

A comparison of the death induced by fungal invasion or toxic chemicals in cowpea epidermal cells. I. Cell death induced by heavy metal salts

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Received June 19, 1987

MEYER, S. L. F., and HEATH, M. C. 1988. A comparison of the death induced by fungal invasion or toxic chemicals in cowpea epidermal cells. I. Cell death induced by heavy metal salts. *Can. J. Bot.* **66**: 613–623.

Death was induced in cowpea leaf epidermal cells by the application of the metal salts CuCl_2 , CuSO_4 , and HgCl_2 . When unfixed, salt-treated cells were observed by light microscopy, their degeneration and death were seen to follow a series of stages: (1) cessation of cytoplasmic streaming, (2) morphological changes in the cytoplasm such as the formation of large vesicles and the appearance of particles in the vacuole, and (3) protoplast collapse. The relative timing of these stages after salt application was affected by the nature and concentration of the chemical used. The application of fluorescein diacetate and plasmolysing sucrose solutions before or after the addition of the copper salts suggested that the semipermeability of the tonoplast was lost during stage 2 and that of the protoplast boundary at stage 3. Following the cessation of cytoplasmic streaming, the changes observed in unfixed cells occurred rapidly and correlated well with the ultrastructural changes observed in the same cells after fixation. First, microtubules appeared to decrease in abundance. Then coiled polyribosomes, endoplasmic reticulum, and Golgi bodies rapidly became undetectable, mitochondrial cristae became dilated, and the cytoplasm consisted of aggregates of ribosome-containing material interspersed with vesicles. However, the study also showed that the first observable sign of degeneration seen in unfixed cells (i.e., the cessation of cytoplasmic streaming) appears to have no clearly diagnostic feature detectable by electron microscopy.

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La mort a été induite chez les cellules épidermiques du dolique par l'application des sels métalliques CuCl_2 , CuSO_4 et HgCl_2 . L'observation au microscope photonique des cellules non-fixées et traitées aux sels a montré que leur dégénérescence et leur mort suivent une série de stades : (1) la cessation des mouvements cytoplasmiques, (2) des changements morphologiques dans le cytoplasme tels que la formation de grandes vésicules et l'apparition de particules dans la vacuole et (3) l'affaissement du protoplaste. Le minutage relatif de ces stades, après l'application des sels était affecté par la nature et la concentration de la substance utilisée. L'application du diacétate de fluorescéine et de solutions plasmolysantes de sucrose avant ou après l'ajout des sels de cuivre a suggéré que l'hémi-perméabilité du tonoplaste était perdue au cours du stade 2 et celle du plasmalemma au stade 3. Après la cessation de la cyclose, les changements observés chez les cellules n'ayant pas subi de fixation se sont faits rapidement et étaient en bonne corrélation avec les changements ultrastructuraux observés dans les mêmes cellules, après fixation. D'abord, les microtubules ont semblé être moins abondants. Ensuite, les polyribosomes spiralés, le réticulum endoplasmique et les corps de Golgi sont devenus rapidement impossible à détecter, les crêtes mitochondriales se sont dilatées; le cytoplasme était alors constitué d'agrégats d'une matière contenant des ribosomes et parsemée de vésicules. Cependant, l'étude a aussi montré que le premier signe observable de la dégénérescence chez les cellules non fixées (c'est-à-dire la cessation de la cyclose) ne semble pas avoir de trait diagnostique clair pouvant être détecté au microscope électronique.

[Traduit par la revue]

Introduction

Cell death is a common feature of both susceptible and resistant plants infected by pathogens (e.g., Heath 1980). However, since studies of plant cell death induced by pathological or mechanical damage are often undertaken only to discover if the plant cells have been damaged or killed (Gahan 1981), neither the mode of elicitation nor the process of cell death in plants is well understood. Davies and Sigeo (1984) have concluded that "... there is no single mechanism to account for either the induction or the progression of cell ageing and death." Such a conclusion seems to be supported by the variety of ultrastructural changes associated with cell death induced by pathogens (Heath 1976, 1980) and various toxins (Arias et al. 1983; Arias 1985). However, studies of cell death in plants lag behind those in animals, where different types of death, involving different processes, have been defined (Wyllie 1981; Beaulaton and Lockshin 1982). Moreover, to our knowledge there have been no studies that compare the ultrastructure of a cell with its appearance in unfixed tissue

during various stages of cell death. The current study was therefore initiated to determine if identifiable and repeatable stages occur during the death of cowpea leaf epidermal cells, to correlate the appearance of unfixed cells with images of the same cells processed for electron microscopy, and to see whether the process of cell death differed with the causal agent of death. Cells were treated with either heavy metal salts or with a fungal plant pathogen for which cowpea is not a host. The present paper reports the effects of metal salts.

Materials and methods

Plant material

Cowpea plants (*Vigna sinensis* (Torner) Savi cv. Early Ramshorn) were grown in sterilized Pro-mix BX (Premier Brands Inc., New Rochelle, NY) in a growth chamber maintained at 20–24°C and illuminated for 16 h/day at about 250 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Plants were treated weekly with 20–20–20 fertilizer. Growing tips were removed to keep the plants at the primary leaf stage. All studies were performed on upper epidermal cells located over the midveins of primary leaves.

Light microscopy

To observe individual healthy and dying cells, tissue pieces (about 7–10 × 5 mm) were cut with razor blades from 12- to 16-day-old

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plants, mounted on slides in distilled water, aqueous CuCl_2 , CuSO_4 , or HgCl_2 , and examined with differential interference contrast optics. Observations were made with either a Reichert Diapan or a Reichert-Jung Polyvar microscope. One to three cells were observed from each tissue piece; a minimum of 11 cells were examined for each CuCl_2 concentration and 4 cells for each concentration of CuSO_4 and HgCl_2 . All observations of CuSO_4 - and HgCl_2 -treated cells were performed on the same batch of plants on the same day, while observations of CuCl_2 -treated cells were done over a period of several weeks, with some concentrations being applied on more than one day. Chemically treated cells were observed until after the cell protoplast had collapsed. Some tissue pieces were immersed in a drop of 0.1 M CuCl_2 in a moist chamber for 4 days, washed in water, and examined.

Effects of CuCl_2 on cells in intact, attached leaves were also observed. A 50- μL drop of 0.003 M CuCl_2 solution was placed above the midvein of each leaf; the experiment was performed twice on a total of 12 leaves. At the time of treatment and at intervals for up to 5 days after treatment, leaf pieces containing treated areas were excised, vacuum infiltrated with distilled water, and examined by light microscopy.

Plasmalemma semipermeability was investigated with a 2 M sucrose solution. Tissue pieces in water and in heavy metal salts were perfused with the solution until the cell protoplasts contracted. The sucrose was then replaced with distilled water and the pieces were soaked for up to 2 h. A total of 18 tissue pieces were examined, and the sucrose was applied (i) while the cytoplasm was still streaming in cells treated with water and 0.04, 0.07, and 0.1 M CuCl_2 (a total of 5 tissue pieces), (ii) immediately or a few minutes after the cell cytoplasm had stopped streaming in cells treated with 0.04, 0.07, and 0.1 M CuCl_2 or 0.2 M CuSO_4 (a total of 11 tissue pieces), and (iii) when cells had structures that resembled vesicles in the cytoplasm in cells treated with 0.2 M CuSO_4 (2 tissue pieces). One to five cells in the center of each tissue piece were examined.

Plasmalemma semipermeability and cell viability also were examined using a 20 $\mu\text{g} \cdot \text{mL}^{-1}$ solution of fluorescein diacetate (FDA) prepared by adding 10 μL of a stock solution in acetone to 1 mL distilled water. This was perfused over tissue mounted on slides either before or after the addition of 0.1 M CuCl_2 . Fluorescence was observed using epifluorescence optics and blue-light irradiation (exciter filter BP 450–495, dichroic mirror DS 510, barrier filter LP 520).

Light micrographs were taken of unfixed and fixed cells, using a microflash and Kodak Panatomic-X film.

Electron microscopy

Pieces were cut from near the tips of leaves of 12- to 13-day-old cowpea plants, and the undersides of the midveins were sliced off with razor blades. Pieces approximately 3 mm² were mounted on slides in either distilled water or 0.1 M CuCl_2 . Some pieces observed in water were remounted in 0.1 M CuCl_2 . Tissue pieces were then fixed at the selected stages of cell death by quickly transferring them to 2.5% glutaraldehyde – 2.0% paraformaldehyde in 0.05 M potassium phosphate buffer pH 6.8. During fixation, samples were placed under a vacuum for a few minutes and then cut into smaller pieces of about 1–2 mm². After 2 h, samples were washed in five to nine 15-min rinses of 0.05 M potassium phosphate buffer – 0.1 M sucrose, postfixed for 2 h in 1.0% OsO_4 – 0.1 M sucrose in the same buffer, and dehydrated in a 5, 10, 20, and 40% ethanol series (10 min each change) followed by 60 and 80% ethanol (20 min each) and four changes of absolute ethanol (20 min, overnight, 30 min, and 30 min). This was followed by ethanol – propylene oxide (1:1, v/v) (1.5 h), propylene oxide (1.5 h), and propylene oxide – Epon 812 (1:1, v/v) (3 h covered, then uncovered overnight). All procedures were performed at room temperature. The resin was changed the next morning, and the samples were flat embedded between plastic sheets several hours later. After incubation in a desiccator overnight, the samples were placed into a 60°C oven for 24–48 h.

Previously photographed cells were identified after embedment, sectioned with a Reichert-Jung ultracut ultramicrotome, and stained in 2% aqueous uranyl acetate for 20 min at 60°C and in lead citrate for 3.5–4 min at room temperature. Serial paradermal sections were cut

from each cell from the adaxial wall to the area where the central vacuole became prominent in the sections. Sections were examined with a Philips 201 transmission electron microscope operated at either 40 or 60 kV.

Stereological analysis

The relative numbers of coiled polyribosomes per unit area and the volume fraction of the cells occupied by microtubules were estimated by stereological analysis of electron micrographs. Four healthy cells, three cells that were treated but had cytoplasmic streaming when fixed, and two cells fixed shortly after streaming stopped were used for this analysis. Counts were made from two micrographs of each cell: one taken from approximately the third and the other from about the sixth section from the adaxial cell wall. The micrographs were magnified 39 000 \times .

To estimate the relative number of coiled polyribosomes per unit area, an acetate sheet with a square (sides 3 cm) marked on it was dropped randomly three times onto each micrograph, and the number of coiled polyribosomes within the square was counted. To estimate the volume fraction of the cell occupied by microtubules, a grid of lines 1.5 cm apart was randomly dropped three times onto each micrograph, the total number of line intersection points within the protoplast (P_{TOT}) was counted, and the number of line intersection points that fell over microtubules (P_{M}) was counted. The volume fraction (V_{M}) of the cell occupied by microtubules was estimated by dividing P_{M} BY P_{TOT} . The numbers obtained for healthy cells and for treated cells fixed while they still showed cytoplasmic streaming were combined, and a *t*-test was performed to compare these values with those obtained from cells fixed just after streaming stopped. The cell wall and large central vacuole (when present in a micrograph) were excluded from all measurements.

Results

Light microscopy

Each healthy leaf epidermal cell of cowpea had peripheral cytoplasm surrounding a large central vacuole (Figs. 1–3). The vacuoles were traversed by cytoplasmic strands (Fig. 2). Streaming was observed in both the peripheral cytoplasm and the transvacuolar strands. The most obvious and easily identifiable organelles observed with the light microscope were chloroplasts and nuclei (Fig. 3). Some of the smaller organelles were discernible as particles in the cytoplasm (Figs. 1, 3).

When tissue pieces were mounted in CuCl_2 , CuSO_4 , or HgCl_2 , the process of cell death was observed to be basically the same regardless of the chemical concentration and could be divided into several broad stages. The first change seen with light microscopy was that streaming slowed and stopped. This was followed by a period when observable structural changes occurred in the cell contents. In the last stage, the protoplast collapsed away from the cell wall.

The average times from the application of the metal salt to the cessation of streaming and to collapse of the protoplast are given in Table 1. The time spans between these stages of cell death were variable, but they generally shortened as the concentration of the chemical was increased. Increasing the CuCl_2 concentration not only decreased the time after addition of the chemical that streaming stopped but also disproportionately decreased the period between cessation of streaming and protoplast collapse; in consequence, a 5-fold change in concentration resulted in a 26-fold reduction in the duration of the death process (Table 1). A similar, but less marked, result was observed with CuSO_4 (Table 1). In contrast, there was only a 2-fold change in the duration of the death process over a 10-fold change in concentration of HgCl_2 (Table 1).

At the lower chemical concentrations, the cytoplasm and organelles streamed sluggishly before actually ceasing to

TABLE 1. Effect of concentration of heavy metal salts on the timing of cytoplasmic morphological events in cowpea epidermal cells

Type of heavy metal salt and concn. (M)	Time (min) after salt addition that:		Relative duration of death process ^a
	streaming stopped	protoplast shrank	
CuCl ₂			
0.04	14 ± 7 ^b	66 ± 14	26
0.07	6 ± 3	38 ± 15	16
0.1	4 ± 1	23 ± 10	10
0.2	4 ± 1	6 ± 6	1
CuSO ₄			
0.2	22 ± 1	95 ± 28	9
0.4	13 ± 4	62 ± 10	6
0.6	13 ± 4	31 ± 6	2
1.1	7 ± 1	15 ± 7	1
HgCl ₂			
0.003	158 ± 10	525 ± 30	2
0.007	29 ± 4	266 ± 60	1
0.015	20 ± 9	255 ± 19	1
0.03	12 ± 2	188 ± 15	1

^aThe time (minutes) between cessation of streaming and protoplast shrinkage divided by the time (minutes) between cessation of streaming and protoplast shrinkage for the highest salt concentration used.

^bMean and standard deviation from a minimum of 4 (CuSO₄ and HgCl₂ data) or 11 (CuCl₂ data) cells.

move; this period of slow movement was followed by a time when there were localized movements of organelles. The actual cessation of streaming was often accompanied by a period when the organelles and any cytoplasmic strands still left were jiggling in a way that resembled Brownian motion. This motion sometimes continued for several minutes after streaming stopped. In some cells, transvacuolar cytoplasmic strands disappeared when streaming stopped; in other cells, the strands stopped streaming but remained in position. Except for the cessation of streaming, the cellular contents observed at this time appeared similar to those of untreated cells.

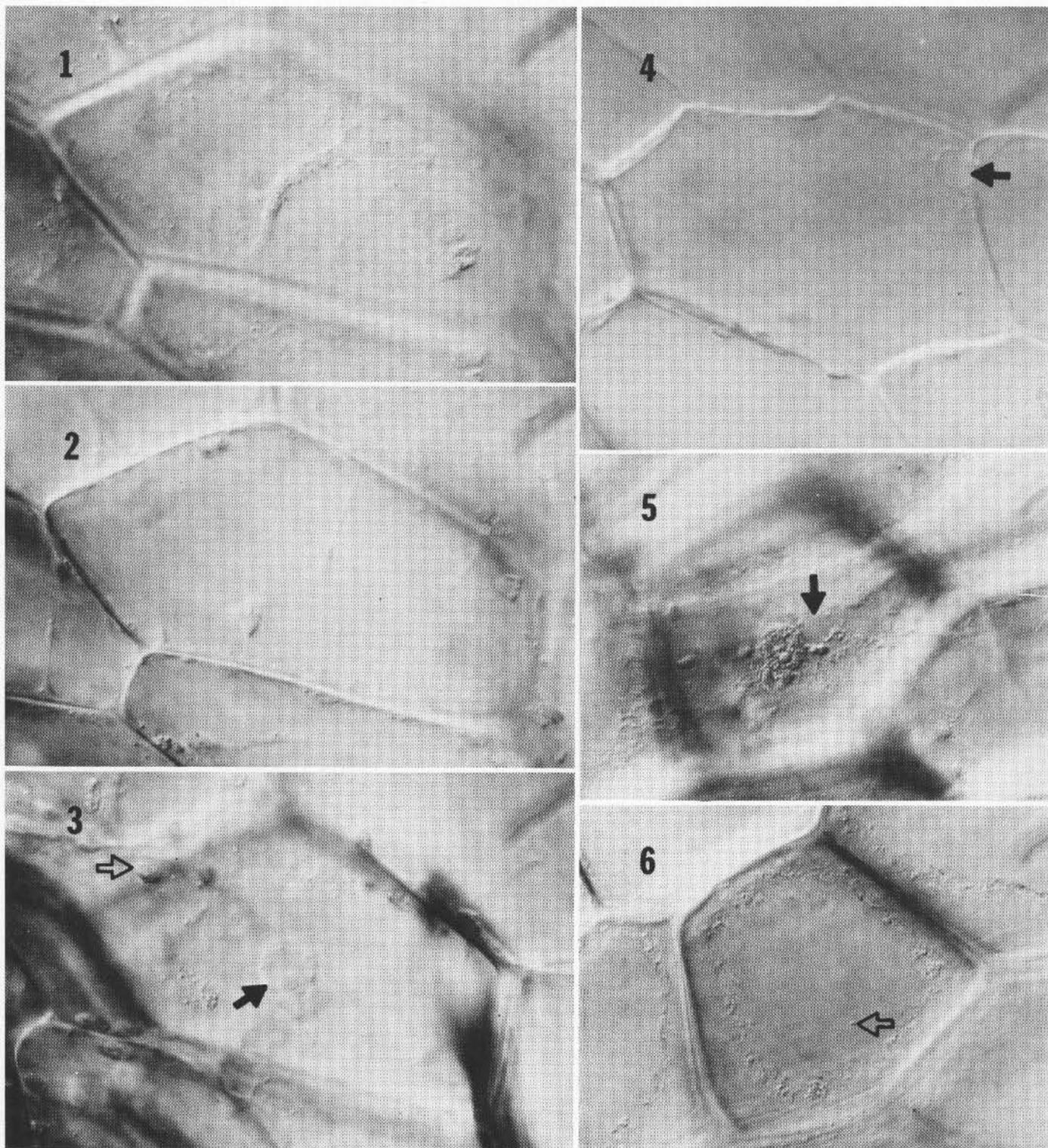
Following the cessation of cytoplasmic streaming, the cytoplasm and nucleoplasm became granular in appearance (Fig. 5). Often, the cytoplasmic strands that had not disappeared looked vesiculated. Large vesicle-like structures also developed throughout the rest of the cytoplasm and sometimes extended into the vacuole (Figs. 4, 5). These structures were largest in HgCl₂-treated cells. Some were formed by transvacuolar strands that remained after the cessation of streaming, but others appeared some time after streaming stopped. Many of the large vesicles were attached to the peripheral cytoplasmic area of dying cells (Fig. 4), but in a few cells, spherical vesicles unattached to the periphery were observed undergoing Brownian motion in the vacuoles. Vesicles were also seen near nuclei (Fig. 5). Some vesicles disappeared with time; others remained. Occasionally, they were seen expanding inside cells. During this cell death stage, small particles showing Brownian motion were present in the vacuoles of cells.

The last major change in appearance of the dying cells was the collapse of the protoplast away from the cell wall (Fig. 6). At the highest CuCl₂ concentration, the collapse sometimes occurred even while streaming was slowing. After collapse, some cells were seen with portions of the protoplast still attached to the cell wall; sometimes only small segments of a cell's protoplast collapsed. In a few cells, strands were observed extending from the collapsed protoplast of a cell to the cell wall. Vesicles that had appeared in the previous stage sometimes remained after protoplast collapse, but in other cells, they were no longer observable. At a concentration of

0.1 M CuCl₂, some cells developed vesicles adjacent to nuclei during this cell death stage. In many cells, small particles and vesicle-like structures undergoing Brownian motion appeared between the collapsed protoplast and the plant cell walls. Particles were also still present in the cell vacuole (Fig. 6).

Browning was not observed in treated tissue pieces mounted on slides, even after up to 4 days in CuCl₂ solution. To see whether this lack of browning was the result of the tissue being detached from the plant, drops of 0.003 M CuCl₂ were applied to veins on undetached leaves. No change was seen on the leaves immediately after application and a cut section observed by light microscopy revealed that the cytoplasm of the cells was streaming and was not discolored. Several hours after treatment the spots began to turn chlorotic. When cut tissue pieces were mounted in water and observed by light microscopy, many treated epidermal cells, especially those at the periphery of the CuCl₂ drops, were straw to brown in color and had collapsed protoplasts. The brown color appeared to be both in the cytoplasm and in the cell walls. Some of the cells with collapsed protoplasts were not discolored.

When tissue pieces cut from healthy plants were mounted in water or in heavy metal salts and then perfused with sucrose solution, the protoplasts of the cells contracted. Cells that showed cytoplasmic streaming when they were treated with sucrose generally regained turgidity after being placed into water, except for one tissue piece that had originally been in 0.7 M CuCl₂, in which the protoplasts remained collapsed even after the tissue was rinsed and soaked in water. However, protoplasts of the other pieces that were initially in CuCl₂ often contracted again after their initial swelling in water. Protoplasts in cells treated with sucrose shortly after cytoplasmic streaming had stopped only rarely regained their normal size after soaking in water. Protoplasts of cells initially given either 0.04 or 0.2 M CuCl₂ remained collapsed or had only a slight increase in protoplast size after water treatment. Less than half of the cells initially in 0.07 or 0.1 M CuCl₂ regained turgidity. The protoplasts of these cells collapsed again later. The CuSO₄-treated cells perfused with sucrose when cytoplasmic streaming stopped or when vesicles had developed showed



FIGS. 1-6. Cowpea epidermal cells; light micrographs of fresh tissue pieces. Figs. 1-3. Untreated cell at successively lower focal planes. $\times 1230$. Fig. 1. Area of the cell under the adaxial cell wall. Particles are visible in the peripheral cytoplasm and in the cytoplasmic strand. Fig. 2. The large central vacuole traversed by cytoplasmic strands and bordered by peripheral cytoplasm. Fig. 3. Nucleus (solid arrow) and chloroplasts (open arrow). Figs. 4-6. Cells treated with copper chloride. Fig. 4. A large peripheral vesicle (arrow) that formed after cytoplasmic streaming had stopped. $\times 1070$. Fig. 5. Large vesicle (arrow) adjacent to the nucleus which has granular nucleoplasm (cf. Fig. 3). This cell was photographed after cytoplasmic streaming had stopped and before the protoplast collapsed. $\times 1100$. Fig. 6. Cell with collapsed protoplast. A particle (arrow) is present in the vacuole. $\times 1100$.

only a slight increase in protoplast size after mounting in water.

FDA, on entering a cell, is cleaved by esterase activity to release fluorescent, polar fluorescein, which is commonly retained within the cell as long as its plasmalemma is intact (see Gahan 1981). Living, untreated cowpea epidermal cells, when mounted in FDA, became fluorescent almost immediately, with the fluorescence primarily in the nuclei and cytoplasm. After the application of CuCl_2 , nuclei and cytoplasm remained fluorescent until after the cytoplasm had stopped streaming and had become vesiculated. Soon thereafter, the fluorescence became evenly distributed throughout the cell, although no change in cytoplasmic structure was observed by interference contrast optics. Fluorescence disappeared from the cells at about the time the protoplast collapsed. If FDA was added to CuCl_2 -treated tissue after morphological changes were observed in the cytoplasm, the cells did not become fluorescent.

Electron microscopy

A preliminary examination was performed of the ultrastructure of two serially sectioned epidermal cells; from this, the cytoplasm under the adaxial walls was selected for succeeding studies, since changes that took place in this area seemed to be representative of those that occurred throughout the cell. Also, this area tended to show the effects of copper chloride more rapidly than areas deeper in the cell, presumably because it was exposed to the chemical sooner. This portion of the cell was also the most useful for comparison with light microscopic studies as it was seen the most clearly with the light microscope. Four cells, two from each of two specimens, were examined from untreated tissue and CuCl_2 -treated tissue that was fixed (1) when the cells still showed cytoplasmic streaming, (2) just after streaming stopped, (3) just after structural changes were observed, and (4) after the protoplast had collapsed. Two cells from one leaf were also fixed a few minutes after streaming stopped when there was no sign of granulation but the cytoplasm contained a few vesicles (i.e., between stages 2 and 3 above).

Untreated cells

Cytoplasm from an untreated cell is shown in Fig. 7. Ribosomes and microtubules were prominent features of the cytoplasm immediately adjacent to the adaxial cell wall. Many ribosomes were arranged in the form of coiled polyribosomes (Fig. 7). Mitochondria, microbodies, vesicles, endoplasmic reticulum (ER), and Golgi bodies were present in the cytoplasm. The ground cytoplasm was fairly uniform in appearance. Although a number of fixation procedures were tried, the plasmalemma and tonoplast could not be properly fixed and often were discontinuous and difficult to find. They were not used as markers of cell death in this study.

Treated cells showing cytoplasmic streaming

Three of these cells could not be distinguished from untreated cells. Ribosomes, frequently in the form of coiled polyribosomes, were observed, as were microtubules, mitochondria, microbodies, vesicles, ER, and Golgi bodies. The ground cytoplasm also appeared as it did in untreated cells. A representative section is shown in Fig. 8. The fourth cell did not look like the cells fixed during this or any time after CuCl_2 treatment. The ground cytoplasm was not uniform in appearance, and a large number of unusual membranous structures were present. This cell may have been unhealthy or damaged

by some means other than CuCl_2 . The other three cells that appeared similar to each other were considered representative of this period after CuCl_2 treatment.

Cells just after streaming had stopped

Ribosomes, microtubules, mitochondria, microbodies, vesicles, ER, and Golgi bodies were present. Two of the cells appeared quite similar to the untreated cells. A section from one of these two cells is shown in Fig. 9. Microtubules appeared less abundant than in untreated cells and this observation was supported by stereological analysis, which gave an average value of 0.03 for untreated and treated cells that showed cytoplasmic streaming and an average value of 0.01 for the cells fixed just after streaming had stopped; this difference was statistically significant ($P = 0.05$). However, the average number of coiled polyribosomes per unit area in these two cells (2.4) was not statistically different at the 95% confidence level from the average number per unit area in the seven cells from the two earlier stages (3.1).

Compared with these relatively normal appearing cells, the other two cells observed at this stage seemed somewhat different (Figs. 10, 11). Coiled polyribosomes, microtubules, and ER seemed less abundant, portions of the ground cytoplasm did not appear quite as uniform, and small clusters of electron-opaque material (Fig. 11) were found in these two cells.

Cells several minutes after cytoplasmic streaming had stopped

Cells were fixed at this time to determine whether the two less healthy looking cells seen in the previous stage were simply slightly farther along in the degeneration process than the two cells that looked similar to untreated cells. This may, indeed, have been the case since in cells fixed several minutes after streaming stopped, the cytoplasm was highly disorganized, indicating that such disorganization occurred rapidly (Fig. 12). Aggregates of dark structures that may have been ribosomes were present and many of these appeared to coat dark lines that were interpreted as microtubules (Fig. 12). Mitochondria were present but looked somewhat more round in section and had inflated cristae. Microbody-like structures and vesicles were present. Both the vesicles and the ER were sometimes difficult to distinguish from membranous structures that seemed to be forming as a result of cell degeneration. Golgi bodies were not seen. The ground cytoplasm was not uniform but was grouped into lighter and darker patches.

Cells after cytoplasmic streaming had stopped but before protoplast collapse

The cellular contents appeared highly disorganized during this stage (Fig. 13). Most of the ribosomes were clustered in disorganized aggregates and in only one section of one cell was a structure seen that resembled a microtubule. Mitochondria were recognizable but had dilated cristae. Microbody-like structures were present and ER was difficult to identify. Golgi bodies were not seen. The ground cytoplasm was not uniform, appearing as light and dark patches. Many large, membrane-bound vesicles were seen in the cytoplasm and one (Fig. 14) was identified as the large vesicle observed by light microscopy in the same cell before fixation.

Cells with collapsed protoplasts

Protoplast was not present just under the adaxial walls of these cells since the cell contents had collapsed away from the walls (Fig. 15). The first paradermal sections to contain protoplast revealed that it closely resembled that seen in cells just

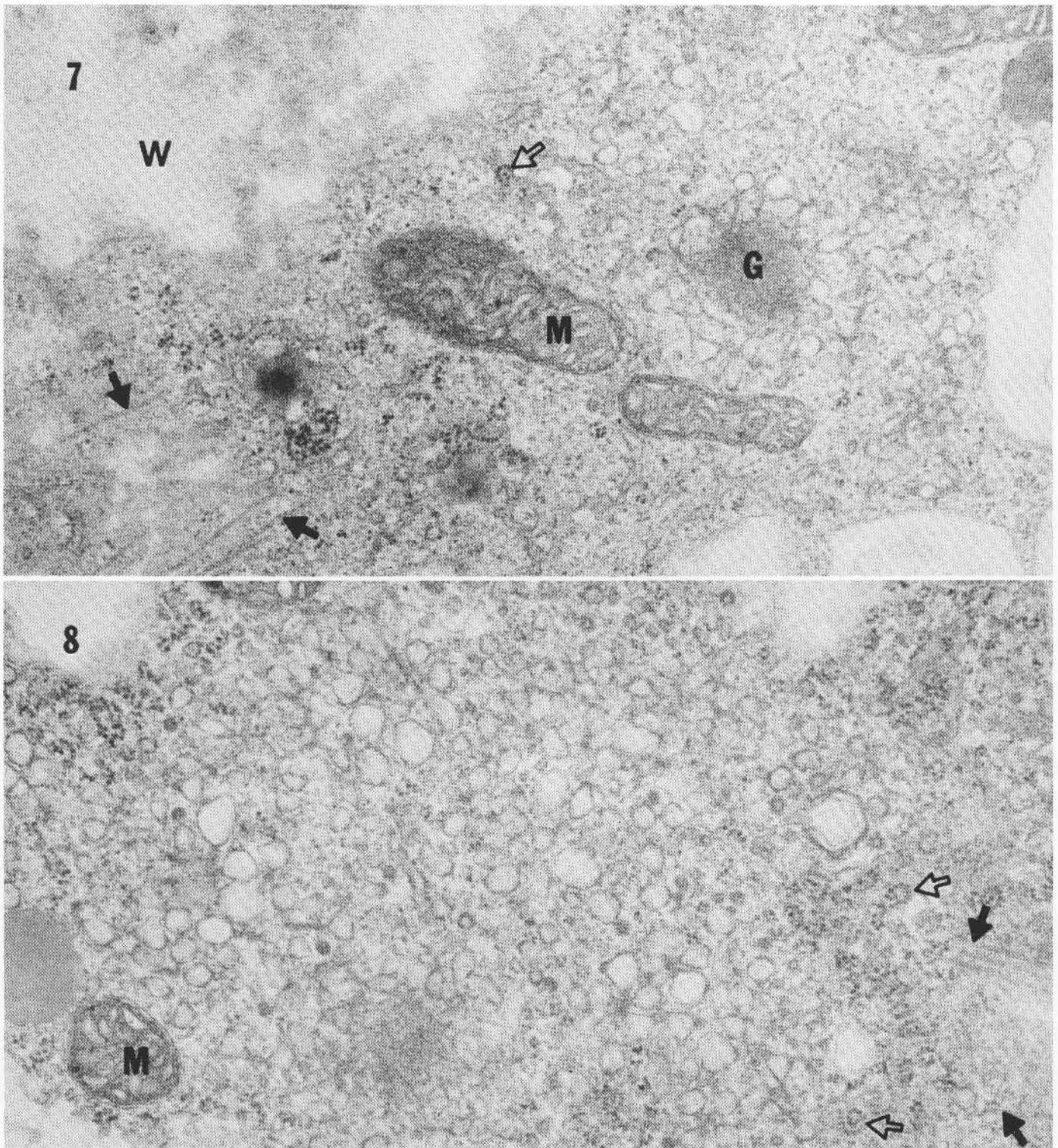
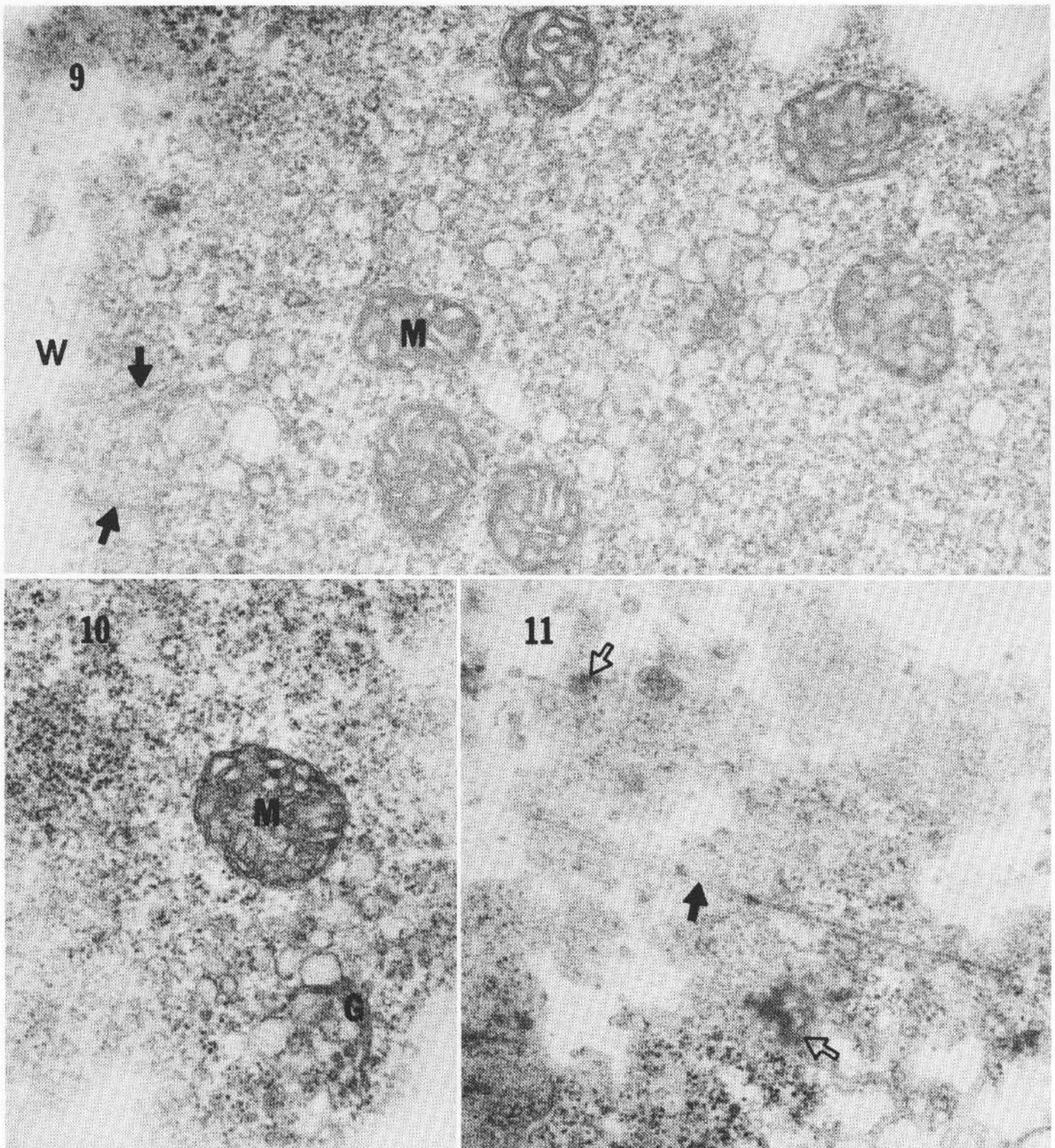


FIG. 7. Electron micrograph of an untreated cell illustrating the normal appearance of a mitochondrion (M), Golgi body (G), and polyribosomes (open arrow). Microtubules (solid arrows) are abundant close to the plant wall (W) seen in grazing section. $\times 38\ 350$. FIG. 8. Cell that was treated with copper chloride but still showed cytoplasmic streaming at the time of fixation. Note the similar appearance of the mitochondria (M), polyribosomes (open arrows), and microtubules (solid arrows) to those in Fig. 7. $\times 43\ 120$.

prior to protoplast collapse (Fig. 16). Membranous structures, possibly corresponding to the particles and vesicles seen by light microscopy, were present between the cell walls and the collapsed protoplasts (Fig. 15).

Discussion

Light microscopic observations indicated that cell death induced in cowpea leaf epidermal cells by the metal salts tested followed an identifiable and similar pattern. The first obvious



FIGS. 9–11. Electron micrographs of cells treated with copper chloride and fixed just after cytoplasmic streaming had stopped. $\times 39\,980$. Fig. 9. A cell that closely resembles an untreated cell (cf. Fig. 7) with respect to mitochondria (M) and other components, but which appears to have less abundant microtubules (solid arrows) adjacent to the plant wall (W). Figs. 10 and 11. A cell with normal mitochondria (M) and Golgi bodies (G) but with markedly fewer microtubules (solid arrow) adjacent to the plant wall (seen in glancing section as white patches in Fig. 11). Note the electron-opaque aggregates (open arrows) in the cytoplasm.

change during the death process was slowing and eventual cessation of cytoplasmic streaming. The cytoplasmic contents then underwent granulation, usually accompanied by the formation (and sometimes disappearance) of large vesicles in the

cell. Particles were seen moving by Brownian motion in the vacuole. Eventually, the protoplast collapsed and particles and small vesicles, all undergoing Brownian motion, appeared between the collapsed protoplast and the cell wall. Progression

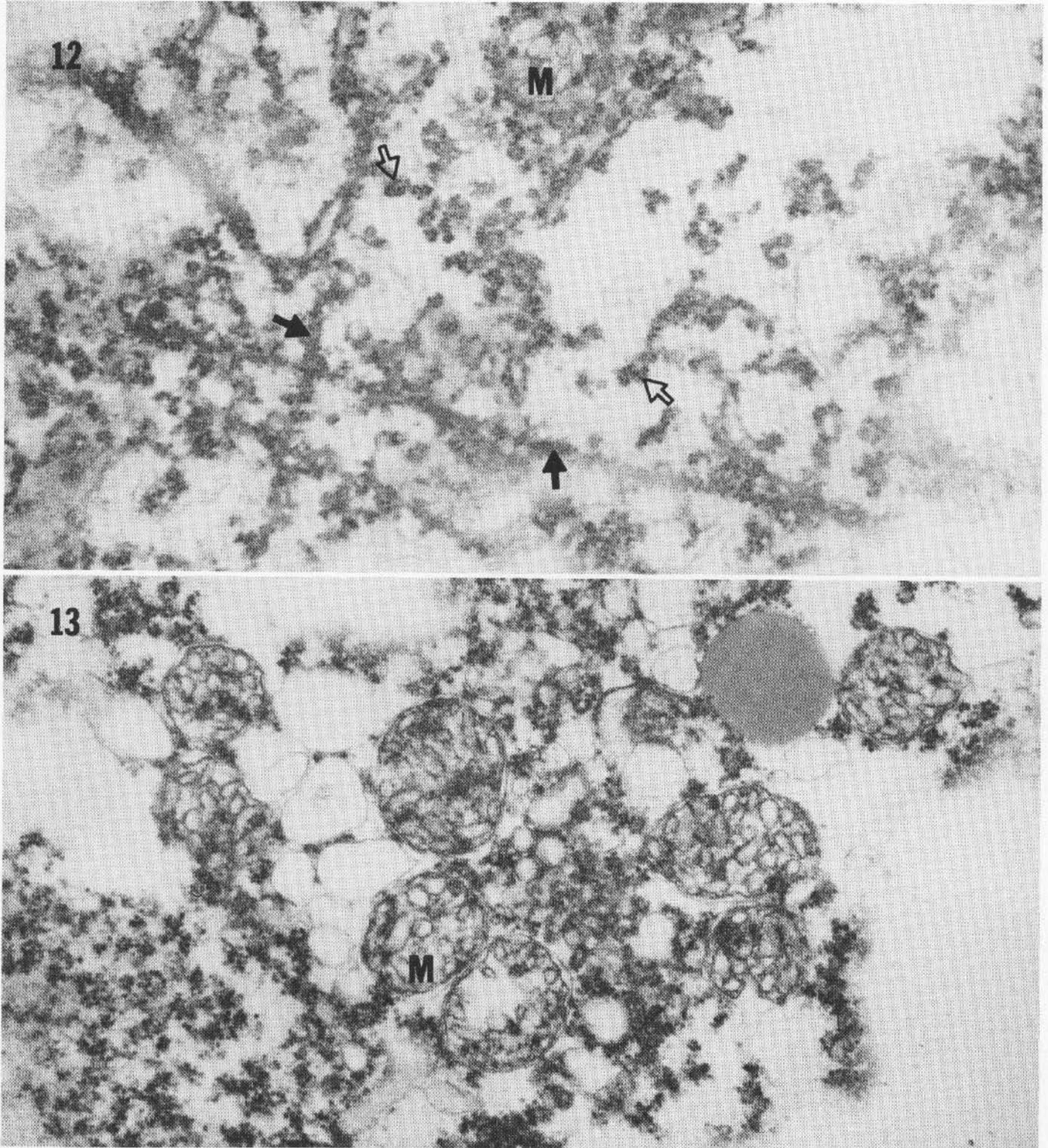


FIG. 12. Electron micrograph of a cell fixed several minutes after streaming had stopped. Ribosomes (open arrows) have aggregated in the cytoplasm and have associated with microtubules (solid arrows). Golgi bodies and endoplasmic reticulum are undetectable and cristae of mitochondria (M) are dilated. $\times 41\ 930$. FIG. 13. Cell fixed after streaming had stopped and during the period when morphological changes were seen by light microscopy. Note the numerous vesicles in the cytoplasm and the dilated cristae of the mitochondria (M). $\times 41\ 650$.

through these stages was faster at higher salt concentration. However, for concentrations that caused the cytoplasm to stop streaming at a similar time after treatment, it took much longer for the protoplasts to collapse in HgCl_2 -treated cells than in those given copper salts. Presumably this difference between the salts reflects the different effects the two metal ions have

on the cell; although both bind to proteins and thus interfere with enzyme action, their binding sites are different (Woolhouse 1983). However, irrespective of the cause of the observations, the data suggest that there is some degree of independence between the timing of certain stages of cell death.

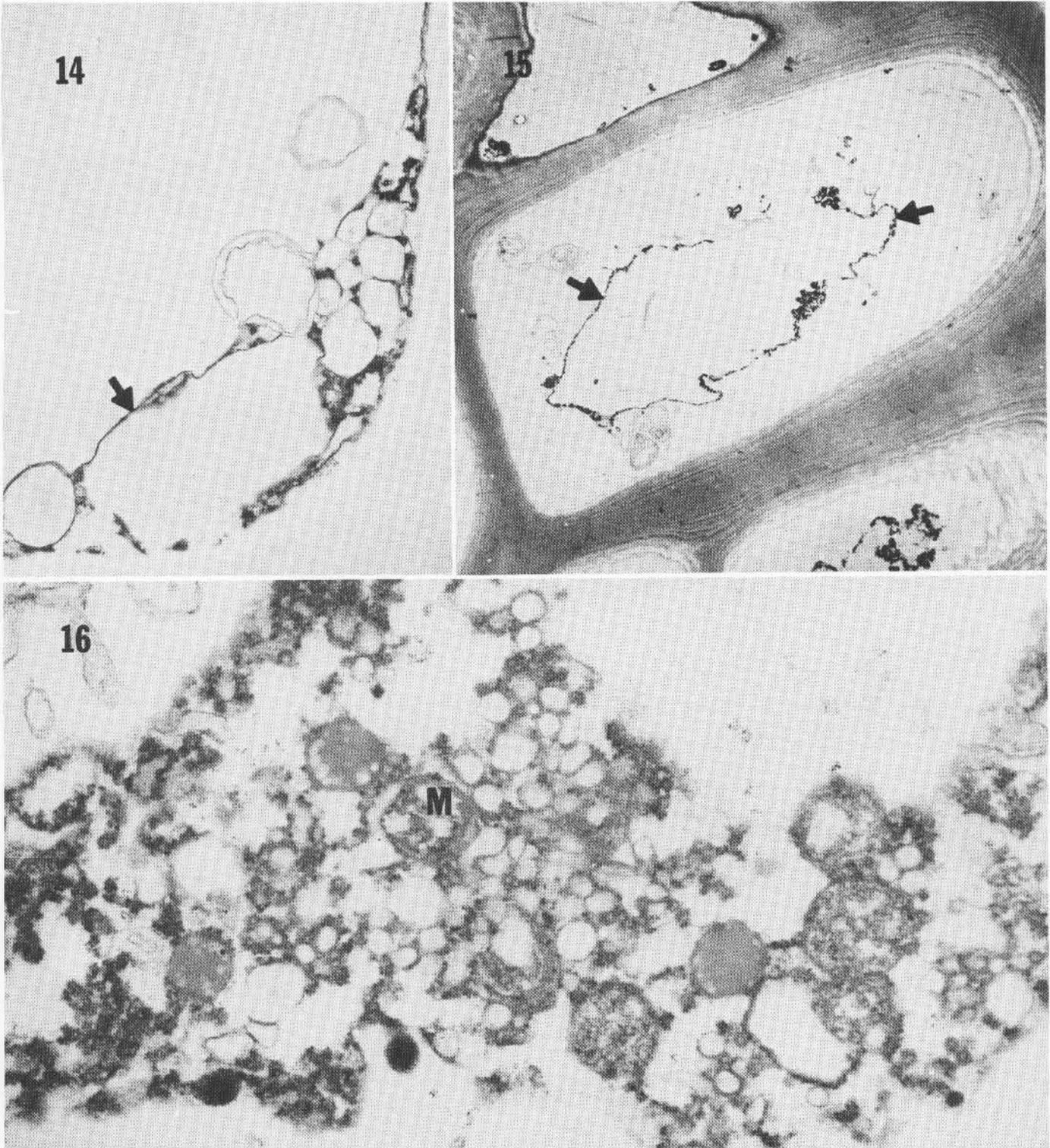


FIG. 14. Electron micrograph of a cell fixed after the morphological changes that follow the cessation of cytoplasmic streaming had taken place. The arrowed structure appeared as a large vesicle when the unfixed cell was observed under the light microscope. $\times 15\ 760$. FIGS. 15 and 16. Cells fixed after protoplast collapse. Fig. 15. Entire cell showing collapsed protoplast (arrows). Membranous structures between the protoplast and the cell wall may be the objects that appeared as particles and small vesicles in the unfixed cell. $\times 2310$. Fig. 16. Detail of Fig. 15. Mitochondria (M) are still recognizable, as are numerous small vesicles. $\times 38\ 880$.

Cessation of cytoplasmic streaming is commonly the first detectable feature of dying cells. Bushnell (1981), studying barley cells inoculated with powdery mildew, worked out a six-level streaming index. The index ranged from "abundant organelles moving rapidly in one or more thick sheets" (level

5), to "organelles showing traces of translational movement in strands or sheets of cytoplasm, or intact cytoplasmic strands showing traces of wiggling" (level 2), to "organelles moving in vibratory fashion, indistinguishable from Brownian motion" (level 1), and finally "no movement" (level 0). This gradation

is very similar to that observed in copper- and mercury-treated cowpea cells.

The granular appearance of the cytoplasm following the cessation of streaming also is a common feature of dying cells (e.g., Bushnell 1981; Tomiyama 1971). Similarly, vesicle formation has also been observed in damaged cells. For example, Endress et al. (1978) saw vesicles in vacuoles of pinto bean leaf cells when the cells had been treated with at least $6.0 \text{ mg HCl gas} \cdot \text{m}^{-3}$.

Protoplast contraction also appears to be a ubiquitous feature of plant cell death. Gahan (1981) noted that no matter what the cause of death, irreversible plasmolysis occurs in dying plant cells. This may also include what Keon (1985) described as pseudoplasmolysis in pectin lyase treated, cultured apple cells. Keon defined pseudoplasmolysis in that study to mean "... irreversible non-osmotic pressure dependent, shrinkage of the protoplast from the cell wall. . . ." The observations in the present study following the application of sucrose or FDA suggest that some degree of semipermeability of the protoplast boundary existed until this stage of death. Perhaps there is a correlation between plasmalemma integrity and its adherence to the cell wall. Such a conclusion is supported by the work of Pennazio and Sapetti (1982), who found that in virus-infected cowpea leaves, the first significant increase in electrolyte leakage, and consequently also in permeability, was measured when cell shrinkage occurred. Interestingly, the fact that in the present study the FDA-induced fluorescence was released from the cytoplasm into the vacuole before it was lost from the cell suggests that the tonoplast may break down before the plasmalemma. Since the vesiculated, but unshrunk, cytoplasm did not become fluorescent if the FDA was added after the CuCl_2 , it appears that esterase activity was inhibited soon after cytoplasmic streaming had stopped.

Browning was a late indicator of cell death in attached leaves but was not observed in tissue immersed in the salt solutions. Most probably, this was because all the cells in the excised tissue were dying. A similar lack of browning has been reported in bean cells killed by freezing unless they are in contact with living cells (Hargreaves and Bailey 1978).

The ultrastructural observations closely reflected those seen with the light microscope in the same cells prior to fixing. Before or just after cytoplasmic streaming stopped, neither the light nor the electron microscope revealed any marked change in the cells treated with a low concentration of CuCl_2 . There did appear to be a decrease in the number of peripheral microtubules in the cells fixed just after streaming had stopped, but more of such cells need to be examined to determine whether this is a constant feature of this stage of death. Even if it is, this may not prove useful as a diagnostic event since changes in numbers and distribution of microtubules may well occur in healthy cells in response to other factors. Therefore, only the lack of cytoplasmic motion seen by light microscopy clearly indicated that the cell was dying. However, both cell morphology and ultrastructure changed very rapidly in the treated cowpea cells after streaming stopped. Vesiculation of the cytoplasm was seen by both light and electron microscopy. Ultrastructural observations indicated that massive degeneration of cell contents occurred, including disruption of coiled polyribosomes and ER, loss of microtubules and Golgi bodies, and alteration of mitochondria. The cellular disruption also involved loss of uniformity of the ground cytoplasm and

clustering of disorganized organelles or parts of organelles into small electron-opaque masses. Either or both of these latter two changes may have accounted for the cytoplasmic granulation observed by light microscopy. There was no indication that such granulation was caused by the swelling of mitochondria, as suggested for fungus-invaded potato cells (Tomiyama 1971).

Protoplast collapse was obvious from both light and electron microscopic observations. The moving particles seen between the collapsed protoplast and the cell wall at this stage, and in the vacuole in this and the preceding stage, appeared to correspond to membranous structures observed by electron microscopy. Presumably, such structures originated either from the disorganized plasmalemma or from other membranes inside the cell.

Such a correlation between light and electron microscopic observations of degenerating cells has not always been observed. For example, Strauss et al. (1982), working with sections of fixed, embedded cowpea leaves, found that abaxial epidermal cells that looked less injured than adaxial epidermal cells under the light microscope appeared more injured by electron microscopy. Such an observation, when compared with those of the present study, suggests that light microscopic observations of fixed material are not as reliable as either ultrastructural examinations or observations of the "living" cell when it comes to determining the state of degeneration of the cell.

The fine-structure study of cowpea cells treated with heavy metals not only correlated positively with the changes seen in unfixed cells but also bore similarities to broad patterns seen in earlier electron microscopic studies of cell death. Butler and Simon (1971), in a review paper on plant cell death, mentioned that whatever the cause of cell death, one of the first occurrences is a decrease in numbers of ribosomes; the free ribosomes degenerate, followed by those attached to ER. In CuCl_2 -treated cowpea cells, loss in numbers of coiled polyribosomes appeared to occur soon after streaming stopped and shortly after microtubules began to decrease in number. Reduction of mitochondrial size and swelling of cristae are other common features of cell death; in the current study, the mitochondria of dying cells tended to be rounded in appearance in thin sections, and the cristae were inflated. Butler and Simon (1971) also noted that mitochondria were still present at later cell death stages, as was the case in this study. They mentioned that ER and Golgi bodies swell, vesiculate, and disappear; this may account for some of the vesicular membranous structures seen in the CuCl_2 -treated cowpea cells.

Plant pathologists commonly use ultrastructural features of infected tissues to determine the nature of the interaction between a pathogen and a plant. For example, electron microscopy has been applied to studies of resistance where the relative timing of fungal and plant cell deaths may be important in determining the mechanism of resistance (Heath 1976; Király and Barna 1985). However, there has been little previous information to indicate at what stage during cell death such ultrastructural changes occur (Heath 1981). The present study suggests that morphological changes in the appearance of the cytoplasm of dying cells are closely reflected in changes in ultrastructure, using standard fixation procedures. Moreover, such changes occur rapidly after cytoplasmic streaming stops. Nevertheless, the first observable sign of degeneration in

living cells, namely the cessation of streaming, appears to have no clearly diagnostic features detectable by electron microscopy.

Acknowledgements

We thank Ms. Kathy Sault for preparation of the plates and for printing many of the micrographs used in this study. Funding for this research and for the first author was provided by a grant from the Natural Sciences and Engineering Research Council of Canada.

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