Protoplasma 98, 163–176 (1979)

PROTOPLASMA

Successive Changes in the Ultrastructure of *Oryza sativa* L. Cells During Growth and Aging

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Received March 12, 1978
Accepted in revised form October 6, 1978

Summary

The ultrastructure of *Oryza sativa* L. cells in suspension was determined as cells developed, matured and senesced at 3, 10, and 17 days, respectively, after transfer to fresh medium. Although cultures of 3-day-old cells contained some senescent cells, the symptoms of cell aging were very conspicuous at 10 days and were most pronounced at 17 days. The amount of cytoplasm decreased as the number of lytic areas, myelin figures and vesicle bodies increased. Other noticeable subcellular changes observed were ultrastructural modifications of mitochondria, proplastids, amyloplasts, and nuclei. Such changes were associated with a general deterioration of the lipoprotein complex of the cell during its growth. A fibrous structure without an external membrane was observed and its reported for the first time for cells grown in suspension culture.

1. Introduction

Suspension cultures of cells of higher plants grown in synthetic media are convenient model systems for the study of biochemical, morphological and ultrastructural changes in cells during growth and aging. The processes of cell aging have been studied extensively by physiologists and biochemists (SHELDRAKE 1974). Ultrastructural aspects of cell aging have received less attention than the biochemical aspects, although changes in ultrastructure of plant cells cultured on liquid or solid medium as a function of time have been reported by many investigators (ISRAEL and STEWARD 1967, FOWKE and SETTERFIELD 1968, SUTTON-JONES and STREET 1968, BLACKWELL, LAETSCH, and HYDE 1969, TULETT, BAGSHAW, and YEOMANN 1969, DAVEY and STREET

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0033-183X/79/0098/0163/$ 02.80

1971, MARES and STONE 1973, SIMOLA 1972, 1973, STREET 1973, GOMEZ, HORIS, and WALNE 1974, and VASIL 1974).

Age-related changes in cellular systems often are not readily identifiable, although at the ultrastructural level sites in certain organelles (lysosomes, microbodies, cytoplasmic vacuoles, plasma membranes and other membrane systems) may reflect such changes. Although many ultrastructural modifications during senescence may appear similar in different plant species, each species has characteristic modifications. The growth of *Oryza sativa* callus (YATAZAWA, FURUHASHI, and SHIMIZU 1967) and of cell suspensions (LIEB, RAY, and STILL 1973) were described previously, but these studies did not include ultrastructural aspects. This paper describes the successive ultrastructural changes in aging *Oryza sativa* cells grown in suspension culture.

2. Material and Methods

Oryza sativa L. "Norin 16" cells were cultured from germinated seeds in R-2 medium (OHIRA, OJIMA, SAIGUSA, and FUJIWARA 1975) with 2 mg, 2,4-D/1. Cell suspensions were cultured in 250 ml Erlenmeyer flasks containing 50 ml autoclaved medium to which 10 ml of a 4- to 5-day-old suspension was added. They were placed on a rotary horizontal shaker at 100 cycles per minute at 26 °C in the dark. Growth was monitored by changes in oven-dry weight at 70 °C. At least 3 flasks were harvested per sampling time. Cells were filtered onto Miracloth, rinsed with water and dried.

For studies with the electron microscope, cells were fixed at 3, 10, and 17 days after inoculation with 3% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) for 1.5 hours, rinsed and suspended in 2.5% agar in buffer, post fixed into 2% OsO_4 in the same buffer for 2 hours at room temperature, dehydrated with acetone and embedded in Spurr resin (SPURR 1969). Thin sections were cut with glass knives on a Tessla ultramicrotome, mounted on uncoated 300 mesh copper grids, poststained with uranyl acetate and lead citrate and viewed with a JEM 7 electron microscope at an accelerating voltage of 80 kV. At least 12 to 15 capsules were made from each sample and at least 200 micrographs were observed for each harvest time.

No samples were taken for electron microscopy at 0 days because the cells had been transferred to fresh medium 5 days before the beginning of the experiments (0 days). Therefore, cells sampled at 0 days would be representative of 5-day-old cells. Presumably, the most actively dividing cells are those most likely to survive and divide for subsequent growth.

3. Results

The growth of *Oryza sativa* cell suspensions was maximum at 10 days, with a gradual reduction of dry matter between 17 and 31 days (Fig. 1).

3.1. The Ultrastructural Aspects of Cells Aging

The ultrastructure of the cells was determined at 3, 10, and 17 days after inoculation. The cell suspensions grew as large clumps of many cells. At 3 days the diversity of cell types was limited and most resembled meristematic cells (Fig. 2 *A*). They were rich in cytoplasm with a large and round (occasionally irregularly-shaped) nucleus that was generally centrally located. Most had a single large nucleolus with a compact inner structure. Some

nucleoli were ring-shaped (in two dimensions) with fine granular fibrillar areas (Fig. 2 *B*). At this stage some of the most numerous and prominent organelles were relatively undifferentiated proplastids (Fig. 3 *A*). Many were similar in size and morphology to mitochondria, so it was sometimes difficult to distinguish between them. The plastids were oval or round with a finely granular stroma and surrounded by a double envelope that was typically more electron dense than that of the mitochondria. Osmiophilic droplets (plastoglobuli) were common. The granular stroma contained small vesicles or thin tubules and cisternae of low electron density that could be interpreted as

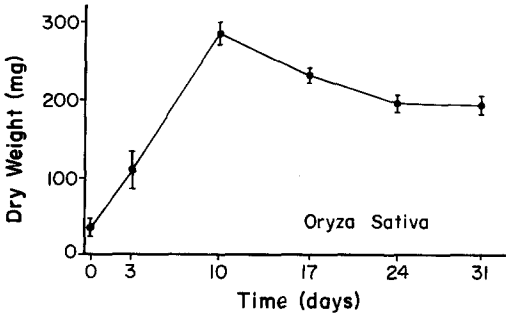

Fig. 1. The growth of *Oryza sativa* cell suspensions in R-2 medium. Data points represent the averages of 3 or more flasks. The entire contents of the flasks were oven dried and weighed. Vertical lines represent standard deviations

thylakoid percursors or prolamellar bodies. Since the cells were grown in the dark, the grana did not differentiate. Endoplasmic reticulum (ER) was abundant and the cisternae contained ribosomes (Fig. 3 *B*). Ribosomes generally were associated preferentially with the ER membranes (RER). Golgi bodies were common and had straight saccules with few vesicles (Fig. 3 *C*).

Amyloplasts contained one or more starch grains (Fig. 3 *C*) and were usually elongated, oval or cup-shaped, with a thin parietal layer of granular stroma surrounding the starch. Amyloplasts rarely contained membrane inclusions and osmiophilic droplets. Some had a clear cavity around the starch grain (Fig. 3 *C*). Proplastids and amyloplasts were in different stages of division. A few vacuoles apparently were autolytic in incipient stages of differentiation (Fig. 3 *D*). Mitochondria were rounded and contained few internal membranes (Fig. 3 *E*).

Fibrous structures were present in cytoplasm of some young cells; sometimes crossing the length of the cell (Fig. 2 *A*). These structures were unbounded by any membrane, and of linear substructure in longitudinal sections (Fig. 3 *E*). They were composed of fibers oriented approximately parallel to one another and were not associated with the ER or ribosomes. Their function is unknown and we are unaware of any previous reports describing similar structures in cells grown in suspension.

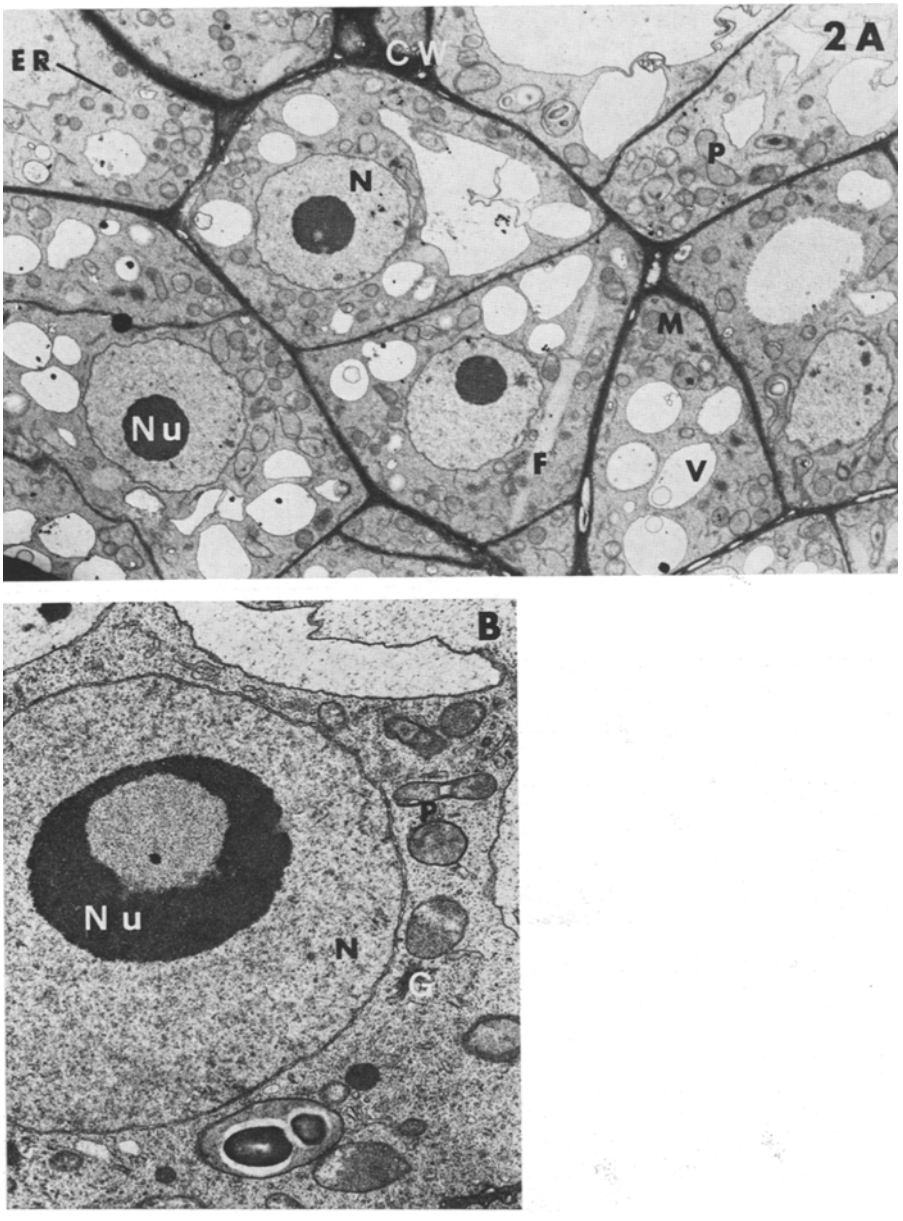

Fig. 2 *A*. Part of a clump of O. *sativa* cells grown 3 days in cell suspension. Typical organelles are nuclei (*N*), nucleoli (*Nu*), mitochondria (*M*), plastids (*P*), vacuoles (*V*), endoplasmic reticulum (*ER*), cell walls (*CW*), and a multifibrillar structure (*F*). (For a detail of the latter see Fig. 3 *E*.) ×5,400

Fig. 2 *B*. Part of a cell showing a typical nucleus (*N*) with a ring shaped nucleolus (*Nu*), plastids (*P*), and Golgi bodies (*G*). ×15,600

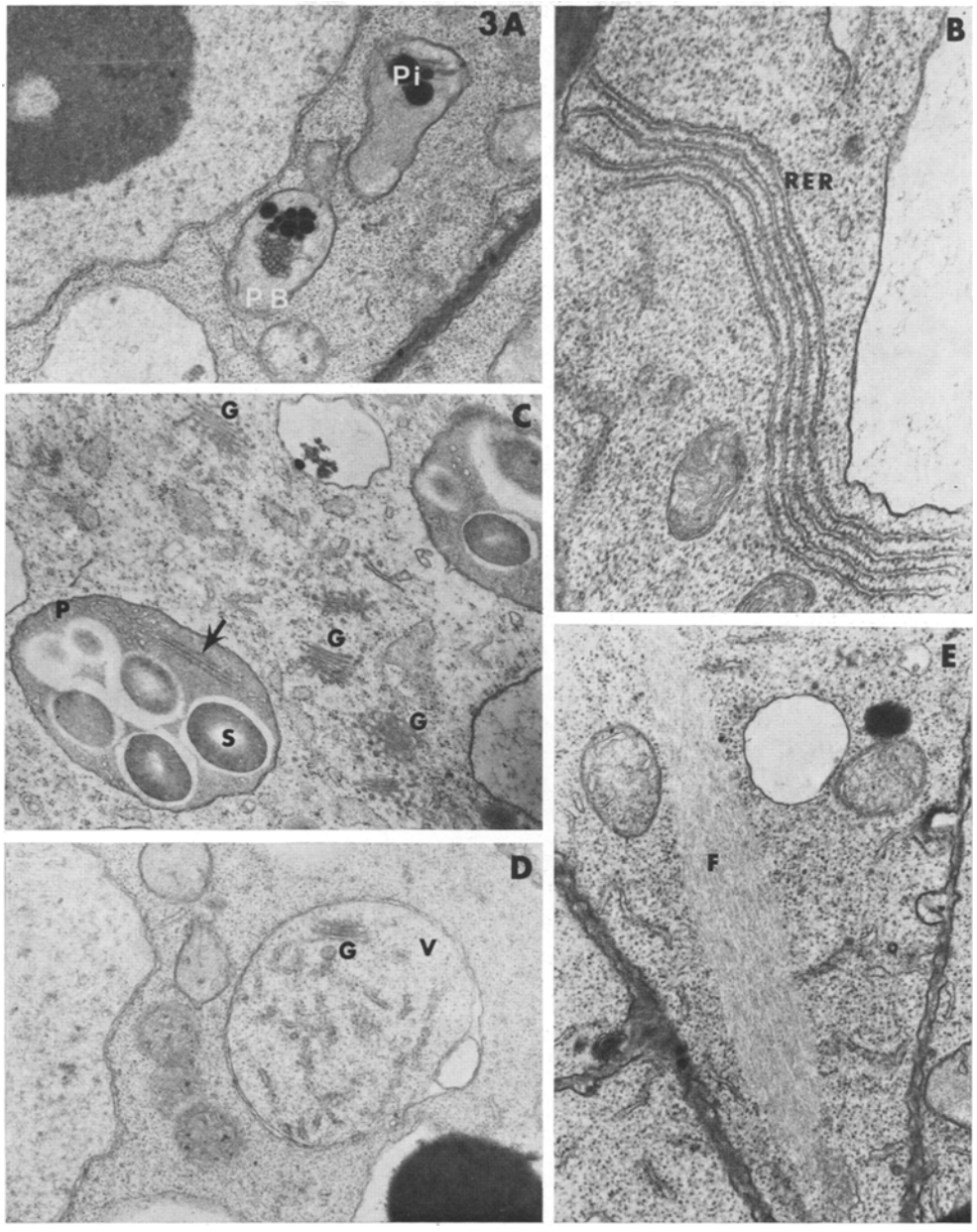

Fig. 3 *A*. Part of a cell showing proplastids with numerous plastoglobuli (*Pi*) and structures that resembled prolamellar bodies (*PB*). ×60,000

Fig. 3 *B*. A profile of rough endoplasmic reticulum (*RER*). ×37,000

Fig. 3 *C*. Many plastids (*P*) contained starch granules (*S*) and membranes (arrow) that did not form into grana. Numerous Golgi bodies (*G*) were present. ×28,600

Fig. 3 *D*. A vacuole (*V*) containing numerous membrane fragments and a Golgi body (*G*). These structures are features of cells in initial stages of senescence. ×55,000

Fig. 3 *E*. Detail of the multifibrillar structure (*F*) shown in Fig. 2 *A*. ×41,700

At 10 days after inoculation, the cell types were more diverse than at 3 days. Most cells contained fewer and relatively larger vacuoles with relatively less cytoplasm and fewer organelles than at 3 days. These cells resembled storage parenchyma cells of intact plants (Fig. 4 *A*). Some cells were in incipient stages of senescence. The number of ribosomes in the cytoplasm appeared to have decreased. Polysomes were frequent and were not membrane bound but others were bound mainly to the ER. Nuclei were oval and nucleoli had distinct fibrillar and granular zones (Fig. 4 *B*). This is in contrast to the more rounded, electron dense nucleoli of younger cells at 3 days.

In cells showing more advanced stages of senescence, the nuclei and other organelles were confined to a small volume appressed next to the cell walls. Amyloplasts were abundant with two to several starch grains (Fig. 4 *C*). Alterations in the mitochondria were minor, consisting mainly of swollen cristae (Figs. 5 *A* and *D*). The latter were sometimes continuous with invaginations of the inner mitochondrial envelope or formed into long cristae in a circular arrangement (Figs. 5 *C* and *E*). Some mitochondria were small and irregularly shaped, and often contained circular inclusions. The ribosomes appeared to decrease substantially in numbers in the cytoplasm but remained associated with the ER membrane (Fig. 5 *A*).

Microbodies were not common. When present they did not contain crystalloid cores or react positively with 3,3-diaminobenzidine (DAB) in the test for catalase such as described in FREDERICK and NEWCOMB (1969). Membranous formations and residual bodies in the cell cytoplasm were conspicuous (Figs. 5 *A* and 6 *C*). Few proplastids were seen and most of them contained thylakoid-like membranes with swollen cisternae not stacked into grana (Fig. 5 *B*). Plastids were greatly modified with age. Frequently, proplastids in the older cells had an irregular ameboid shape, with reduced inner inclusions as compared to young cells.

The cell wall was more electron dense in old cells than in young cells. Most of the O. *sativa* cells at 17 days of culture had typical senescence features, with single large central vacuoles that occupied most of the cell volume (Fig. 6 *A*). The osmiophilic material from the vacuoles disappeared completely. Only a very thin layer of cytoplasm adjacent to the cell wall was present. Amyloplasts increased in size in older cells and large starch grains occupied almost the entire amyloplast (Fig. 6 *A*). In the more advance stages of senescence, plastid stroma was very much reduced and less dense than in young cells. Plastid envelopes were broken in some places. Also, the starch grains were dispersed in the cytoplasm in an unorganized manner. The plasma membrane had secondary vacuoles of variable sizes and shapes that often contained membranous configurations or fibrous materials. These structures appeared less frequently in young cells but were extremely abundant in old cells. Secondary vacuoles protruded into the primary vacuole but were delimited from the tonoplast by an intermembranous zone of variable width.

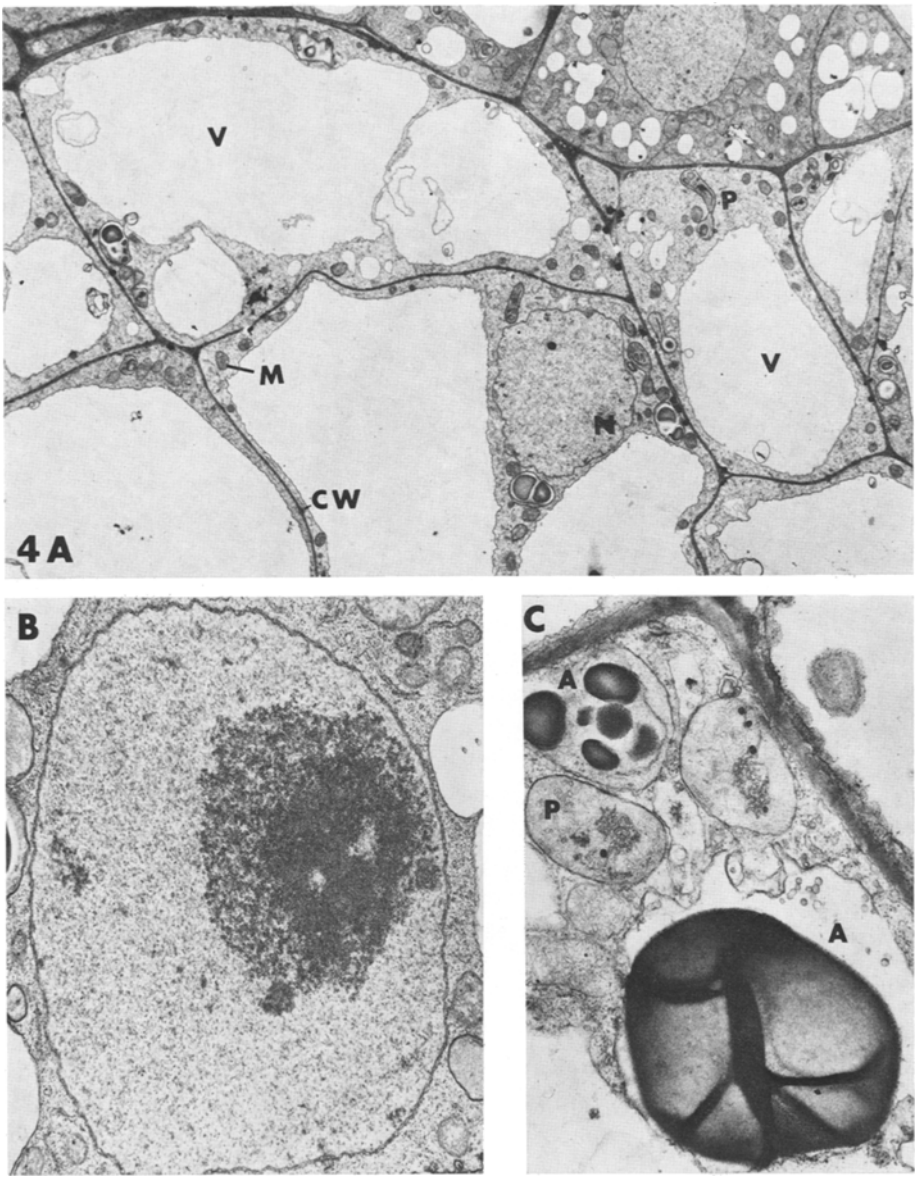

Fig. 4 *A*. Ten-day-old cells. Most cells contained larger and fewer vacuoles (*V*) with proportionately less cytoplasm per cell volume than at 3 days. Cell wall (*CW*), nucleus (*N*), plastid (*P*), mitochondria (*M*). ×6,600

Fig. 4 *B*. A nucleus with a nucleolus that shows distinct fibrillar and granular zones intermingled but easily recognizable. ×32,000

Fig. 4 *C*. Part of a cell showing proplastids (*P*) and conspicuous amyloplasts (*A*) with few starch granules or a single large one. ×25,000

In many cases secondary vacuoles were detached from the plasma membrane and formed a single membrane-bound compartment; usually possessing membranous inclusions. Many myelinic figures were present, with irregular and vesicular shapes (Fig. 5 *B*). Most of the cell organelles were modified. The nucleus was usually present until the most advance stages of senescence and, in some cases, was normal. However, in many cells it was altered seriously. The perinuclear space increased and contained some osmiophilic material (Figs. 6 *B* and *D*). Also, the nuclear envelope was limited by numerous small vesicles that were of electron dense material near the ER (Figs. 6 *B* and *D*). The outer membrane of the nuclear envelope often had numerous large vesicles surrounding the nucleus (Fig. 6 *B*). The appearance of the nucleoli ranged from no apparent change to less compact than in young cells.

4. Discussion

Cells of varying ages are found in suspensions grown asynchronously. These cells range from those actively dividing to those totally degraded by the normal senescence process as the nutrients of the medium are depleted and its composition is changed. Proper sampling is a major problem when asynchronous cell suspensions are used. Sampling must be adequate to determine which anatomical features are most representative of individual cells at each particular age. This, of course, would be unnecessary in synchronous cultures.

On the other hand, cell suspensions are uniquely adapted to studies of senescence because differentiation does not occur or sometimes can be controlled by chemical manipulation. Therefore, the aspects of senescence can be separated from those of differentiation. An important phenomenon of aging in rice cells was the reduction in quantity of cytoplasm relative to the cell size. Lytic areas within the cytoplasm, myelin figures, multivesicular bodies and residual bodies all occurred more frequently in older cells than in younger cells. The appearance of myelin figures and other membranous arrays are evidence of intracellular digestion of lipoprotein complexes, with resultant accumulation of undigested residues.

A greater mitochondrial population was not observed with age as reported

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Fig. 5 *A*. Part of a cell in an advanced stage of senescence showing numerous areas and reduced numbers of ribosomes within the cytoplasm. Mitochondria (*M*) and Golgi bodies (*G*). Ribosomes are still associated with the ER (*RER*). ×20,000

Fig. 5 *B*. A proplastid (*P*) with an irregular ameboid shape. Numerous myelin figures (arrows) or multivesicular bodies from the tonoplast have become conspicuous. ×25,000

Fig. 5 *C*. A mitochondrion (*M*) containing atypical cristae. This structure might also be interpreted as an abnormal plastid with a single thylakoid and numerous plastoglobuli (arrow). ×25,000

Fig. 5 *D*. A mitochondrion (*M*) with an irregular shape. ×59,000

Fig. 5 *E*. A mitochondrion (*M*) with very long cristae arranged in a circular fashion. ×60,000

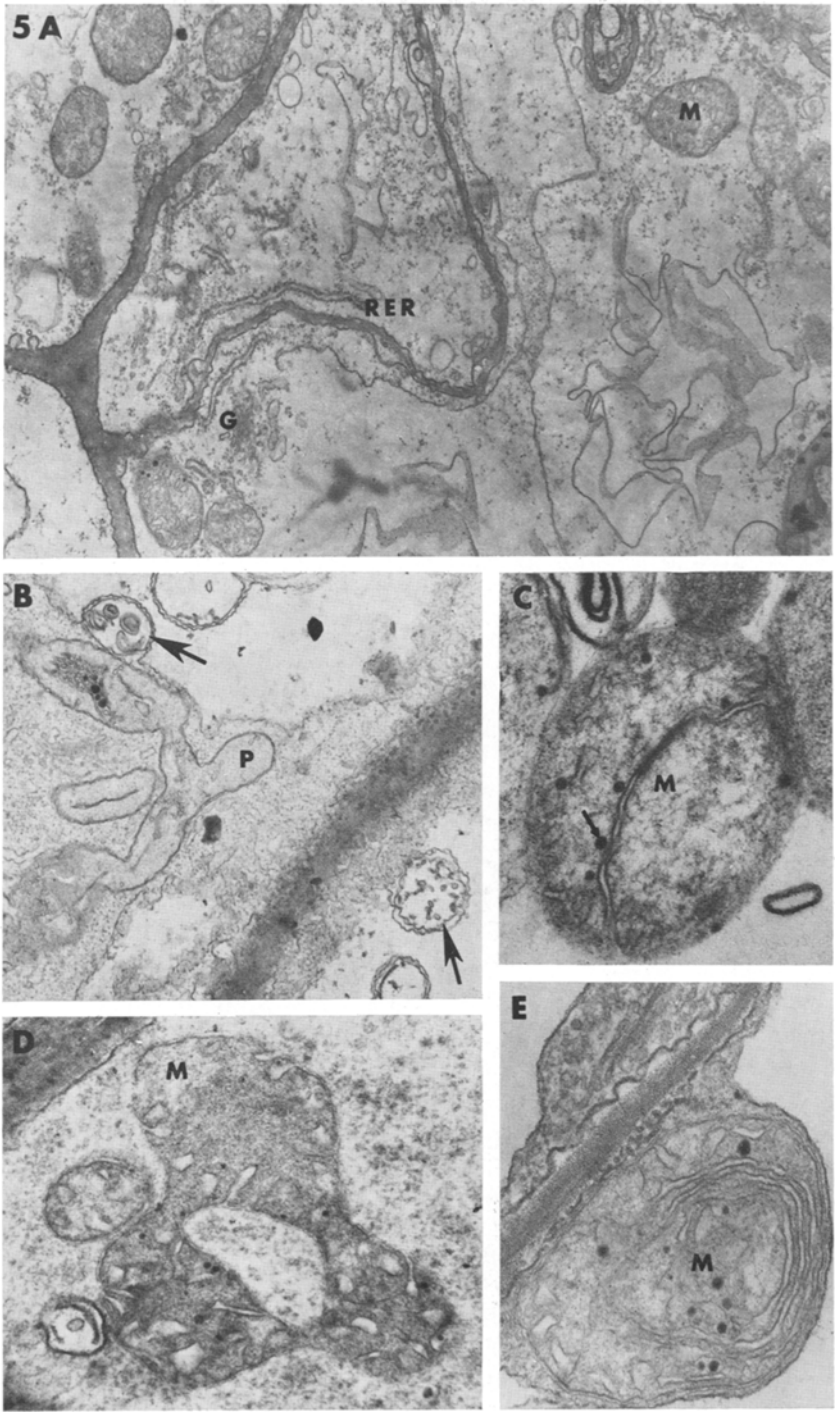

Fig. 5 *A–E*

by LEE and CHASSON (1966) for potato tuber tissue. However, we observed modifications in mitochondrial inner structure (*i.e.*, atypical cristae that were swollen or arranged in a circular pattern) similar to those in root tips of *Phaseolus vulgaris* L. (NEWCOMB, STEER, HEPLER, and WERGIN 1968) or *Acer pseudoplatanus* L. cells cultured in suspension (DAVEY and STREET 1971). SUTTON-JONES and STREET (1968) also described the circular pattern of cristae as a characteristic modification in older *A. pseudoplatanus* cells. These morphologic anomalies occurred simultaneously with the loss of structural organization and probably are associated with mitochondrial protein hydrolysis. Proplastids and amyloplasts had numerous modifications that might have resulted from inhibition of lamellae system differentiation and hydrolysis of their structural proteins. The synthesis of thylakoid structural proteins is under the control of chloroplast DNA so the inhibition of differentiation of the lamellae system in the senescent cells could have resulted from changes in the transcription of plastid DNA.

The amount of ER and numbers of ribosomes attached to the ER increased with age. FOWKE and SETTERFIELD (1968) described the same phenomenon during aging of Jerusalem artichoke tuber slices and suggested that this was due to gene repression and establishment of an organized protein synthesizing complex. Microbodies were seldom observed in *O. sativa* although CRONSHAW (1964), DAVEY and STREET (1971), SUTTON-JONES and STREET (1968), and MATSUSHIMA (1971 a, 1971 b) described crystal-containing bodies corresponding in detail to microbodies in other cell cultures. FOWKE and SETTERFIELD (1968) indicated that the crystal containing bodies in Jerusalem artichoke tuber slices lost their crystalline core, and the amorphous bodies practically disappeared after 2,4-D was added as a growth factor. These bodies were thought to disappear during auxin-stimulated expansion. The *O. sativa* culture medium contained 2,4-D as a growth regulator which may account for the lack of microbodies or their presence only as amorphous bodies. The 2,4-D may have inhibited microbody differentiation or catalase accumulation.

The elongated fibrous structures in *O. sativa* cells resembled the crystalline p-protein components previously described in sieve elements and companion cells by WERGIN and NEWCOMB (1970) and by PALEVITS and NEWCOMB (1971). The appearance of fibrous proteinaceous structures and their association with ER and ribosomes suggested that such structures may be involved in protein synthesis that may be extremely active at 3 days when a substantial increase in cytoplasm occurs. These inclusions have no known role. They resemble structures that WILSON, ISRAEL, and STEWARD (1974) observed in carrot (*Daucus carota* L.) cultures. These workers indicated that the "pinwheel" and fibrous structures in their cultures were not virus particles, although they do resemble somewhat structures reported to be induced by viruses (MARTELLI and RUSSO 1977) or even virus particles themselves

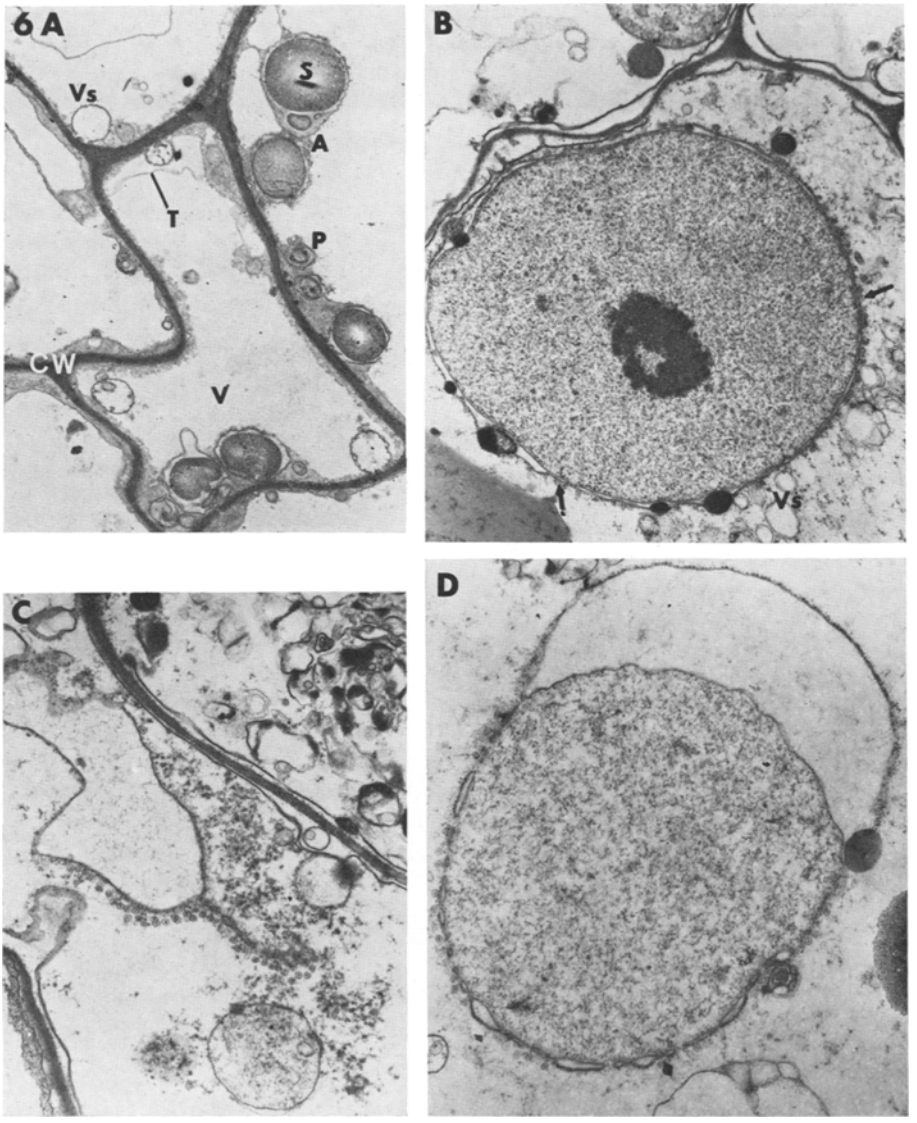

Fig. 6 *A*. A typical senescent cell with a large central vacuole (*V*) and most of the cytoplasm next to the cell walls (*CW*). Most of the organelles are in various stage of degradation; *i.e.*, amyloplasts (*A*) contain large starch granules (*S*), tonoplasts (*T*) frequently become separated from the cytoplasm, numerous vesicles (*Vs*) are formed. Some organelles resemble proplastids (*P*) in various stages of development. ×15,000

Fig. 6 *B*. A nucleus that is relatively intact, except for the separation of the inner and outer membranes of the nuclear envelope and the presence of the electron dense formations inside of the perinuclear space (arrows). Vesicles (*Vs*) that may have originated from the nuclear envelope are common. ×21,000

Fig. 6 *C*. Part of the cytoplasm from a senescent cell with numerous vesicles and membrane formations. ×32,500

Fig. 6 *D*. A modified nucleus of a senescent cell with increased perinuclear spaces and swollen nuclear pores and the outer membrane of the nuclear envelope has separated from the inner one. Numerous vesicles and membrane formations are also present. ×25,000

(CHRISTIE and EDWARDSON 1977). We have no evidence to suggest the fibrous structures in *O. sativa* cell suspensions are viral inclusions.

Secondary vacuoles appeared frequently. These structures have been called "lomasomes" (SUTTON-JONES and STREET 1968), "multivesicular bodies" (HALPERIN and JENSEN 1967) or "plasmalemmasomes" (ROBARDS and KIDWAI 1969). MARCHANT and ROBARDS (1968) suggested that membranes external to the plasma membrane may be classified as either lomasomes or plasmalemmasomes, both of which they interpreted as having exocytotic functions. MAHLBERG, TURNER, WALKINSHAW, and VENKETESWARAN (1974) studied these structures in aging cultures of *Euglena gracilis*. Their results suggested that the diverse membranous contents may become integrated and thus no longer represent separate structures. They indicated that secondary vacuoles may detach from the plasma membrane to form a single membrane-bound compartment, usually possessing membranous inclusions. Endocytosis may complement reported exocytosis and contribute to a cycling process of membranes within cells.

The vacuoles that appeared at incipient stages of senescence may form from the cytoplasm, with ribosome, ER profiles and Golgi bodies protruding into the vacuoles, followed by the start of the digestion process. Alternately, the smooth ER membrane may surround portions of the cytoplasm, followed by digestion or autolysis. This is representative of the processes in senescing cells found in cell suspensions of all ages.

Senescence is accompanied by all of the different phenomena described. They apparently are part of the general biological process of deterioration of the cellular lipoprotein complex and characteristic for each species.

In the growth and development of the cell, numerous metabolites, such as proteins, glycoproteins, amino acids and their breakdown products, nucleic acids and their breakdown products, lipids, and other substances, are released within the cytoplasm. Some of the metabolites are degraded by autolysis and recycled in the metabolic pool, but others may remain sequestered inside the cell and become toxic.

Acknowledgements

We thank Ing. C. DIMITRIU, Mrs. ROSA STOLZENBERG, and Misses M. FILISIAN and V. MOLEA for their technical assistance, and Dr. K. OHIRA for the rice cultures. The work of Dr. A. BREZEANU in the United States was funded by the International Research Exchange (IREX) Foundation.

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