ELECTRON-MICROSCOPE OBSERVATIONS OF MITOSIS AND CYTOKINESIS IN MULTINUCLEATE PROTOPLASTS OF SOYBEAN

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SUMMARY
Multinucleate soybean protoplasts produced by spontaneous fusion during enzyme digestion of the cell wall initiated cell division after approximately 40 h in culture. The structure of these protoplasts during mitosis and cytokinesis was studied with both light and electron microscopes. Most nuclei did not fuse but divided synchronously. Interphase nuclei were commonly connected by short narrow nuclear bridges. At prophase and metaphase the nuclei appeared typical of those in most higher plants; technical difficulties prevented an adequate examination of protoplasts at anaphase. Telophase was characterized by cytokinesis involving phragmoplast and cell plate formation; however, complete partitioning of the cytoplasm by cell plates was not observed. Numerous coated vesicles were present near to or continuous with the cell plate and plasmalemma. The presence of a few dividing protoplasts with at least double the normal chromosome number suggests that some nuclear fusion occurred prior to mitosis. Very little cell wall material was detected at the margin of the dividing protoplasts.

INTRODUCTION
Multinucleate protoplasts have been examined with both the light microscope (e.g. Eriksson & Jonasson, 1969; Bawa & Torrey, 1971; Miller, Gamborg, Keller & Kao, 1971; Motoyoshi, 1971; Grambow, Kao, Miller & Gamborg, 1972; Van der Valk & Wessels, 1973; Reinert & Hellmann, 1973; Fowke, Bech-Hansen & Gamborg, 1974b) and electron microscope (Withers & Cocking, 1972; Fowke, Bech-Hansen, Gamborg & Shyluk, 1973). Mitosis has been reported in multinucleate protoplasts but has only been clearly illustrated at the light-microscope level (Miller et al. 1971; Motoyoshi, 1971; Van der Valk & Wessels, 1973). It is not apparent from these studies whether nuclear division and cytokinesis in multinucleate protoplasts are typical of division in other plant cells. A recent electron-microscope study indicated that the division process in uninucleate soybean protoplasts is essentially the same as in cultured soybean cells and cells of most higher plants (Fowke, Bech-Hansen, Constabel & Gamborg, 1974a). The present report describes the fine structure of mitosis and cytokinesis in multinucleate soybean protoplasts.
MATERIALS AND METHODS

Isolation and culture of protoplasts

Cells of soybean (Glycine max (L.) Merr.) were cultured as described previously (Fowke et al. 1974a). Protoplasts were produced from cells sampled 45 h after transfer to fresh B5 medium (Gamborg, Miller & Ojima, 1968). Two millilitres of suspension culture were mixed with 2 ml of enzyme solution consisting of 2 % cellulase (Onozuka SS 1500, desalted on Sephadex G-25), 1 % pectinase and 0.5 M sorbitol in B5 medium without sucrose and growth hormones. The incubation was carried out in Petri dishes on a slowly rotating shaker at room temperature for 5 h. The released protoplasts were collected by centrifugation at 100 g for 3 min. After pipetting off the supernatant, the pellet was washed twice in B5 medium containing 0.3 M sorbitol. The protoplasts were then cultured in 200-μl droplets of B5 medium with 0.36 M sorbitol in Falcon dishes. The dishes were sealed with Parafilm and stored in humidified plastic boxes at room temperature.

Sampling for microscopy

Protoplast samples were collected immediately after enzyme treatment and at regular intervals thereafter, fixed in glacial acetic:ethanol (1:3, v/v), stained with carbol fuchsin in 45 % acetic acid (Miller et al. 1971) and examined in the light microscope to determine the frequency of mitoses in multinucleates. Protoplasts were sampled and prepared for subsequent light and electron microscopy when multinucleate divisions were most frequent.

Microscopy

Protoplasts were fixed in 1 % glutaraldehyde for 1 h at room temperature followed by 3 % glutaraldehyde for 2 h at room temperature. The glutaraldehyde was prepared in B5 medium + 0.3 M sorbitol. They were then washed briefly with the B5 medium + sorbitol, transferred to 0.05 M sodium phosphate buffer (pH 6.8), cooled to 0 °C and given 2 changes of buffer over 2-3 h. The protoplasts were postfixed in 1 % osmium tetroxide in the same buffer overnight at 0 °C.

Following a brief wash in distilled water at 0 °C the protoplasts were dehydrated slowly in ethanol to propylene oxide at 0 °C and infiltrated with Araldite at room temperature (see Fowke et al. 1973; Fowke, 1975, for details). They were finally embedded in glass Petri dishes which had previously been coated with releasing agent ARC7 (Acheson Colloids Canada Ltd, Brantford, Ontario), dried, and wiped thoroughly with tissues. Following polymerization, the Petri dishes usually had to be broken to remove the thin hardened Araldite blocks. These blocks were examined directly in the light microscope and dividing multinucleates were selected for subsequent electron microscopy.

RESULTS

Multinucleate protoplasts were observed immediately after enzyme treatment. Mitoses were first noted after approximately 40 h and were recorded for a further 40-h period.

Interphase

Eight multinucleate protoplasts containing interphase nuclei (7 binucleates, 1 trinucleate) were examined and in all cases the nuclei were linked by short narrow nuclear bridges. The nuclei in the binucleates were joined by 1–3 bridges while in the trinucleate 2 bridges connected the 3 nuclei in series (Figs. 2A–C). All 13 bridges observed in this study were approximately 0.2 μm in diameter. The nuclei of one of
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the binucleates and the trinucleate may have been in early prophase rather than in interphase since chromosome condensation had occurred and microtubules were observed in the cytoplasm surrounding the nuclei.

Prophase

Almost all multinucleate protoplasts entered mitosis synchronously and maintained synchrony throughout the division cycle. Only binucleates were observed at prophase. Prophase nuclei were almost entirely surrounded by a distinct clear zone containing microtubules, endoplasmic reticulum and a few larger organelles (Figs. 3A, B). Chromosome condensation was accompanied by disappearance of the nucleoli, breakdown of the nuclear envelope and movement of microtubules into the nuclei (Fig. 3B).

Metaphase

In multinucleate protoplasts at metaphase the individual spindles did not appear to be in contact with one another. Serial sections of the protoplast in Fig. 4A indicated the presence of 6 such spindles. Each spindle contained paired chromosomes aligned on the equatorial plates, microtubules and dilated endoplasmic reticulum profiles (Fig. 4B). The chromosomal microtubules were embedded in rather diffuse granular kinetochores (Fig. 4C).

Anaphase

Only one multinucleate protoplast at anaphase was found. At least three planes of division were present but the limited number of micrographs obtained were difficult to interpret.

Telophase

Four different multinucleate protoplasts at telophase were obtained and in all cases daughter nuclei were completely formed. At least 4 nuclei were present in each and serial sections indicated that the protoplast in Fig. 5A contained 10 nuclei. Cell plates which appeared to form by phragmoplasts were present between most of the nuclei in the 4 telophase multinucleates examined (Figs. 5A, B). The cell plates contained fine fibrils and were irregularly thickened (Fig. 5C); the phragmoplasts at the edges of the cell plates consisted of vesicles, microtubules and some electron-dense granular material (Fig. 5D). Numerous coated vesicles were observed in the cytoplasm near the forming cell plate and were often continuous with vesicles of the cell plate (Fig. 5E).

Other observations

A few multinucleate protoplasts at metaphase and anaphase appeared to contain at least twice the normal number of chromosomes (Fig. 6). These protoplasts seemed to undergo quite normal mitosis.
Very little cell wall material was detected at the surface of the dividing multinucleate protoplasts. Fig. 7 illustrates the cell wall present on a multinucleate protoplast at metaphase after 65 h in culture. Coated vesicles were frequently observed adjacent to or continuous with the plasmalemma.

The sequence of events leading from cells in culture through formation and subsequent division of multinucleate protoplasts is summarized in diagrammatic form in Fig. 1.

Fig. 1. Diagrammatic representation of multinucleate protoplast formation and division in soybean. A, cultured cells; B, spontaneous fusion during enzyme treatment; C, multinucleate protoplast with interphase nuclei linked by bridges; D, prophase; E, metaphase; F, anaphase; G, telophase; D1, protoplast containing fused nuclei.
DISCUSSION

The flat-embedding technique used in this study proved essential for subsequent selection of dividing multinucleate protoplasts with the light microscope. Embedding in glass Petri dishes yielded thin blocks of Araldite with both surfaces glassy smooth; such blocks showed a minimum of distortion when examined in the light microscope and facilitated selection of protoplasts for electron microscopy. Even when using this technique, dividing multinucleates were difficult to identify, particularly metaphases and anaphases. In addition the overall frequency of dividing multinucleate protoplasts was very low. As a result of these problems the current study does not describe mitosis in multinucleate protoplasts in the same detail as was reported for uninucleate protoplasts of soybean (Fowke et al. 1974a).

There appears to be some variability in the mode of origin of multinucleate protoplasts with different cell types. Nuclear division without cytokinesis has been reported by Eriksson & Jonasson (1969), Van der Valk & Wessels (1973) and Reinert & Hellmann (1973). This study and previous work (Miller et al. 1971) indicates that multinucleate protoplasts of soybean arise by spontaneous fusion during the enzyme treatment. Similarly, multinucleate protoplasts of Ammi (Fowke et al. 1973, 1974b), carrot (Grambow et al. 1972), tobacco (Withers & Cocking, 1972) and maize (Motoyoshi, 1971) are formed during protoplast isolation. The process of spontaneous fusion in tobacco has been examined at the ultrastructural level (Withers & Cocking, 1972). Our results demonstrate that multinucleates resulting from spontaneous fusion can undergo mitosis which resembles that occurring in cultured cells and uninucleate protoplasts of soybean (Fowke et al. 1974a).

The significance of the nuclear bridges connecting interphase nuclei is not known. It is possible that these bridges may play a role in synchronizing the nuclei prior to mitosis. The vast majority of multinucleates examined divided synchronously; synchronous divisions seem to be common in multinucleate protoplasts (Miller et al. 1971; Motoyoshi, 1971; Van der Valk & Wessels, 1973). In fact synchronous divisions are typical of most naturally occurring and artificially created multinucleate cells (Johnson & Rao, 1971). The basis of this synchrony is not really understood. Evidence from a number of systems suggests that proteins in the cytoplasm may induce synchrony.

It is also possible that the bridges may precede and facilitate nuclear fusion. Nuclear bridges have been reported between fusing nuclei during fertilization in plants (Jensen, 1964; Van Went, 1970).

Prophase and metaphase in the multinucleates seemed identical to prophase and metaphase in the uninucleate protoplasts of soybean (cf. Fowke et al. 1974a). A comparison was not possible for anaphase since we were able to detect this stage only rarely in the multinucleates. This may be due to the difficulty in recognizing anaphases in the Araldite block. In addition, anaphase is usually a rapid phase in mitosis (Mazia, 1961) and this would further reduce the chances of detecting multinucleates at this stage.

In a number of plant protoplasts nuclear division is apparently not followed by
cytokinesis (see above). Our results demonstrate clearly that cytokinesis, involving typical cell plate and phragmoplast formation, occurs in multinucleate protoplasts. Cytokinesis seems to resemble the process in uninucleate soybean protoplasts (Fowke et al. 1974a). We have not been able to determine whether all nuclei are finally separated by cell plates with the subsequent formation of a multicellular aggregate.

Miller et al. (1971) illustrated that nuclear fusion can occur prior to mitosis in soybean multinucleates. The protoplasts containing division figures with at least double the number of chromosomes (Fig. 6) are thus believed to be a result of such fusions. There was no evidence of polyploidy in the cultured cells from which the multinucleates were derived.

It seems quite evident that both uninucleate protoplasts (Fowke et al. 1974a) and multinucleate protoplasts of soybean are capable of nuclear division and cytokinesis with little detectable external cell wall. At telophase it is likely that wall material is being synthesized for 2 distinct sites, external cell walls and cell plates. Coated vesicles are thought to be involved in cell wall deposition (Bonnert & Newcomb, 1966) and the observed association of numerous coated vesicles with both the cell plate and plasmalemma is consistent with this idea.

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REFERENCES


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VAN DER VALK, P. & WESSELS, J. G. H. (1973). Mitotic synchrony in multinucleate Schizo-


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**ABBREVIATIONS ON PLATES**

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<th>Symbol</th>
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<tr>
<td>ch</td>
<td>chromosome</td>
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<tr>
<td>cz</td>
<td>clear zone</td>
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<tr>
<td>m</td>
<td>mitochondrion</td>
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<tr>
<td>n</td>
<td>nucleus</td>
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<td>nu</td>
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The figures in a series (e.g. 2A, 2B) represent micrographs of the same protoplast.

Fig. 2A. Light micrograph showing a trinucleate protoplast. Adjacent thin sections (Figs. 2B, C) illustrate bridges connecting the nuclei in the regions indicated by the arrowheads. × 1200.

Fig. 2B. Electron micrograph illustrating same nuclei as in Fig. 1. Nucleus 1 (n1) and nucleus 2 (n2) are connected by a nuclear bridge (single arrow). The second bridge linking nucleus 2 (n2) with nucleus 3 (n3) in the region of the double arrow is shown in Fig. 2C. × 17700.

Fig. 2C. Serial section in the region indicated by the double arrowhead in Fig. 2B showing nuclear bridge (arrow) connecting n2 with n3. × 18000.
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Fig. 3A. Light micrograph of a binucleate protoplast at prophase showing parts of both nuclei (arrowheads). A clear zone is present around the nuclei and some chromosome condensation is evident. An electron micrograph of the nucleus on the right is shown in Fig. 3B. × 1000.

Fig. 3B. Electron micrograph of the prophase nucleus indicated by the double arrowhead in Fig. 3A. The nucleus is almost entirely surrounded by a clear zone (cz) containing endoplasmic reticulum (large arrowheads) and many microtubules. Chromosomes (ch) and a few microtubules (small arrowheads) are evident within the nucleus. × 13600.

Fig. 4A. Light micrograph of a multinucleate protoplast at metaphase showing 4 separate metaphase plates (arrowheads). Note that the individual spindles do not appear to be in contact with one another. Serial sections demonstrated 2 additional metaphase plates in this protoplast. × 1000.
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Fig. 4B. Electron micrograph of one of the spindles shown in Fig. 4A. Note the kinetochores (large arrowheads) with associated microtubules and the endoplasmic reticulum (small arrowheads) within the spindle. An enlargement of the area outlined in black is shown below in Fig. 4C. × 15000.

Fig. 4C. Enlargement from Fig. 4B showing microtubules embedded in the diffuse kinetochores (arrowhead). × 28000.

Fig. 5A. Light micrograph of a multinucleate protoplast at telophase. Five nuclei (arrowheads) from a total of 10 are evident in this section. × 1000.
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Fig. 5A. Electron micrograph of the protoplast in Fig. 5A showing 7 nuclei (n). Cell plates (large arrowheads) are present amongst the nuclei. Note the phragmoplasts (small arrowheads) at the margins of the cell plates. Enlargements of the areas outlined in black are shown in Figs. 5C, D. × 3600.

Fig. 5C. Enlargement from Fig. 5A showing the irregularly thickened cell plate (arrowheads). × 17000.
Fig. 5D. Enlargement from Fig. 5B showing a phragmoplast containing vesicles, microtubules (arrowheads) and some electron-dense granular material. × 18,400.

Fig. 5E. Electron micrograph showing part of a cell plate in face view (large arrowheads) from the same protoplast as in Fig. 4. Numerous coated vesicles are evident (small arrowheads) some of which appear continuous with vesicles of the cell plate (double arrowheads). × 28,700.

Fig. 6. Light micrograph of a protoplast at anaphase. Nuclear fusion prior to mitosis is thought to be responsible for the unusually large number of chromosomes. × 1000.

Fig. 7. Electron micrograph showing loosely organized cell wall material (arrowheads) at the margin of a multinucleate protoplast at metaphase. × 25,000.
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