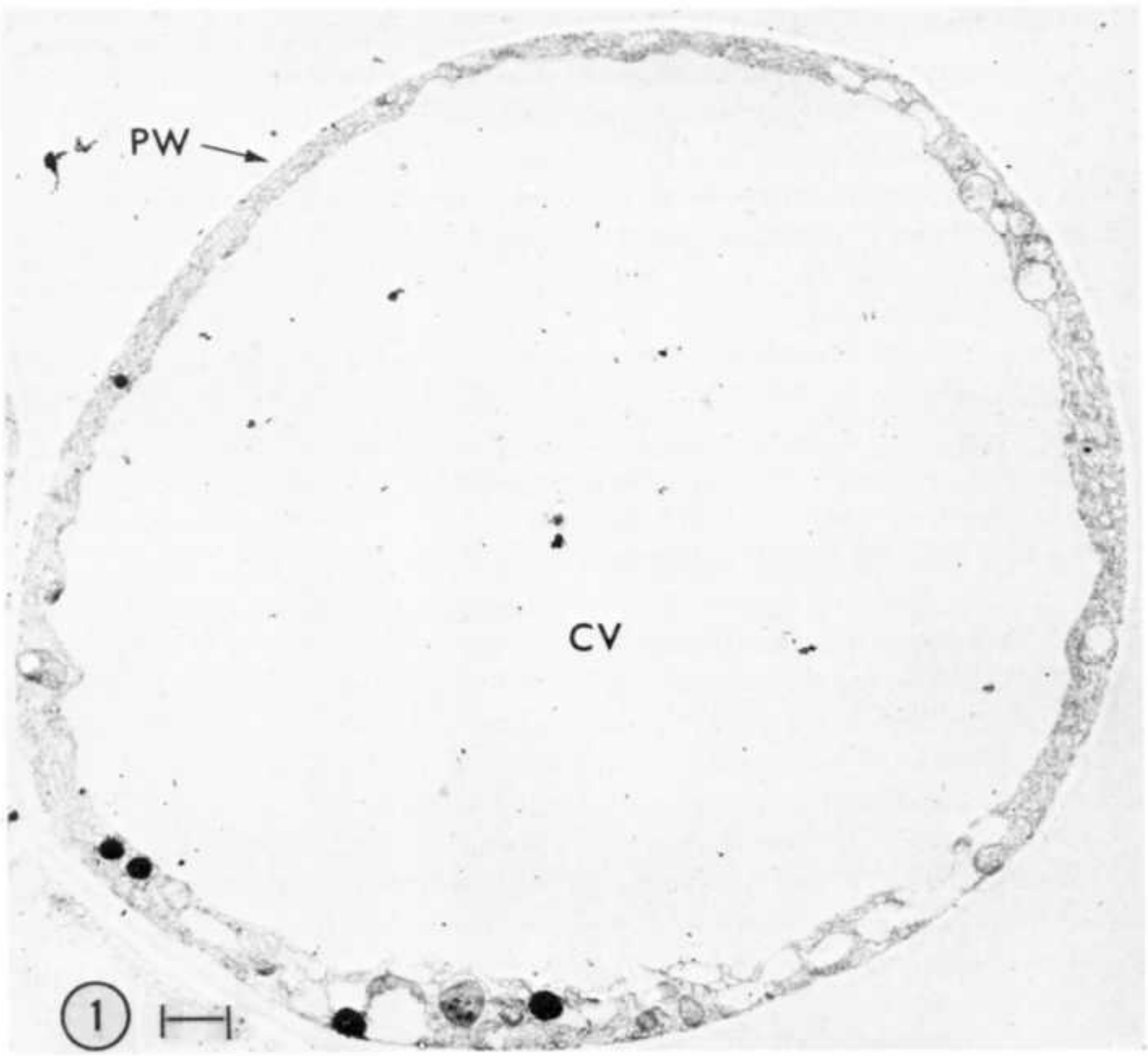


Figure 1 A cotton fibre in cross section at 10 days post-anthesis (DPA). Note the relative thinness of the cytoplasm containing typical organelles. Note the thin primary wall (PW). Fixation A. CV = central vacuole. Bar represents 1 μm.

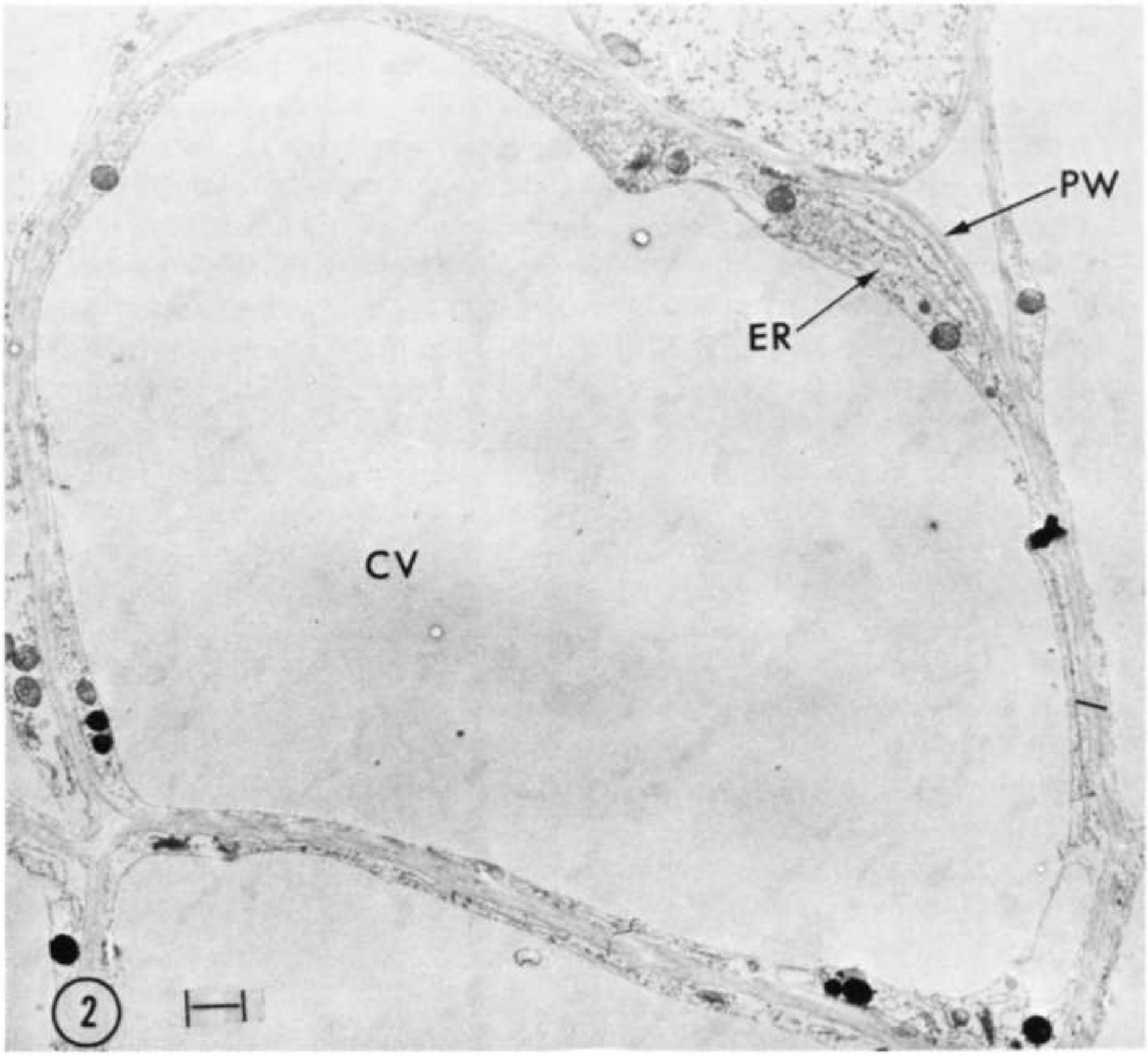


Figure 2 Cross section of a cotton fibre at 10 DPA, with neighbouring fibres pressed against it. Note the similarity of ultrastructural features in Figures 1 and 2. Fixation B. ER = endoplasmic reticulum. Bar represents 1 μm.

material normally obtained from fertilized ovules after one month (Beasley, 1974). In this study, both fertilized and unfertilized ovules were grown in culture with success, but only fertilized ovule cultures were examined with the electron microscope.

A Fiske Osmometer (model G-66A) was used to assay the osmolarity of solutions with fixations. With samples of 3 ml, three osmolarity readings were made for each of the sample solutions in order to establish the accuracy and reliability of the values obtained.

Numerous fixation procedures were tested, but fixation A (below) provided the best results. (1) Glutaraldehyde fixative: 1% glutaraldehyde (Serva), prepared from an 8% aqueous stock with pH pre-adjusted to 7.2 with barium carbonate, 0.025 M sodium cacodylate buffer (pH = 7.2), 0.1 M D-glucose (0.1 M sucrose may also be used), final pH = 7.2. (2) Wash solution (for both post-glutaraldehyde and post-osmication wash): 0.025 M sodium cacodylate buffer (pH = 7.2), 0.2 M D-glucose (0.1 M sucrose may be substituted), final pH = 7.2. (3) Post-osmication solution: 1% osmium tetroxide, 0.025 M sodium cacodylate buffer (pH = 7.2), final pH = 7.2.

Ovaries were cut from the plant, and the external ovary wall was dissected away, one carpel at a time, exposing the ovule. Young ovules, at less than 3 days post-anthesis,

were individually removed and placed into the glutaraldehyde fixative solution. After 3 days post-anthesis, the fibres of ovules in the same carpel become greatly intertwined, making it impossible to isolate an ovule and its fibres without damaging the fibres; therefore, older ovules were removed from the carpel as an intact cluster and immersed directly in the fixative solution. Some ovules at 20 or more days post-anthesis were sliced into smaller pieces to facilitate fixative penetration and specimen handling. In such cases, it was essential that the entire specimen be immersed in the fixative solution while being sliced. It was important to avoid touching, pulling, and cutting the fibres unless absolutely necessary. Cultured ovules were lifted directly from the culture dish with sterile tweezers or a sterile transfer loop and immersed immediately in the fixative solution.

Specimens were initially fixed with glutaraldehyde fixative solution A at room temperature for 30 min after which they were transferred to a refrigerator at 5°C. After 7 to 17 h, they were rinsed in three changes of the wash solution at 5°C for 10 min each. This was followed by post-osmication at 5°C in the dark for 1 to 2 h. The ovules were then washed at 5°C in full strength wash solution twice for 10 min each; in half strength wash solution for 10 min, and in distilled water for 5 min.

Fibres were also fixed according to the method of Berlin (Schubert *et al.*, 1973). This method has been designated as the fixation B series.

A simultaneous glutaraldehyde osmium tetroxide fixation (Franke *et al.*, 1969) was also used with some success (fixative C). The methods used were the same as described for fixation A except that fibres were fixed for 1 h in the dark at 4°C. An ethanol-acetone dehydration series followed the post-fixative washes. Ovules were dehydrated 5 min in each series with cold 20%, 40%, 60%, 80%, 90% and 95% ethanol followed by 10 min in cold anhydrous 100% acetone, and 10 min in anhydrous 100% acetone at room temperature.

Using a 6A:4B mixture of Epon 812 (WPE 150) (Luft, 1961), specimens were infiltrated at room temperature with constant rotation. The following infiltration series was used: 25% Epon (diluted with anhydrous acetone) for 4 h; 50% Epon for 4 h; 15% Epon for 12 h; and 100% Epon for 12 h. After infiltration specimens were embedded in fresh 100% Epon. Some were embedded whole in large moulds, and others were sliced into

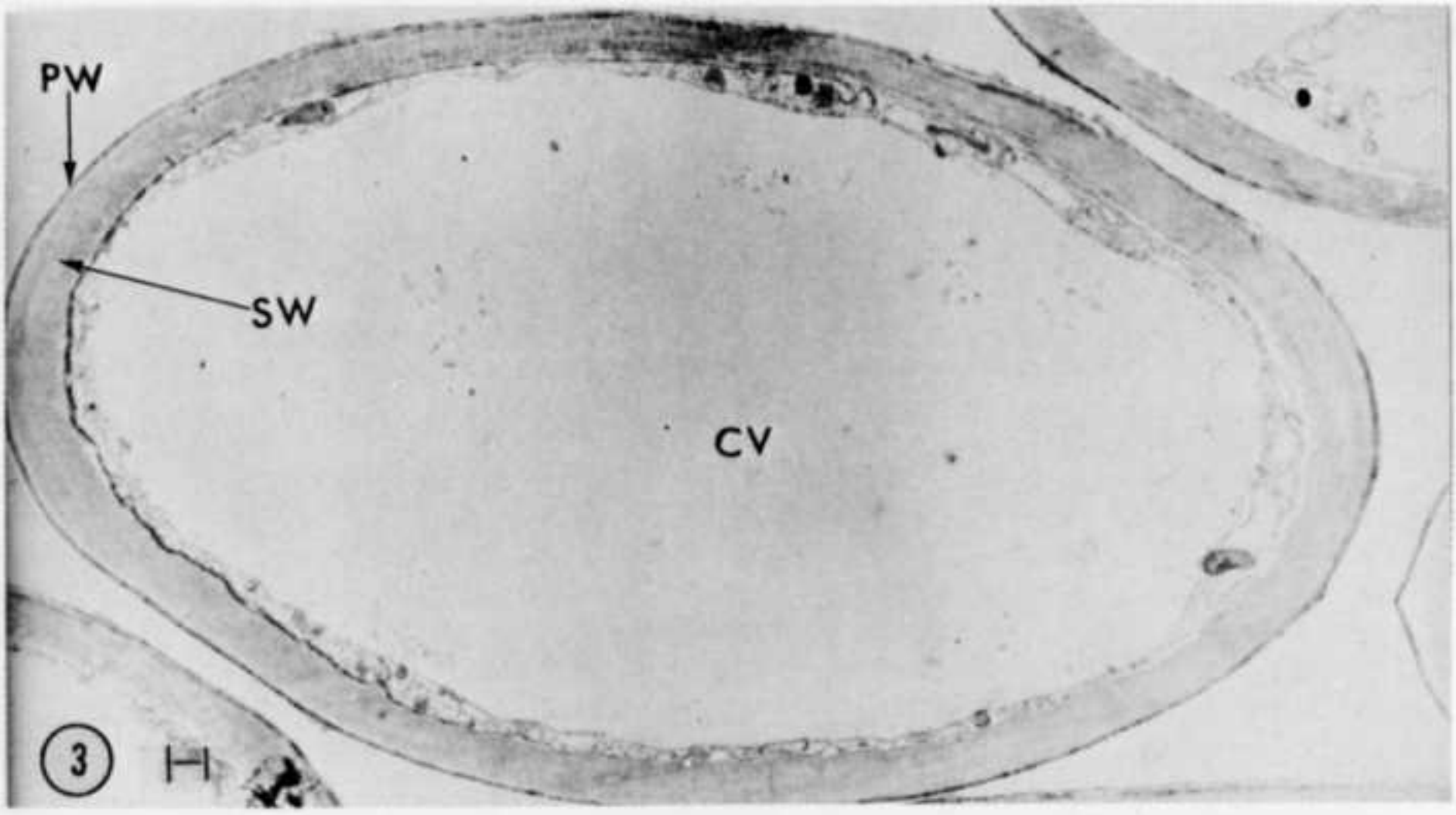
Figure 3 Cotton fibre in cross section at 22 DPA during which secondary wall synthesis is active. Note the thick secondary wall (SW). The primary wall (PW) is the dark, thin outermost layer. Fixation A. Bar represents 1 μm.

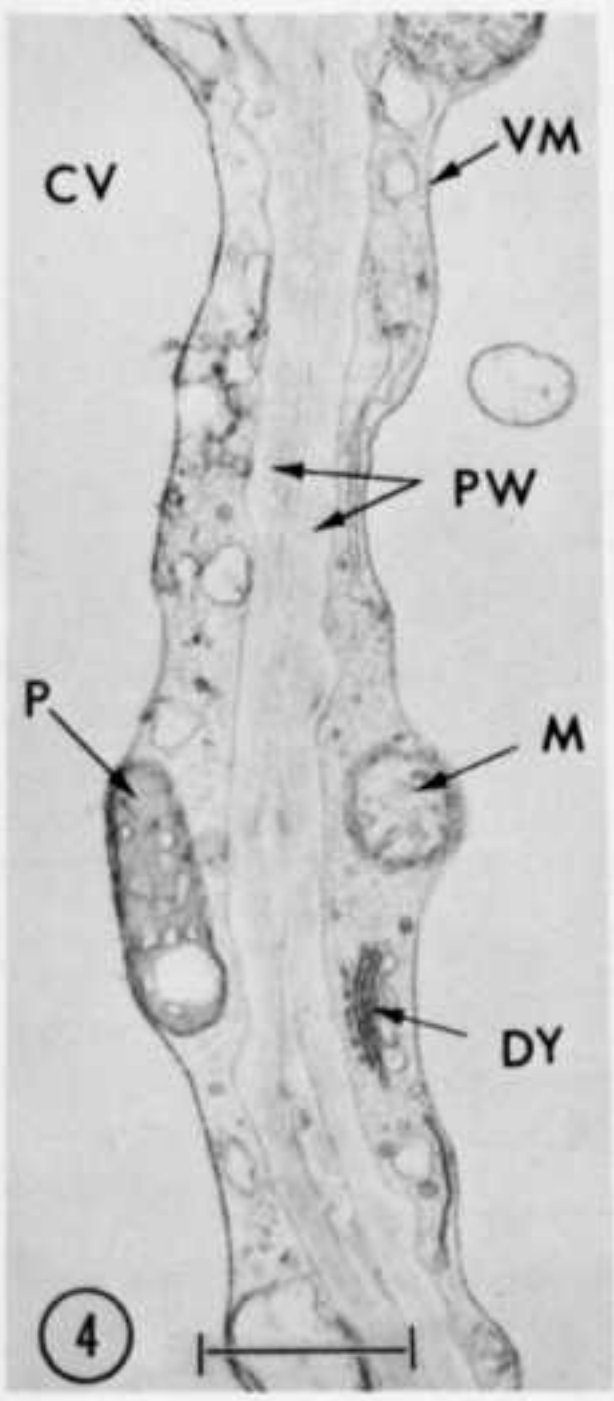
Figure 4 Cross section through two adjacent fibres from a cultured ovule at 10 DPA. Note dictyosome (DY), plastid (P), and mitochondrion (M). VM = vacuolar membrane. Fixation A. Bar represents 1 μm.

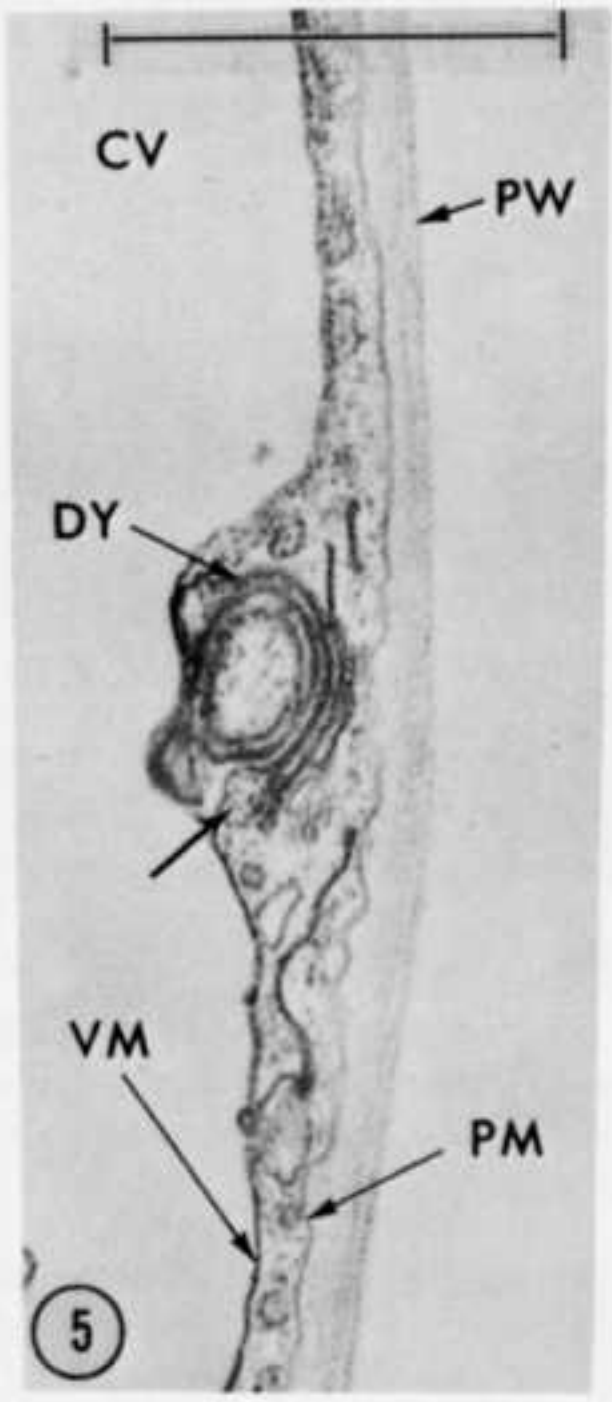
Figure 5 Transverse section through fibre at 10 DPA. Note the dictyosome (DY), and vesicle containing fibrillar material blebbing from a cisterna (arrow). Fixation B. Bar represents 1 μm.

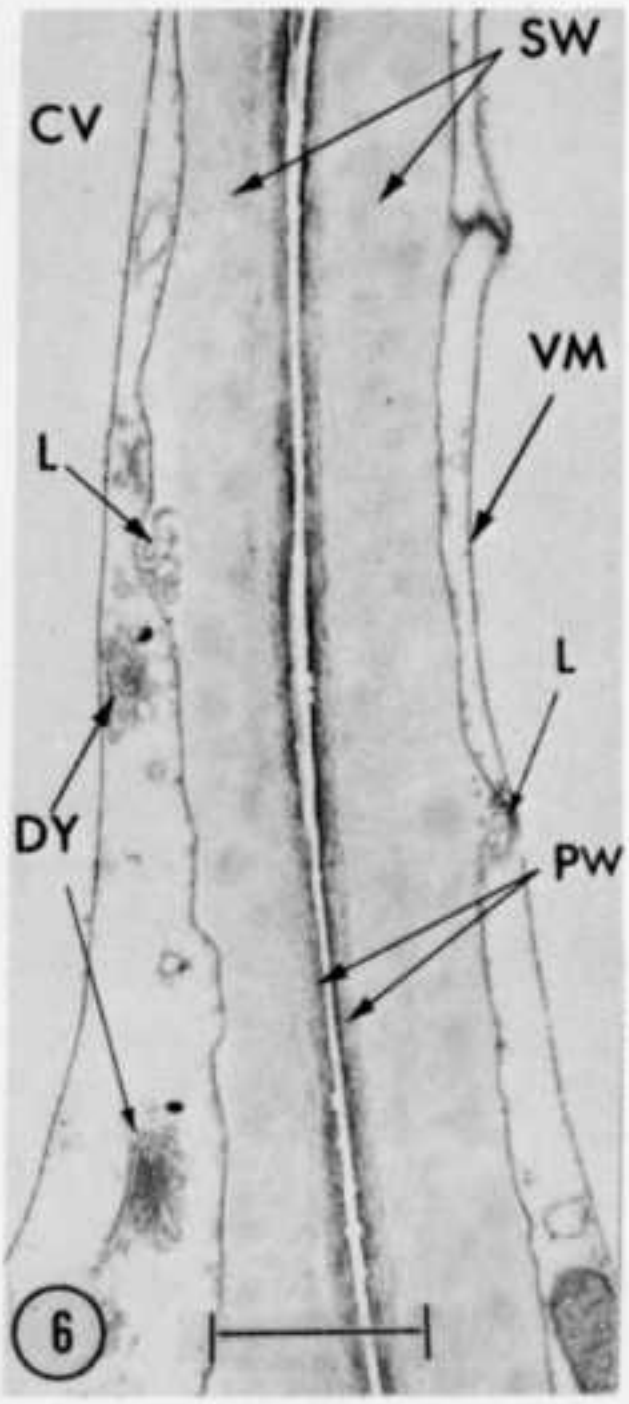
Figure 6 Cross sectional view of two adjacent fibres at 19 DPA. The secondary wall (SW) is being synthesized at this stage. The primary wall (PW) remains as a thin, darker layer on the periphery. Note the 'lomasome-like' structures (L). Altered dictyosomes (DY) also are present. Fixation B. Bar represents 1 μm.

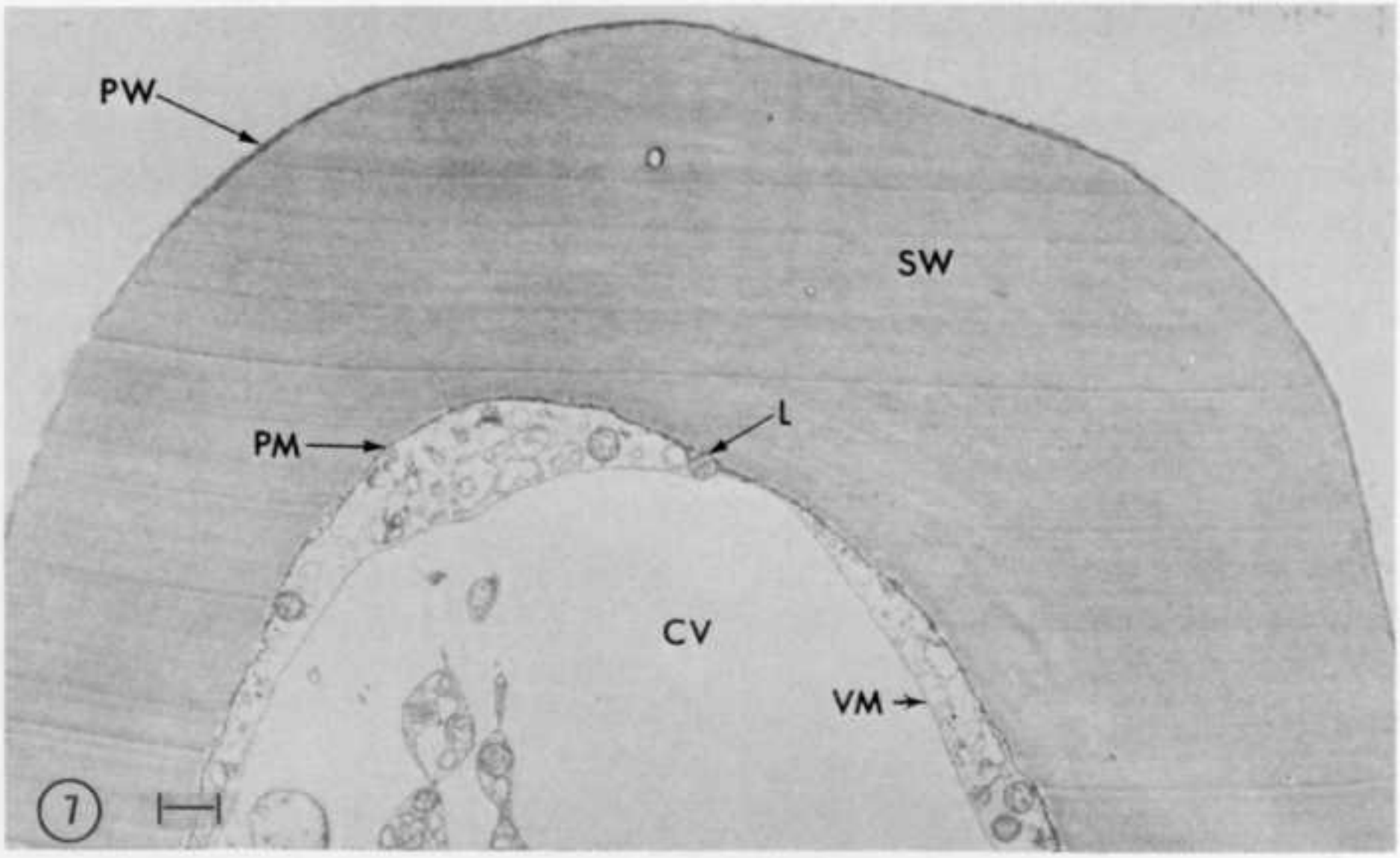
Figures 3, 4, 5 and 6











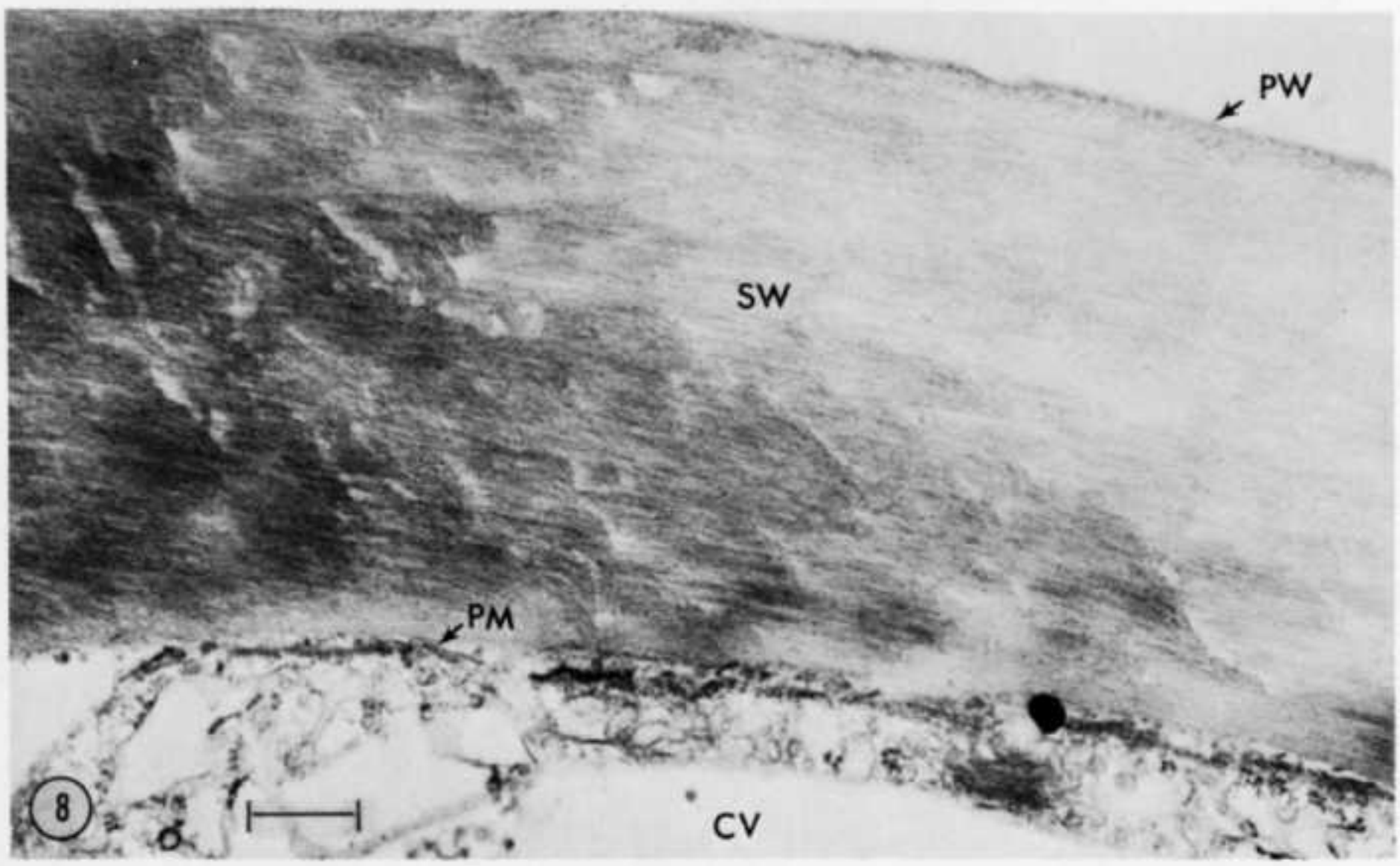


Figure 7 Cross section of cotton fibre at 40 DPA. The secondary wall (SW) is very thick, and the primary wall (PW) is quite thin in comparison. Note the 'lomasome-like' structure (L). Fixation A. Bar represents 1 μm.

Figure 8 Slightly oblique section at 29 DPA. Note the cellulosic microfibrils in negative contrast. Fixation C. Bar represents 1 μm.

segments of grouped fibres and embedded in smaller moulds. Embedded fibres were polymerized for 48 h in a 60°C oven. Silver sections were made with a diamond knife on a Reichert Om U2 Ultramicrotome. Sections were post-stained 10 min with 2% aqueous uranyl acetate and 15 min with lead citrate (Reynolds, 1963). All electron micrographs were made with an Hitachi HU-11E Electron Microscope at an accelerating voltage of 75 kV.

Carbohydrates of the cell were characterized by the periodic acid-silver methenamine cytochemical stain (Pickett-Heaps, 1967a; Brown and Romanovicz, 1976; Romanovicz and Brown, 1976). The phosphotungstic acid-chromic acid post-stain (Roland *et al.*, 1972) was used to differentiate the plasma membrane from other components of the endomembrane system during secondary wall formation.

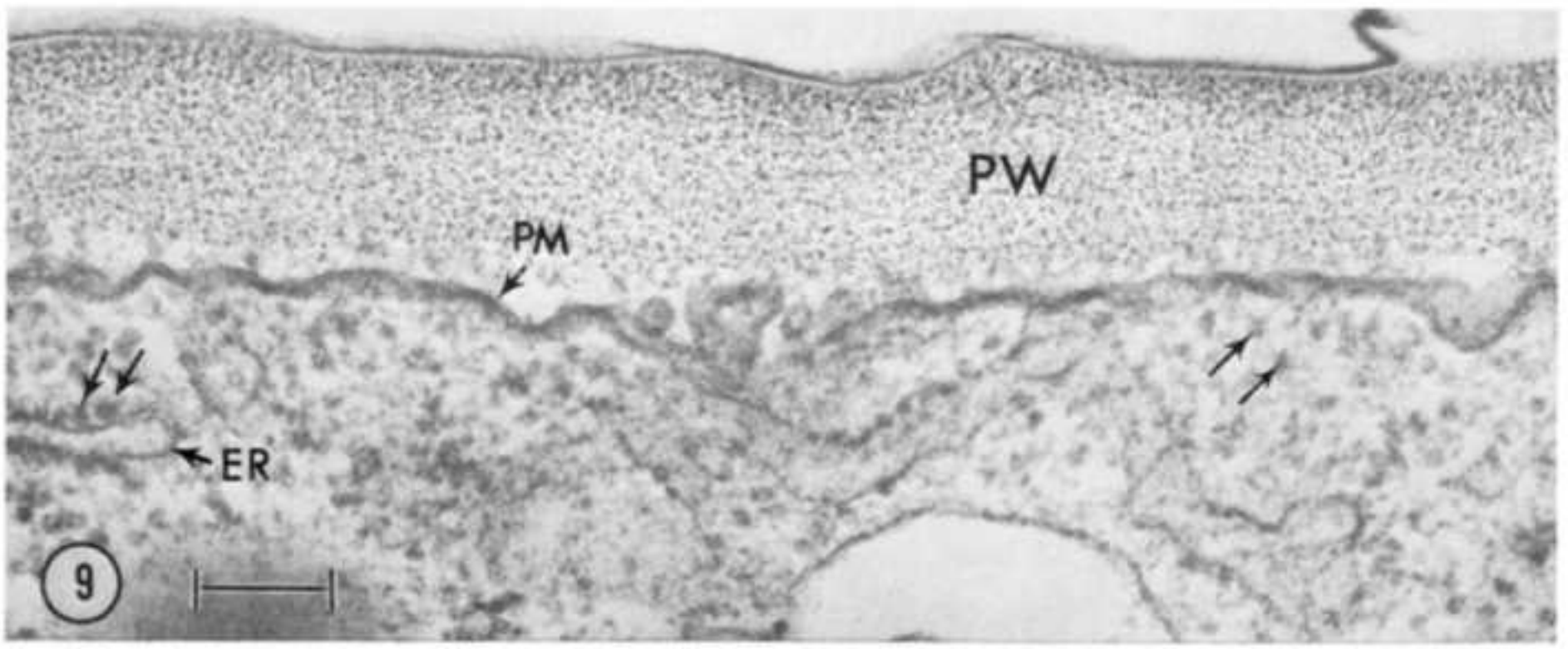


Figure 9 Cross section at 12 DPA. The cell wall consists only of primary wall (PW). Numerous free ribosomes are present in the cytoplasm (arrows, right) as well as some ribosomes bound to the endoplasmic reticulum (ER, arrows, left). Note the undulatory plasmalemma. Fixation A. Bar represents 1 μm.

Figure 10 A slightly oblique section through a fibre at 22 DPA. The primary and secondary walls are easily distinguished. Microtubules (MT) are visible, lining the plasma membrane (PM). Fixation A. Bar represents 1 μm.

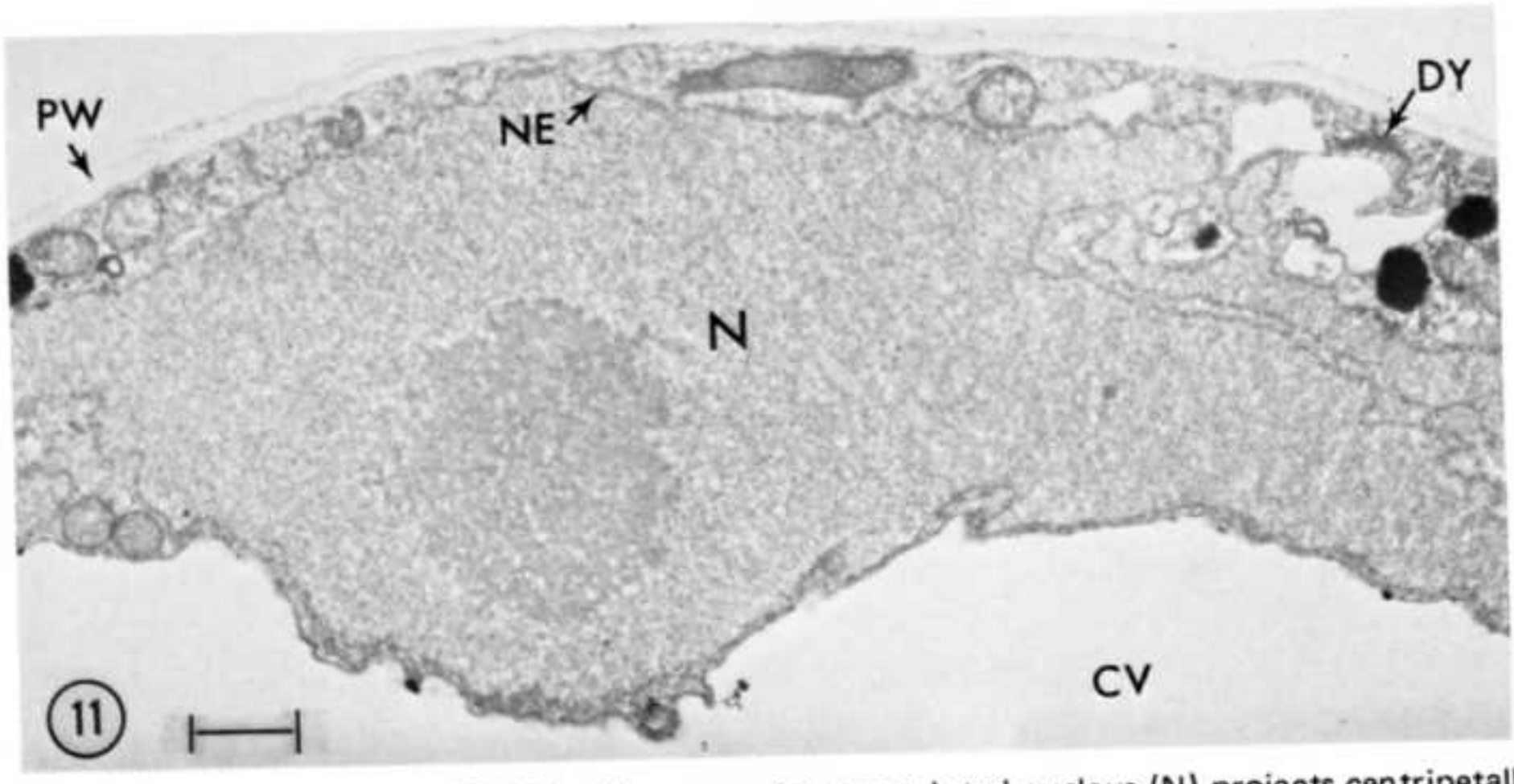


Figure 11 Cross section at 10 DPA. The somewhat convoluted nucleus (N) projects centripetally into the central vacuole (CV). Fixation A. NE = nuclear envelope. Bar represents 1 μm.

Results

Fixation studies

When this reseach was begun, there were few ultrastructural studies of the cotton fibre protoplasm by which to evaluate adequately ultrastructure preservation. Therefore, it was necessary to arrive at a suitable fixation procedure independently. Initial fixations were unsuccessful due to plasmolysis and rupture of the highly labile vacuolar membrane of the cotton fibre. After individual changes in pH, fixative concentration, duration of fixation, etc, failed to prevent plasmolysis, analyses of the osmotic pressures of various fixation solutions were undertaken. It had been suggested (McLean, 1960) that in fixing plant tissues one could reduce plasmolysis by immersing specimens in a series of glucose solutions of increasing osmolarity, thereby reducing the turgor of the tonoplast (central vacuole). Thus plasmolysis was prevented by matching the osmolarities of fixation solutions (= 220 milliosmols) to the osmolarity of the Beasley and Ting culture medium

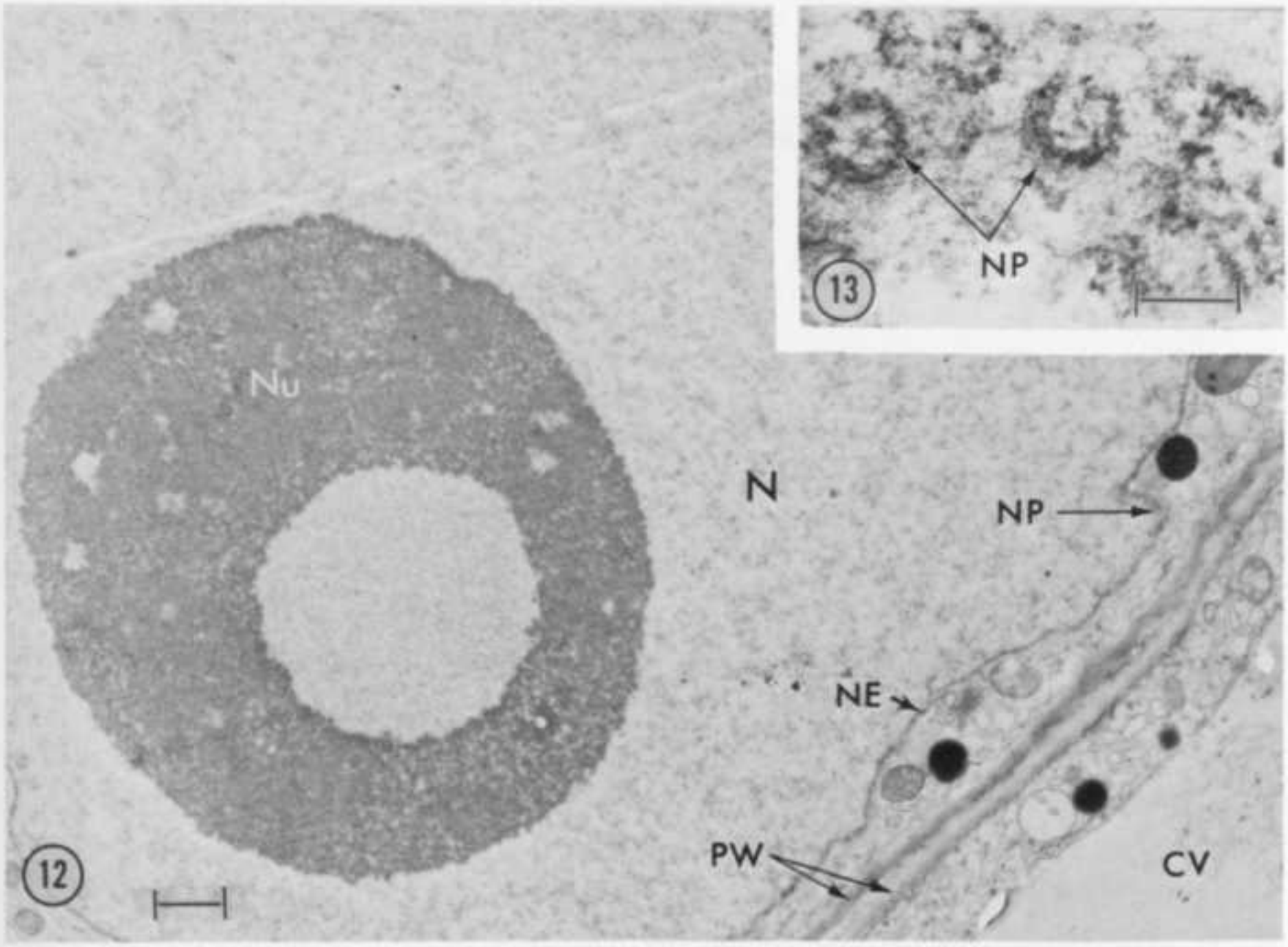
Figure 12 Cross section of fibre at 15 DPA. Note nucleus (N) with prominent nucleolus (Nu). The nuclear envelope (NE) and pores can be seen in tangential section. Fixation A. Bar represents 1 μm.

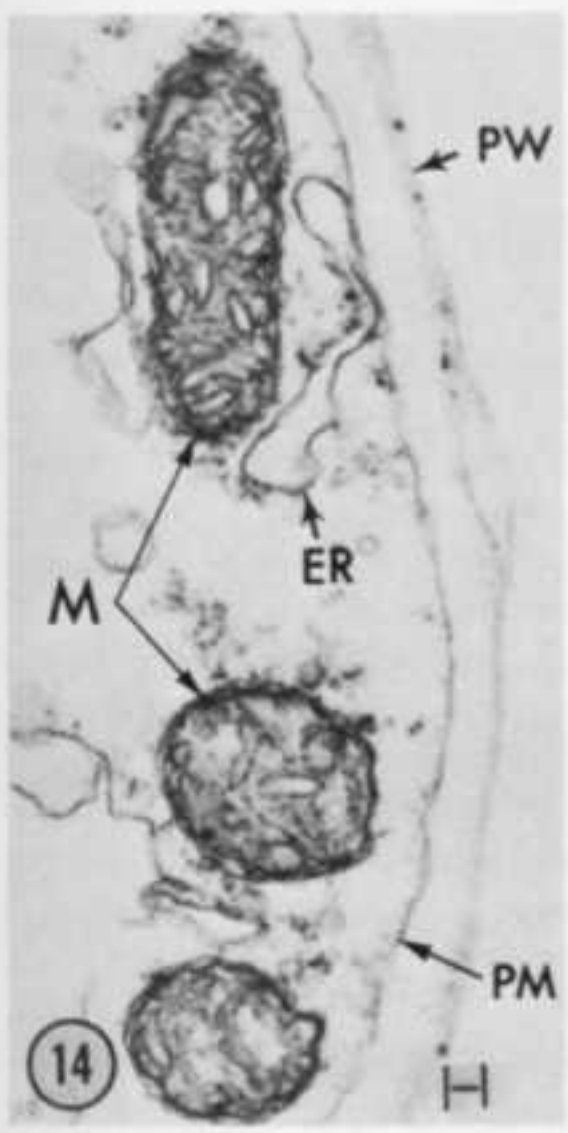
Figure 13 Tangential section through nuclear envelope (NE) of cotton fibre at 15 DPA. The nuclear pores (NP) consist of an annular complex of subunits with spoke-like arms connecting them with a core at the centre. Fixation A. Bar represents 0.1 μm.

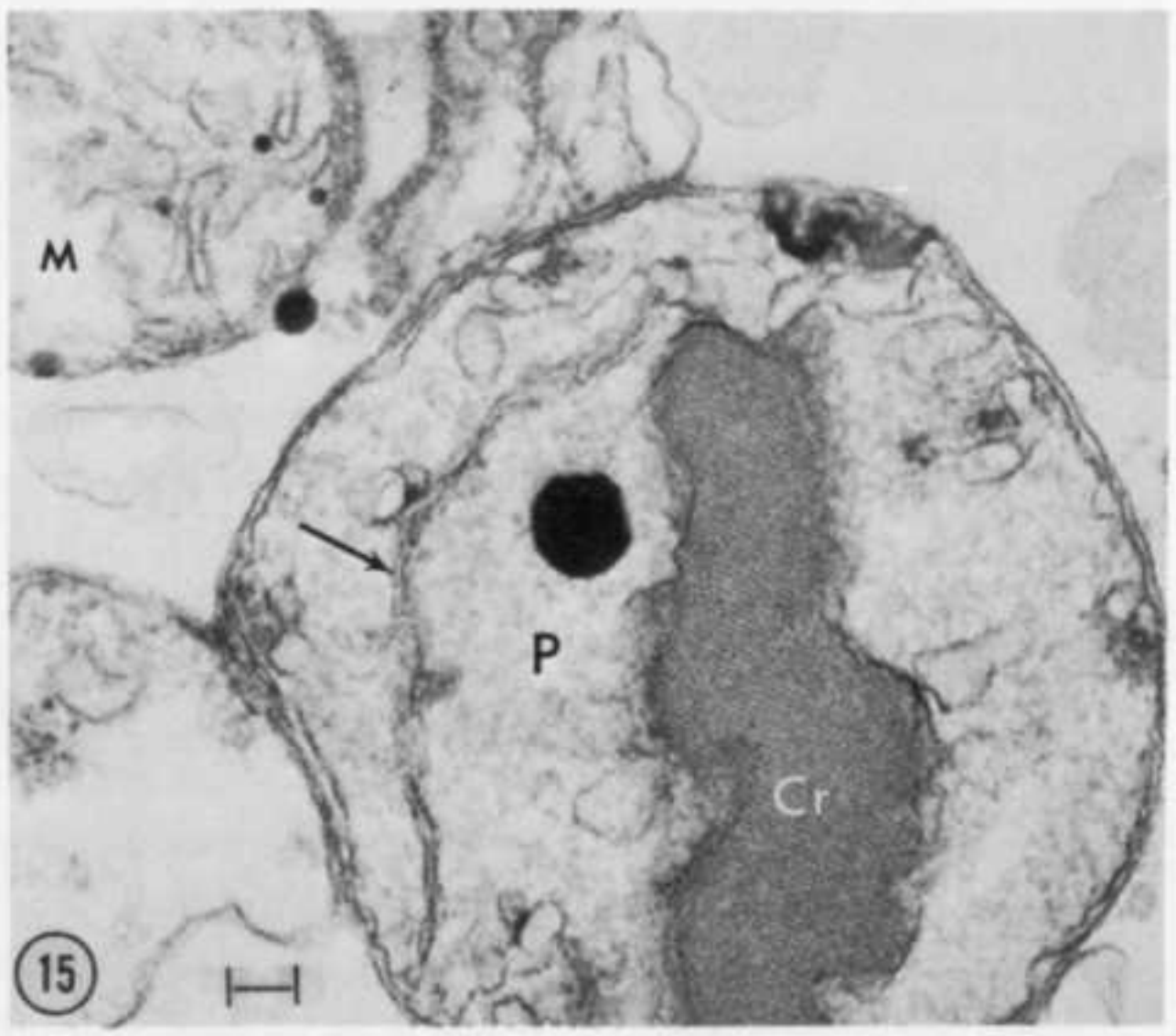
Figure 14 Cross section at 10 DPA showing mitochondria (M) with tubular cristae. Fixation A. Bar represents 0.1 μm.

Figure 15 Cross section 25 DPA through a proplastid containing a crystalline inclusion. Note the inner lamella (arrow) and the double, limiting membrane. Fixation A. Bar represents 0.1 μm.

Figures 12, 13, 14 and 15







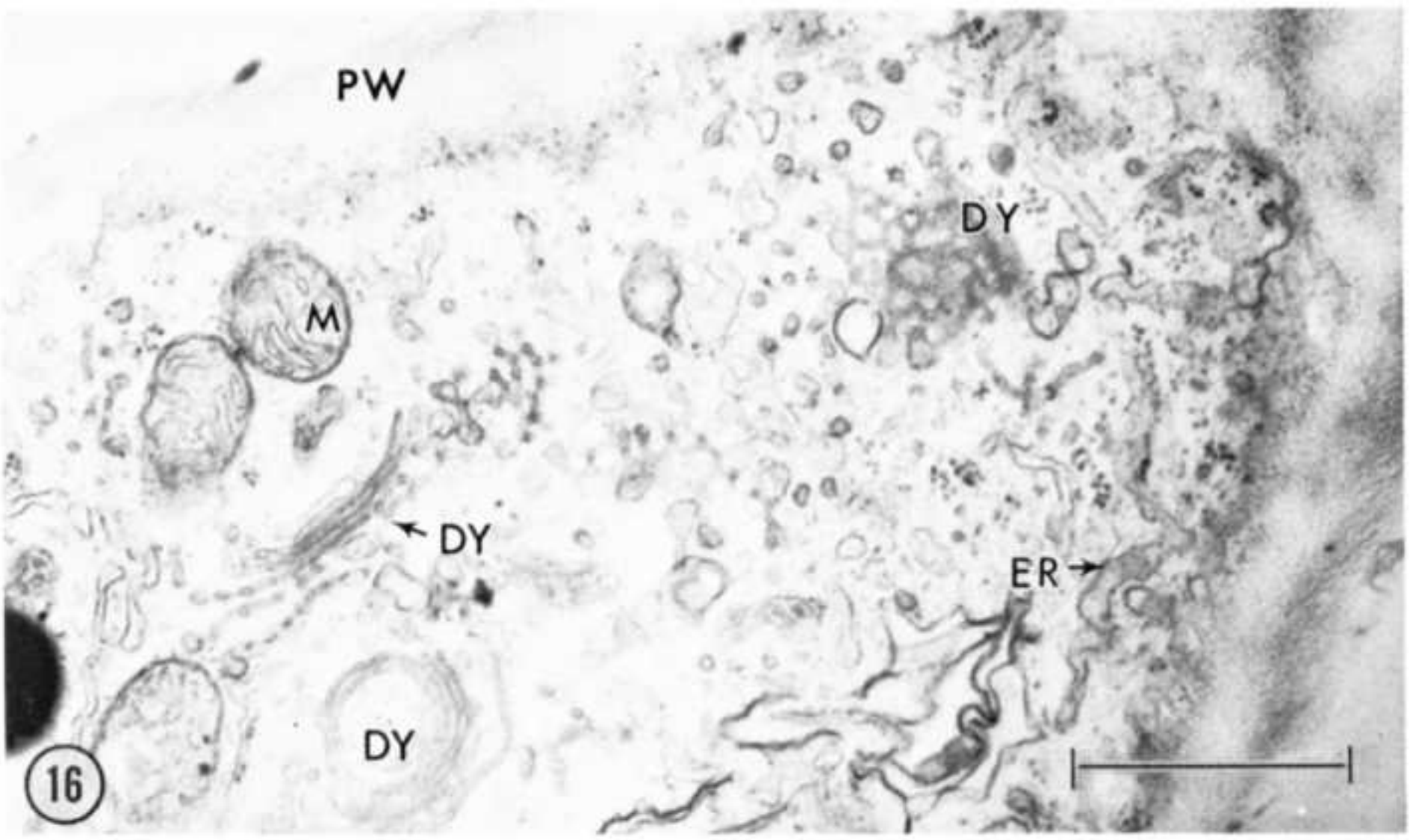


Figure 16 An oblique section at 16 DPA. Note the dictyosome in tangential section revealing fenestrations in the periphery of cisternae. Fixation A. Bar represents 1 μm.

for cotton ovules (Beasley and Ting, 1973). The osmolarities of solutions in fixation A were adjusted to approximately 220 mOsM, yielding superior results.

Later it was discovered that Berlin and co-workers (Berlin and Ramsey, 1970; Watson and Berlin, 1973; Schubert *et al.*, 1973) had already developed an adequate fixation method (fixation B) for cotton fibres. Subsequently the two fixations (A and B) were compared using specimens from the same ovary, with ovaries collected at different times post-anthesis, and using specimens from the same culture. The general ultrastructure observed is quite similar for these two fixations (Figures 1–7), except that fixation A tends to give a denser cytoplasm and, perhaps, better membrane preservation.

The simultaneous glutaraldehyde-osmium tetroxide fixation (fixation C) was not perfected in this study, but it appears likely that adjustments in the osmolarity of the glutaraldehyde-osmium tetroxide solution would yield improved results. Figure 8 shows the excellent preservation of the cell wall provided by this fixation, the microfibrils being clearly visible in negative contrast.

Cytoplasmic ultrastructure

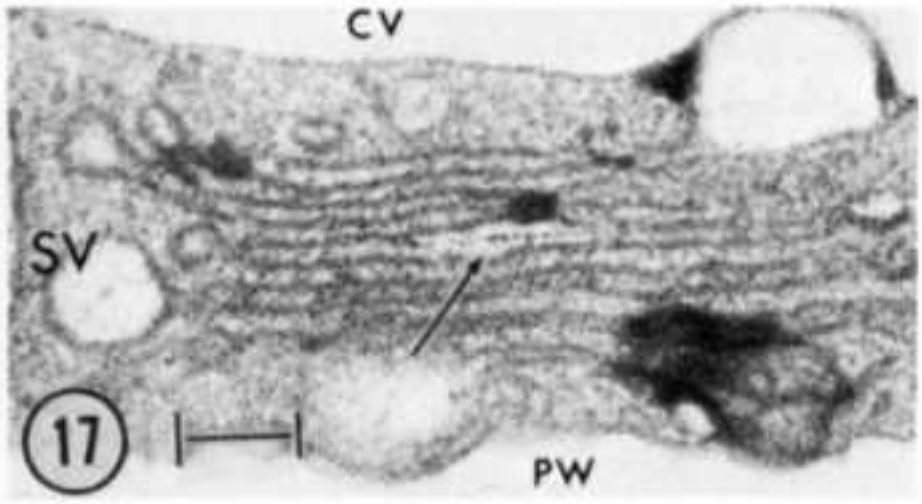
Transverse sections of the cotton fibre show the protoplasm to be a thin layer (0.1–0.5 μm) surrounding a large central vacuole (10–30 μm in diameter) (Figures 1–3). Exterior to the protoplasm is a cell wall the thickness of which depends upon fibre age. During the first 16 days post-anthesis, a relatively thin (0.1–0.5 μm), primary wall is synthesized (Figure 9). Secondary wall synthesis begins around 20 days post-anthesis. At this stage, the thin outermost layer is the primary wall, and the thick, innermost layer is the secondary wall (Figure 10). Secondary walls attain a thickness of 8 μm in older fibres (Figure 7). These older fibres are more difficult to preserve perhaps because the increase in cell wall thickness retards penetration of the fixative into the cytoplasm.

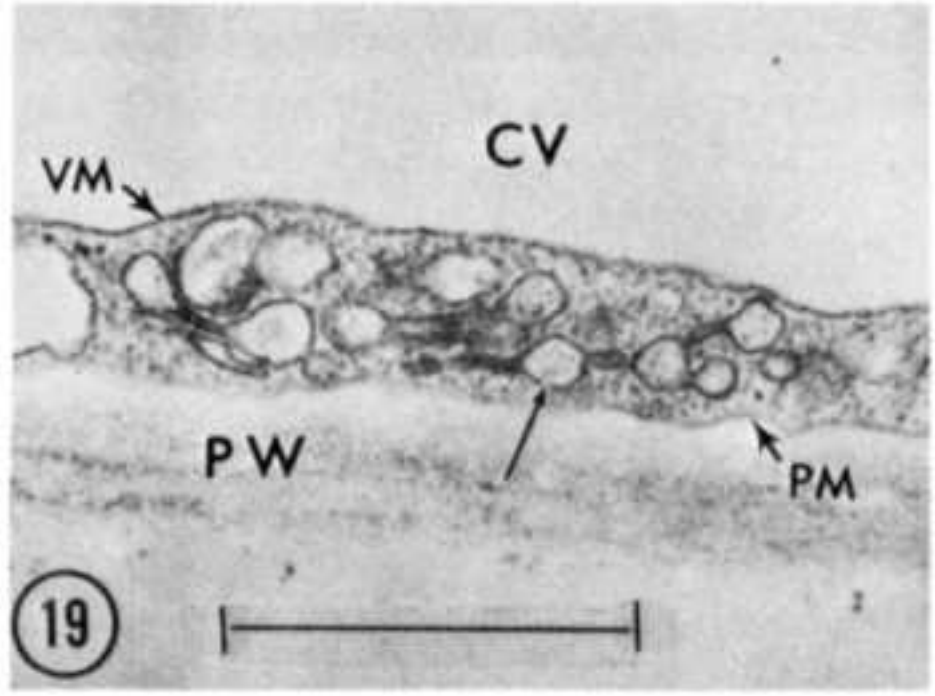
Among the protoplasmic features common to both the elongation and secondary wall thickening phases of cotton fibre development are nuclei, mitochondria, plastids,

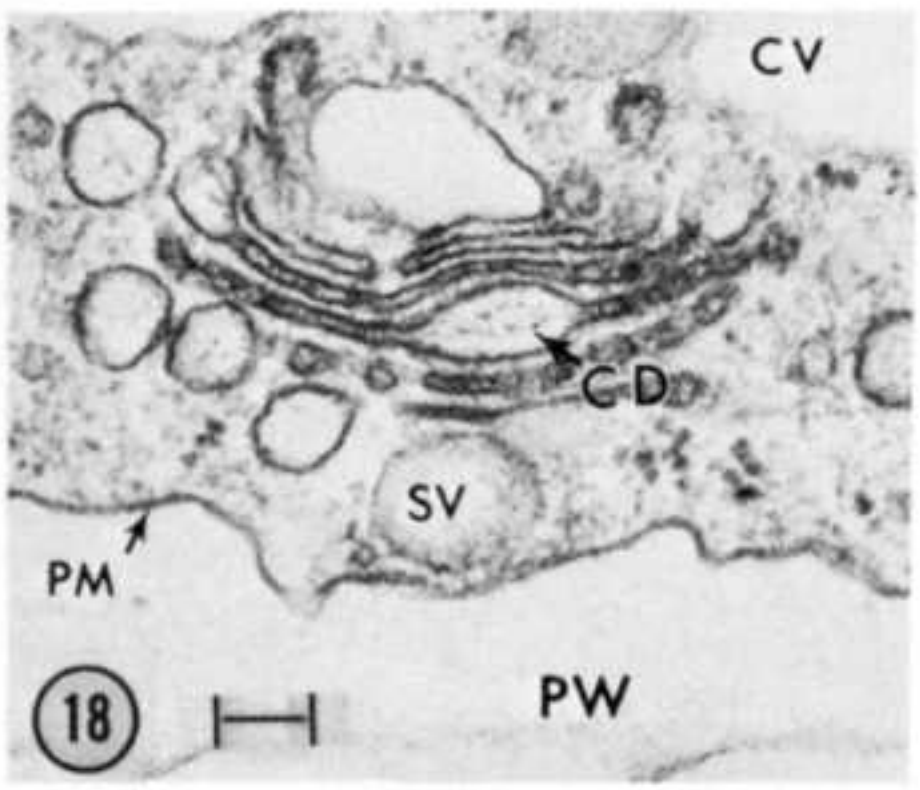
lipid bodies, microtubules, and the endomembrane system (Morré and Mollenhauer, 1974). The latter is composed of nuclear, vacuolar, and plasma membranes as well as membranes of the endoplasmic reticulum (ER) and the Golgi apparatus. Some of these elements demonstrate structural changes as the fibre ceases elongation and primary wall synthesis and then inititates massive secondary wall deposition.

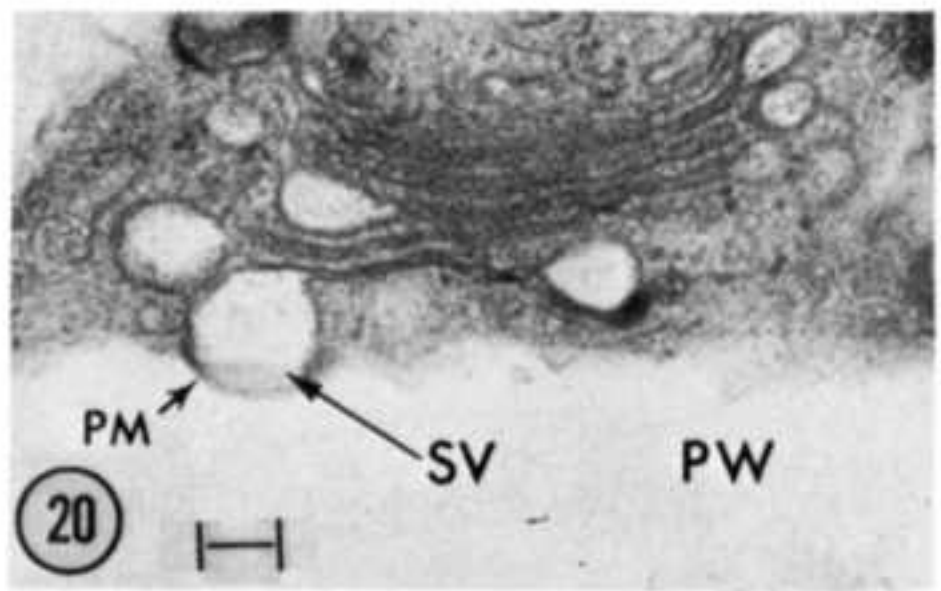
Nucleus

The cotton fibre contains a typical eukaryotic nucleus (Figure 11). Nuclear pores are numerous, and in tangential section they clearly demonstrate annular complexes with spoke-like strands radiating from the centre to each of the eight subunits surrounding the pore (Franke, 1966) (Figure 13).









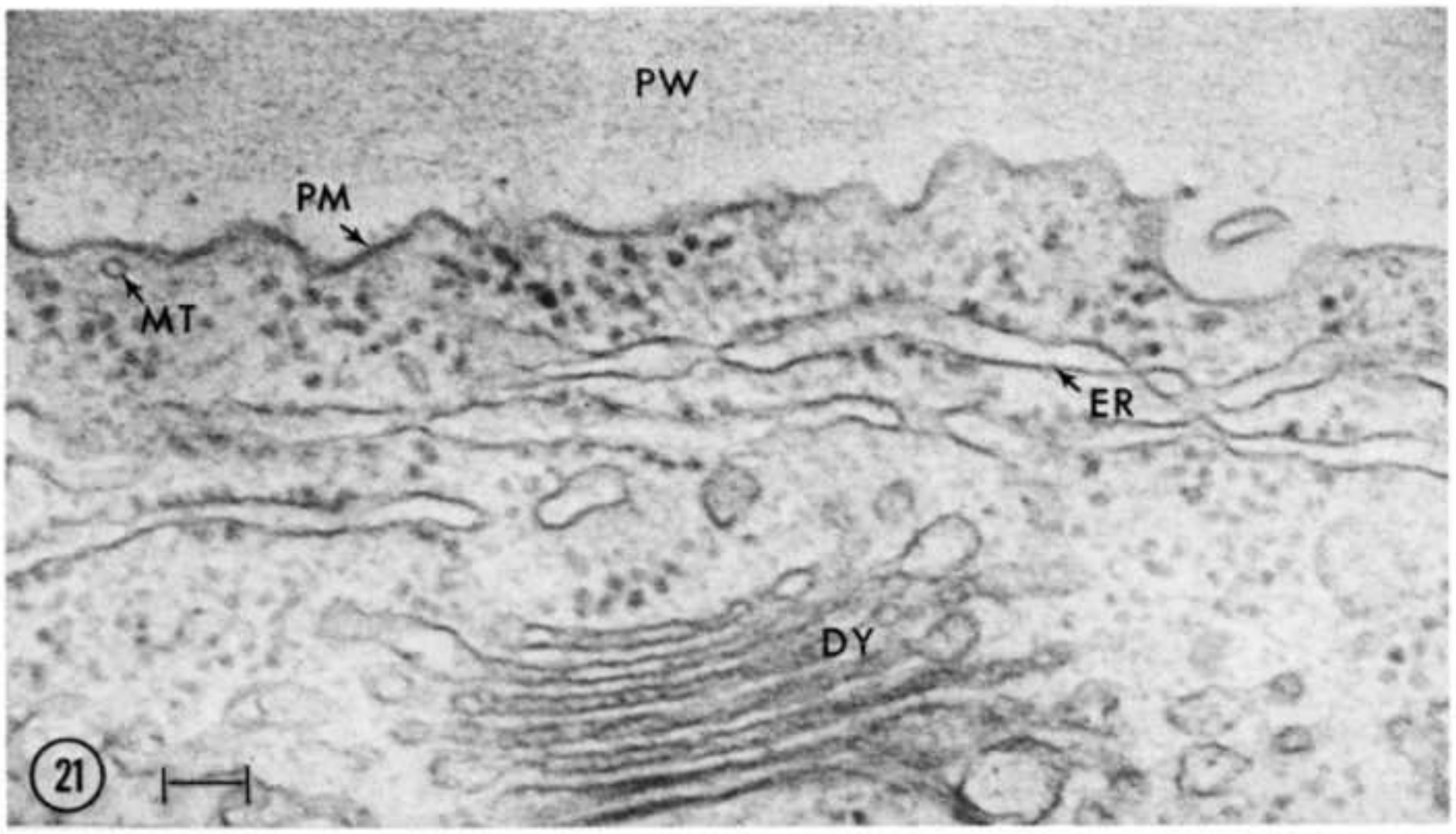
Figures 17–20 These represent evidence of Golgi apparatus involvement in production of primary wall material. Each of the four Figures is a cross section through a different dictyosome in a different cotton fibre, all at 10 DPA. Fixation A.

Figure 17 A thin layer of dictyosomal product is visible within an intercalary cisterna (arrow). Note nascent secretory vesicle (SV) containing product budding from a cisterna. Bar represents 0.1 μm.

Figure 18 Note the central dilation (CD) containing fibrillar material. Secretory vesicles (SV) released from the periphery of the cisternae also are visible. Bar represents 0.1 μm.

Figure 19 The apparent lateral movement of a central dilation toward the periphery of a cisterna is visible (arrow). Bar represents 1 μm.

Figure 20 A nascent secretory vesicle (SV) budding from a cisterna is visible appressed to the plasma membrane prior to expected exocytosis. Bar represents 0.1 μm.



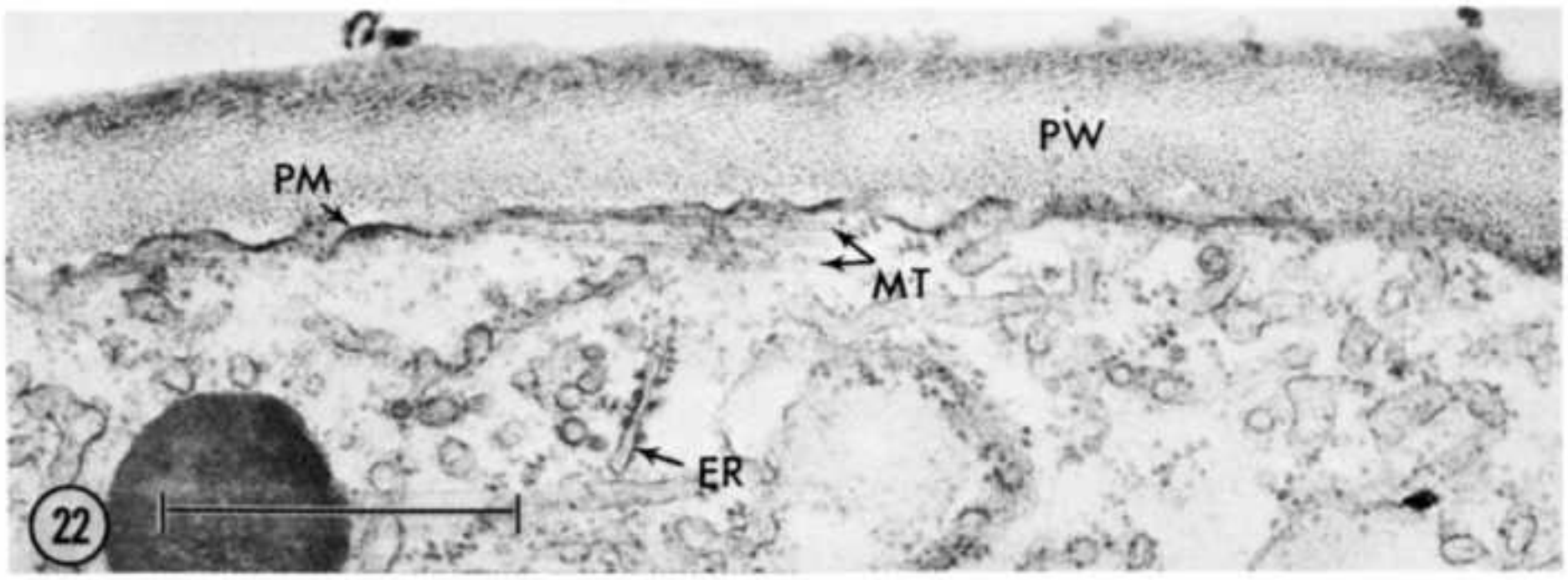


Figure 21 A cross section of the primary wall (PW) and cytoplasm of a cotton fibre at 15 DPA. Microtubules (MT) are present as singlets at this stage, and they are few in number. Note the numerous free ribosomes and those attached to the endoplasmic reticulum (ER). Fixation A. Bar represents 0.1 μm.

Figure 22 A longitudinal section of a cotton fibre at 15 DPA. The polysomes and bundle of cortical microtubules (MT) suggest that this is an intermediate stage in the conversion from primary to secondary wall synthesis. Fixation A. Bar represents 1 μm.

Mitochondria

Mitochondria are consistent in appearance throughout both the elongation and the secondary wall thickening phases. They appear circular in cross section to the long axis of the fibre (Figure 14) and sometimes are paired. The cristae are tubular.

Amyloplasts

The plastids are similar in appearance to the mitochondria, but they tend to have a denser matrix, and they frequently contain starch or crystalline inclusions (Figure 15). Note the double limiting membrane and inner lamellae.

Lipid bodies

Highly electron dense bodies which average about 0.4 μm in diameter occur in the cytoplasm and are interpreted as lipid bodies. No limiting membrane is visible. These are most abundant during the elongation phase (Figures 1 and 2).

Microtubules

Cortical microtubules are present in both phases of cotton fibre development, but striking differences in number and order of microtubules exist between the two phases. In the elongation phase (Figures 21 and 22) microtubules are sparsely distributed and generally run parallel to the long axis of the fibre. In the secondary wall thickening phase, microtubules become numerous and line the inner surface of the plasma membrane. These cortical microtubules clearly are oriented parallel to the fibre's long axis (Figure 23) and appear to parallel the layer of secondary wall microfibrils immediately adjacent to the plasma membrane (Figure 26). Cross-links between the cortical microtubules and the plasmalemma are common in the secondary wall thickening phase (Figure 23, inset B).

Endoplasmic reticulum

The endoplasmic reticulum in cross section appears as two parallel membranes which may bear ribosomes on the exterior surfaces (Figures 9 and 22). Oblique sections reveal that the ER appears to connect or associate with fenestrated cisternae of the Golgi apparatus (Figure 16). During primary wall formation most of the ribosomes exist free in the cytoplasm, although some are attached to the ER (Figure 21). During secondary wall formation, however, most ribosomes exist in a spiral, polysomal configuration of some six to ten ribosomes attached to the endoplasmic reticulum (Figures 25 and 26). These observations confirm similar data reported in a study of the cotton fibre (Berlin and Ramsey, 1970) and research on other plant cells (Cronshaw, 1965).

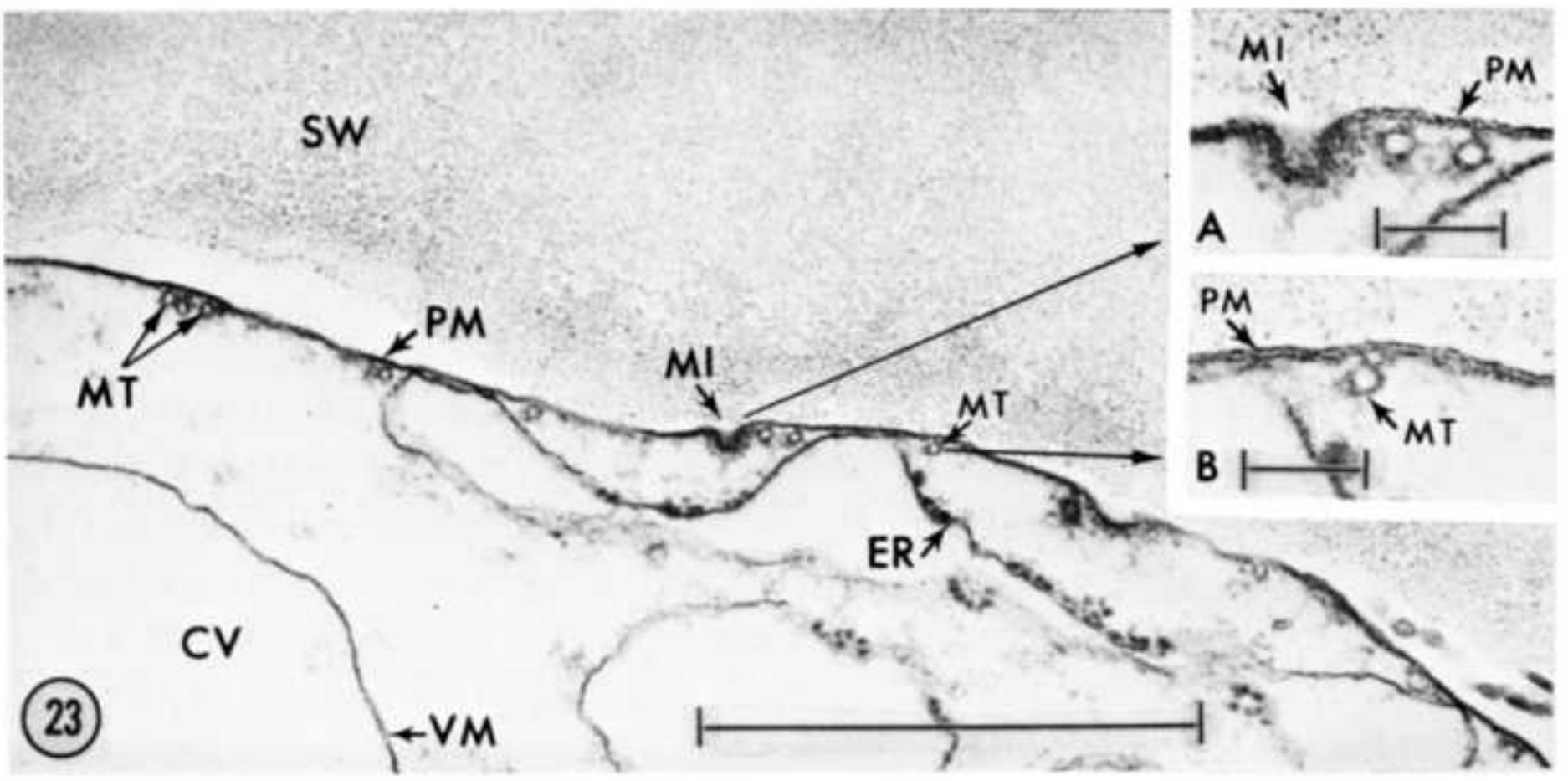


Figure 23 Cross section through fibre at 40 DPA. Note the micro-invaginations (MI, arrow) and the microtubules (MT) with apparent cross-links to the plasma membrane (PM). Much of the endoplasmic reticulum (ER) is coated with polysomes, while some smooth portions subtend the plasma membrane (PM) near the micro-invagination. Fixation A. Bar represents 1 μm.

Figure 23A Detail of micro-invagination (MI). Note subunits attached to the cytoplasmic surface of the invaginated plasma membrane (PM). Adjacent to it are two microtubules. Bar represents 1 μm.

Figure 23B Detail of cross-linkage between a cortical microtubule (MT) and the plasma membrane (PM). Bar represents 0.1 μm.

As the secondary wall thickening phase begins and spiral polysomes become the dominant ribosomal configuration, the ER cisternae are frequently swollen (Figure 23). Additionally, the ER may subtend micro-invaginations (see below) of the plasma membrane, structures which are observed only in fibres synthesizing secondary wall and which have not been reported before in the literature (Figure 23, inset A). Additionally, microvesicles seem to be derived from the endoplasmic reticulum (Figure 24). The microvesicles subtend plasmalemma micro-invaginations (Figure 24).

Golgi apparatus

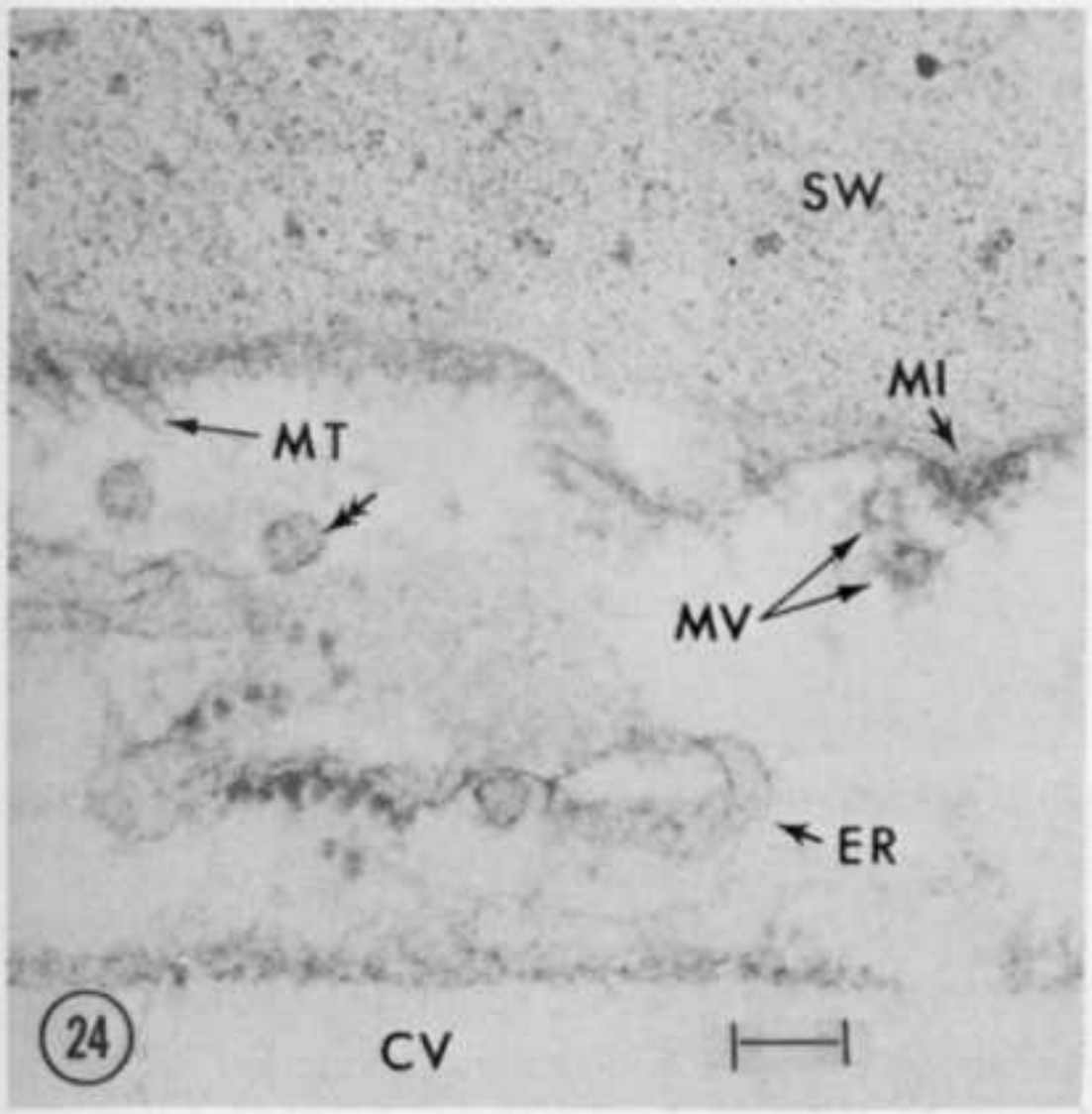
During primary wall formation, the Golgi apparatus consists of numerous dictyosomes (Figures 1–3). Calculations based on a fibre length of 2 cm and an average section thickness of 800 Å indicate that during primary wall synthesis there may be as many as 75,000 dictyosomes per cotton fibre. Each dictyosome is composed of five to seven cisternae (Figures 17 and 18), which in tangential section appear as plate-like vesicles with solid centres and fenestrated peripheries of anastomosing tubules (Figure 16). In cross sections of the cotton fibre, vesicles can be seen budding off the periphery of cisternae during the elongation phase (Figures 17–20). These vesicles can be seen appressed to the inner face of the plasma membrane (Figure 20) and presumably fusion of some vesicles with the plasma membrane does occur. The role of the Golgi apparatus in primary wall formation will be discussed below. In the secondary wall thickening phase, dictyosomes are less numerous and are altered in morphology but their function is not clear. They appear to be swollen with no product visible inside the cisternae.

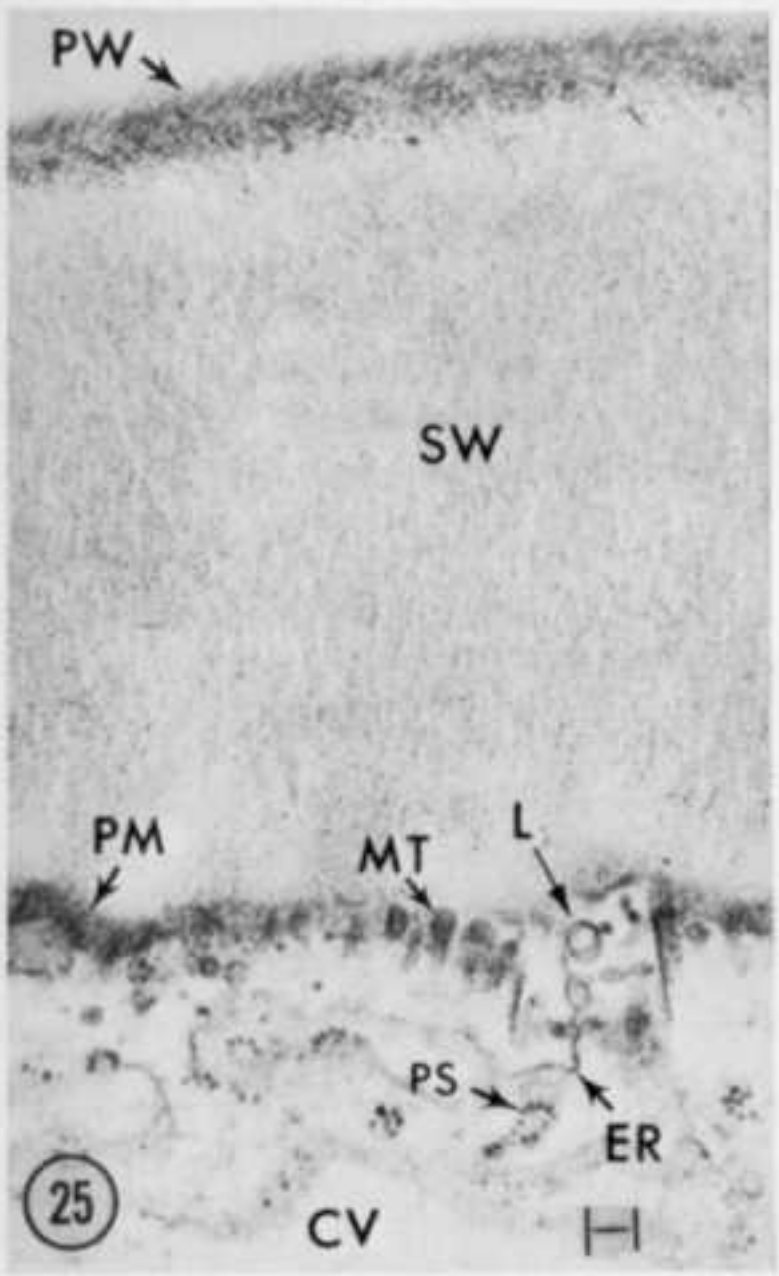
Plasma membrane

The plasma membrane is appressed to the inner surface of the cell wall during all phases of fibre development, but some noticeable differences between the cell surface of the elongation phase and the secondary wall thickening phases have been observed. The plasma membrane fibres fixed at 10 days post-anthesis appears to be continuous and smooth except for small bulges and indentations which give the cell surface a rippled appearance, and occasional small, convoluted regions (Figure 9).

At 22 days post-anthesis, two significant differences in the plasma membrane morphology were observed: (1) small micro-invaginations with densely staining subunits attached to the cytoplasmic surface are fairly common (Figures 23, 24 and 28). These micro-invaginations are 300–500 Å in diameter, penetrating about 400 Å into the cytoplasm (Figure 23, inset A). The subunits lining the inner surface are each about 170 Å in length, extending from the inner surface of the plasmalemma into the cytoplasm, and they appear to be composed of a globular unit about 70 Å in diameter, which is linked to the plasma membrane by a narrow strand approximately 100 Å in length, and; (2) large invaginated areas (Figures 30, 32, 33, and 34) containing numerous tubular convolutions are prominent in the secondary wall thickening phase. Similar structures have been reported in other plant cells such as xylem (Cronshaw, 1965). All of these resemble a structure, first identified in fungi as a lomasome (border body) by Moore and McAlear (1961). Figure 31 shows a multivesicular body containing tubular subunits similar in size to those in the 'lomasome-like' bodies.

The plasma membrane of the secondary wall thickening stage stains intensely with the PTA-chromic acid post-stain as expected (Figures 36–38). Furthermore, the membranes of the 'lomasome-like' bodies demonstrate an affinity for this stain equivalent to that of the plasma membrane. The cortical microtubules do not stain (Figure 36).





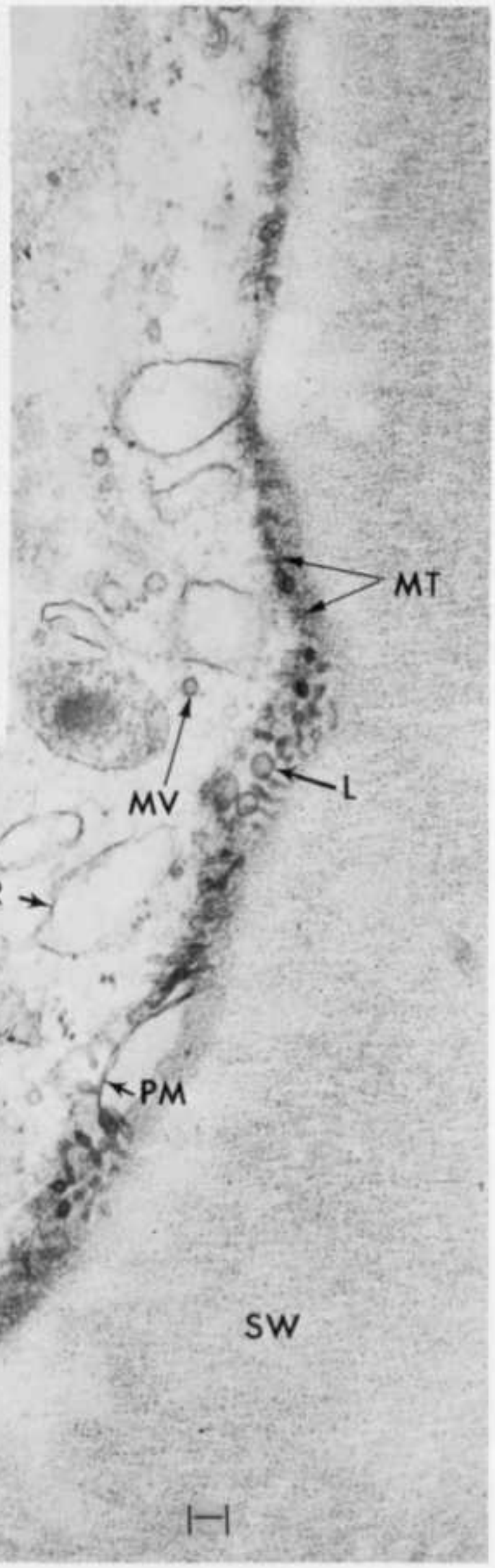
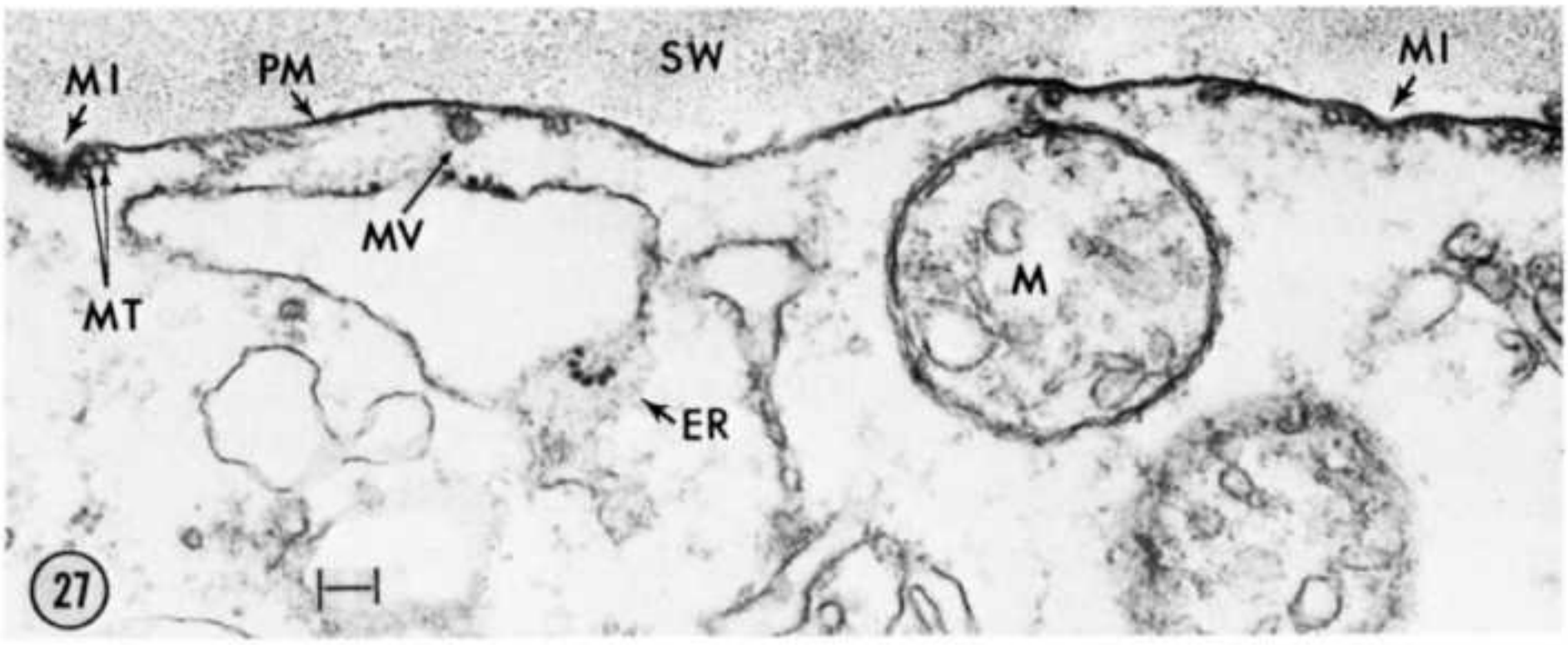


Figure 24 Slightly oblique section at 22 DPA. Two microvesicles (MV), possibly ER-derived, lie near a micro-invagination. Note microvesicle which appears to be budding from a smooth region of the ER (double arrow). Fixation A. Bar represents 0.1 μm.

Figure 25 Slightly oblique section at 22 DPA. Polysomes (PS) are visible attached to the ER. MIcrotubules (MT) flank a 'lomasome-like' region (L) of the plasma membrane (PM). Fixation A. Bar represents 0.1 μm.

Figure 26 Slightly oblique section at 22 DPA. The ER is coated with spiral polysomes (PS). Cortical microtubules (MT) lie adjacent to the plasma membrane (PM) and parallel to the orientation of microfibrils in the secondary wall. Fixation A. Bar represents 0.1 μm.



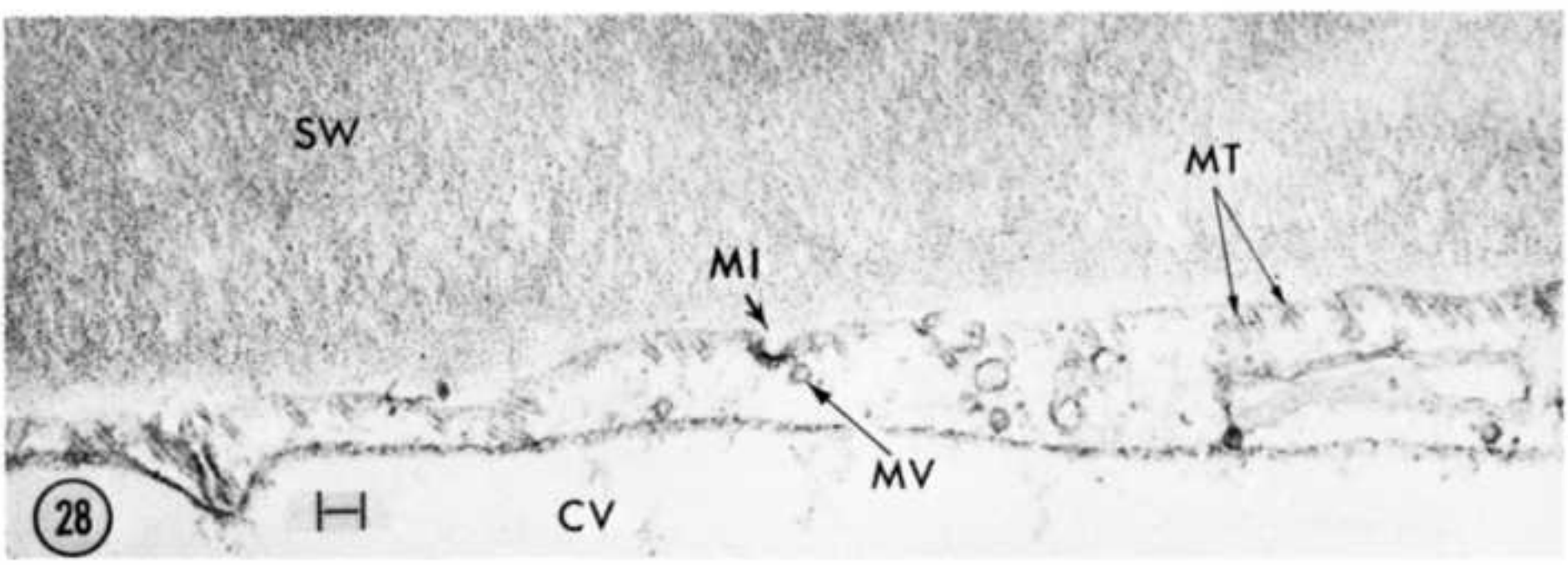


Figure 27 Cross section at 40 DPA. A micro-invagination (MI) is visible at the far left. It appears to be flanked by a pair of microtubules (MT) at its right. The adjacent plasma membrane (PM) is subtended by smooth ER. Note the incipient micro-invagination (MI) at the far right. Fixation A. Bar represents 0.1 μm.

Figure 28 An oblique section at 22 DPA. Note the micro-invagination (MV) subtended by a micro-vesicle (MV), probably ER-derived. Fixation A. Bar represents 0.1 μm.

Primary cell wall

In thin sections, the primary wall appears as a darkly staining, fibrillar material from 0.1 μm to 0.5 μm thick. Individual cellulosic microfibrils are sometimes difficult to visualize since they do stain themselves, and since the primary wall is not dense enough to permit visualization of them in negative contrast to the darkly staining matrix materials.

The Golgi apparatus of the elongating cotton fibre appears to be involved in primary wall formation. Both fixation A and fixation B demonstrate fibrillar materials inside vesicles budding from the cisternae of dictyosomes of specimens fixed 10 days post-anthesis (Figures 19 and 5 respectively). The fibrillar substance inside these vesicles is very similar in stain intensity and appearance to the material in the primary wall. The dictyosome cisternae are typically oriented parallel to the long axis of the fibre, and in specimens prepared according to fixation A, a product is visible within intercalary cisternae. In Figure 17 a thin layer of product can be seen inside the centre of a cisterna. This product might be confused with the intercisternal elements sometimes seen in dictyosomes (Turner and Whaley, 1965), but careful analysis shows that the structure seen here lies inside the cisterna rather than between cisternae. In Figure 18 strands of product appear within a central dilation of a cisterna. Lateral dilations containing

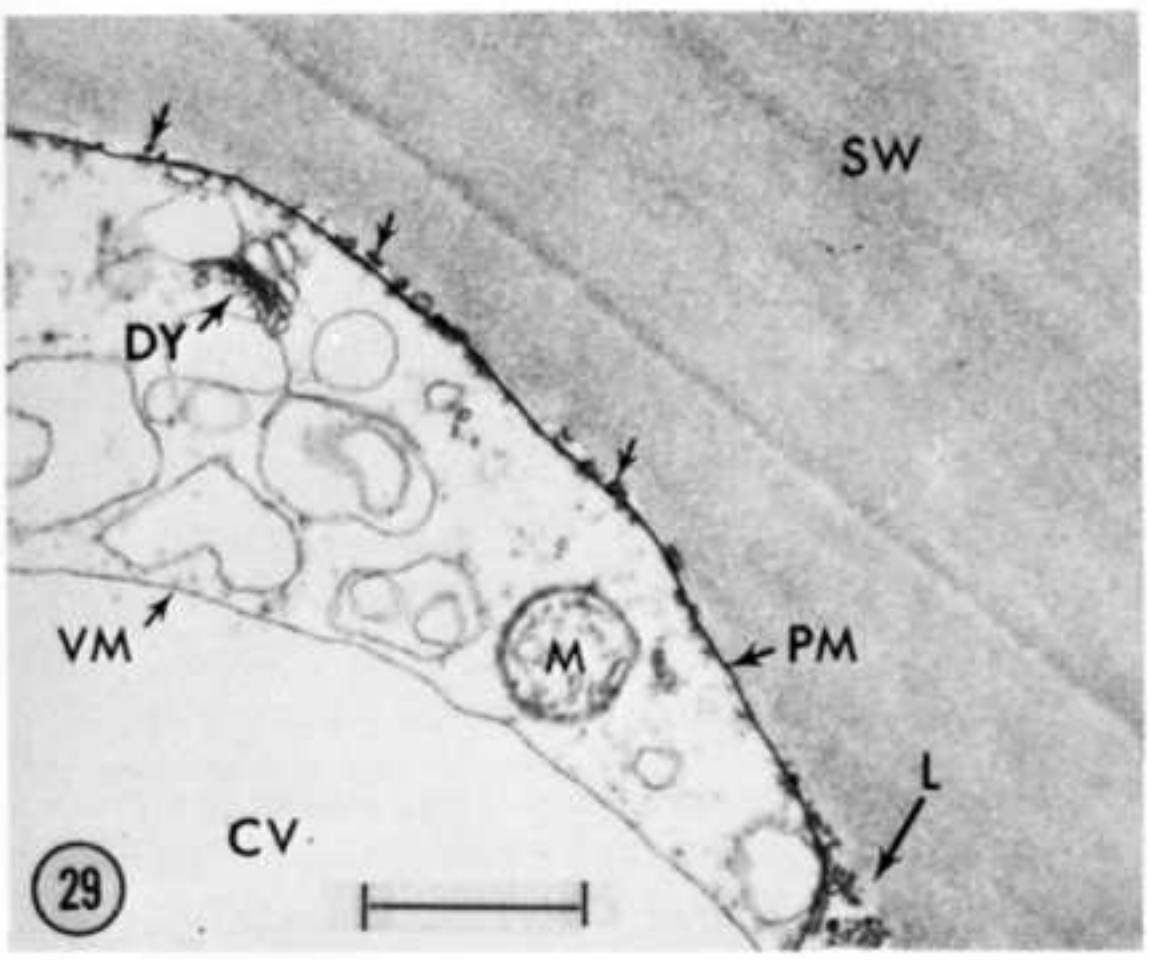
product also occur (Figure 19). Vesicles blebbing from the periphery of cisternae contain the fibrillar material, and these secretory vesicles are sometimes observed appressed to the inner surface of the plasma membrane (Figure 20). Although examples of fusion with the plasma membrane and exocytosis of the Golgi product have not been observed thus far, it is consistent with observations in other plant tissues (Cronshaw, 1965; Pickett-Heaps, 1966; Brown *et al.*, 1969) to assume that fusion and exocytosis of the product do occur, contributing new material to the primary cell wall.

Preliminary results from experiments with the periodic acid-silver methenamine stain for carbohydrates show a strong positive reaction at the primary wall as one would expect, and there is some evidence of a positive reaction inside vesicles budding from cisternae of the Golgi apparatus.

Judging from the appearance of microfibril layers (Figures 10, 22 and 25) it appears that the primary wall is made of several layers which may spiral along the length of the cotton fibre in alternative directions; a layer spiralling in an alpha helix pitched at an angle to the long axis of the fibre; followed by a layer spiralling at the same pitch but in a beta helix; followed by another alpha helix and so on. The mechanism controlling this primary wall orientation is unknown. Cortical microtubules may be involved, but they are fewer in number during primary wall synthesis than during secondary wall synthesis. No extensive study of tip growth versus intercalary growth of the primary wall was undertaken. However, since dictyosomes with product are found all along the length of the cotton fibre, it is not unreasonable to infer that intercalary growth occurs.

Secondary cell wall

Secondary walls up to 8 μm thick were observed (Figure 7). The secondary wall does not stain so intensely as the primary wall since it contains fewer matrix substances such



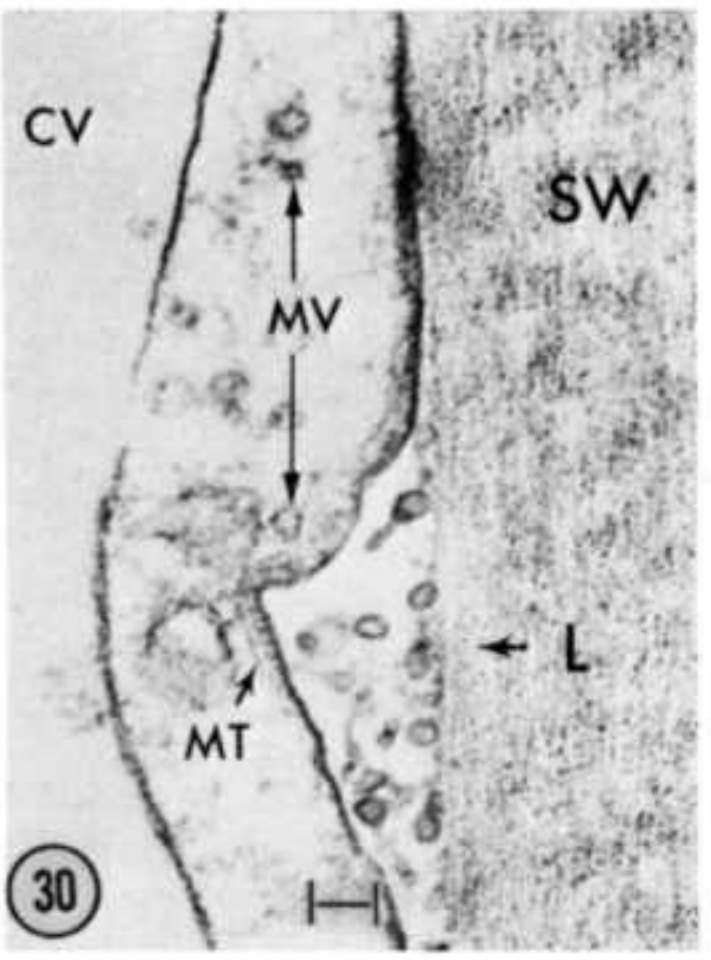
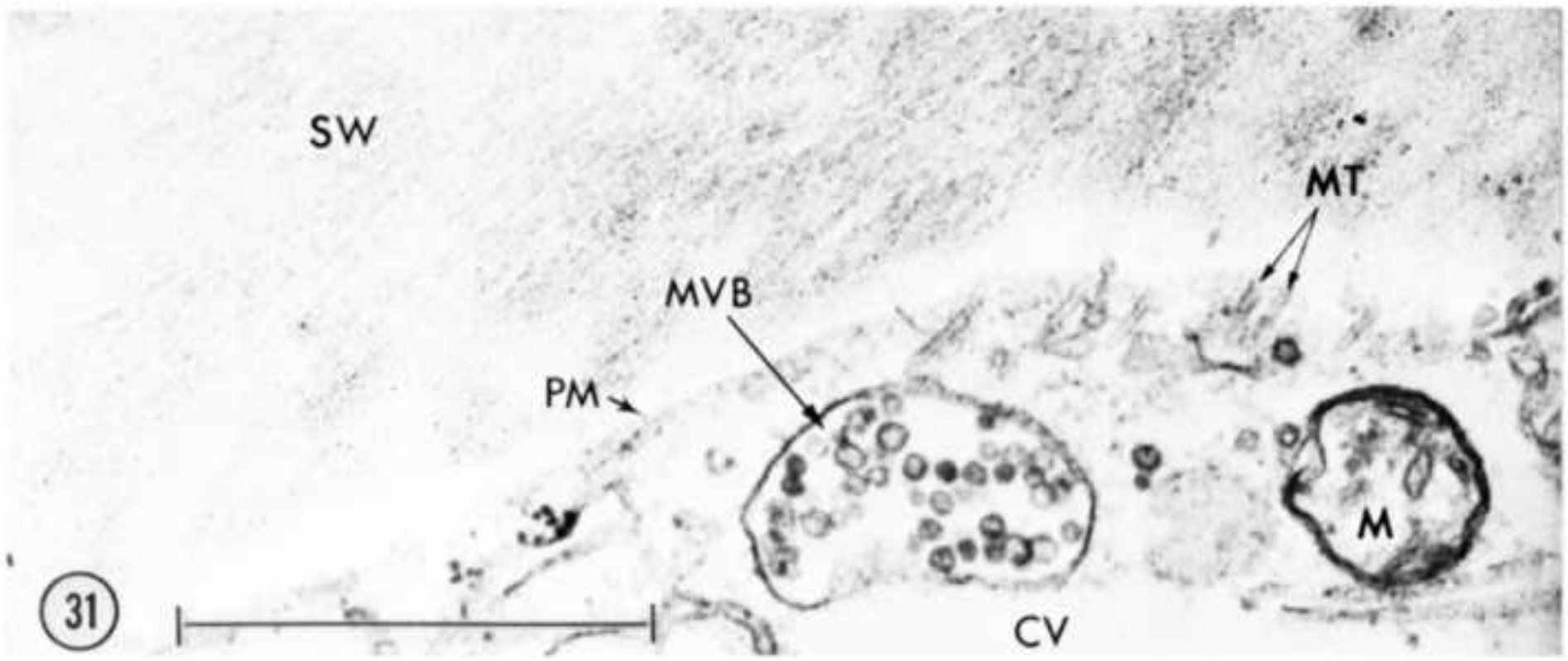
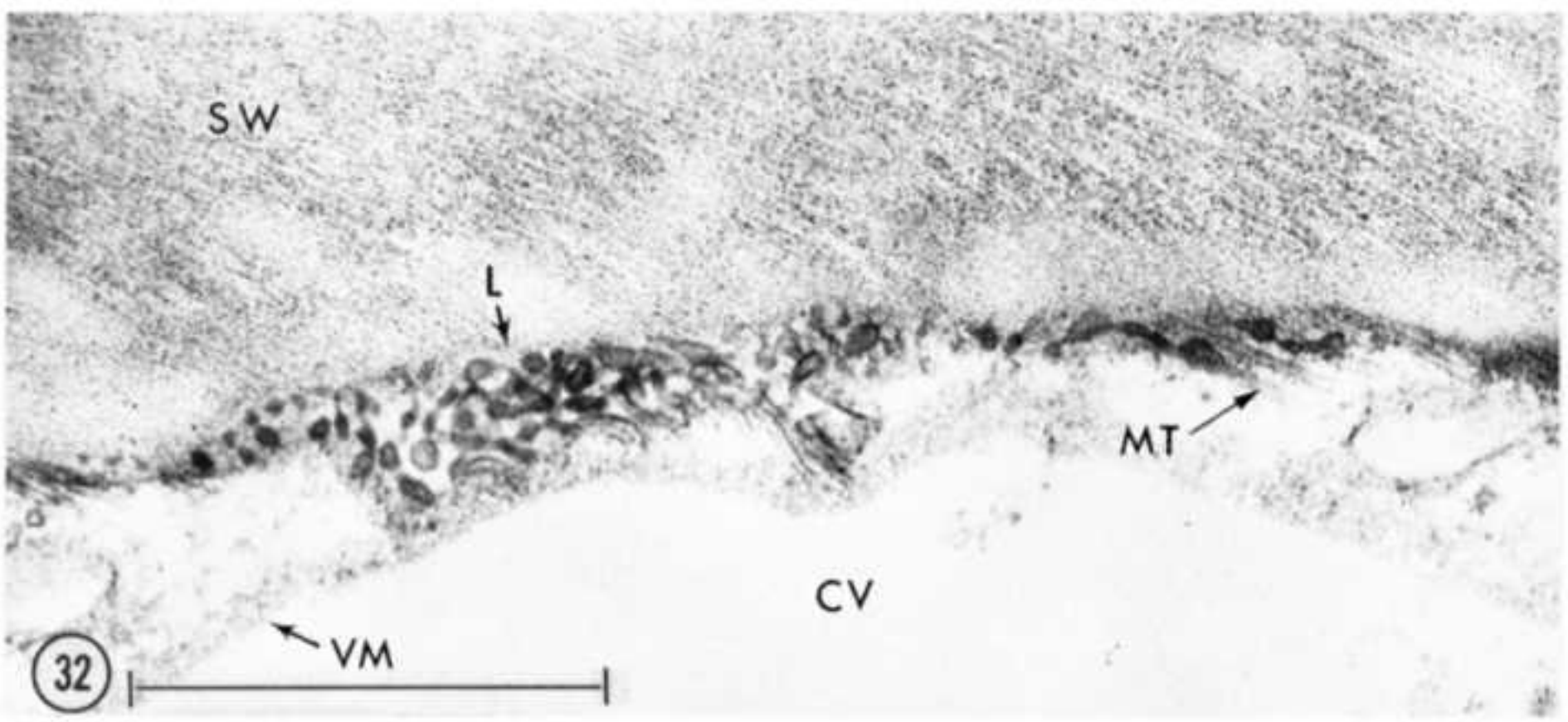


Figure 29 Cross section at 40 DPA. Note the 'lomasome-like' body (L, lower right) and the membrane-like material between the plasmalemma and the secondary wall (arrows). Fixation A. M = mitochondrion. Bar represents 1 μm.

Figure 30 A longitudinal section at 22 DPA. A 'lomasome-like' body (L) with numerous vesicles is subtended by a microtubule (MT) which appears to follow the contour of the invagination of the plasma membrane to which it is cross-linked. Fixation A. Bar represents 0.1 μm.





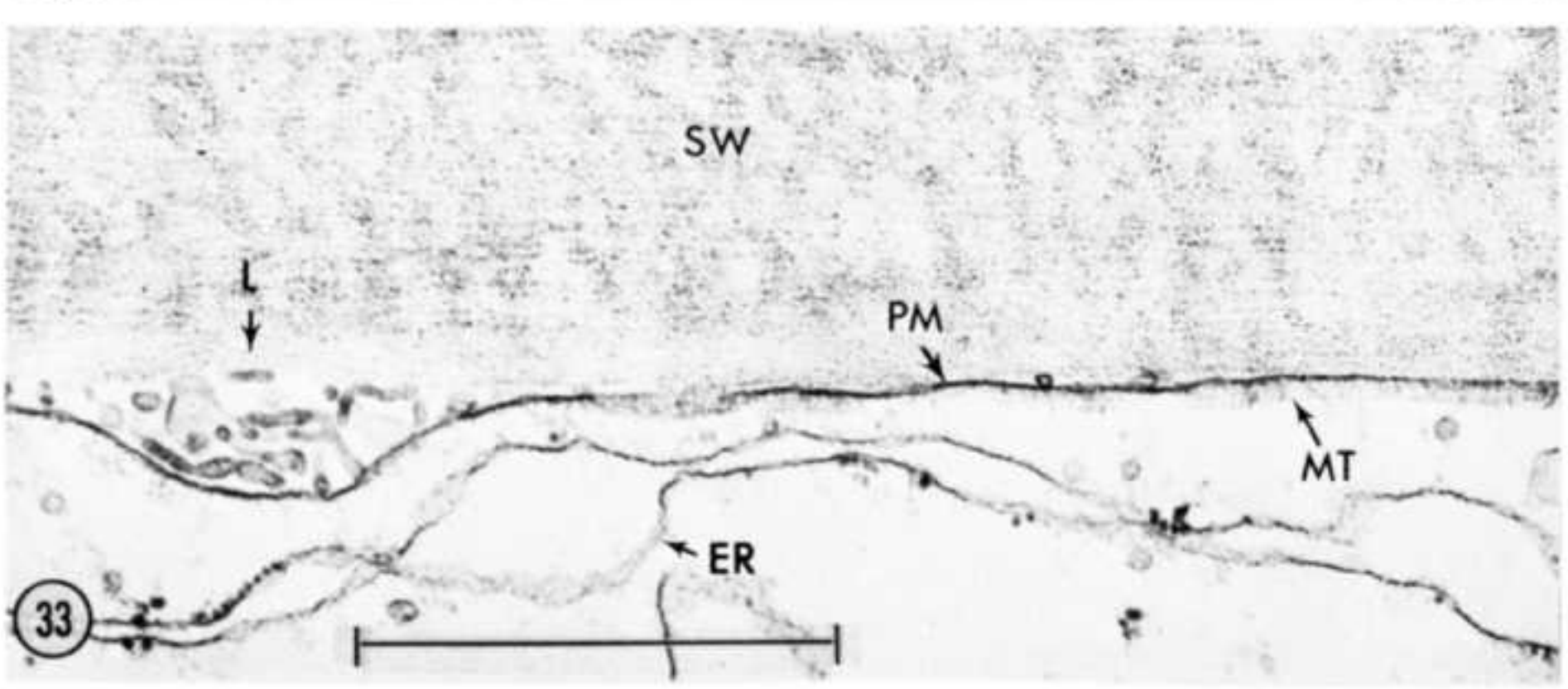
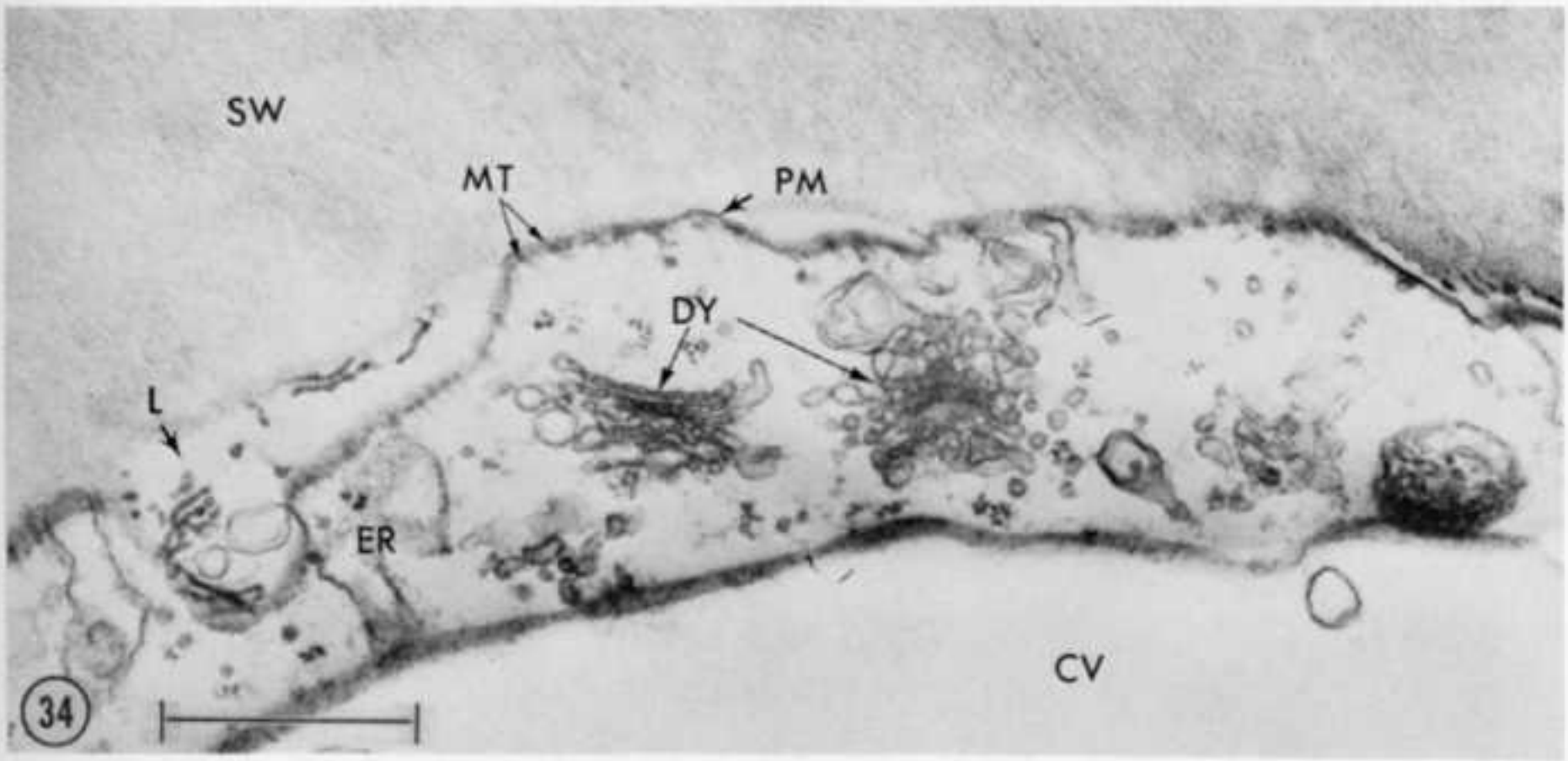


Figure 31 An oblique section at 22 DPA. Note the multivesicular body (MVB) which contains many 'lomasome-like' vesicles. This structure may be a section artifact in which an invagination of the plasma membrane may be mistaken for a sub-surface vesicle. Fixation A. Bar represents 1 μm.

Figure 32 Oblique section at 22 DPA. Note the 'lomasome-like' figure and the cortical microtubules. The microfibrils are visible in negative contrast. Note the parallel orientation of microtubules, 'lomasome-like' vesicles, and secondary wall microfibrils. Fixation A. Bar represents 1 μm.

Figure 33 A longitudinal section at 22 DPA. The ER subtends the plasma membrane near a 'lomasome-like' body (L). A microtubule (MT) is visible immediately adjacent to the plasmalemma. Fixation A. Bar represents 1 μm.



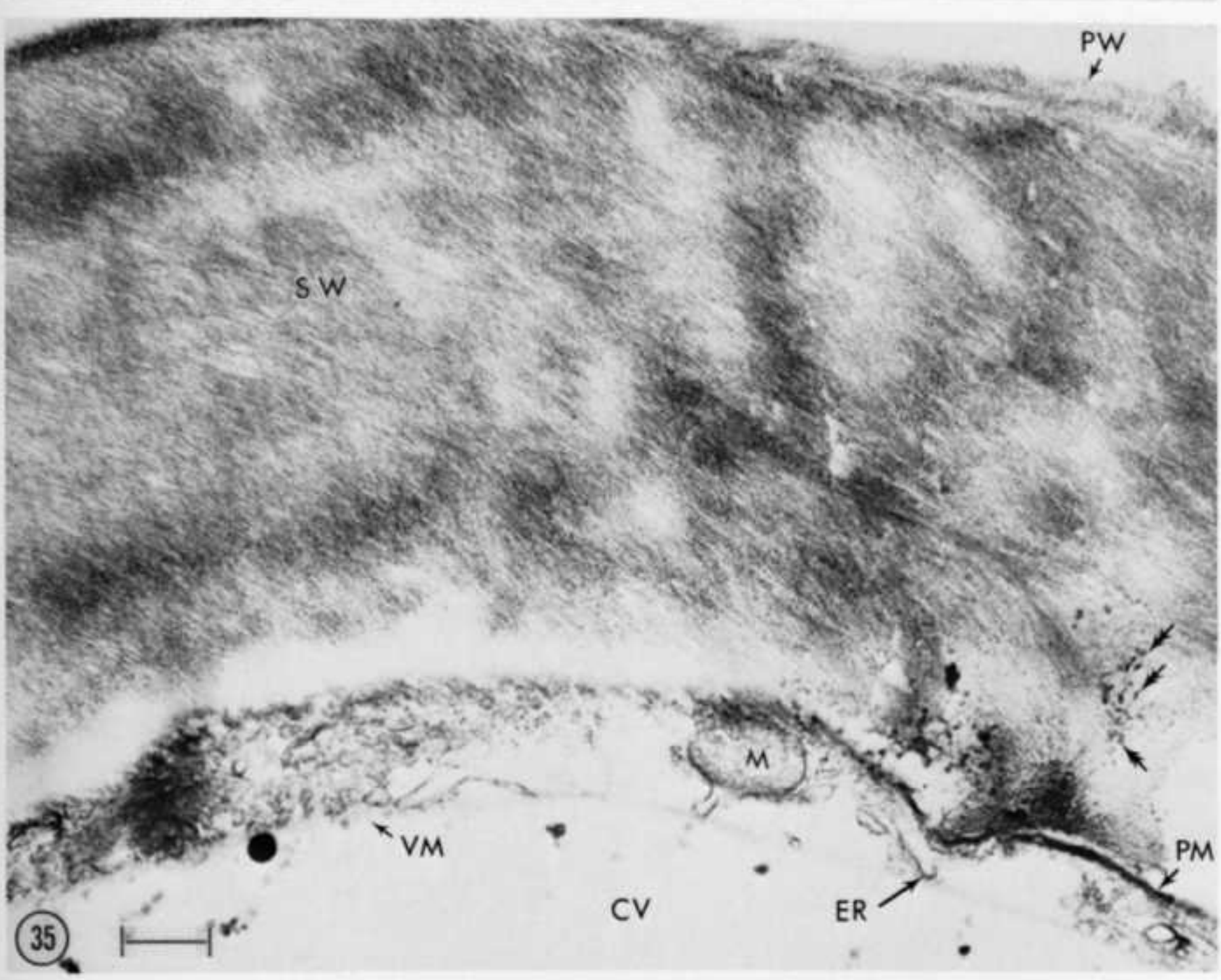
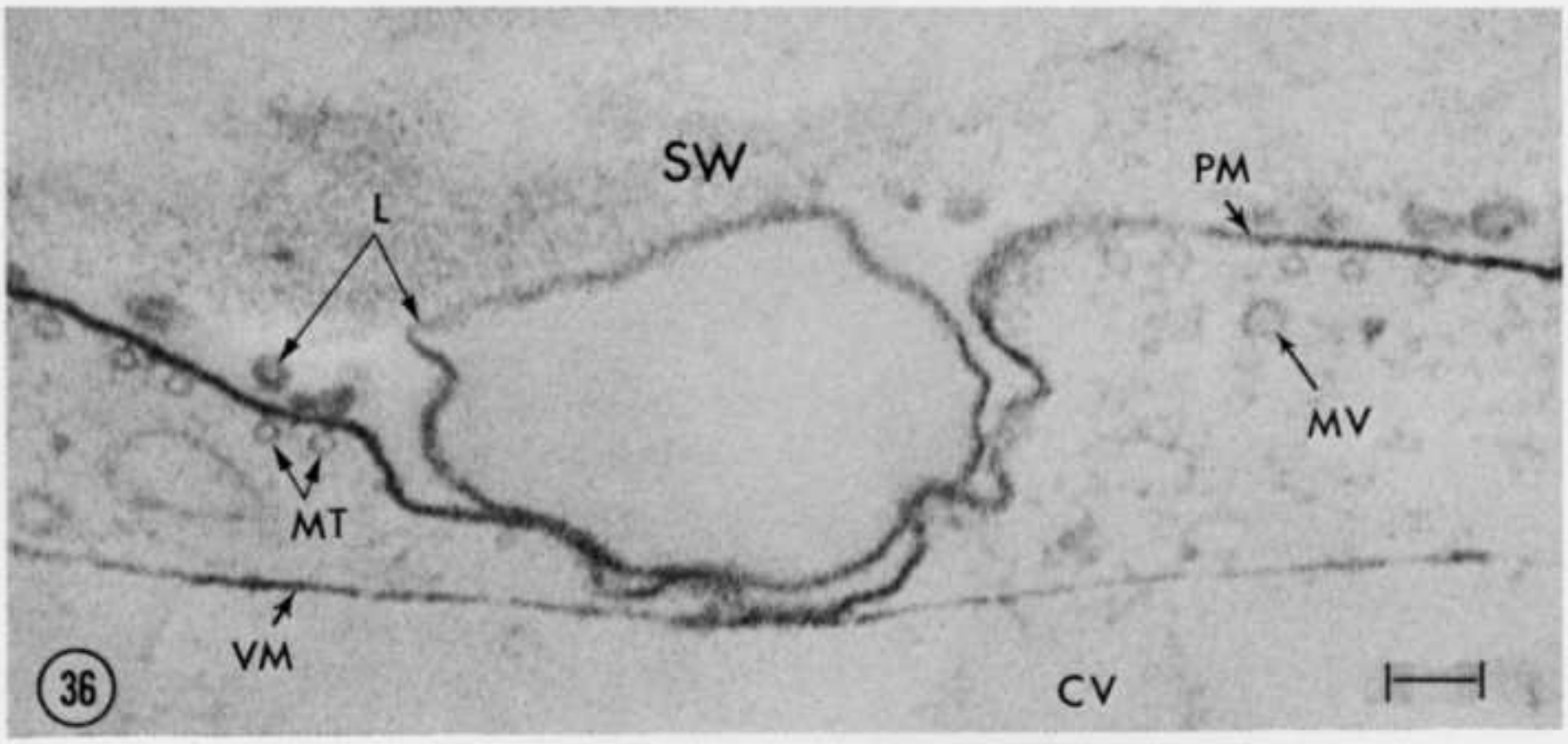
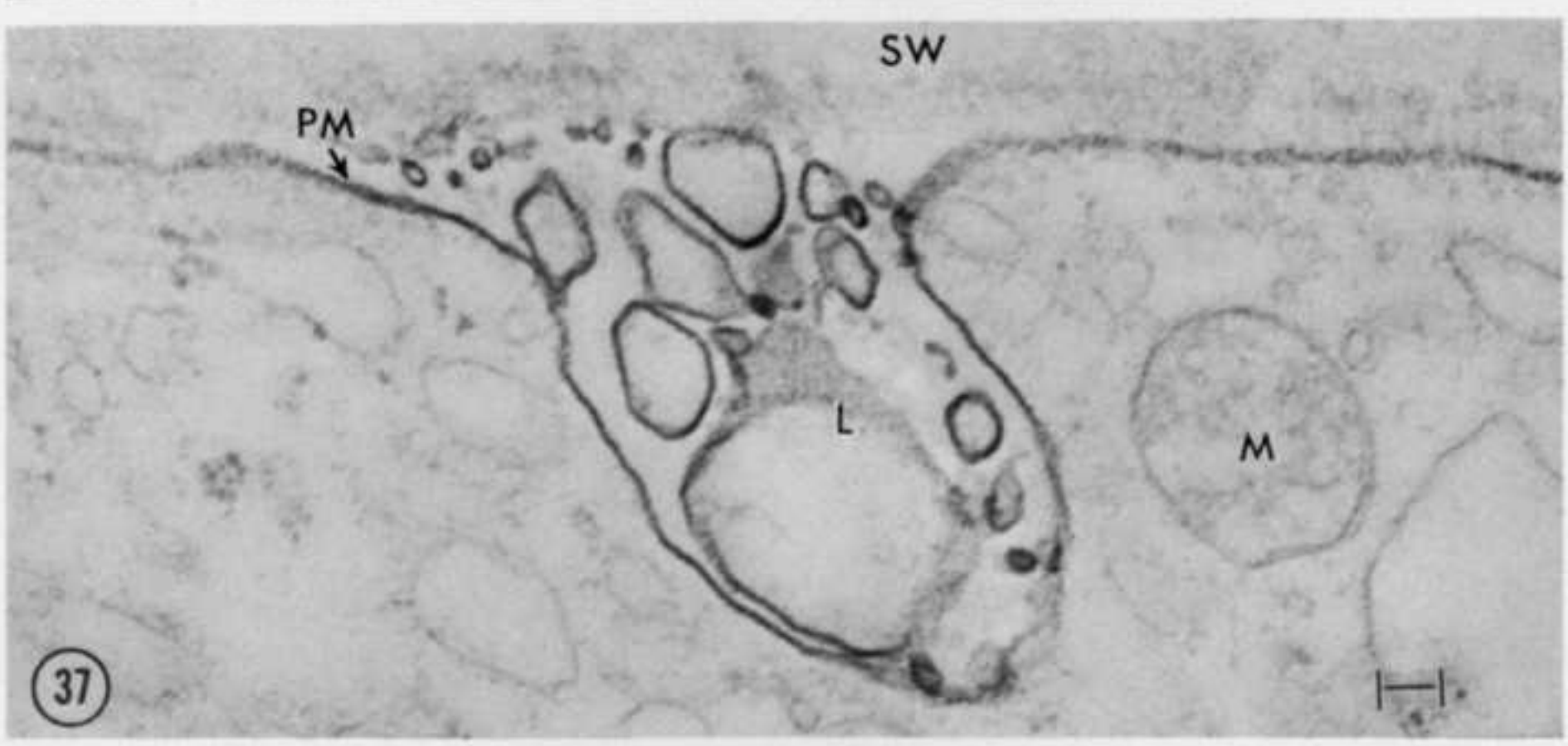


Figure 34 An oblique section at 22 DPA showing two altered dictyosomes (DY) during the secondary wall (SW) stage and a 'lomasome-like' structure (L) subtended by the ER. Fixation A. Bar represents 1 μm.

Figure 35 An oblique section at 29 DPA reveals a 'lomasome-like' area from which microfibrils appear to extend into the secondary wall in a whorled pattern. Note 'lomasome-like' material (L) incorporated in the wall (arrows). Fixation C. Bar represents 1 μm.





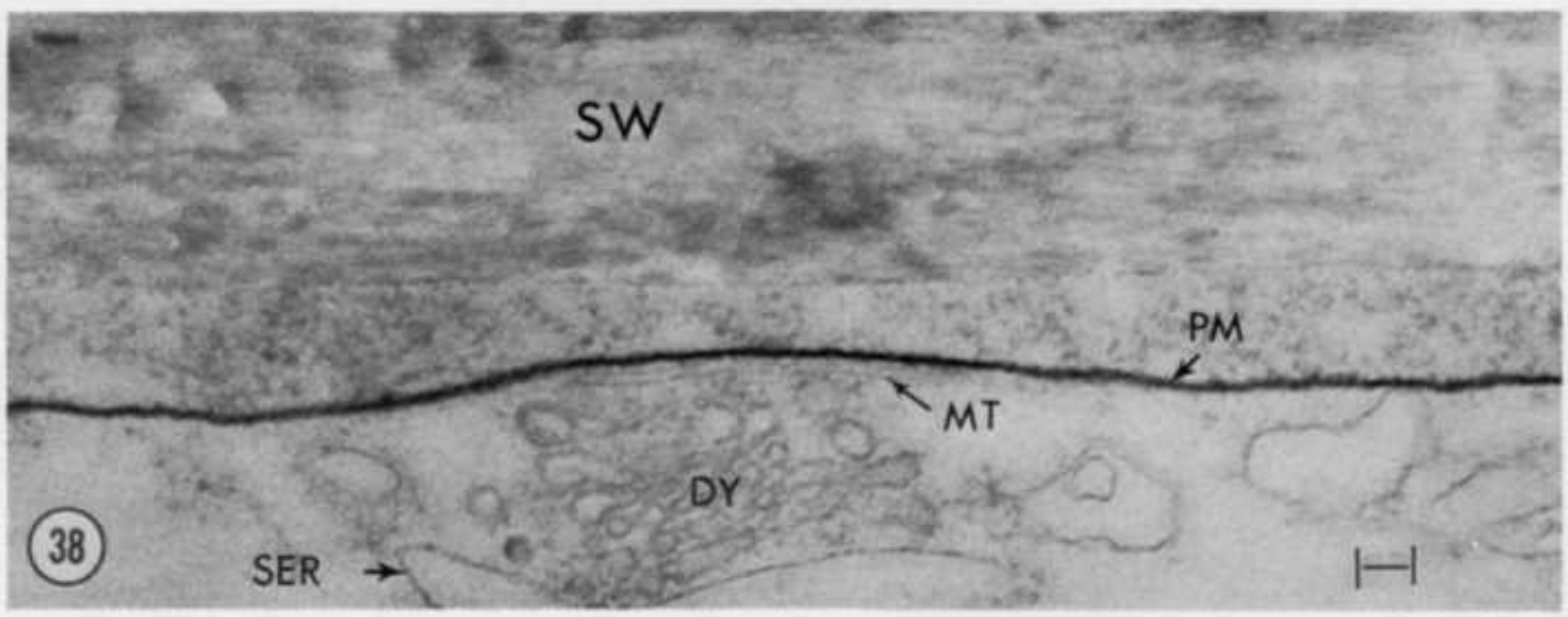


Figure 36 A cross section at 22 DPA post-stained with PTA-chromic acid. The 'lomasome-like' body and the plasma membrane stain with equal intensity. Note that the cortical microtubules (MT) do not stain. The vacuolar membrane (VM) does stain but not as intensely as the plasma membrane (PM). Fixation A. Bar represents 0.1 μm.

Figure 37 An oblique section at 22 DPA post-stained with PTA-chromic acid. The 'lomasome-like' body (L) stains with an intensity equivalent to that of the plasma membrane (PM). Other membranes stain less intensely. Fixation A. Bar represents 0.1 μm.

Figure 38 A longitudinal section at 22 DPA post-stained with PTA-chromic acid. The plasma membrane stains very intensely. Note unstained microtubule. Fixation A. SER = smooth endoplasmic reticulum. Bar represents 0.1 μm.

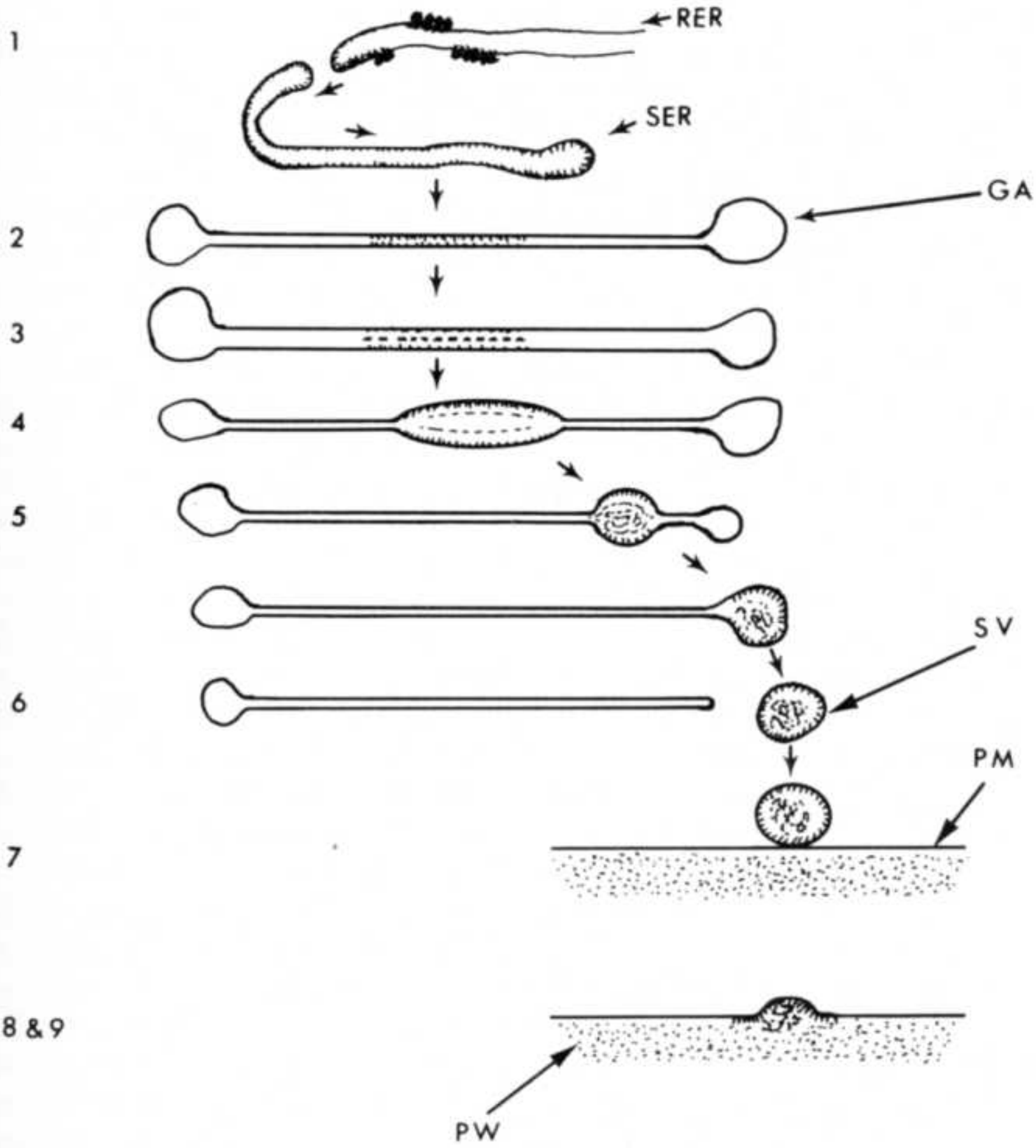


Figure 39 Cross-sectional model of primary wall synthesis in the cotton fibre. For details see text.

as pectins and hemicelluloses. However, the high density and the high degree of order of the cellulosic microfibrils of the secondary wall enhance the negative contrast effect provided by the staining of matrix materials surrounding the microfibrils (Figures 8 and 35) (Heyn, 1966). Microfibrils appear to be oriented nearly parallel to the long axis of the cotton fibre at a pitch of about 22°. Figure 35 shows a slightly oblique section of a 29-day-old fibre fixed with the simultaneous glutaraldehyde-osmium tetroxide fixation (fixation C). In this section, microfibrils appear to lie in a whorled pattern originating from a well-defined locus or micro-invagination on the plasma membrane. Membrane-like material appears to be associated directly with the secondary wall at this locus. It is tempting to speculate that this may be a localized site of secondary wall microfibril synthesis in the cotton fibre, but the fact remains that future research is necessary to determine whether or not this configuration of microfibrils arising from a definitive locus on the plasma membrane is an artifact of fixation.

Discussion

This study provides a foundation for understanding the pathways of cell wall biogenesis in the cotton fibre. The cytological evidence clearly demonstrates distinctive ultrastructural modifications accompanying the conversion from primary wall synthesis and cell elongation to massive secondary wall synthesis.

The ultrastructure of the cotton fibre is similar in many respects to other higher plant cells. Especially striking are similarities to elongate, highly vacuolate plant cells in which differentiation is culminated with the production of a thick secondary wall. The structure, alignment, and function of subcellular components of the cotton fibre are similar to those of xylem and phloem (Cronshaw and Wardrop, 1964; Cronshaw, 1965; Wardrop, 1965; Srivastava and O'Brien, 1966a,b; Robards, 1968). In these cell types the subcellular components undergo considerable change as the cells differentiate. Vacuolation is believed to be associated with secondary wall thickening in xylem (Wardrop, 1965), as well as in cambium and phloem (Srivastava and O'Brien, 1966a,b). It has been suggested that rapid and extensive vacuolation results in the alignment of the endoplasmic reticulum and the Golgi apparatus with their membranes oriented parallel to the cell wall (Cronshaw and Wardrop, 1964; Wardrop, 1965). These suggestions are supported in the present study. Furthermore, the conversion from free ribosomes during primary wall synthesis to spiral polysomes during secondary wall synthesis (Berlin and Ramsey, 1970) implies that differentiation is coupled through gene amplification with a specialization in protein synthesis of glucan synthetases, microtubular protein synthetases or other specialized enzymes required for the terminal synthesis of secondary wall.

Primary cell wall

A model for the biogenesis of the primary cell wall of the cotton fibre is presented in Figure 39. From this model, and in combination with data from other systems, it is postulated that: (1) synthetases for matrix substances and, perhaps, for cellulose are made in the endoplasmic reticulum. (2) These enzymes are transferred to the Golgi apparatus via membrane flow (Franke *et al.*, 1971; Morré and Mollenhauer, 1974) and are incorporated within the inner surfaces of the cisternal membranes. (3) Substrates in the cisternae of the Golgi apparatus react with enzymes bound to the cisternal membranes, forming incipient secretory products. (4) The products are released from the inner surfaces of the cisternal membrane into a central dilation. (5) The dilation moves laterally, transporting the products and membrane to the periphery of the cisterna forming a nascent secretory vesicle. (6) The secretory vesicle separates from the cisterna and migrates to the plasma membrane. (7) The membranes of the secretory vesicles and the plasma membrane fuse and the product is released via exocytosis. (8) The products are incorporated into the growing primary cell wall; and (9) enzymes (perhaps including cellulose synthetases) transported within the secretory vesicle become exposed to the cell exterior where they initiate synthesis while attached to the membrane, or existing free in the periplasm, or attached to components of the cell wall itself.

In addition to the synthesis and transport of cell wall materials, the Golgi apparatus contributes new membrane to the plasmalemma and tonoplast thereby increasing the surface area of the cell during primary wall biogenesis and cell elongation. The abundance of dictyosomes throughout the length of the cotton fibre during elongation supports the concept of intercalary addition of new membrane as well as primary wall materials along the entire length of the cotton fibre as opposed to isolated, tip growth (O'Kelley, 1953; Delmer, 1974).

It must be emphasized that the composition of the product observed in the Golgi apparatus of the cotton fibre is unknown. Since the primary wall contains a large percentage of matrix materials such as pectins and hemicelluloses, and a smaller percentage of cellulose (Roelofsen, 1959; Siegel, 1962), it seems likely that the product visible in the Golgi apparatus may consist of some or all of these substances. Autoradiographic evidence from the root cap cells of wheat implicates the Golgi apparatus as a possible site of pectin and hemicellulose biosynthesis (Northcote and Pickett-Heaps, 1966). In a study of wheat xylem, Pickett-Heaps (1966) found tritiated glucose label in the Golgi apparatus, indicating polysaccharide biosynthesis in that organelle.

Convincing evidence for the involvement of the Golgi apparatus in cellulose biosynthesis has come from the research of Brown and co-workers (Brown *et al.*, 1969, 1970, 1973; Herth *et al.*, 1972). It has been postulated that the Golgi apparatus may be active in carrying membrane-bound enzymes and intermediates to the plasma membrane for cellulosic microfibril biosynthesis (Kiermayer and Dobberstein, 1973; Roland and Pilet, 1974). Although most investigations have focused on the involvement of the plasma membrane alone in cellulose biogenesis, recent evidence that the Golgi apparatus membranes carry a B(1–4) glucan synthetase suggests that this organelle may act in concert with the plasma membrane in the process of cellulose biogenesis in higher plants (Van Der Woude *et al.*, 1974).

Central dilations of dictyosome cisternae have been reported in other plant cells (Franke, 1970), but they have not been treated as functional structures involved in primary cell wall formation. The occurrence of central dilations containing product is analogous to some aspects of scale biogenesis in the alga *Pleurochrysis* (Brown *et al.*, 1969, 1970, 1973). Nascent scales are formed appressed to the inner surfaces for dilated membranes. Cytochemical enzyme localization has demonstrated certain enzymes for specific steps of scale biogenesis in dilated regions (Romanovicz and Brown, 1974). Further elucidation of the composition of the Golgi apparatus product awaits the use and development of suitable autoradiographic and cytochemical staining procedures capable of differentiating between different polysaccharides inside the cell.

Secondary cell wall

The ultrastructure of the secondary cell wall raises some interesting questions, but there is insufficient evidence at this point to support any specific model for secondary wall biogenesis. One intriguing possibility suggested by this research is that the endoplasmic reticulum may become the major vehicle for synthesis and transport of enzymes or other materials required for secondary cell wall formation. When secondary wall deposition begins, an increase in the number of ER-associated polysomes is observed. Further, the ER cisternae frequently subtend the plasma membrane, and smooth regions of the ER gives rise to microvesicles which appear to associate with certain areas of the plasma membrane. This association was not observed during primary wall formation. This suggests that the activity of the endoplasmic reticulum may increase as the cell shifts from primary to secondary wall formation. In addition, the PTA-chromic acid post-stain suggests that some ER membranes undergo differentiation, acquiring characteristics which may permit fusion with the plasmalemma. Inferring from this evidence and with suggestions that the activity of the Golgi apparatus may decrease or even cease during secondary wall formation, it is possible that the ER could play a more direct role in the synthesis and transport of materials required for secondary wall formation, thus by-passing the Golgi pathway of exocytosis.

Evidence from other plant tissues supports some of these conclusions. Robards (1968) noted that the endoplasmic reticulum of willow xylem plays a more active role in secondary wall formation than in primary wall formation. Other investigators have proposed that the ER has some direct function in cell wall synthesis (Buvat, 1964; Cronshaw, 1965; Cronshaw and Bouck, 1965; Pickett-Heaps and Northcote, 1966; Bowles and Northcote, 1972, 1974). Although not observed in this study, products have been reported inside the ER cisternae of regenerating *Coleus* xylem (Hepler and Newcomb, 1964) and in differentiating willow xylem (Robards, 1968).

Autoradiographic evidence from wheat xylem suggests that the ER is involved in transport or synthesis of cell wall material (Pickett-Heaps, 1966). In the same study, Pickett-Heaps noted that the ER forms a definitive pattern with cortical microtubules. In this pattern, the ER cisternae and groups of microtubules subtending the plasma membrane alternate along the circumference of the cell. The shift from primary to secondary wall synthesis appears to involve a shift in cytomembrane flow as well as plasmalemma morphology. Changes observed in the endomembrane system include: (1) formation of 'lomasome-like' structures associated with the plasma membrane, (2) formation of micro-invaginations of the plasma membrane, which have a coating on the cytoplasmic side, and (3) increased endoplasmic reticulum activity accompanied by decreased Golgi apparatus activity.

Many studies have demonstrated membrane-like vesicular elements outside the plasma membranes of plant cells (Wardrop, 1965; Esau *et al.*, 1966). The postulated functions of these 'lomasome-like' bodies are numerous (see Marchant and Robards, 1968, for review), ranging from the endocytotic function of secondary vacuole formation (Mahlberg *et al.*, 1974) to the exocytotic function of transporting cell wall materials and enzymes to the periplasm (Buvat, 1964; Robards, 1968). Even if these structures are fixation artifacts, as some researchers contend, the fact that they occur only during secondary wall formation could suggest that plasmalemma cellulose enzyme complexes while bound to the microfibrillar product, may be pulled from the plasma membrane during fixation, yielding the 'lomasome-like' fragment. Presumably some characteristics of the plasma membrane may change as the ER contributes and incorporates protein (perhaps in the form of enzyme complexes) into the plasma membrane. Conceivably, such an addition could occur without the necessity of new membrane synthesis.

The micro-invaginations are a significant part of the plasma membrane structure as the cotton fibre begins secondary wall biosynthesis. The possibility that these may be artifacts remains, but it would seem doubtful due to the frequency of their occurrence and their consistent morphology. Perhaps micro-invaginations may be membrane receptor sites for exocytosis of microvesicles (Brown, 1975) since microvesicles frequently are found near the micro-invaginations. An alternative function for the micro-invaginations is that they may represent glucan synthetase complexes embedded in the membrane and hence would be the sites of microfibril synthesis. This concept is similar to the recently described plasma membrane-bound cellulose enzyme complex in *Oocystis* (Brown and Montezinos, 1976).

Groups of microtubules lie adjacent to the micro-invaginations, and it is inviting to speculate that through the cross-linking elements, the microtubules direct the pattern of microfibril deposition. Cortical microtubules increase both in number and specificity of orientation during secondary wall biogenesis. All cortical microtubules assume a common orientation approximately parallel to the long axis of the cotton fibre. The

change in cortical microtubules parallels an increase in the degree of order and specificity of orientation of cellulosic microfibrils in the secondary cell wall. This coincidence suggest that cortical microtubules may control the directionality of microfibril deposition. Many investigators have noted a similar change in the distribution of cortical microtubules in differentiating xylem (Hepler and Newcomb, 1964; Wooding and Northcote, 1964; Newcomb, 1969; Hepler and Foskett, 1971). A number of colchicine studies show that disruption of microtubules causes cell wall deposition to become disordered (Bunning *et al.*, 1956; Pickett-Heaps, 1967b). The mechanism by which microtubules control the direction of microfibril deposition remains a topic of speculation. The cross-links between microtubules and the plasma membrane shown here and other studies (Cronshaw, 1967; Robards, 1968; Kiermayer, 1968) suggest that directionality of microfibril deposition may be effected by plasmalemmal motion associated with microtubular movement. These considerations remain viable in a system of microfibril orientation based on protoplasmic streaming. Movement of the protoplast was used to explain patterns in cell wall microfibril orientation in plants nearly a century ago (Crüger, 1885). Opponents of the protoplasmic streaming model contend that microfibrils are oriented purely by forces such as charge interactions between microfibrils (Ben-Hayyim and Ohad, 1965; Colvin, 1965, 1966; Carson *et al.*, 1967). Ordered microfibril deposition, as well as maintenance of a specific orientation, may be due to directed protoplasmic movement as well as intermolecular forces between microfibrils. The inertia of newly synthesized microfibrils might cause them to flow in a direction opposite to that of protoplasmic streaming, while charge interactions between microfibrils may cause them to lie parallel and to adhere to each other. Reversals of the spiralling protoplast might account for the occasional reversals observed in the direction of microfibril orientation along the length of the cotton fibre.

The cotton fibre has great potential as a model system for understanding primary and secondary cell wall formation in higher plants. Its advantages are numerous: (1) it is one of the purest sources of cellulose known to man; (2) it is ultrastructurally similar to other higher plant cells; (3) the developmental pattern of the cotton fibre is predictable, making it an ideal subject for studying cell differentiation and how it relates to cell wall formation; and (4) cotton ovules may be grown in culture, producing essentially normal fibres. This facilitates experiments requiring the application of radioactive precursors or other external factors, an example being the study of hormonal regulation of plant cell functions (Beasley, 1973; Beasley and Ting, 1973. 1974).

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