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BIOLOGICAL EFFECTS OF SURFACTANTS
II. INFLUENCE ON THE ULTRASTRUCTURE OF ORCHID SEEDLINGS

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Summary

Effects of a nonionic surface active agent (lauroyl/myristoyl di (2-hydroxyethyl) amide) and one anionic surfactant (sodium (linear) dodecylbenzene sulphonate) on cell ultrastructure were determined using *Phalaenopsis* protocorms. There were no observable effects after 5 days exposure to 1000 ppm of the nonionic. However, the anionic caused severe damage. Drastic changes in morphology, loss of membranes, swelling of thylakoids and appearance of dense osmophilic granules were evident in chloroplasts. Other effects include disintegration of polysomes into monosomes; swelling of mitochondria; dispersion of chromatin in the nucleus; appearance of unidentified vesicles in the cytoplasm and plasmolysis of cells. These ultrastructural changes can be attributed to the possible emulsification of membrane lipids as well as precipitation and dispersion of cellular proteins.

Introduction

Surfactants inhibit the growth of orchid seedlings (Ernst, Arditti and Healey, 1971; Ernst and Arditti, 1968), elongation of corn roots and germination of oat seeds (Buchanan, 1965) and possess phytotoxic qualities (Furmidge, 1959a, b; Parr and Norman, 1964). Although the reasons for surfactant phytotoxicity are complex, there is some evidence to suggest that it may be, at least partially, due to their emulsifying effects on cytomembranes or other lipid-containing structures. Some workers (Deamer and Crofts, 1967; Kellner, Correll and Ladd, 1951; Scanu *et al.*, 1961; Swanson and Whitney, 1953; Parr and Norman, 1964; Kondo and Tomizawa, 1966, 1968) have shown that processes located on cytomembranes or substances making up their structure are disrupted.

This paper presents evidence regarding ultrastructural changes which accompany the inhibition of growth and decrease in survival caused by selected surfactants.

Materials and methods

Plant material. Protocorms of *Phalaenopsis* cv. Alice Gloria × *Phalaenopsis* cv. Francine were grown on Knudson C culture medium (Knudson, 1946) as described previously (Ernst *et al.*, 1971; Ernst and Arditti, 1968) and transferred to test and control media for periods ranging from 1.2 hours to 5 days.

Culture conditions. Screw cap tubes (150 × 25 mm) were used as culture vessels. Absorbent cotton platforms, instead of agar, served as support for the protocorms. All

cultures were maintained at a constant temperature of 22° C under 12-hour photoperiods and a light intensity of 283 $\mu W/cm^2$ (120 ft-candles) produced by Gro-Lux lamps.

Surfactants. A highly purified anionic, sodium (linear) dodecylbenzene sulphonate (LAS), or the nonionic, lauroyl/myristoyl di(2-hydroxyethyl) amide (LDA), were added to agar-free Knudson C culture medium at concentrations of 1000 ppm. Purification and determinations of stability as well as surface and interfacial tension measurements were carried out by previously described methods (Ernst *et al.*, 1971, Appendix).

Fixation of tissues. First leaf primordia were excised from treated and control protocorms and placed in 2% glutaraldehyde in veronal acetate buffer (pH 7.2) containing 1.5% sucrose. Leaf primordia were further fixed in either (1) 2% $KMnO_4$ for 1 hour, or (2) 2% OsO_4 for 12 hours. Both $KMnO_4$ and OsO_4 were prepared using the same buffer and pH as was used for glutaraldehyde. A glutaraldehyde/$KMnO_4$ combination (J. Kiethe, Department of Developmental and Cell Biology, University of California, Irvine, unpublished) has most of the advantages of $KMnO_4$ for emphasizing membrane structure without accompanying membrane damage so often seen with $KMnO_4$ alone. Tissues were dehydrated in a graded concentration series of acetone, embedded in Epon 812 (Luft, 1961), sectioned with a diamond knife and both observed and photographed on a Zeiss EM 9A electron microscope.

RESULTS

Untreated seedling

Tissues from two regions of the first leaf primordium of the *Phalaenopsis* protocorm were examined. One was just below the protoderm of the apical ridge while the other was from the more central portion of the presumptive mesophyll.

Presumptive mesophyll cells were larger than apical subdermal ones. This size difference was primarily due to the greater vacuolar size in mesophyll cells. In addition, chloroplasts of apical subdermal cells had well-developed grana and stroma lamellae (Plate 1, No. 1), while those of the presumptive mesophyll had less well-developed membranes and large starch grains (Plate 1, No. 2). Mitochondria, dictyosomes, nuclei, endoplasmic reticulum, plasmalemma and tonoplast were normal for plant cells and no differences were observable between tissues of the two regions. Well-defined polysomes were seen in both tissues (Plate 1, No. 3).

Effects of LDA

Protocorms for electron microscopic examination were collected only up to 5 days after treatment. Some were left on the surfactant for up to 1 month and, when replated on basal medium (Knudson C) without LDA, grew as well as control plants. This surfactant did not induce detectable cytological effects.

Effects of LAS

Four-hour treatment. Initial cytological changes were detectable after 4 hours exposure to LAS. Chloroplast membranes became thin and granular in appearance (Plate 2, Nos. 4 and 5) and less distinct when compared to the control. In many cells of the presumptive mesophyll the limiting double membrane of the chloroplast was missing (Plate 2, No. 5). A large number of the chloroplast thylakoids showed swelling in both stroma and grana areas (Plate 2, Nos. 4 and 5). There were no apparent morphological changes in membrane systems other than the chloroplast. Although ribosomes did not

appear to decrease in absolute concentration, they were seldom seen in polysomal configurations (Plate 2, No. 6).

The changes elecited by a 4-hour LAS treatment were more extensive in the presumptive mesophyll (Plate 2, No. 5) than in the subdermal areas (Plate 2, No. 4). However, the changes were not qualitatively different in the two regions.

Forty-eight-hour treatment. Extensive cellular changes took place following a 48-hour treatment with LAS. The plasmalemma was usually intact although often pulled away from the cell wall (plasmolysis, Plate 3, No. 7). However, the tonoplast was generally not visible. Mitochondria still showed a limiting membrane, but their substructure was not always clearly double. Some cristae were seen within the mitochondria. They were greatly swollen when compared to the control (Plate 1, No. 2, Plate 3, No. 8). Dictyosomes were not observable after this treatment. The nuclear envelope was still visible as a double membrane structure but the chromatin appeared dispersed and the entire nucleus took on a homogeneous, granular appearance (Plate 3, No. 9). Chloroplasts, in many cases, changed their shape from discoid and biconvex in the control plantlets (Plate 1, Nos. 1 and 2), to cup-like and concavo-convex (Plate 3, Nos. 9 and 10). Actual structure of membranes within the chloroplast was less distinct than those after 4 hours of treatment with LAS, but grana areas remained clearly visible although somewhat flattened (Plate 3, Nos. 9 and 10). Numerous osmophilic dense granules were seen in the chloroplasts (Plate 3, No. 10). They appeared mainly at the margins.

In addition to the organelles, already mentioned, numerous unidentifiable vesicles appear in the cytoplasm (Plate 3, No. 8). These structures may have represented swollen dictyosome vesicles, cisternae or mitochondria.

Five-day treatment. There are no detectable cytological differences between protocorms treated for 2 days (48 hours) and those treated for 5 days when treatment is carried out in the light. When plantlets were kept in the dark during the 5 days of treatment, cells appeared totally necrotic, as shown by massive adsorption of stain and a complete lack of organized membranes or other identifiable protoplasmic structures.

Discussion

No observable ultrastructural changes occurred in protocorms treated for 5 days with the nonionic LDA but it did bring about growth retardation and killing after several months (Ernst *et al.*, 1971). This indicates that it does not damage the tissues for a considerable length of time although its actual activity may have been lowered by partial insolubility since stratification of this surfactant in agar-free culture medium was observed. No information is available on what finally causes the LDA to act phytotoxically.

Several changes were noted in protocorm tissues treated for various periods of time with the anionic, LAS;

After 4 hours:

Swelling of chloroplast thylakoids;
Loss of chloroplast limiting membrane;
Disintegration of polysomes into monosomes.

After 48 hours:

Plasmolysis of cells;
Swelling of mitochondrial cristae;
Dispersion of chromatin in the nucleus;
Appearance of osmophilic dense granules in the chloroplast;
Severe changes in chloroplast morphology; and

Appearance of unidentifiable vesicles in the cytoplasm.

In addition to these changes, differences were noted in the survival of protocorm cells held for 5 days in the light or in the dark. The effects of the LAS occurred very quickly. Damage was detected after only 4 hours and protocorm cells showed almost complete cytological disintegration after 5 days of treatment. Damage fell roughly into two classes —membrane disruption with probable subsequent loss of permeability control and disaggregation of polysomes and nuclear chromatin, likely due to protein precipitation. Structural disintegration is obvious from the loss of integrity or complete disappearance of membranes. Chloroplasts of protocorm cells treated for only short periods of time (4 hours) showed effects similar to those observed when these organelles were subjected to a low salt medium (Izawa and Good, 1966). In both cases, double limiting membranes seem to become discontinuous and the internal lamellar structure becomes swollen and disoriented due to water influx. The chloroplasts become cup-shaped but they differ in that when protocorm cells are treated with LAS, plastids do not lose their grana, appearing similar to those treated with low salt concentrations and then returned to a solution of physiological osmotic concentrations (Izawa and Good, 1966). Additional support that cytomembranes are the site of surfactant activity comes from the effect of Triton X-100 (octylphenol ethoxylate) on chloroplast membranes. There, it was postulated that the surfactant produced small pores in the outer limiting membrane. These in turn destroyed the ability to maintain a light dependent pH gradient and caused swelling due to osmotically active substances inside the chloroplasts (Deamer and Crofts, 1967).

Evidence that membrane lipids are extracted from cytomembranes and emulsification occurs is given by the appearance of the small osmophilic dense droplets in chloroplasts treated with LAS for 48 hours. Other workers have found release of membrane lipids occurring after treatment with surfactants. Striking and sustained elevations of blood cholesterol, phospholipids and neutral fats were observed following intravenous injection of Tween 80 (polyoxyethylene sorbitan monooleate) and Triton A20 (alkylphenol ethoxylate) into rabbits (Kellner *et al.*, 1951). Similar results were obtained with Triton-WR 1339 (alkylphenol ethoxylate) injected into dogs at the rate of 250 mg/kg body weight (Scanu *et al.*, 1961). In both dogs (Scanu *et al.*, 1961) and rabbits (Kellner *et al.*, 1951) lipid concentration in blood serum increased as did its opacity. If opacity was caused by emulsification of lipids from blood cell membranes and formation of small lipid bodies this effect may be similar to the increase of osmophilic granules observed in *Phalaenopsis* chloroplasts.

Although we have not assayed the physiological aspects of the membranes, there are strong indications that membrane-bound functions are disrupted by surfactant treatment. Triton X-100 at 60–120 μM (40–80 ppm) completely inhibited light induced conformational changes and photophosphorylation in spinach chloroplasts (Deamer and Crofts, 1967). Reduction of ^{32}P uptake by Tween 80 (Swanson and Whitney, 1953) and of potassium by the same surfactant, as well as Tween 20 (polyoxyethylene sorbitan monolaurate; Parr and Norman, 1964) may well be due to surfactant mediated membrane changes.

The breakdown of polysomes to monosomes is a common cellular reaction to detergent which also occurs in *Phalaenopsis* (Plate 2, No. 6). The general cellular breakdown is also indicated by dispersion of the nuclear chromatin and formation of non-identifiable vesicles. This phenomenon may be due, in part, to action of the surfactant on the nucleic acids. But, it is more probably due to the ability of surfactants to precipitate the nucleic acid-associated proteins (Parr and Norman, 1965).

Based on our findings with polar lipid–surfactant interfaces (Ernst *et al.*, 1971), and the correlation between increase of surfactant concentration and phytotoxicity we suggest that the highly polar lipids of cytomembranes emulsify as concentration is increased. A change of the type induced by surfactants in interfacial tension may lead to changes in membrane permeability brought about by micellar rearrangements (Seufert, 1965). These, in turn, could lead to loss of membrane integrity and disruption in cellular organization and function. In addition to the disruption of the membrane by LAS, the precipitation of protein is probably a strong contributor to cellular deterioration.

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EXPLANATION OF PLATES

Key to lettering: C, chloroplast; N, nucleus; M, mitochondrion; S, starch; V, vacuole; CW, cell wall; X, unidentifiable vesicle; DG, osmophilic dense granule.

PLATE 1

Untreated *Phalaenopsis* cells.

No. 1. Chloroplast and nucleus of sub-apical cell showing internal organization including well-developed internal membranes and no starch (glutaraldehyde–$KMnO_4$ fixation). × 22,800.

No. 2. Portion of cell from the presumptive mesophyll of an untreated *Phalaenopsis* protocorm. Chloroplast distended by large starch deposits. Extensive vacuole is also seen (glutaraldehyde–$KMnO_4$ fixation). × 13,800.

No. 3. Polyribosomes (arrows) are seen in the cells of untreated protocorms (glutaraldehyde–osmium fixation). × 53,200.

PLATE 2

Portions of cells treated with 1000 ppm LAS for 4 hours (all glutaraldehyde–osmium fixation).

No. 4. Chloroplast from a sub-apical cell showing extensive swelling of the thylakoids. Outer envelope is still intact. × 51,300.

No. 5. Chloroplast from a presumptive mesophyll cell showing not only the extensive swelling of the thylakoids but also the indistinct nature of the outer envelope. × 38,000.

No. 6. Short exposures dissociate the polysomes into their component monosomes (arrows). × 38,000.

PLATE 3

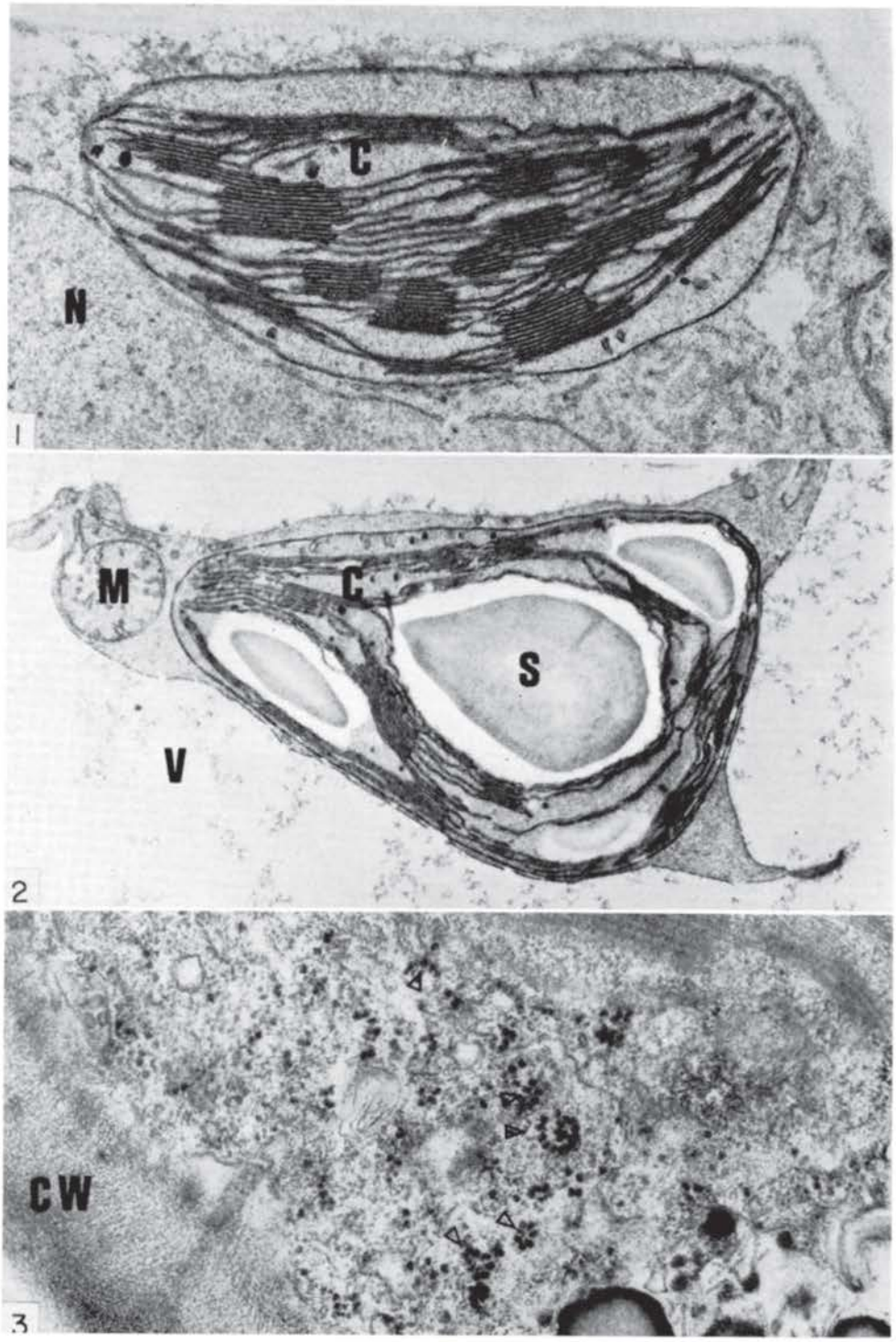
Portions of cells treated with 1000 ppm LAS for 48 hours (all glutaraldehyde–osmium fixation).

No. 7. Plasma membrane (arrows) remains intact after 48 hours of treatment although plasmolysis is extensive. × 14,400.

No. 8. Mitochondrion shows swelling of cristae (arrows). Cells contain many unidentifiable vesicles, some of which have double membrane envelopes. × 30,000.

No. 9. Chromatin is dispersed in the nucleus rather than concentrated near the nuclear envelope. Chloroplasts become highly cup-shaped after 48-hour treatment. × 4840.

No. 10. Chloroplasts contain osmophilic dense granules at their margins. As in No. 9, chloroplasts have taken on a cup-shaped appearance. × 4830.



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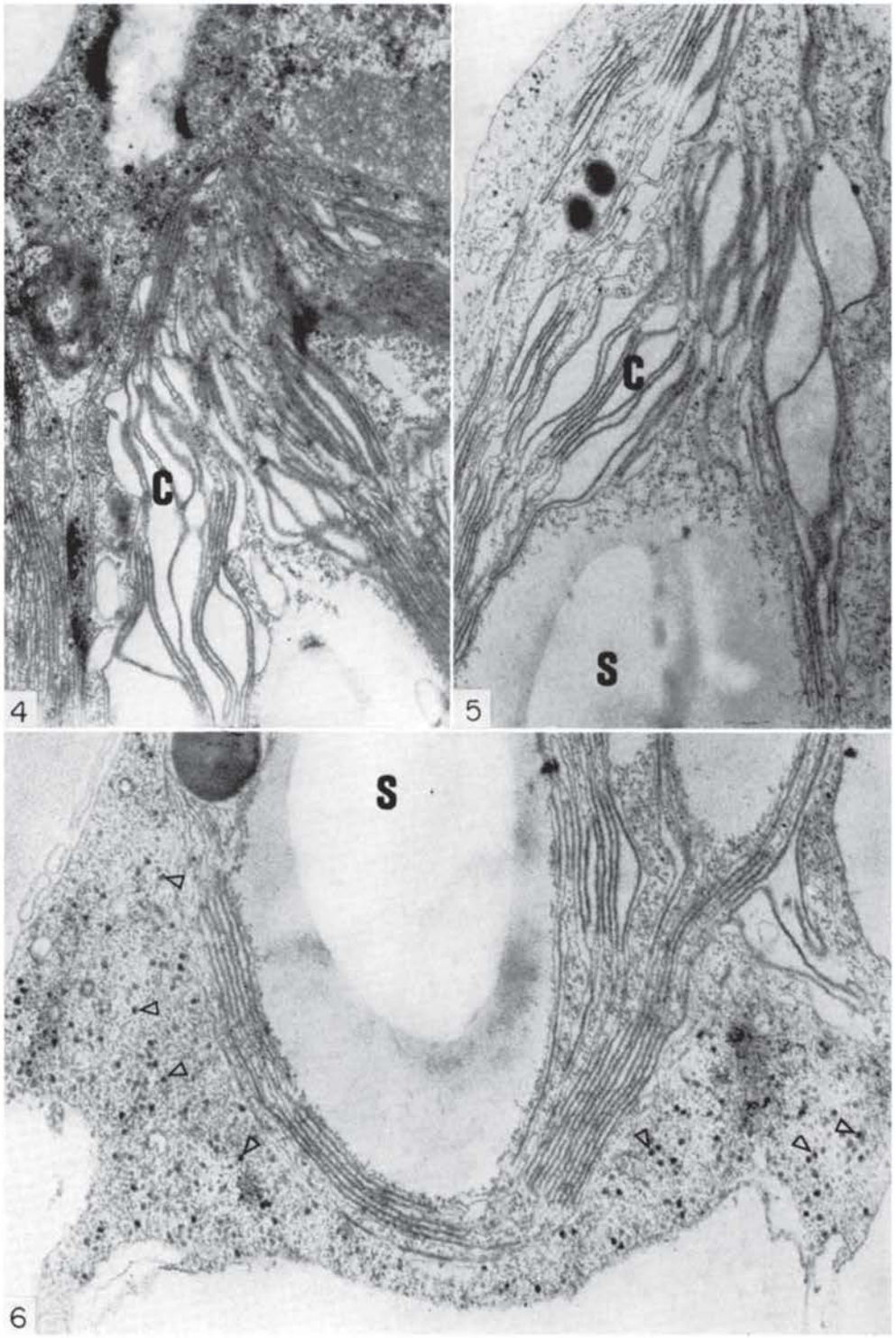
(*facing page* 482)



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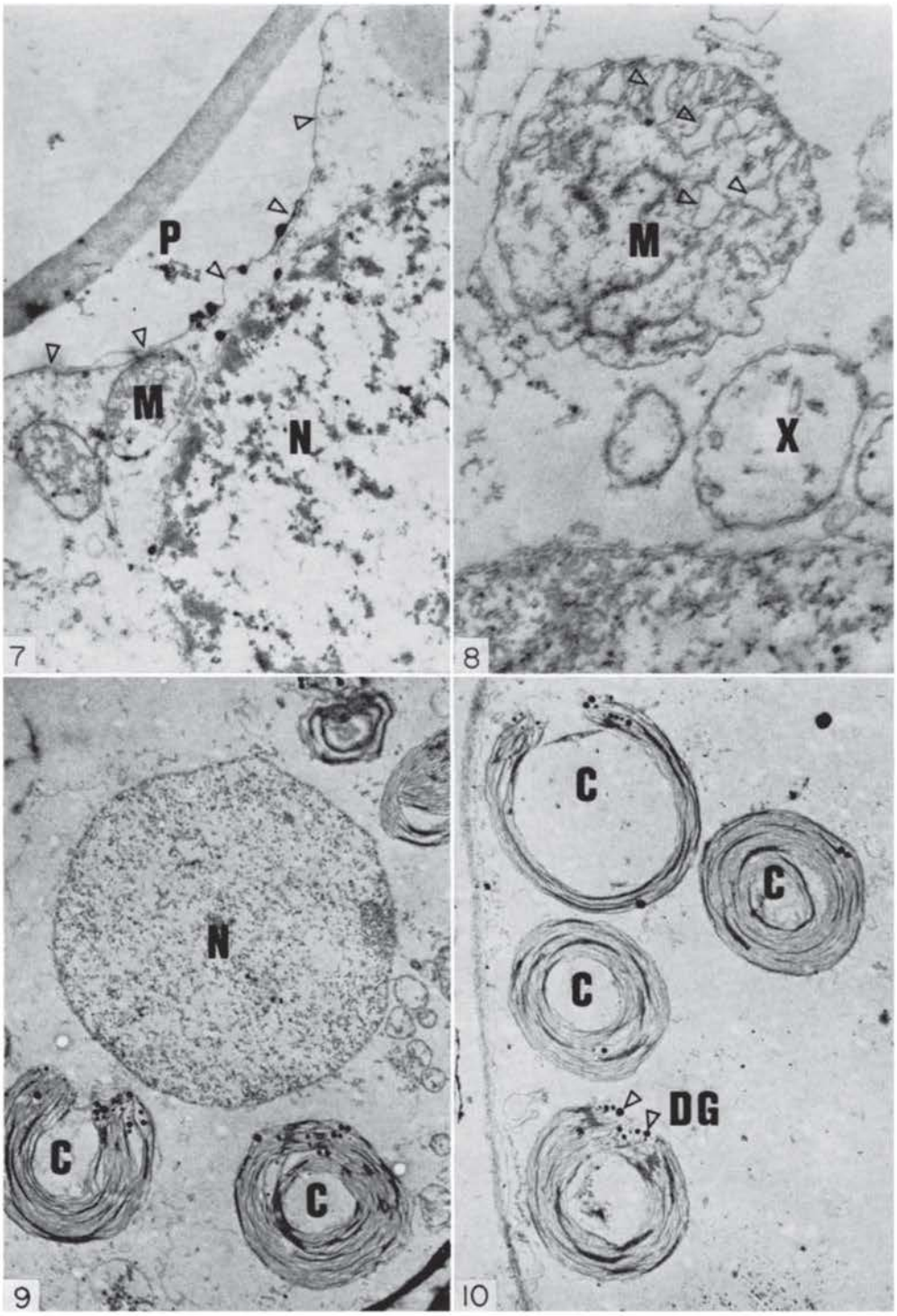

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