MICROTUBULES AND EARLY STAGES OF CELL-PLATE FORMATION IN THE ENDOSPERM OF *HAEMANTHUS KATHERINAE* BAKER

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ABSTRACT

A fine structure study of the phragmoplast and developing cell plate has been made on glutaraldehyde-osmium tetroxide-fixed, dividing, cultured cells of the liquid endosperm of *Haemanthus katherinae* Baker. The phragmoplast arises between the telophase nuclei, usually in association with a remnant strand of spindle elements, and consists of an accumulation of microtubules oriented at right angles to the plane of the future cell plate. The microtubules, which are 200–240 A in diameter, occur in small clusters spaced at approximately 0.2–0.3 μ intervals along the plate. Short interconnections interpreted as "cross-bridges" have been observed between individual microtubules. Within each cluster there is an electron-opaque zone about 0.3 μ in width which can be attributed in part to an overlap of microtubules from both sides of the plate and in part to a local accumulation of an amorphous electron-opaque material. During development these dense zones become aligned in a plane which itself defines the plane of the plate. Vesicles, commonly observed in long files, are derived from a cytoplasmic matrix rich in elements of the endoplasmic reticulum and sparse in dictyosomes. They aggregate between the clusters of microtubules and eventually coalesce to form the cell plate.

INTRODUCTION

The formation of the cell plate is easily viewed in the living cultured cells of the endosperm of the African blood lily, *Haemanthus katherinae* Baker, and has been the subject of several light microscopic investigations, most notably those of Inoué and Bajer (12), Bajer (1), Bajer and Allen (3), and more recently Jackson (13). Observations made with a variety of different optical systems (polarized light, phase contrast, and Nomarski differential interference contrast) have revealed that the cell plate complex or phragmoplast is composed of fibrillar elements which arise at the plate and are oriented at right angles to its plane, and numerous cytoplasmic droplets which eventually accumulate and fuse in the mid region of the cell (1, 3, 12).

Analysis of the fine structure of cell-plate formation has been largely confined to the dividing root tip cell (7, 10, 14, 16, 20, 21, 24, 25). These studies have demonstrated that the birefringent, fibrillar elements noted previously in the polarizing light microscope are microtubules similar in structure to those of the spindle (10, 14, 24). In root tips the plate itself is composed of vesicles which presumably originate from dictyosomes and migrate to the mid region of the dividing cell

where they fuse with one another, supplying both material for the plate matrix and membrane for the new plasmalemma (7, 10, 24, 25).

In contrast to cells of the root tip, those of the liquid endosperm can be removed from the seed at an early stage of development and cultured as single cells. Under culture conditions they normally undergo a further division, and, since they flatten to approximately 5–7 μ, they are easily observed throughout mitosis with the light microscope. The endosperm, therefore, affords the unique opportunity of comparing in the same cell the light microscopic morphology and the fine structure of phragmoplast development.

Certain structural conformations thought to be involved in cell-plate development have been observed in these cells which had not been seen in previous studies of root tip cells. Most notable are the interdigitation of microtubules and the accumulation of an amorphous dense material in the plane of the plate. A close morphological relationship of elements of the endoplasmic reticulum (ER) to the vesicles which comprise the plate is also demonstrated.

MATERIALS AND METHODS

Liquid endosperm cells of *Haemanthus katherinae* Baker were expressed from immature seeds and plated on plastic cover slips which had been coated with a 5–10 μ thick layer of 0.5% agar containing 3.5% sucrose. The cells were allowed to flatten for 20 min and then were examined with a phase-contrast microscope. (For details of the preparation procedure see Jackson, reference 13.) Cells in the later stages of mitosis, i.e. anaphase and early telophase, were selected for analysis in the electron microscope.

Initial fixation was accomplished by placing the cover slips in a porcelain holder in an atmosphere in equilibrium with a 25% glutaraldehyde solution at room temperature. After 5–10 min in glutaraldehyde vapor, the cover slips were transferred to an aqueous atmosphere for removal of the residual glutaraldehyde. The cells were then postfixed in osmium vapors above a 2% aqueous solution of OsO_4. Following postfixation, the cells were dehydrated at 1 min per change in solutions of ethanol (50, 70, 90, 95, and 100% four times). After the last change the cover slips were blotted quickly on an edge for removal of excess ethanol. An Epon-embedding mixture containing DMP-30 accelerator, 1.5% by volume, was then placed directly on the fixed dehydrated cells and allowed to infiltrate under vacuum at room temperature for 1–3 hr. The cover slips were then placed in a 60°C oven for 48 hr.

The polymerized embedding mixture was cleaved free from the plastic cover slip; this yielded a wafer of Epon in which the flattened cells were oriented in a plane approximately 5–10 μ beneath the surface. With a phase-contrast microscope equipped with a Leitz object marker, well-fixed cells in the desired stage of mitosis were selected, photographed, and circled. These individual cells were then isolated, mounted on blank blocks, trimmed, and sectioned on a Porter-Blum MT-2 ultramicrotome. Sections were mounted on 200–400-mesh copper grids and counterstained with uranyl acetate and lead citrate (22). Examination was carried out in either a Siemens Elmiskop I or a Hitachi HU-11C electron microscope.

OBSERVATIONS

Early stages of cell-plate formation in the dividing endosperm cell are characterized by the appearance of large numbers of microtubules in the mid region of the cell. First they arise at an edge of the zone previously defined by the spindle apparatus, usually in association with a few remaining spindle microtubules (Fig. 1). Cinemicrographic studies have shown that the plate may form in two or more places simultaneously and then undergo lateral growth centripetally until the central portion of the cell is traversed by the plate (1). Subsequently, the plate grows centrifugally as it does in most dividing plant cells. Our observations are confined mainly to the centrifugal phase of growth, during which there is a massive accumulation of oriented microtubules at the plate and during which there is no evidence of the spindle structure.

Careful examination of the phragmoplast reveals that the microtubules, 200–240 A in diameter, are organized in small clusters spaced approximately at 0.2–0.3-μ intervals along the plate (Figs. 2, 4, 7). Within each cluster there is an electron-opaque zone about 0.3 μ in width which can be attributed in part to an overlap of microtubules from both sides of the plate and in part to the local accumulation of an amorphous material (Figs. 2, 4, 5). Initially the dense zones of the clusters of microtubules do not lie in a plane. Later, however, they become aligned in a plane which itself defines the plane of the future plate (Fig. 7). The microtubules in each cluster are tightly packed and, in many instances, are superimposed on each other; thus it is difficult to visualize their overlap. At higher magnifications, though, it is possible to see that individual microtubules enter the overlap zone from both sides of

FIGURE 1 A low magnification micrograph of a flattened endosperm cell in telophase shows the beginning of cell-plate formation. The phragmoplast (*Ph*), which is offset towards the right-hand side of this cell, is evident as a local accumulation of microtubules. A few remaining spindle tubules can be observed extending between the phragmoplast and a trailing arm of a chromosome (*C*). During development the cell plate first grows centripetally (to the left) until the central portion of the cell is traversed, and subsequently grows centrifugally (to the right) towards the edge of the cell. Some clustering can be noted in the phragmoplast microtubules. Cytoplasmic organelles are absent from the spindle and phragmoplast zones, and can be found at the poles of the cell. × 8,000.

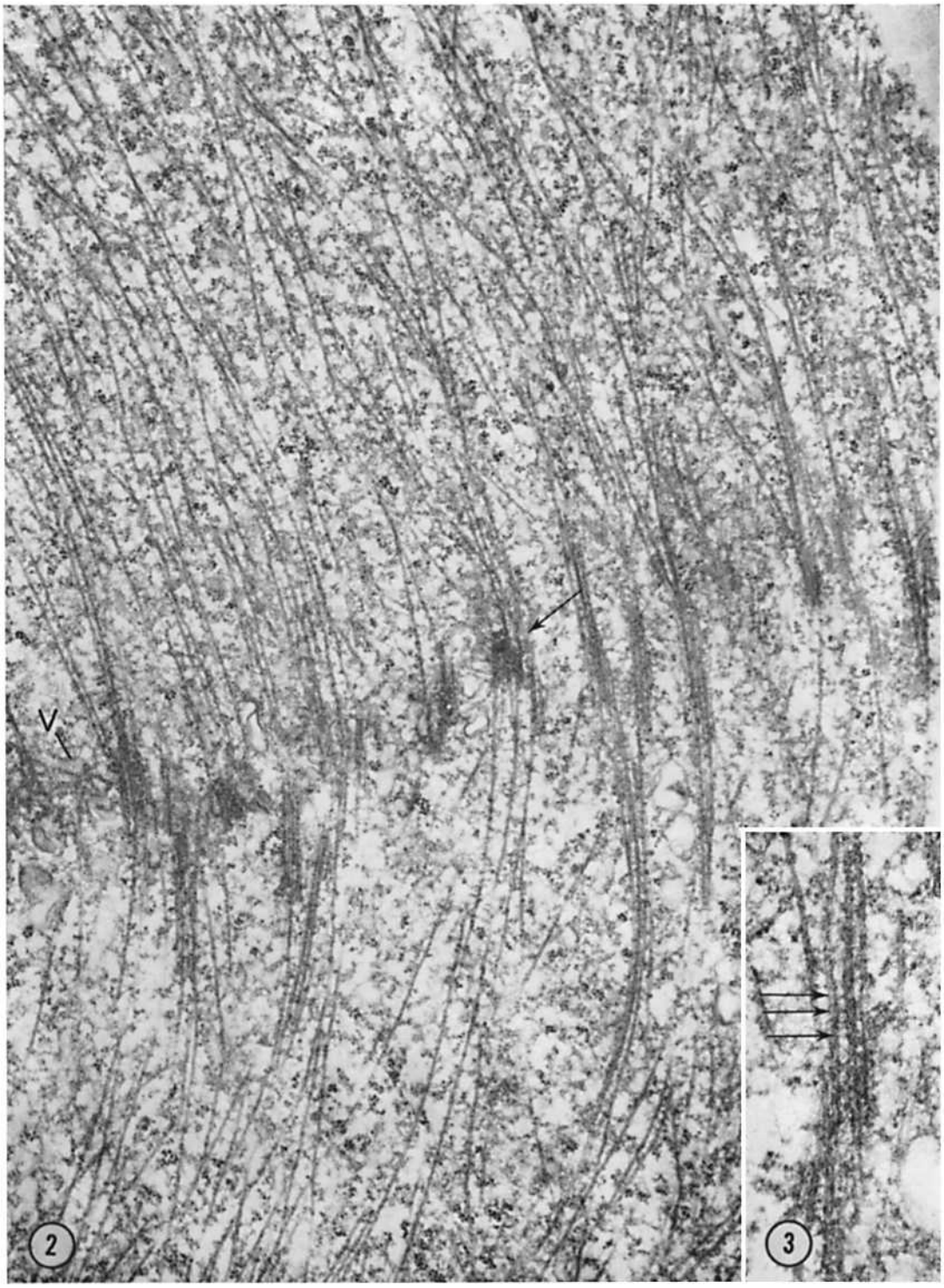

FIGURE 2 This figure shows the edge of a centrifugally growing cell plate in a later stage than in Fig. 1. The phragmoplast microtubules are clustered, and within each cluster a denser region can be noted which falls, roughly, in the plane of the plate. Microtubules from both sides of the plate appear to overlap within the clusters (arrow); a dense, amorphous material can also be noted. In more advanced portions of the plate (on the left), a few vesicles (*V*) can be observed between the clusters, while in younger portions (on the right) no vesicles are evident. × 26,000.

FIGURE 3 A cluster of microtubules at higher magnification showing the short periodically spaced interconnections or cross-bridges (arrows) between two closely adjacent microtubules. × 54,000.

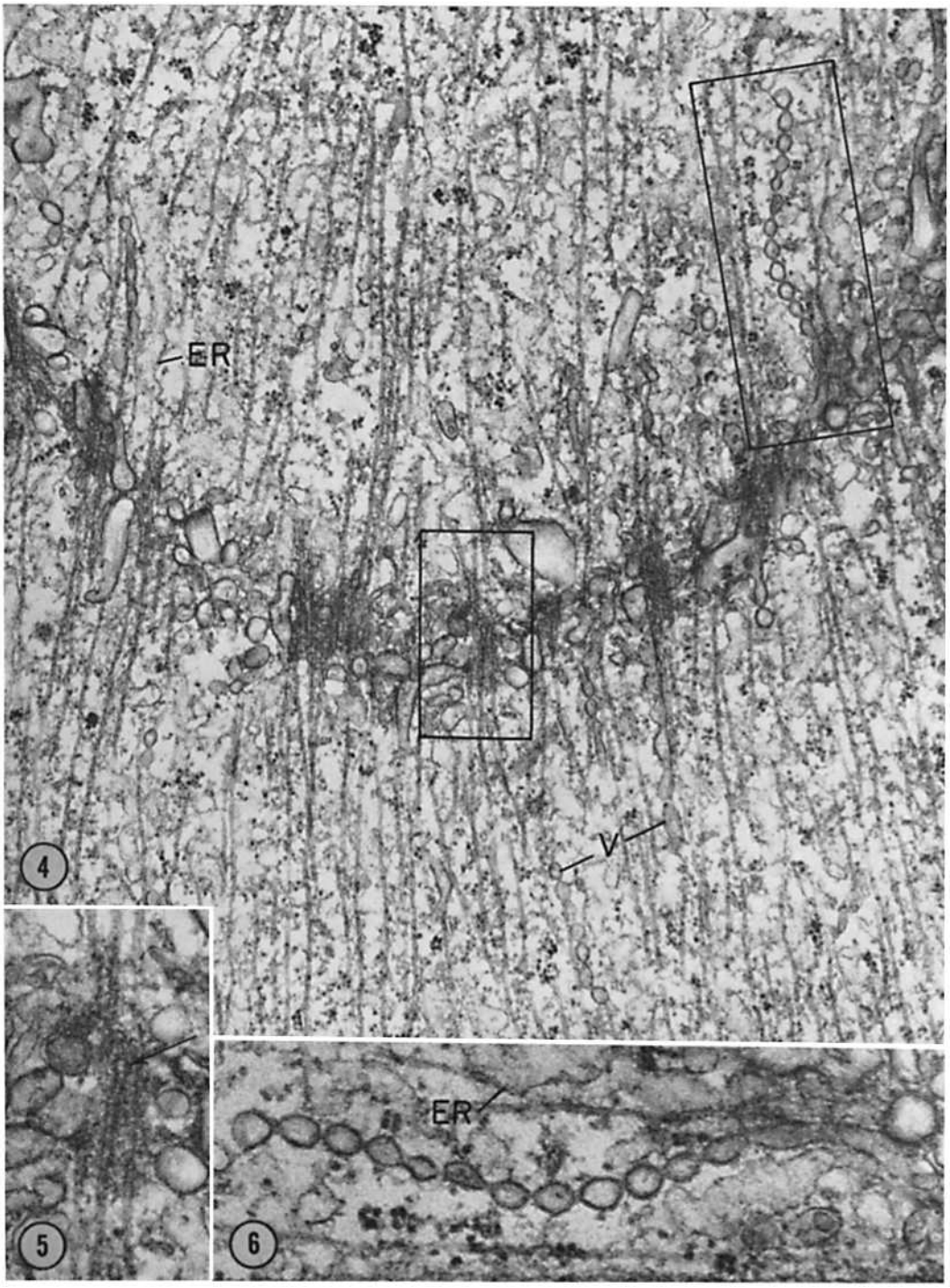

FIGURE 4 A portion of a developing cell plate in a later stage of development. Vesicles are aggregating in large numbers between clusters of microtubules. Of particular interest are chains of vesicles (*V*) extending from the plate for some distance into the cytoplasm. Numerous elements of ER (*ER*) can be seen throughout the phragmoplast matrix. × 35,000.

FIGURE 5 Enlargement of one cluster on Fig. 4. Microtubules can be observed entering the overlap zone from both sides of the plate. A single microtubule from one side does not extend beyond the overlap zone into the cytoplasm on the other side. A microtubule with a bulb-shaped end appears to have terminated in the overlap zone (arrow). Dense, amorphous material is evident between microtubules. × 72,000.

FIGURE 6 A chain of vesicles on Fig. 4. is shown at higher magnification. The vesicles are bounded by triple-layered unit membrane of which the inner leaflet stains more densely than the outer. The vesicles, which appear to have arisen by periodic constriction of a large lamella, are still attached to each other. Elements of ER are evident (*ER*). × 72,000.

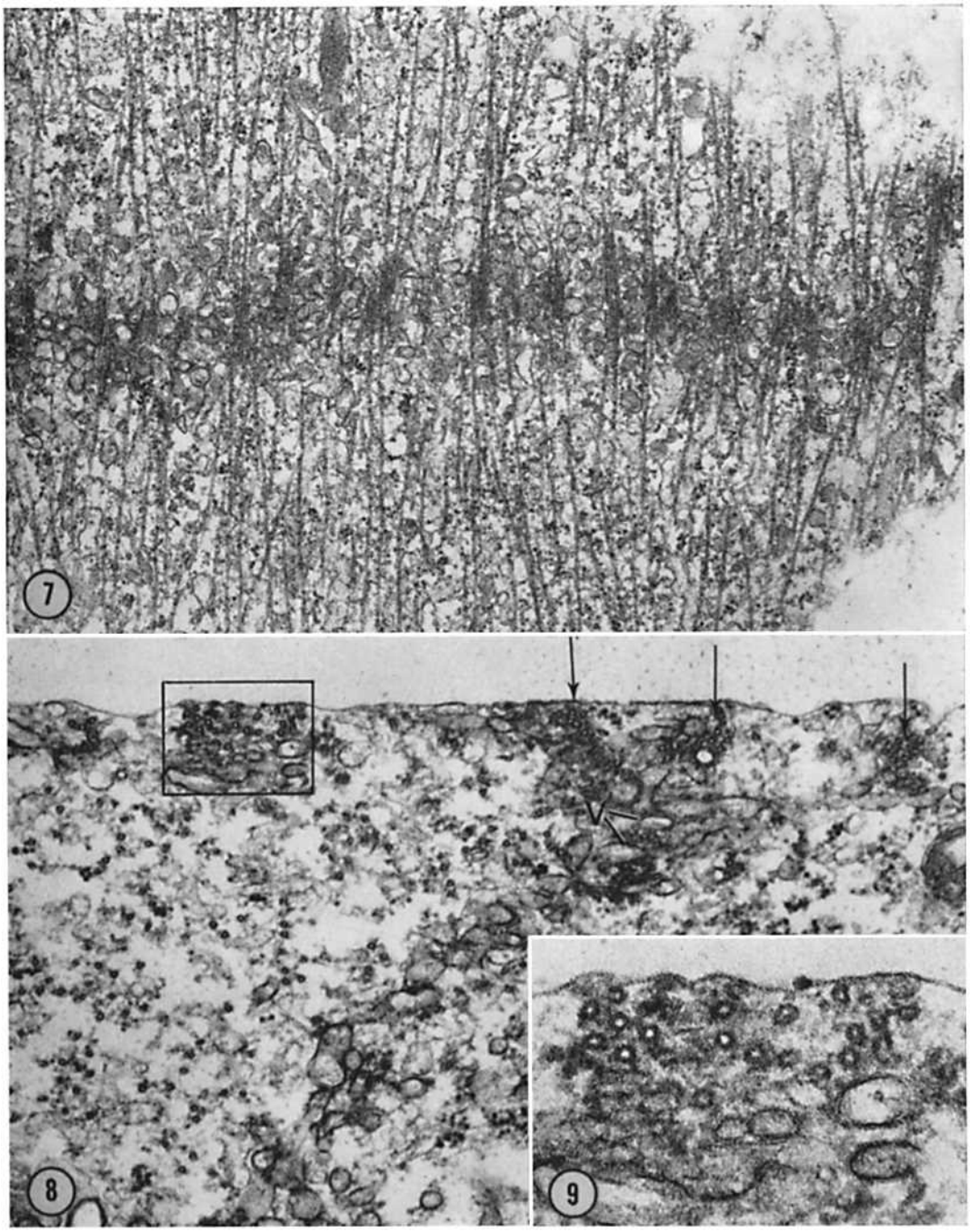

FIGURE 7 A portion of a developing plate in approximately the same stage of development as the plate in Fig. 4. The clusters are aligned in a plane and spaced at approximately 0.2–0.3-μ intervals. Vesicles are quite numerous in the intervals between the clusters. × 29,000.

FIGURE 8 A transverse section of the developing plate showing the clusters of microtubules in face view. When the plane of the section passes out of the plane of the plate, a uniform dispersion of microtubules is observed. Within the plane of the plate the microtubules are more tightly packed (arrows). While individual clusters are found close to vesicles (*V*), no specific grouping or structural relationship between them can be noted. × 36,000.

FIGURE 9 A higher magnification view of a portion of Fig. 8 shows a microtubule cluster with a hexagonal pattern. The center-to-center distance between microtubules within this cluster is approximately 400 A. An amorphous matrix is evident between the microtubules. × 105,000.

the plate, but do not extend beyond it (Fig. 5). One microtubule in Fig. 5 appears to have terminated within this region with a slightly bulb-shaped end. From observations of numerous cell plates it is our impression that a single microtubule from the cytoplasm of one developing daughter cell does not extend beyond the overlap zone into the cytoplasm of the other cell.

Studying the clusters at higher magnification, we have been able to demonstrate short, periodically spaced interconnections between closely adjacent microtubules. These interconnections are interpreted as cross-bridges which may function in stabilizing the tightly packed microtubules (Fig. 3).

When the cell plate is sectioned parallel to its plane, the clusters of microtubules are revealed in face view (Figs. 8, 9). The number of microtubules per cluster varies, but is usually less than seven. While in most cases the microtubules do not appear to be grouped in a specific manner, occasionally they are arranged in close-packed, hexagonal pattern (Fig. 9). Of particular interest is the electron-opaque, amorphous material, noted earlier in the longitudinal sections, which appears between the closely clustered microtubules and in some cases seems to adhere to the microtubular walls (Fig. 9). The phragmoplast microtubules differ in this respect from the cortical microtubules noted by Ledbetter and Porter (14) to have clear, unstained halos.

As development of the phragmoplast proceeds, vesicles accumulate and fuse between the clusters of microtubules in the established plane of the plate. Examination of the processes of vesicle fusion and aggregate growth in transverse sections has failed to reveal the planar, branched bodies noted in a recent study of cell-plate formation in bean root tip cells (10). However, since the present observations are mainly confined to early phases of plate formation, and since the branched bodies were largely seen at later stages, the possibility remains that they also appear in the endosperm cells.

In longitudinal sections vesicles are often seen attached to one another in long chains; this gives the impression that they are flowing into the plate (Figs. 4, 6). Similar rows of vesicles have been noted by Esau and Gill (6) in the phragmoplast of dividing procambial cells of *Beta vulgaris* L. In *Haemanthus katherinae* the chains of vesicles are derived from a cytoplasmic matrix rich in elements of the ER and sparse in numbers of dictyosomes. It is recognized, though, that the vesicle membrane has a unit structure approximately 90 A wide, the inner leaflet of which is more densely stained than the outer leaflet, while the ER membrane is only about 50 A wide and is rarely resolved into its unit components (Fig. 6). Transition states between the vesicular membrane and the ER membrane, if they occur, have not been observed.

The accumulation and fusion of many vesicles form a continuous zone of separation between the daughter nuclei. Microtubules at this stage are considerably reduced in number and in length, and are not found in the conspicuous clusters evident at earlier stages (Fig. 10).

DISCUSSION

The massive accumulation of microtubules which we have found in association with the developing cell plate can be correlated with the occurrence of birefringent material seen by Inoué and Bajer (12) using polarized light microscopy. The detail provided by the electron micrographs, however, gives us a clearer picture of the organization of the birefringent elements, or the microtubules, within the phragmoplast.

It is apparent from this study and also from others (14, 17, 18) that the microtubules of the phragmoplast are structurally identical with those of the spindle. Our measurements of both the phragmoplast and the spindle microtubules in *Haemanthus katherinae* are 200–240 A; Harris and Bajer (8) reported structures 150–200 A in diameter in the same material. In their staining and in their lability in fixation, the microtubules of the phragmoplast are indistinguishable from those of the spindle. While individual elements are structurally similar, their manner of association is considerably different. The phragmoplast microtubules cluster in groups of seven or less, while as many as 50–100 spindle microtubules may emanate from a single kinetochore (2, 8).

The similarity in structure of phragmoplast and spindle microtubules suggests that they may be composed of the same protein subunits. Observations with the polarizing light microscope (12) and from our own electron micrographs, which indicate that the phragmoplast microtubules arise directly at the plate, suggest that near the end of mitosis the spindle microtubules may

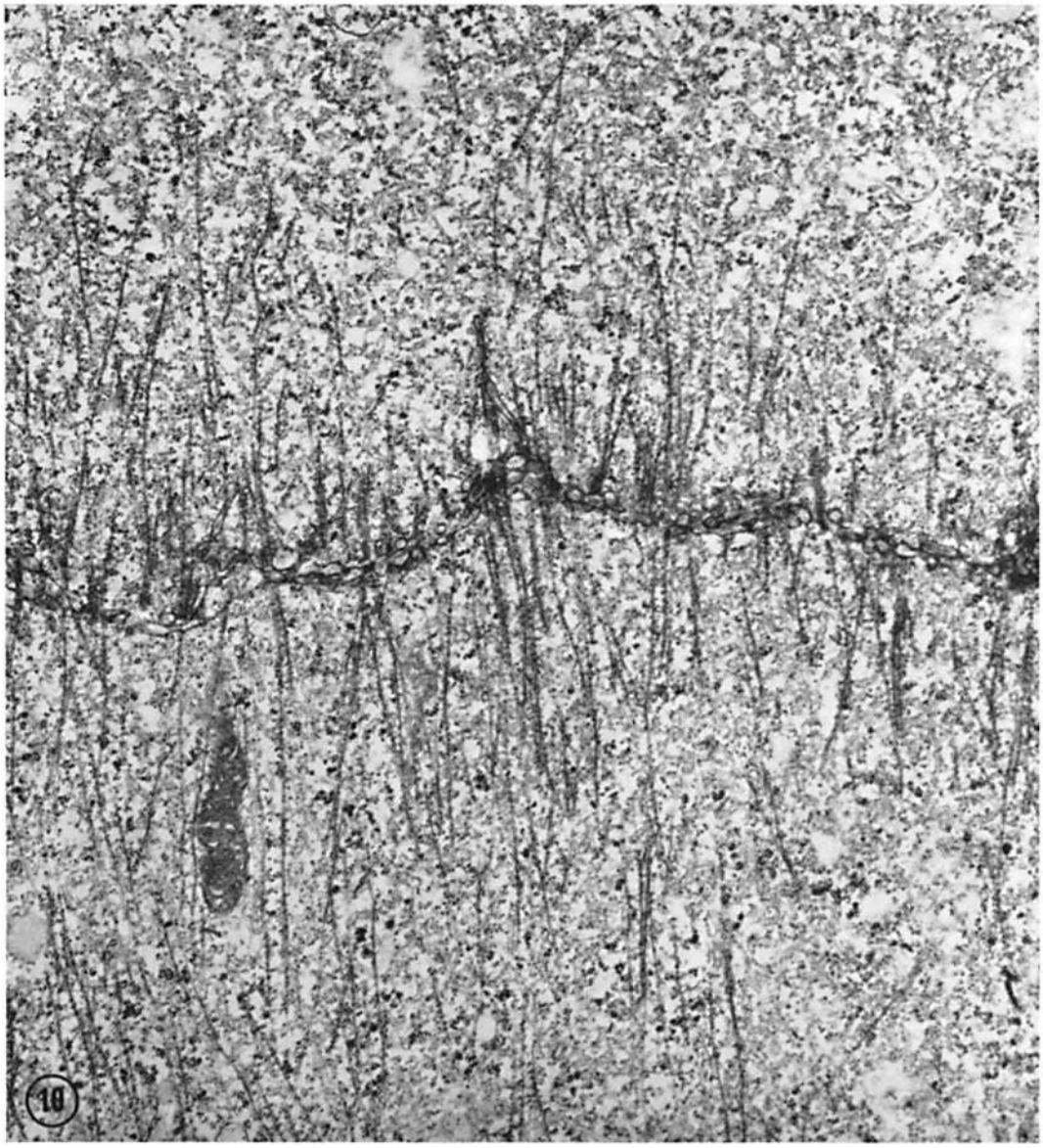

FIGURE 10 A longitudinal section of a more advanced cell plate. Microtubules, while still present, are reduced in length and number, and do not show the regular clustering evident in earlier phases of development. The vesicles have undergone considerable fusion and have formed a more continuous phase of separation between the daughter cells. × 12,000.

depolymerize into protein subunits and that these subunits move to the region of the young cell plate where they reassemble as phragmoplast microtubules. A similar process may occur during plate development itself. As the cell plate grows centrifugally, new microtubules are continuously being formed at its edge. They are long compared with the microtubules observed in more advanced portions of the plate and show a degree of organization in their clustering and spacing which is not evident in microtubules in older regions of the plate (compare Figs. 2 and 10). It seems

possible, then, that microtubules associated with the plate might depolymerize once the plate is established and subsequently reform *de novo* at the site of new phragmoplast activity.

The interdigitation of phragmoplast microtubules in growing portions of the plate, we believe, plays an important role in establishing the place of division. Overlapped microtubules, to the best of our knowledge, have not been reported previously in the phragmoplast of dividing plant cells but, interestingly, they have been noted in the mid body of dividing HeLa cells where they are thought to play a role in the final stage of cleavage (15). Because the clusters of overlapping microtubules appear very early in the developing plate and become aligned in a plane at a time when only a few or no vesicles have aggregated, they may determine or affect the position to which the vesicles migrate.

As important, perhaps, as the interdigitation of microtubules in the organization of the phragmoplast is the amorphous material observed around the clusters of microtubules at their region of overlap. A similar amorphous material has been seen associated with microtubules in centrioles (5, 23) and in kinetochores (4). In the formation of new centrioles microtubules appear to arise from aggregations of amorphous densities which are of unknown composition but which are thought to be active in the formation and stabilization of the microtubules (5, 23). That the region within the phragmoplast where the amorphous material is localized and where the microtubules interdigitate is the place of microtubule origin and growth is supported by the ultraviolet microbeam experiments of Inoué (11). He showed that irradiation of the phragmoplast specifically at the plate destroyed both the birefringent elements and capability for the formation of new elements, while irradiation of the phragmoplast in areas distant from the plate destroyed only the birefringent elements but not the capability for their reformation.

The vesicles which accumulate between the clusters of microtubules and eventually fuse to form the continuous phase of separation between the two daughter nuclei appear to be derived from the ER. It was anticipated at the start of this project, on the basis of many studies of dividing plant cells (7, 10, 24, 25), that dictyosomes would be the predominantly active organelle in providing vesicles for the growing cell plate. Although dictyosomes are present in endosperm cells, they are few in number, they show no obvious spatial relationship to the plate, and they appear to produce only a few vesicles. Since the ER is so abundant throughout the phragmoplast, we postulate that it is the organelle most active in the formation of the new plate and that it provides membrane for the plasmalemma and material for the plate matrix. Dictyosomes may also play a role in supplying material needed in smaller amounts for the plate.

Porter and Caulfield (20) and Porter and Machado (21) first drew attention to the close morphological relationship of the ER to the developing plate. Recently, Pickett-Heaps (16), in an electron microscopic, radioautographic analysis of the incorporation of tritiated glucose into the cell plate, showed that there was as much or more label over elements of the ER as there was over dictyosomes. Our results and those of Pickett-Heaps cast doubt upon the universality of the dictyosome–cell plate theory and suggest that elements of the ER may participate more actively in the formation of the cell plate than was formerly supposed.

The plate vesicles in *Haemanthus* frequently occur in long chains as if they were formed by a periodic constriction along the length of a larger lamella. Constriction does not completely separate the vesicles, but leaves them attached to each other. These rows of vesicles may correspond to the swellings or droplets which Bajer (1) observed, in cinemicrographs, to slide along the phragmoplast filaments and fuse with the cell plate.

Electron microscopy has not been able to tell us whether microtubules generate a force which moves the vesicles. It has, however, revealed a number of structural features of the phragmoplast, namely the interdigitation of microtubules, the presence of cross-bridges between them, and the amorphous material at the zone of overlap, which may be involved in the processes of cell-plate formation. It seems likely, as suggested in other studies (6, 10, 17–19), that the microtubules determine the channels along which vesicles move. The zone of overlapped microtubules and amorphous material which we have observed, we believe, provides a focus for aggregation of the migrating vesicles and thereby defines the plane of the plate.

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