

STUDIES ON *LOLIUM MULTIFLORUM* ENDOSPERM IN TISSUE CULTURE

II.* FINE STRUCTURE OF CELLS AND CELL WALLS AND THE DEVELOPMENT OF CELL WALLS

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Abstract

Log phase cells of *L. multiflorum* endosperm grown in liquid suspension cultures contain large nuclei, mitochondria, protein bodies, compound starch granules, small vacuoles, and multilayered membranous structures. At later stages of growth the cell organelles, protein bodies, and starch granules disappear and in senescent cultures many empty cells are seen.

The endosperm cell walls are simple in appearance, having a microfibrillar network embedded in an amorphous matrix. The development of cell walls in dividing cells follows a pattern similar to that for meristematic and other cells in tissue culture.

Endosperm cells in culture have thicker walls than their counterparts from seeds, but at the stage of maximum growth they contain much less starch and protein than mature endosperm cells.

I. INTRODUCTION

This paper describes the morphology of *Lolium multiflorum* endosperm cells grown in suspension culture (Smith and Stone 1973*a*). Cells have been examined by electron microscopy at different phases of growth and compared with endosperm cells taken directly from developing *L. multiflorum* grains.

The surface appearance and ultrastructure of cultured endosperm cell walls *in situ* and following isolation have been examined and the sequence of events leading to the formation of new cell walls in dividing cells in culture has been followed by electron microscopy. The chemical composition of the cell walls is described elsewhere (Smith and Stone 1973*b*).

II. METHODS

(a) *Cell Culture*

L. multiflorum endosperm cells were cultured as described by Smith and Stone (1973*a*). A modified White's medium with sucrose as a carbon source was used throughout.

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(b) Isolation of Cell Walls

Cell aggregates from liquid suspension cultures were removed from the culture medium by filtration through two layers of cheesecloth, washed with 70% ethanol, and disrupted in a French pressure cell at 4 tons in^{-2} ($0.62 \times 10^7 \text{ N m}^{-2}$). The suspension of cell walls and cytoplasmic components was filtered with suction through a bed of No. 10 Ballotini glass beads on a sintered-glass funnel. The cell walls trapped in the bed were washed by repeated suspension of the top layers of the bed in 70% ethanol. The walls were decanted from the glass beads and collected by low-speed centrifugation. Membrane fragments and cytoplasmic components adhering to the cell walls were removed by repeating the glass bead filtration after first disaggregating the resuspended cell walls by ultrasonic disruption. Phase-contrast microscopy indicated that the final wall preparation was almost completely free of adherent cytoplasmic components. The walls were stored at 4°C in absolute ethanol.

(c) Collection of Developing Endosperm Tissue

Immature grains were cut from the pollinated heads of glasshouse-grown *L. multiflorum* at various times after anthesis, the time of anthesis of each kernel being noted since this time varied for the developing grains on the same head. Development was followed by examining 1 μm sections of Araldite-embedded embryo sacs in the light microscope and thin sections were examined by transmission electron microscopy.

(d) Transmission Electron Microscopy

The usual method of fixation was that described by Mares and Stone (1973). Alternatively, tissue was fixed in 1% KMnO_4 in sodium barbiturate buffer (pH 7) for 1 hr at 4°C and was then washed in distilled water, dehydrated, and embedded as previously described.

(e) Scanning Electron Microscopy

Aggregates of tissue cultured endosperm cells were fixed with glutaraldehyde and osmium tetroxide as described by Mares and Stone (1973), freeze-dried onto metal stubs, coated with a 200 Å film of gold in a vacuum evaporator, and examined in a JEOLCO JSM-U3 scanning electron microscope. Acceleration voltages of 12–15 kV were employed.

(f) Light Microscopy

The methods used were those described by Mares and Stone (1973).

III. RESULTS

*(a) Morphology of Cells in Culture**(i) Morphological Changes Accompanying Growth*

Typical growth curves showing the increase of dry weight of the culture with time are shown in Part I of this series (Smith and Stone 1973a). These results are very similar to those obtained by Henshaw *et al.* (1965) for several plant cell cultures. It is likely that cell division took place during the latter part of the lag phase and the early part of the log phase, since dividing cells were not observed after day 9 or 10.

The sequence of morphological changes which accompanies growth of the culture was investigated by taking samples at 7-, 14-, 21-, and 45-days after inoculation and examining them as thin sections in the transmission electron microscope. Cells

were assigned to one of four types designated A, B, C, or D depending on the appearance of the cytoplasm and the abundance of organelles. Electron micrographs of representative cells of each type are shown in Figures 1-4.

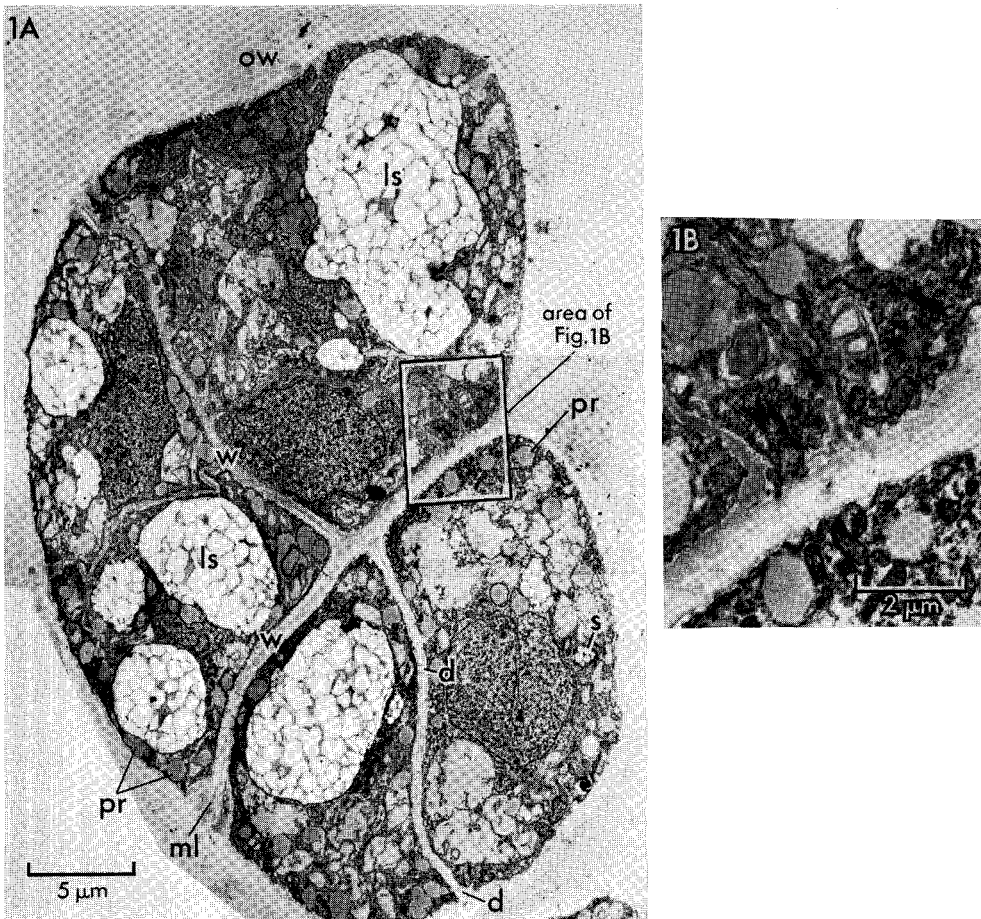


Fig. 1A.—Type A cells in an aggregate from a 7-day *L. multiflorum* culture. Young cell walls (*w*) stained more heavily than older cell walls (*ow*). In some areas the boundary of the older cell walls is difficult to distinguish from the embedding medium. Middle lamellae (*ml*) and plasmodesmata (*d*) are evident. Large multiple starch granules (*ls*), developing starch granules (*s*), and protein bodies (*pr*) are found in the cytoplasm. Tissues were fixed with glutaraldehyde and osmium tetroxide and stained with alkaline lead citrate and uranyl acetate.

Fig. 1B.—Enlargement of portion of Figure 1A showing the irregular plasma membrane-cell wall boundary in a young cell.

Cell aggregates of 5-day-old cultures contained predominantly type A cells with a small percentage of type B and a variable proportion of type D cells, the latter presumably being present at the time of inoculation. The approximate proportions

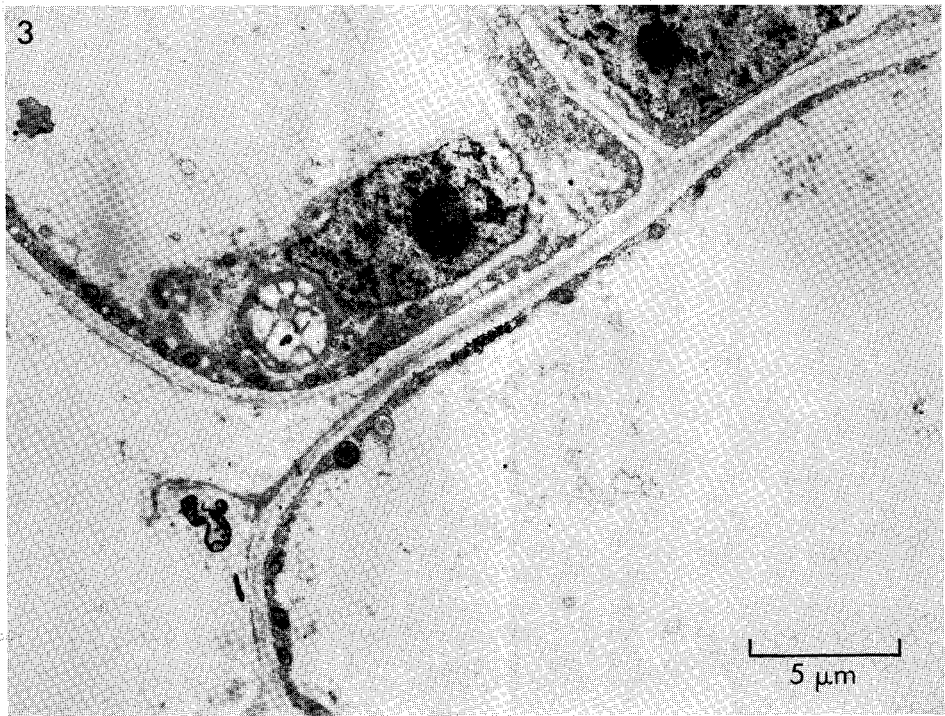
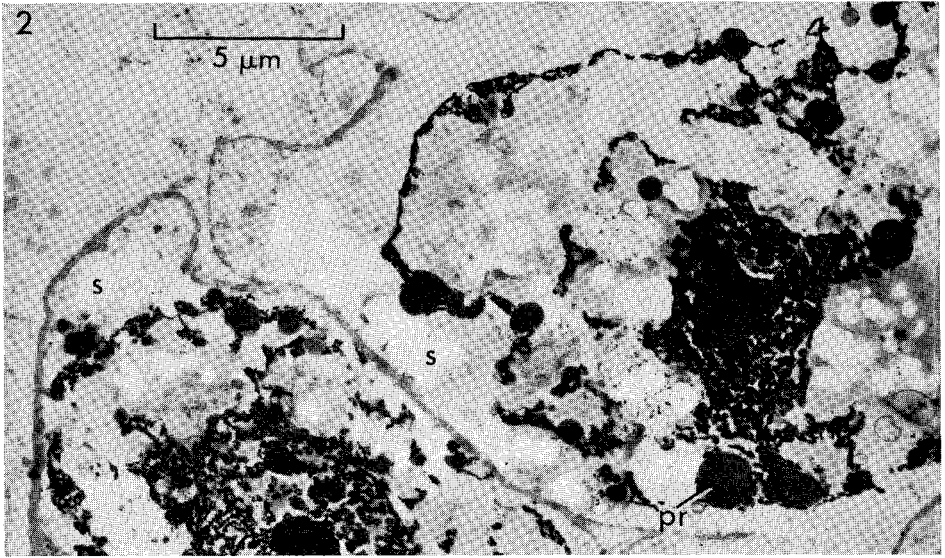


Fig. 2.—Type B cells. The contents of these cells are apparently undergoing autolysis; membranes, protein bodies (*pr*), fragments of disrupted starch granules (*s*), and necrotic nuclei are evident. In Figures 2–4, cells were fixed with glutaraldehyde and osmium tetroxide and sections stained with uranyl acetate and alkaline lead citrate.

Fig. 3.—Type C cells from a 14-day culture, showing an attenuated layer of cytoplasm lining the cell walls and containing organelles, small vesicles, nuclei, and, occasionally, small starch granules.

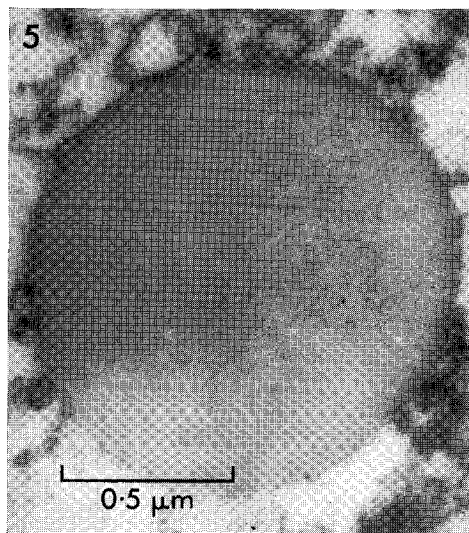
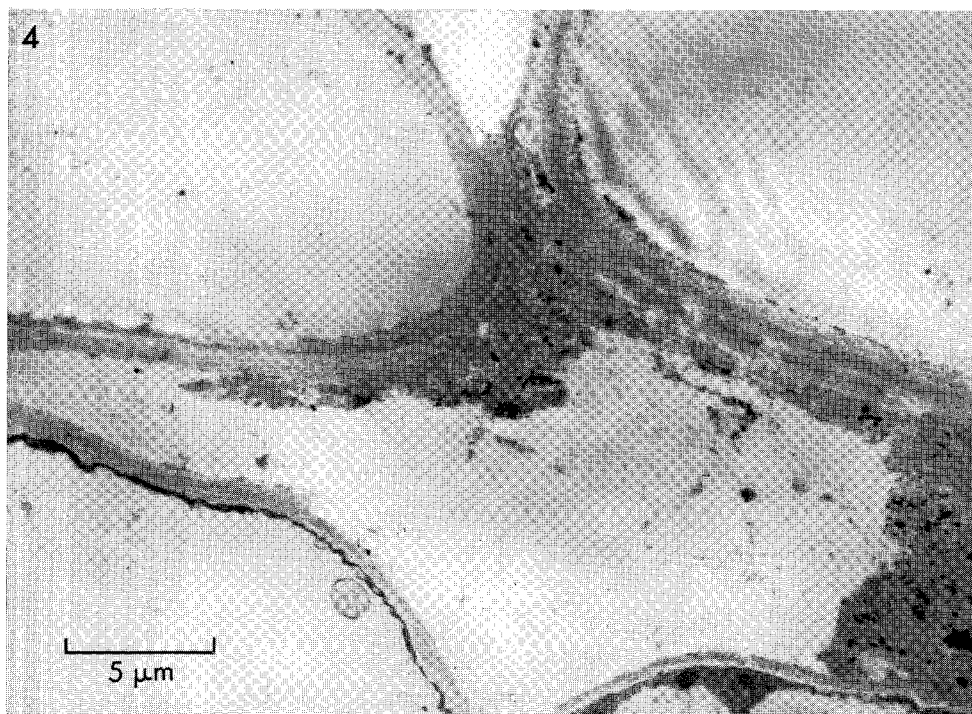


Fig. 4.—Type D cells from a 14-day culture; cellular components almost completely absent, plasma membrane absent or discontinuous.

Fig. 5.—A protein body apparently bounded by a membrane. Fixation: glutaraldehyde and osmium tetroxide; staining: uranyl acetate and lead citrate.

Fig. 6.—Starch granules developing within the membrane system of an amyloplast. Fixation: potassium permanganate.

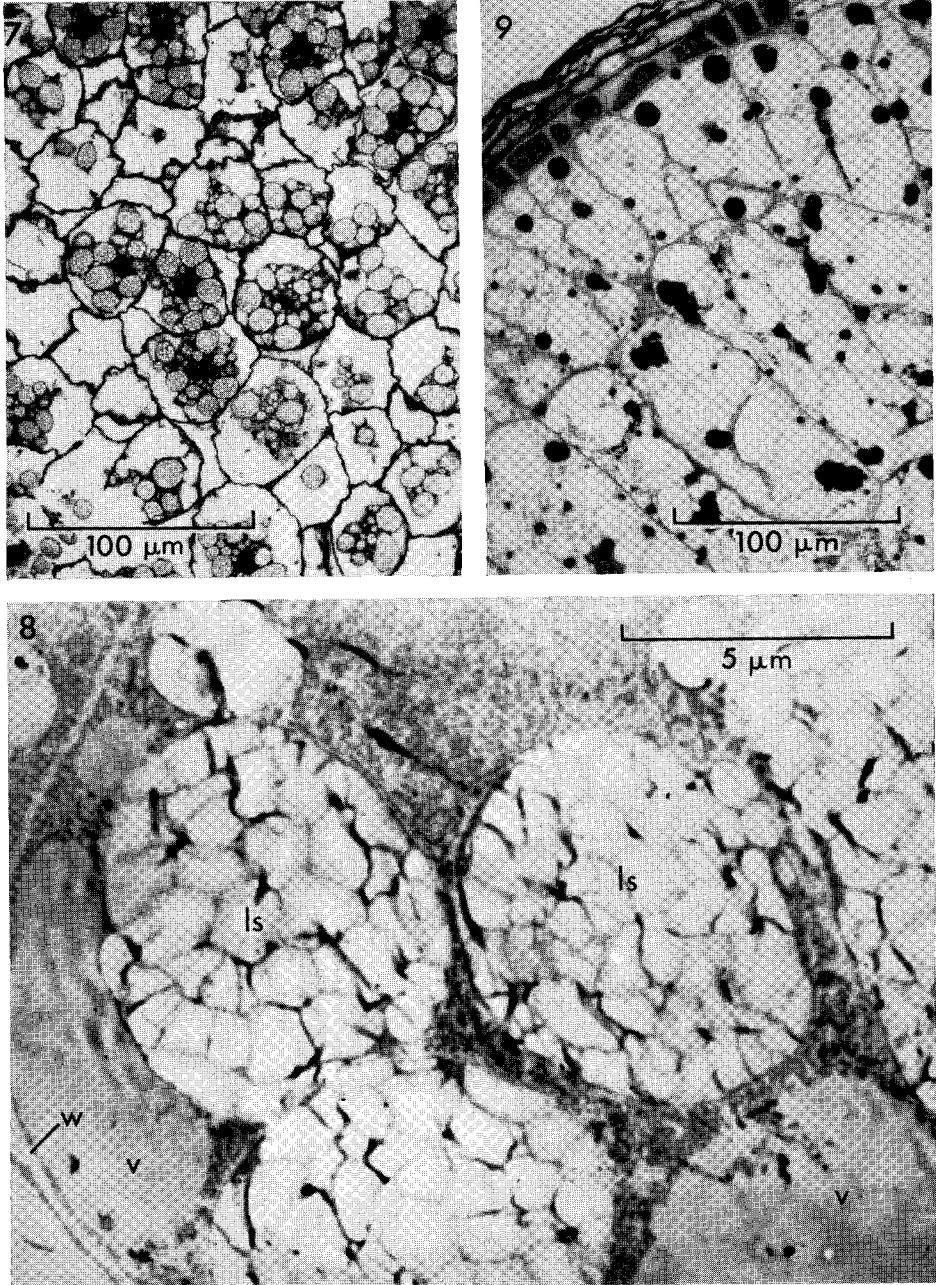


Fig. 7.—Light micrograph of endosperm tissue from 6-day *L. multiflorum* kernels. Large multiple starch granules are evident in the large, thin-walled cells. Fixation: glutaraldehyde and osmium tetroxide; staining: toluidine blue.

Fig. 8.—Transmission electron micrograph of endosperm cells from 6-day *L. multiflorum* kernel. Large multiple starch granules (*ls*), large vacuoles (*v*), and areas of dense cytoplasm are enclosed by thin endosperm cell walls (*w*). Fixation: glutaraldehyde and osmium tetroxide; staining: uranyl acetate and alkaline lead citrate.

of the cell types in 7-, 14-, 21-, and 45-day cultures are shown in Table 1, these values being estimated from an electron microscope study of approximately 500 cell profiles made up from sections prepared from three tissue blocks for each stage of growth.

TABLE 1
DISTRIBUTION OF CELL TYPES IN CULTURED ENDOSPERM AT VARIOUS STAGES OF GROWTH
Electron micrographs of the four types of cells are shown in Figures 1-4

Days after inoculation	Cell type (%)				Notes
	A	B	C	D	
7	50	5	5	40	A and C cells were found together
14	9	27	27	37	} A cells at this stage had no protein bodies and few starch granules
21	4	20	34	52	
45	—	10	20	70	

(ii) Appearance of Protein Bodies and Starch Granules

Protein bodies and starch granules were observed in cultured endosperm cells up to the later stages of the log phase of growth when they disappeared. Protein bodies were round, uniformly electron-dense organelles and in some cases were associated with membranes over part of their surfaces (Figs. 1A and 5). Compound starch granules developed within the dense membrane system of the amyloplasts (Fig. 6) and stages of starch granule development ranging from the first appearance of a single granule in the matrix of the amyloplast to large multilocular structures with only a thin membranous boundary layer (Fig. 1A) were observed.

(b) Development of Cells during Maturation of the Seed Endosperm

Light micrographs of developing grains 6 days after anthesis showed endosperm cells containing large compound starch granules (Fig. 7). Transmission electron micrographs of sections from the same grains showed thin-walled cells with large compound starch granules embedded in cytoplasm containing some membranes and mitochondria, but very few protein bodies (Fig. 8). At the 12-15-day stage, the endosperm cells were so densely packed with starch that it was practically impossible to prepare thin sections. However, thick sections examined in the light microscope clearly showed the tight packing of the cells with compound starch granules (Fig. 9).

(c) Electron Microscopy of Cell Walls of Cultured Cells

Cross-sections of aggregates of cultured cells showed walls of variable thickness and appearance. The intensity of staining seemed to decrease with increasing age of

Fig. 9.—Light micrograph of endosperm from 12-15-day *L. multiflorum* kernels. The large, thin-walled endosperm cells are densely packed with multiple starch granules. The smaller, thick-walled cells of the aleurone layer are seen at the surface of the endosperm. Fixation: glutaraldehyde and osmium tetroxide; staining: toluidine blue.

the walls until the outer cell wall boundary became difficult to distinguish from the embedding medium (Fig. 1A). Older cell walls often appeared to consist of several layers with slightly different staining characteristics and where cell contents and plasma membranes had disappeared, there was occasional evidence of cell wall disruption (Fig. 10).

The middle lamella and plasmodesmata could frequently be distinguished in cell walls between adjoining cells (Figs. 1A and 3). In electron micrographs of material fixed with potassium permanganate, the middle lamellae, the complex nature of older cell walls, intercellular spaces, and indications of cell wall disruption became much more evident (Figs. 11 and 12, and also Figs. 3 and 4).

Irregular small protrusions of the plasma membrane into the cytoplasmic matrix of the cell were often observed, particularly in young cells (Fig. 1B). Cell wall material was occasionally detected in these invaginations but in many other instances they appeared to be devoid of electron-opaque material. It was difficult to decide whether the protrusions represented real structural features or fixation artifacts.

In cell aggregates, details of the microstructure of the walls were not readily apparent but a fibrous texture was sometimes observed (Fig. 4) and was more evident in isolated walls (Fig. 13), presumably due to swelling of the walls during their isolation.

A randomly oriented microfibrillar network was observed on the surface of metal-shadowed, isolated cell walls (Fig. 14). The random orientation of microfibrils throughout the depth of the cell wall became more evident following the sequential removal of matrix material with 7M urea and 4M KOH solutions (Figs. 15 and 16). Small oval granules, similar to those observed on the surface of wheat endosperm cells (Mares and Stone 1973) were sometimes seen on *Lolium* cell wall fragments (Fig. 14).

Sections of isolated walls were stained with the silver-hexamine reagent (Ramborg and Leblond 1967) and showed an even distribution of silver grains throughout the cell walls, indicating that periodate-sensitive polysaccharides are present in all parts of the wall.

Scanning electron micrographs of cell aggregates showed that the cells had a wrinkled appearance (Fig. 17), and at higher magnifications the coarse fibrous texture of the surfaces was apparent.

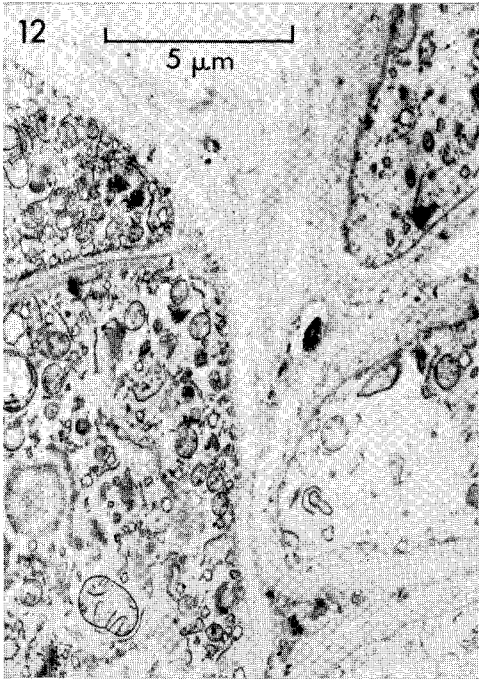
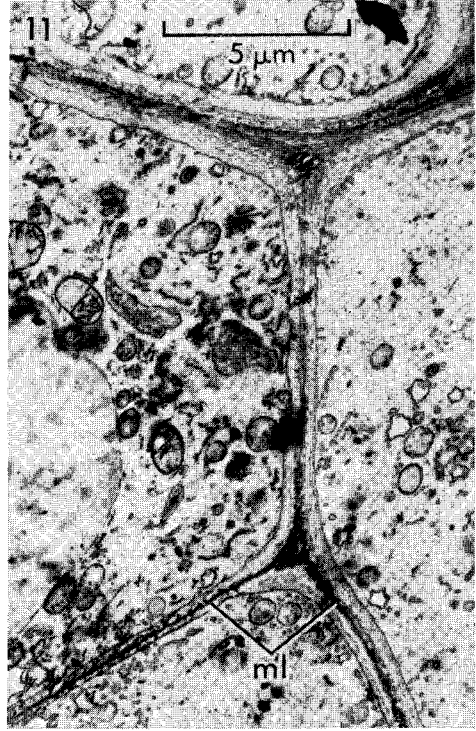
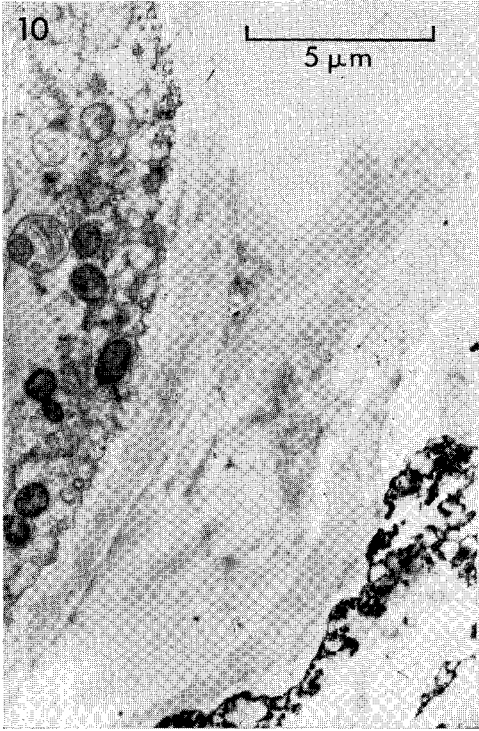
(d) Formation of Cell Walls in *L. multiflorum* Endosperm Cultures

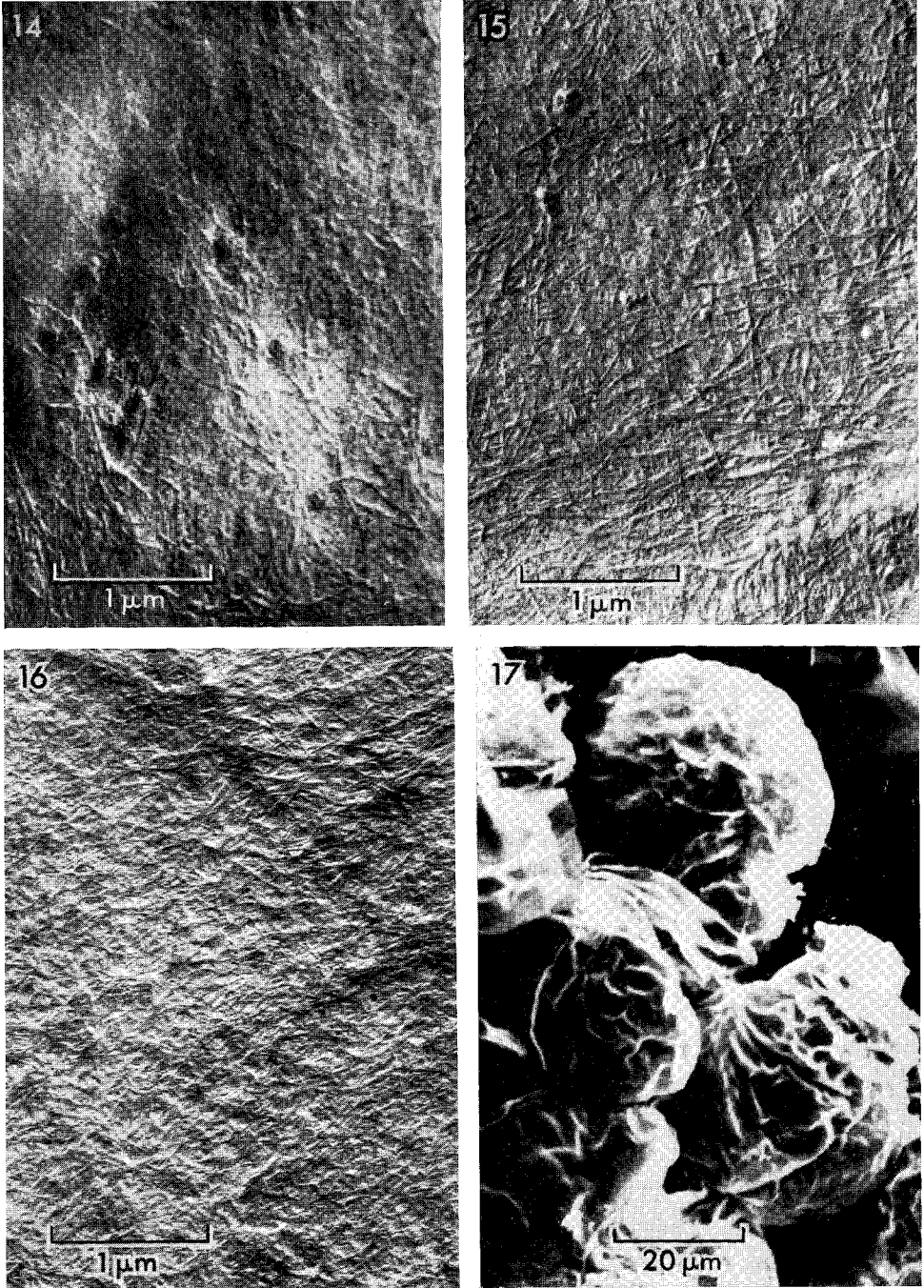
Dividing cells, especially those showing stages of new wall formation, were examined in thin sections of cells from 6-7-day cultures. The earliest sign of wall development observed was a line of small, membrane-bound vesicles in the centre of the phragmoplast between the chromosomes, which by this stage had moved towards

Fig. 10.—Partially disrupted cell walls from a section of a 14-day aggregate of cultured cells. Fixation: glutaraldehyde and osmium tetroxide; staining: uranyl acetate and alkaline lead citrate.

Figs. 11 and 12.—Sections of 7-day culture cell aggregates, fixed with potassium permanganate. Middle lamellae (*ml*), stratified wall structures, intracellular wall inclusions, and partially disrupted cell walls are readily seen.

Fig. 13.—Fibrillar texture of a sectioned, isolated cell wall. Fixation: glutaraldehyde and osmium tetroxide; staining: uranyl acetate and alkaline lead citrate.





Figs. 14–16.—Surface texture of isolated *L. multiflorum* cell walls, metal-shadowed with platinum/palladium alloy. 14, Cell wall isolated in 70% ethanol. 15, Cell wall extracted with 7M urea. 16, Cell wall extracted successively with 7M urea and 4M KOH.

Fig. 17.—Scanning electron micrograph of an aggregate of cells from a 7-day culture. Cells were fixed with glutaraldehyde and osmium tetroxide then freeze-dried onto metal stubs and coated with gold.

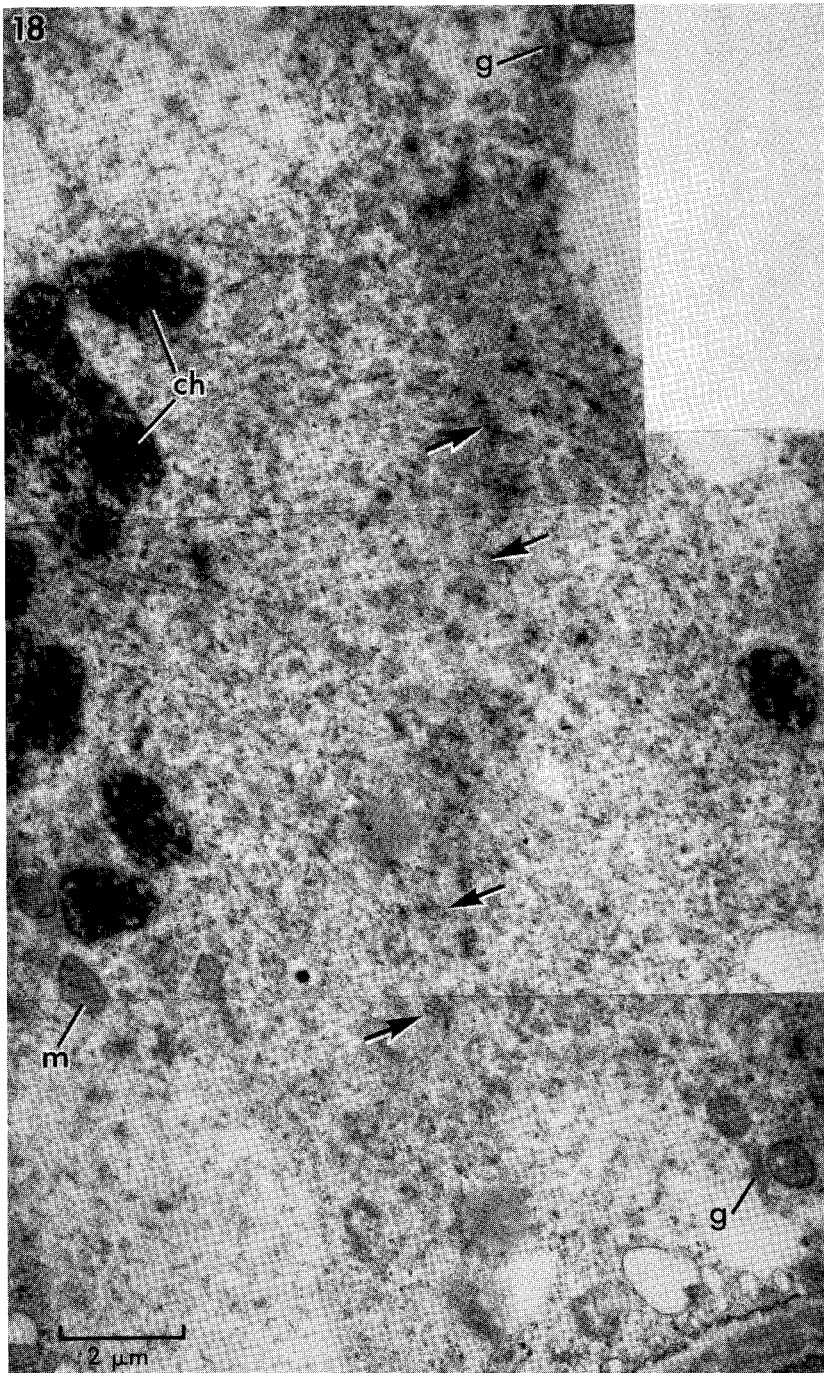


Fig. 18.—Early stage of cell plate formation in cultured cells of *L. multiflorum* endosperm. Vesicles are aligned in the phragmoplast (arrows) between chromosomes (*ch*) which have moved towards the poles of the parent cell. Short segments of phragmoplast microtubules are visible among the vesicles and Golgi apparatus (*g*), mitochondria (*m*), and spindle microtubules can be seen at the edges of the phragmoplast region. Cells fixed with glutaraldehyde and osmium tetroxide, then stained with uranyl acetate and alkaline lead citrate.

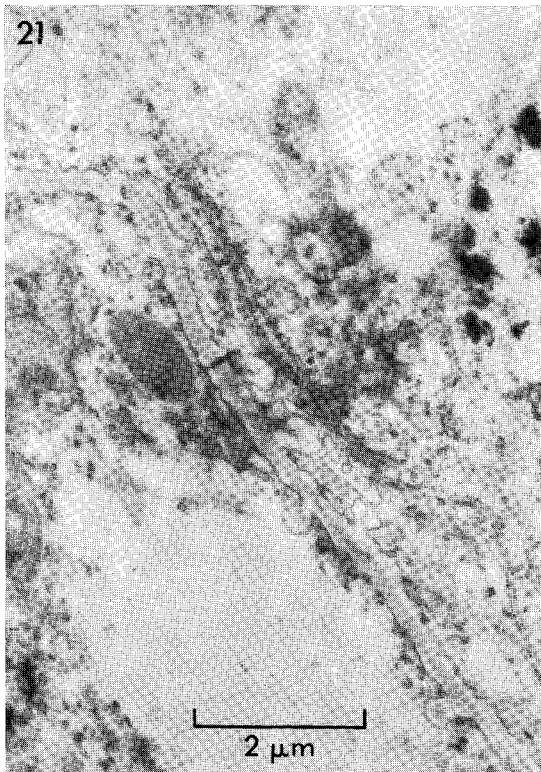
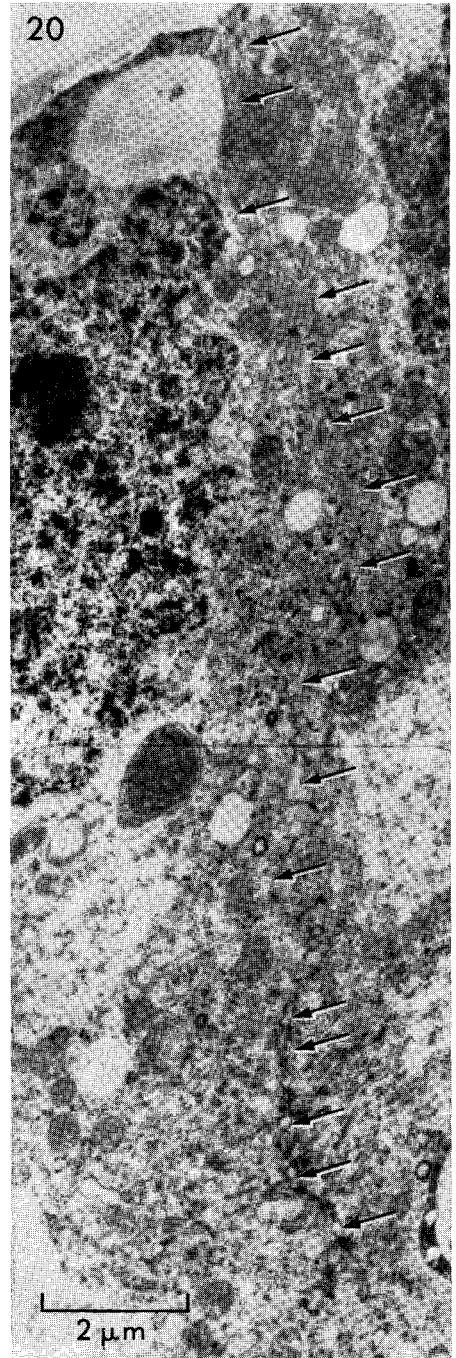
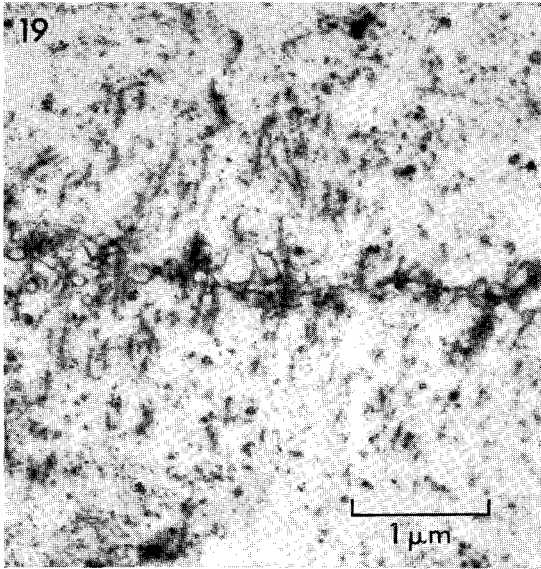


Fig. 19.—Phragmoplast microtubules and small and large vesicles in the line of the future cell plate. In Figures 19–21, cells were fixed with glutaraldehyde and osmium tetroxide and stained with uranyl acetate and alkaline lead citrate.

the poles of the dividing cell (Fig. 18). Generally, the new nuclear membranes appeared around distinct daughter nuclei just after this stage. Numerous microtubules were observed in the phragmoplast, extending into the cytoplasm on either side of the line of vesicles (Fig. 19). Mitochondria, elements of endoplasmic reticulum, some Golgi bodies, and a few residual spindle microtubules were observed at the edges of the phragmoplast.

Although the vesicular components of the phragmoplast were morphologically similar to those being produced by the few Golgi bodies seen in the sections, it could not be conclusively demonstrated that these vesicles were identical or that vesicles did not also arise from other membranous elements. Golgi bodies were relatively few in number immediately prior to cell division and also produced vesicles similar to the phragmoplast vesicles in cells which were not undergoing division. There was no evidence of increased Golgi activity prior to or during cell division. In one cell section vesicular bodies were observed which were apparently being extruded from endoplasmic reticulum membranes. However, as with Golgi vesicles, no definite correlation with phragmoplast vesicles could be made.

At later stages in wall development other vesicles, some of which were larger than those discussed above, could be seen in the line of the future cell plate and later these apparently coalesced. In regions of the cell plate where fusion of these vesicles had occurred, microtubules were no longer seen, but they were apparent at the margins of the cell plate as it grew towards the wall of the parent cell. Even after a continuous zone had been formed in the region of the cell plate, vesicles could be seen apparently fusing with the wall matrix. Frequently the outer margins of the growing cell plate did not reach the opposite walls of the parent cell simultaneously. This was especially common in cells where the nuclei were displaced to one side of the cell and in extreme cases the fusion of one margin with the parent cell wall had occurred while the other margin was in a very early stage of development (Fig. 20). No unusual features could be distinguished in the parent cell wall just prior to or after fusion with the developing cell plate.

The young cell wall, which was often observed to follow a zig-zag path between the cell walls of the parent cell (Fig. 21), appeared to contain a fibrous central region and continued to grow in width by fusion with vesicles originating from the cytoplasm. Although microtubules were rarely seen to be associated with new cell walls after they had become continuous, in two instances microtubules running obliquely towards cell walls were observed. The cell walls in these cases were well-developed structures and the significance of the associated microtubules is unexplained.

Fig. 20.—Section of a dividing *L. multiflorum* culture cell showing the cell plate developing between the two daughter nuclei which in this case are displaced to one side of the parent cell. Fusion of the cell plate with the parent cell wall has occurred while the other cell plate margin is still in an early stage of development. Organelles and membranes are found close to the more advanced regions of the developing cell plate but are completely absent from the cytoplasm which surrounds the growing margin.

Fig. 21.—Developing cell wall at a stage just after the wall had become continuous. The central regions of these walls have a fibrous appearance and vesicles in the adjacent cytoplasm appear to fuse with the wall matrix.

IV. DISCUSSION

(a) *Morphology of Cells in Culture*

Any aggregate of cells taken from a suspension culture of *L. multiflorum* contained a spectrum of cell types, ranging from those which were densely packed with cytoplasm and organelles, including small vacuoles, to those with a large vacuole and a thin attenuated layer of cytoplasm or to others which were empty. The proportion of empty cells increased with age of the culture. Actively growing cells, which contained abundant cytoplasm, mitochondria, endoplasmic reticulum elements, and Golgi bodies, also contained starch granules and protein bodies of various sizes and at various stages of development.

The protein bodies were uniformly electron dense, appeared to be bounded by a membrane and were very similar to the protein bodies observed in developing wheat endosperm (Buttrose 1963; Jennings *et al.* 1963). There was no evidence of a layered structure such as has been reported in other endosperm protein bodies (Mitsuda *et al.* 1969; Rost 1971). The compound starch granules were seen to develop in amyloplasts, which initially were similar in size to mitochondria although they were distinguishable from them by their much denser internal membrane system. As the number of granules in the amyloplast increased, the internal membranes disappeared and at maturity the starch granules were encapsulated in a membranous envelope.

(b) *Comparison of the Morphology of Cultured and Native L. multiflorum Endosperm Cells*

Several authors (Steward 1961; Tulecke 1964; Halperin 1969) have observed that the morphology of plant cells grown in tissue culture does not necessarily resemble that of the parent tissue from which they were derived. In *L. multiflorum* a number of differences have been noted between the developmental features of actively growing endosperm cultures and seed endosperm tissue. Clumps of cultured cells contained cells at widely different stages of maturation, in contrast to the approximately synchronous nature of endosperm cell development in *L. multiflorum* seeds and in cereal grains in general. In addition, cultured cells never reached a stage where the cytoplasm was densely packed with starch granules and protein bodies. The maximum accumulation of starch which was observed in cultured cells corresponded to that found in seed endosperm at about 7 days after anthesis.

The development of starch grains and protein bodies in seed endosperm appeared to follow a pattern similar to that demonstrated in wheat endosperm by Jennings *et al.* (1963). These workers observed starch granules as early as 2 days after pollination and suggested that rapid synthesis of starch took place between days 5 and 12. Protein synthesis lagged behind that of starch, starting at approximately day 14 and continuing almost to maturity. However, in cultured endosperm, starch and protein were apparently synthesized concurrently and prior to days 7-8, indicating that the mechanisms controlling the time of initiation of protein and possibly starch synthesis had been altered. Starch granules and protein bodies did not persist in cultured cells, but disappeared during the latter half of the log phase of growth. The resorption of the starch and protein may have been a consequence of a decrease in nutrient levels in the culture medium (Smith and Stone 1973a) or perhaps of a

decreased ability of the cultured cells to store reserve materials as has been suggested by Norstog *et al.* (1969). Morphologically, the starch granules and protein bodies observed in cultured cells and in seed endosperm cells appeared similar. In summary the chief differences between cultured cells and those from endosperm developing on the plant were in the quantity of starch and protein inclusions, the relative time of appearance of these two storage compounds and their later resorption.

(c) Appearance of Cell Walls

Cell walls of cultured *L. multiflorum* endosperm cells appeared in electron micrographs, both in surface-shadowed preparations and in cross-sections, to be similar to typical primary plant cell walls. Microfibrils were visible in isolated walls and these became more clearly visible following successive extraction with water, 7M urea, and 1M KOH. The same type of microfibrillar framework has been observed in wheat endosperm cell walls (Mares and Stone 1973). Middle lamellae and plasmodesmata were readily distinguished in electron micrographs of cross-sections of cell walls. No obvious areas of localized thickening were seen on the walls and although older cell walls did show some evidence of stratification, this was not comparable to the layering observed by MacLeod *et al.* (1964) in thickened endosperm walls from *Bromus sterilis* or by Meier (1958) in ivory nut cell walls. Older cell walls also showed evidence of disruption and, in cells from which the contents and plasma membrane had disappeared, the walls were only lightly stained and variations in wall thickness and discontinuities in the wall structure were visible. These changes could have been caused by enzyme action during the autolysis of senescent cells, or by the partial disruption of cell aggregates due to collisions in the shaken cell suspension. The partial disruption of cell aggregates and the sloughing of material from cell wall surfaces have been noted in other culture systems by Henshaw *et al.* (1965) and Halperin and Jensen (1967).

In carrot cell cultures, Halperin and Jensen (1967) observed that following division, a new cell wall grew around each newly divided cell forming a complete layer inside the old cell wall. No observations of similar behaviour in dividing *L. multiflorum* endosperm culture cells were made, although cells in older cultures sometimes had compound walls. This would be compatible with a wall growth pattern similar to that observed by Halperin and Jensen (1967), but more likely arose by adpression of younger cell walls to the adjacent walls of senescent cells or to partially degraded walls.

Walls of cultured *L. multiflorum* endosperm cells were noticeably thicker than those of endosperm cells from seeds. Norstog (1956) observed that the walls of cultured *L. perenne* endosperm cells grown on coconut milk medium were thicker than those grown on yeast extract medium, suggesting that the composition of the culture medium and possibly the physical environment of the cells have an influence on wall thickness.

(d) Development of Cell Walls

The pattern of development of new cell walls in cultured endosperm is very similar to that described for meristematic cells and for other plant cells in culture (Porter and Machado 1960; Whaley and Mollenhauer 1963; Frey-Wyssling *et al.*

1964; Bajer 1965; and Hepler and Jackson 1968). Nuclear division is always accompanied by cell wall formation in endosperm cells in tissue culture as is the case in the later stages of endosperm development in the maturing grain. However, in grasses the earliest phase of endosperm nuclear division is not accompanied by wall formation (Brenchley 1909; Sandstedt 1946; Buttrose 1963; Evers 1970; Mares and Stone, unpublished data), and the formation of walls around the endosperm nuclei has been shown for developing wheat endosperm (Mares and Stone, unpublished data) to follow a course which is quite distinct from the normal pattern.

V. ACKNOWLEDGMENTS

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