

ELECTRON MICROSCOPIC OBSERVATIONS OF CULTURED CELLS AND PROTOPLASTS OF AMMI VISNAGA¹

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ABSTRACT

Protoplasts were prepared from cultured cells of *Ammi visnaga* (Umbelliferae) by enzymatic digestion of the cell walls and examined microscopically. Staining of fresh protoplasts with Calcofluor and silver hexamine demonstrated the apparent absence of wall material. Protoplasts contained more cell organelles than the whole cells, particularly endoplasmic reticulum and associated polysomes. The plasmalemma of most protoplasts appeared smooth; some protoplasts were connected by structures resembling plasmodesmata. Multinucleates resulting from fusion were frequently observed.

PROTOPLASTS HAVE been produced from a variety of plant cell cultures by enzymatic digestion of the cell walls and have been observed with the light microscope, but not the electron microscope (e.g., Eriksson and Jonasson, 1969; Schenk and Hildebrandt, 1969; Miller et al., 1971; Grambow et al., 1972). Some information is available concerning the fine structure of protoplasts isolated from fruits, leaves, and roots by enzymatic removal of the cell walls (Pojnar, Willison, and Cocking, 1967; Power and Cocking, 1970; Benbadis, 1971; Withers and Cocking, 1972) and from roots by mechanical techniques (Prat and Roland, 1970). It could be expected that ultrastructural changes would occur as a result of enzymatic digestion of the cell walls. Using both light and electron microscopes, this study compares the structure of cultured cells and of protoplasts from *Ammi visnaga*. The *A. visnaga* cells were chosen because protoplasts are easily obtained and the methods for cell regeneration had been developed.

MATERIALS AND METHODS—Cell culture—The cell suspension cultures of *Ammi visnaga* were established from sterile seedlings as described previously for carrot (Grambow et al., 1972) and grown in mineral salt-sucrose-vitamin medium (B5) with 0.1 mg/liter of 2,4-dichlorophenoxyacetic acid (2,4-D) (Gamborg, Miller, and Ojima, 1968). The cultures used for protoplast production were

grown under continuous light at 28 C in 250 ml DeLong flasks with a total volume of 40 ml of cells and medium. The cells were subcultured at 2-3 day intervals.

Protoplast formation—The cells were collected on a 44- μ m stainless steel filter and washed in (2,4-D and sucrose)-free B5 medium containing 0.56 M mannitol (B5-M). Approximately 2 ml of cell suspension containing about 100 mg cells (fresh weight) were mixed with an equal volume of washing medium containing 1% w/v desalted cellulase "Onozuka P5000" (All Japan Biochemical Co. Ltd.) and 0.5% desalted hemicellulase "Rhozyme HP 150" (Rohm and Haas Co. of Canada). Protoplast production from all cells was completed within 6-7 hr. The protoplast suspension was passed through a 44- μ m filter and then washed by filtration through an 8- μ m Millipore filter (Grambow et al., 1972). Approximately 100-150 ml of B5-M medium were passed through the filter; the liquid on the filter was never allowed to drain completely. The protoplasts then were suspended in the same medium and fixed for microscopy as described below.

Microscopy—The microscopical techniques were modified from those used previously with highly vacuolated plant cells (Fowke and Setterfield, 1968; Fowke and Pickett-Heaps, 1969).

a) **Fixation**—Three percent glutaraldehyde was prepared in both B5 and B5-M media and adjusted to pH 5.5. These fixatives were added to the cell and protoplast suspensions, respectively, to give a final glutaraldehyde concentration of 1%. After 1-2 hr at room temperature the initial fixa-

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tives were replaced by 3% glutaraldehyde in the appropriate media (B5 for cells and B5-M for protoplasts, pH 5.5) for a further 1–3 hours. Following a brief rinse in their respective media, the cells and protoplasts were washed at 0°C in three changes of sodium phosphate buffer, pH 6.8 over 2–3 hr (cells, 0.05 M buffer; protoplasts, 0.3 M buffer). They were then postfixed in 1% osmium tetroxide prepared in above buffers at 0°C for 11–17 hr.

b) Dehydration and embedding—Following a brief wash in cold distilled water all specimens were slowly run to absolute ethanol by 10% increments at 0°C and then washed overnight in this solvent (with three changes); occasionally they were stored in ethanol for up to 6 days at –25°C. They were then slowly passed through propylene oxide and finally embedded in Araldite (Fowke and Pickett-Heaps, 1969). Both cells and protoplasts were embedded in a thin layer of Araldite in glass Petri dishes previously coated with the releasing agent ARC7 (Acheson Colloids Canada Ltd., Brantford). The final hardened blocks could be examined directly with the light microscope

(phase optics) thus facilitating selection of specimens for subsequent microscopy.

c) Sectioning and staining—The embedded cells and protoplasts were removed and mounted for sectioning with either glass or diamond knives. Silver sections were picked up on coated grids, stained with uranyl acetate and lead citrate, and examined in a Zeiss 9A electron microscope. Adjacent thick sections (0.5–1.0 μm) were mounted on glass slides and stained with toluidine blue (Fowke and Pickett-Heaps, 1969) for examination by the light microscope. Live protoplasts were also observed with the phase microscope (Fig. 6).

d) Cell wall staining—Cultured cells and fresh protoplasts were stained with 0.1% Calcofluor white (American Cyanamid Co., Bound Brook, New Jersey) to detect the presence of cell walls (Nagata and Takebe, 1970). In addition, wall polysaccharides were stained in electron microscope preparations with silver hexamine (Pickett-Heaps, 1967; Rambourg, 1967). Briefly, pale-gold sections of both cells and protoplasts were

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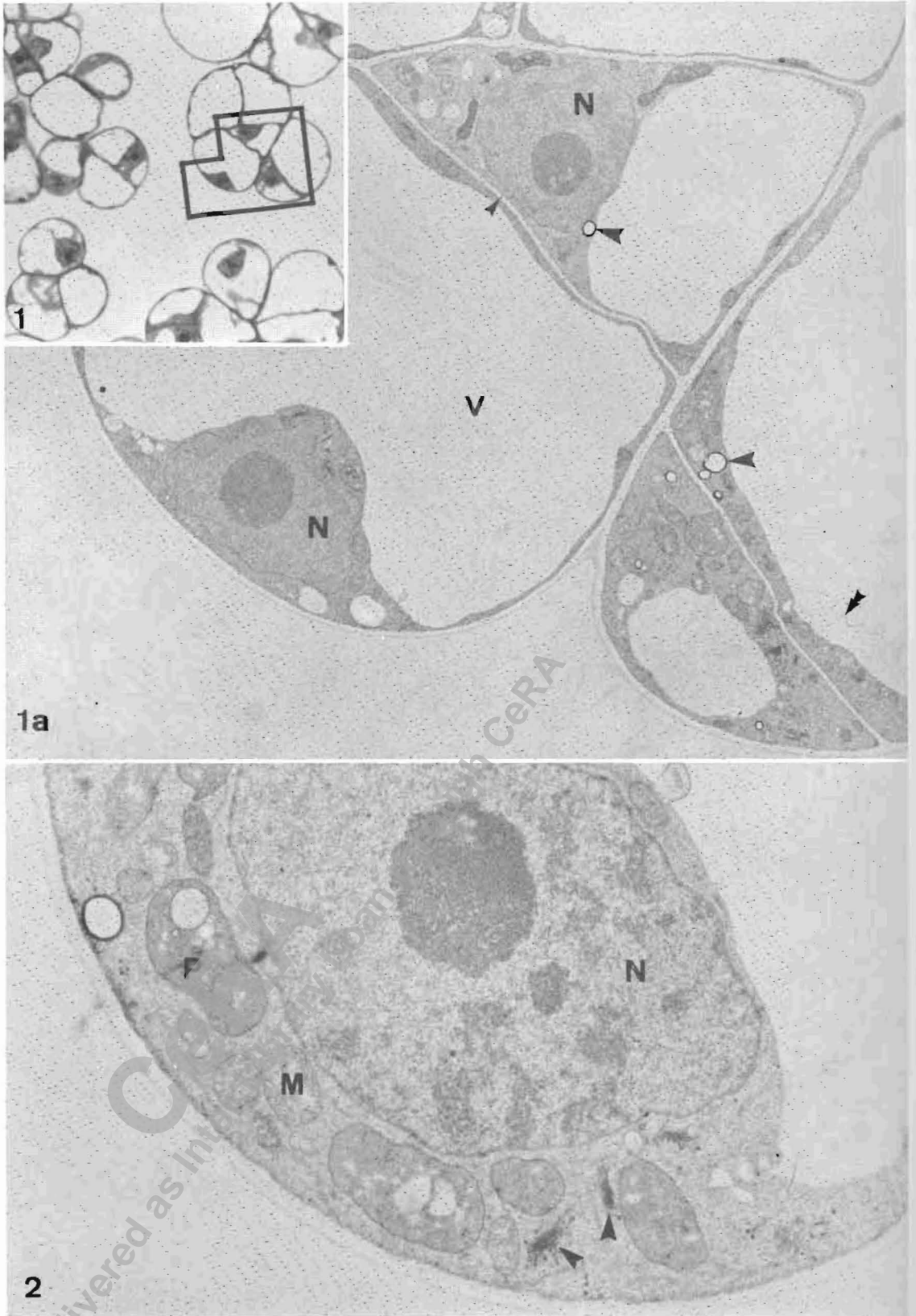
KEY TO LABELING: *G*, Golgi body; *M*, mitochondrion; *N*, nucleus; *P*, plastid; *V*, vacuole. The figures in series (e.g. 7–7b) represent micrographs of the same cell(s).

Fig. 1–2. **1.** Light micrograph of cultured cells. An electron micrograph of the cells outlined in black is shown in Fig. 1a. × 560. **1a.** Electron micrograph of cells outlined in Fig. 1. The highly vacuolated cells seem well preserved; only slight tonoplast damage is evident (small double arrow). Cell organelles are localized in the cytoplasm surrounding the nuclei. The lipid bodies (large arrows) have an electron translucent core. Note the single plasmodesma (small single arrow). × 5,400. **2.** Electron micrograph of cultured cell, showing the accumulation of cell organelles near the nucleus. The plastids exhibit a relatively simple structure. Small Golgi bodies (arrows) surrounded by tiny vesicles are present. × 12,000.

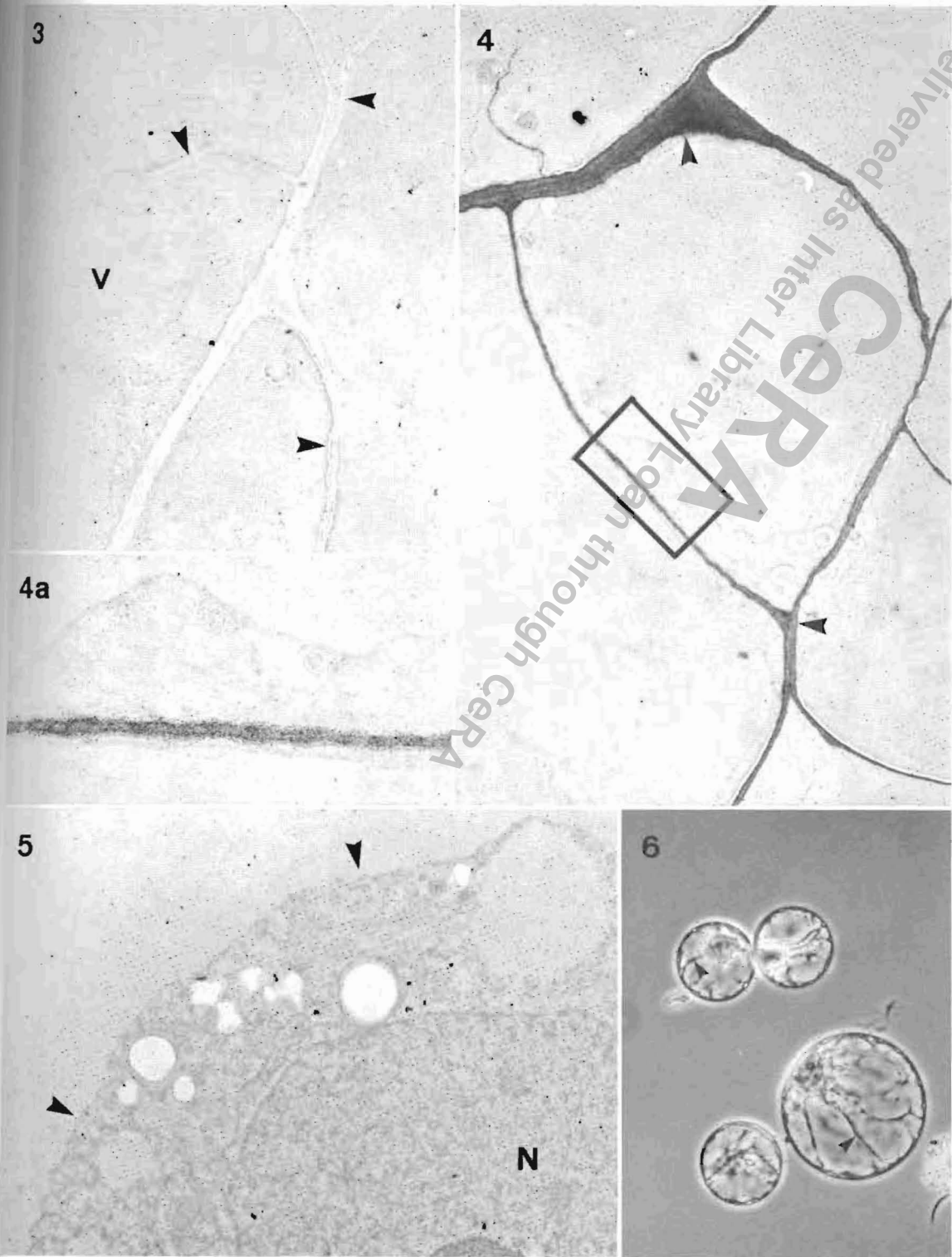
Fig. 3–6. **3.** Electron micrograph of cells blocked with chlorous acid and then stained with silver hexamine without prior oxidation with periodic acid. The cell walls (arrows) are virtually unreactive. × 4,500. **4.** Electron micrograph of cells blocked with chlorous acid, oxidized with periodic acid, and then stained with silver hexamine. The cell walls (arrows) have reacted strongly (cf. Fig. 3). The area outlined in black is shown in Fig. 4a at higher magnification. × 4,500. **4a.** Enlargement from Fig. 4, showing the stained cell wall. × 15,600. **5.** Electron micrograph of newly formed protoplast blocked with chlorous acid, oxidized, and then stained with silver hexamine. Cell wall material is not evident at the margin of the protoplast (arrows). × 9,600. **6.** Light micrograph (phase contrast optics) of live protoplasts. Note the cytoplasmic strands (arrows) within the spherical protoplasts. × 360.

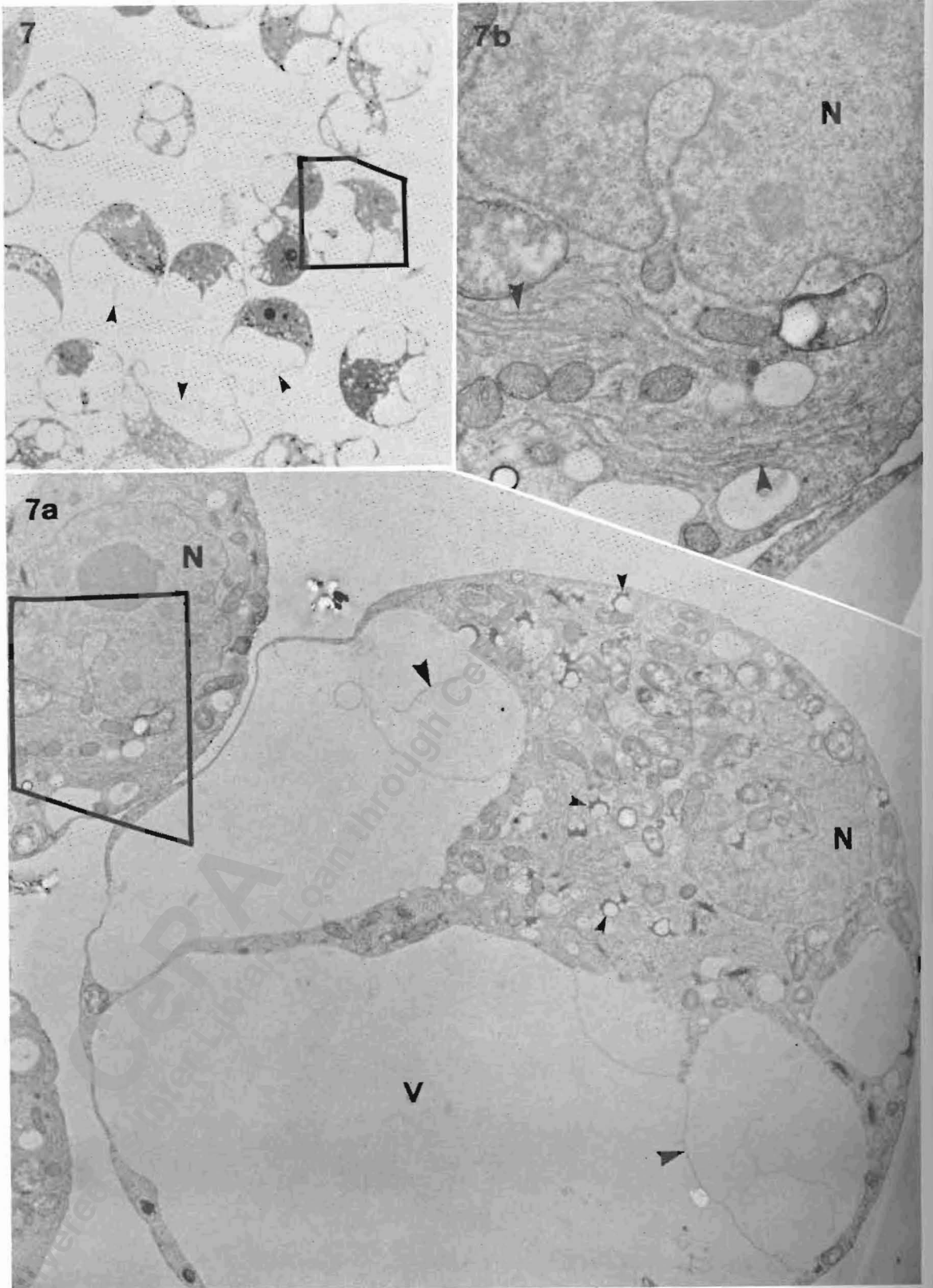
Fig. 7–7b. **7.** Light micrograph of newly formed protoplasts. Most of the smaller protoplasts have retained their spherical shape (cf. Fig. 6) but some of the larger ones have collapsed (arrows). An electron micrograph of the protoplasts outlined in black is shown below. × 560. **7a.** Electron micrograph of protoplasts outlined in Fig. 7. The protoplasts seem quite well preserved although some membrane damage is evident (large arrows). The apparent striking increase in cell organelles (cf. Fig. 1a) is particularly noticeable in the cytoplasm surrounding the nuclei. Note the irregularly shaped lipid bodies (small arrows). The area outlined in black is shown in Fig. 7b at higher magnification. × 5,400. **7b.** Enlargement from Fig. 7a showing the stacks of rough endoplasmic reticulum (arrows) near the nucleus. × 13,000.

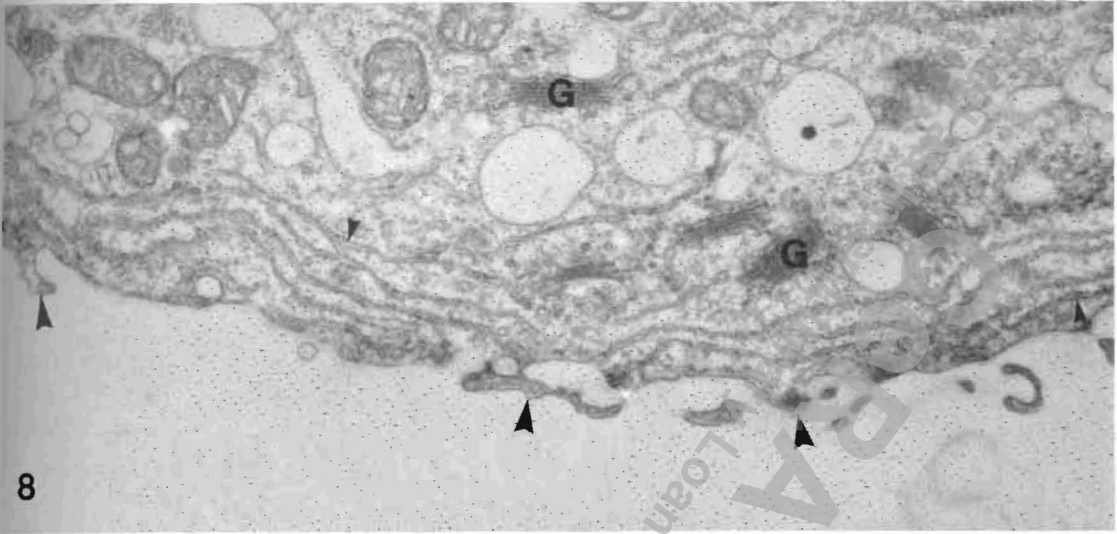
Fig. 8–9b. **8.** Electron micrograph, showing the irregular plasmalemma (large arrows) seen occasionally on protoplasts (cf. Fig. 7a, 9a). Note the rough endoplasmic reticulum (small arrows) paralleling the margin of the protoplast. × 18,600. **9.** Light micrograph, showing two spherical protoplasts which remained attached following enzyme treatment. The connections present are illustrated in Fig. 9a and 9b. × 1,400. **9a.** Electron micrograph of two protoplasts shown in Fig. 9. The cytoplasm of both protoplasts contains numerous cell organelles (cf. Fig. 1a). Starch granules (arrows) are present in some of the plastids. The area outlined in black is shown in Fig. 9b at higher magnification. × 5,400. **9b.** Enlargement from Fig. 9a, showing the dense structures (large arrows) connecting the two protoplasts. Note the microtubules (small arrow) near the plasmalemma. × 34,000.



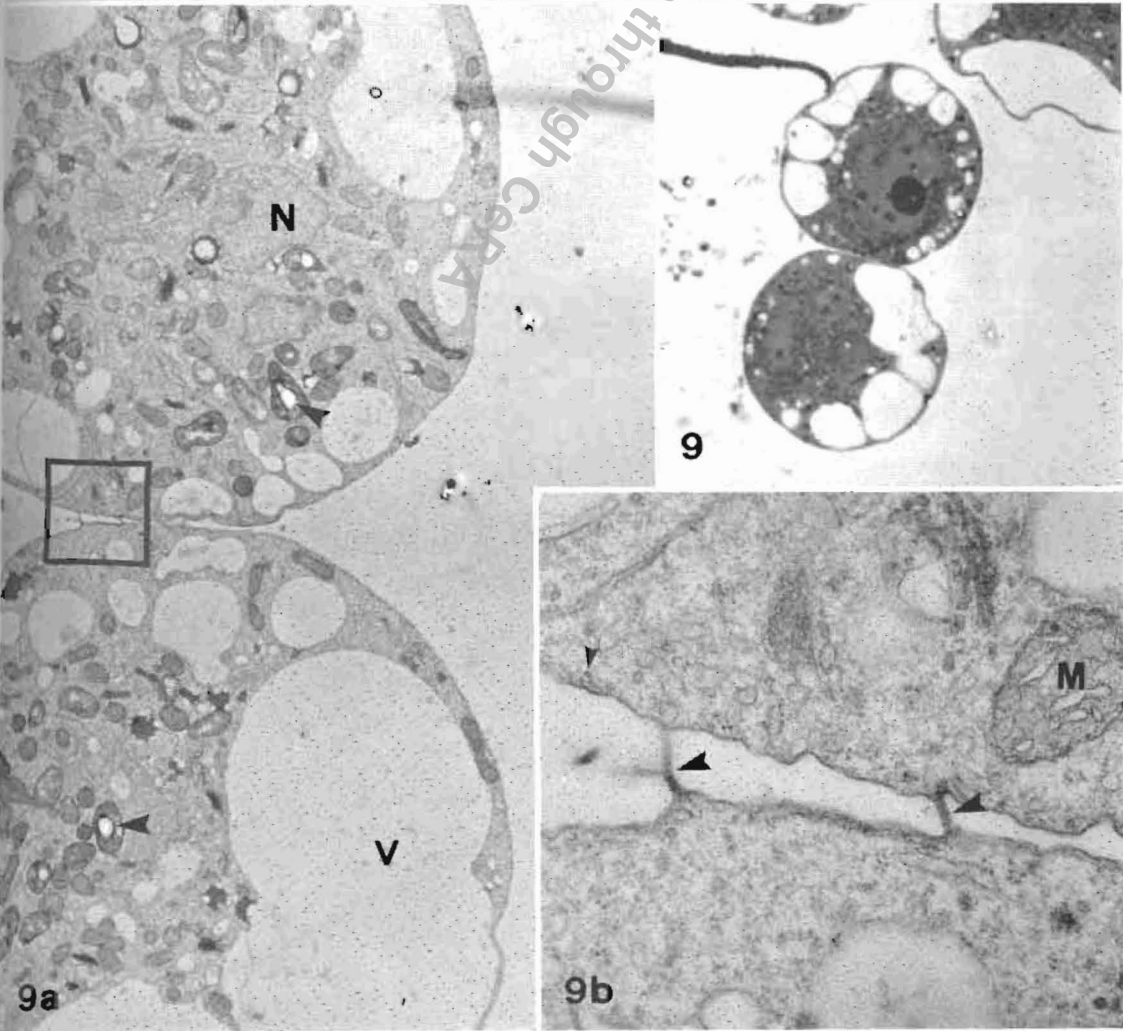
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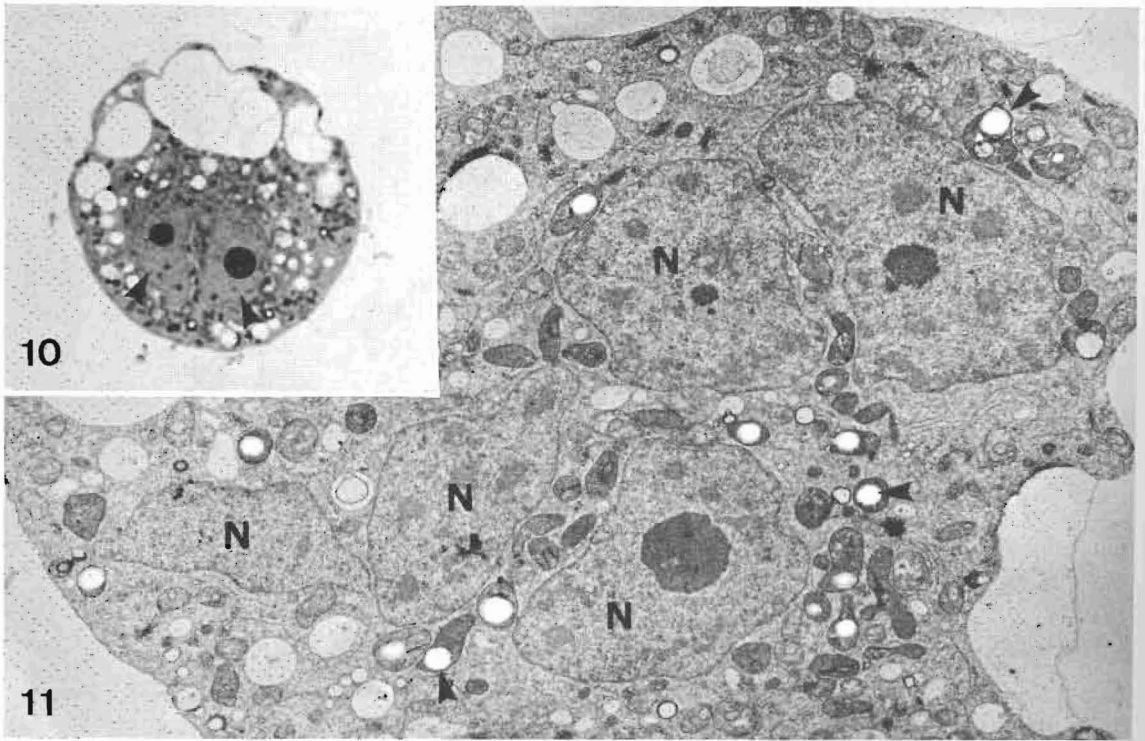


Fig. 10, 11. **10.** Light micrograph of protoplast containing two nuclei (arrows). $\times 1,400$. **11.** Electron micrograph of protoplast apparently containing five nuclei. Note the starch granules (arrows) in the plastids. $\times 5,400$. See p. 305 for Key to Labeling.

collected in polythene washers to facilitate all subsequent manipulations (Pickett-Heaps, 1967). The sections were treated as follows:

1. Blocked with chlorous acid (0.2 M sodium chlorite in N acetic acid) for 1–1¼ hr at 60 C (Rappay and van Duijn, 1965; Pickett-Heaps and Fowke, 1970) and then stained with the silver hexamine reagent for 30–40 min at 60 C (Rambourg, 1967). When sections were not blocked, a general nonspecific staining was obtained with silver hexamine.
2. Blocked as above but oxidized with 1% periodic acid for 30 min at room temperature, and then stained with the silver hexamine reagent.

After staining with silver hexamine, clearing with thiosulphate, and washing, the sections were mounted on coated grids and stained very lightly with uranyl acetate.

RESULTS—Quality of fixation—The vacuolated cells of *Ammi visnaga* appeared to be well preserved (Fig. 1–2); only occasionally was slight tonoplast damage evident (Fig. 1a). During the preservation of freshly prepared protoplasts, slight shrinkage of small protoplasts and some collapse of larger protoplasts occurred (Fig. 7). Disrup-

tion of the tonoplast occurred frequently (Fig. 7a, 9a, 10, 11), but otherwise the protoplasts remained intact (Fig. 7, 7a, 9, 9a, 10). In general the organelles seemed well preserved.

Cultured cells—The cultured cells were characterized by a thin layer of peripheral cytoplasm surrounding a large central vacuole (Fig. 1, 1a). A single nucleus was present in each cell and most other cell organelles were localized in the thickened region of cytoplasm enclosing the nucleus (Fig. 2). The plastids showed very little development (Fig. 2); a few scattered starch granules were evident in the homogeneous plastid matrix. Prolamellar bodies and grana were never seen in the plastids. Golgi bodies were typically small and appeared to be producing tiny vesicles (Fig. 2). The cytoplasm contained numerous ribosomes but almost no polysomes. Very few endoplasmic reticulum profiles were present and thus most ribosomes were free in the cell. A few (presumed) lipid bodies were scattered in the cytoplasm; they were either uniformly electron dense or exhibited an electron-translucent core (Fig. 1a, 2).

The cells were enclosed by a typical cell wall easily detectable at the light microscope level with Calcofluor; similarly, the walls reacted strongly when stained for polysaccharides with silver hexa-

mine (Fig. 3-4a). Plasmodesmata were observed between the cultured cells (Fig. 1a), but only infrequently.

Protoplasts—Cell walls seemed completely lacking on freshly prepared protoplasts as indicated by negative reactions with both Calcofluor and silver hexamine (Fig. 5). Consequently, the live protoplasts were typically spherical (Fig. 6). As mentioned above, when these protoplasts were processed for microscopy some of the larger ones collapsed (Fig. 7) and all lost their perfectly spherical shape.

In the electron microscope these protoplasts were characterized by an apparent increase in number of most of the cell organelles (Golgi bodies, mitochondria, plastids, lipid bodies, endoplasmic reticulum profiles, and ribosomes). As in the cultured cells, the organelles were primarily localized near the nuclei (Fig. 7a, 7b, 9a, 11). The change in the number of endoplasmic reticulum profiles was particularly striking; stacks of rough endoplasmic reticulum were observed throughout the cytoplasm enclosing the nucleus (Fig. 7a, 7b) as well as paralleling the plasmalemma (Fig. 8). There were numerous polysomes. The structure of the plastids and the Golgi bodies remained relatively unchanged following removal of the cell walls, although a slight loss of plastid matrix was evident (Fig. 7a, 7b). In many protoplasts the lipid bodies assumed a very irregular shape (Fig. 7a, 9a).

Almost all protoplasts were enclosed by a smooth plasmalemma (Fig. 7a, 9a); however, occasionally the plasmalemma was very irregular (Fig. 8). Despite the lack of cell walls, protoplasts sometimes remained attached to each other by dense projections (Fig. 9-9b).

Unlike the cultured cells, the protoplasts often contained two nuclei (Fig. 10) and, in fact, in a few cases multinucleates contained at least five nuclei (Fig. 11). Multinucleates were easily observed with the light microscope in live or embedded protoplasts.

DISCUSSION—The techniques described proved very satisfactory for preserving both cultured cells and the very fragile fresh protoplasts of *Ammi visnaga* isolated from the cultured cells. To assure optimal results, fixation with glutaraldehyde in the culture medium and very gradual dehydration of specimens were essential. The short washing period following glutaraldehyde fixation also was critical; when stored for extended periods at this stage the background cytoplasm of cells and protoplasts appeared extremely dense. Consequently, during prolonged experiments the tissue was run to absolute ethanol and then stored at -25 C (M. McCully, personal communication)

with no detectable deterioration in the quality of fixation. The cells and protoplasts were not centrifuged until after the initial fixation with glutaraldehyde, thus minimizing organelle displacement.

The cultured cells did not appear to be intensely active as indicated by the number of mitochondria and endoplasmic reticulum profiles and the almost total absence of polysomes (cf. protoplasts below). The plastids were characteristically very simple in structure; despite continuous illumination, chloroplasts never developed in these cells. Similarly, plastids with a relatively simple structure have been reported in other cultured cells; however, under certain culture conditions these plastids developed grana (e.g., carrot—Halperin and Jensen, 1967; Israel and Steward, 1967; artichoke—Tullett, Bagshaw, and Yoeman, 1969). The general scarcity of plasmodesmata in *Ammi* cells is similar to that observed for carrot cells cultured in a similar medium (Leppard and Colvin, 1971).

Enzyme treatment of these cells apparently removed the cell wall completely (Fig. 7a, 9a; cf. Fig. 1a, 2). The absence of wall material was confirmed in the light microscope with Calcofluor and in the electron microscope with silver hexamine.

The apparent increase in most cell organelles during the enzyme treatment (Fig. 7a, 9a cf. Fig. 1a) suggests a general activation of the protoplasts, presumably in preparation for cell wall synthesis; protoplasts of *Ammi visnaga* will regenerate new cell walls and undergo mitosis within 7 days when cultured (Fowke et al., unpublished). The striking increase in endoplasmic reticulum and associated polysomes is consistent with an upsurge in protein synthesis; similar changes accompany the activation of dormant storage tissues in preparation for rapid cell expansion involving wall growth (Fowke and Setterfield, 1968). Since Golgi bodies are thought to be responsible for cell wall deposition in a variety of plant cells (Fowke and Pickett-Heaps, 1972), it is not surprising that more Golgi are present in protoplasts preparing for wall synthesis. The small Golgi vesicles present did not, however, stain with silver hexamine, indicating that in fresh protoplasts of *Ammi* the Golgi apparently had not become active in depositing wall polysaccharides. In contrast, the tiny Golgi vesicles observed in mechanically produced protoplasts of onion apparently do contain polysaccharide and are thought to be involved in formation of new cell walls (Prat and Roland, 1970). The irregularity of the plasmalemma is also thought to be indicative of active cell wall formation (Pojnar et al., 1967; Prat and Roland, 1970); Pojnar et al., (1967) noted a relatively smooth plasmalemma on freshly prepared proto-

plasts of tomato fruit, whereas marked infolding of the plasmalemma was evident on protoplasts during deposition of new cell wall material. However, in both freshly prepared protoplasts of *Ammi* and those synthesizing new cell walls (Fowke et al., unpublished) irregularities of the plasmalemma were rare. The nature of the connections between certain protoplasts (Fig. 9a, 9b) is uncertain but they bear a strong resemblance to plasmodesmata. The retention of plasmodesmata between freshly prepared protoplasts has recently been reported (Withers and Cocking, 1972).

The formation of many multinucleate protoplasts during the enzyme treatment is likely a result of fusion during wall degradation. Multinucleates have been observed in newly formed protoplasts of various plants (Miller et al., 1971; Grambow et al., 1972; Withers and Cocking, 1972) and in all cases were attributed to fusion. Alternatively, multinucleate protoplasts might be formed by nuclear division without cytokinesis. It is highly unlikely that such a mechanism could account for the proliferation of nuclei in *Ammi* during the relatively short enzyme treatment. Nuclear division has been suggested as a source of multinucleate protoplasts where they were maintained in culture for various periods of time (Eriksson and Jonasson, 1969; Bawa and Torrey, 1971; Hellmann and Reinert, 1971). Protoplast fusion during enzyme treatment is not responsible for the marked increase in cell organelles (see above) since all uninucleate protoplasts displayed a similar change.

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