CHAPTER 4

Further Observations of the Ultrastructure of Avocado Fruits, Some Comments on Techniques, and Conclusions of the Dissertation

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INTRODUCTION

During the present investigation of the ultrastructure of mesocarp parenchyma cells of ripening avocados, I also studied the oil cells and made some detailed observations on other aspects of the cell ultrastructure. This chapter will consist of a series of discussions of the following topics: A) The structure of the specialized oil cells or idioblasts, and a comparison of these with similar structures described in the stems and leaves of other species; B) Observations on the structure of some of the internal membranes as well as the envelope of plastids from avocado mesocarp as revealed by freeze fracture techniques; C) Further discussion of the changes in particle density of the plasmamembrane during ripening, and additional data on this topic will be presented; and D) A discussion of new techniques developed or improvements on old techniques which allowed the conclusion that ripening is not senescence and does not involve cell degradation. The chapter will then conclude with E) A summary of the conclusions of the Dissertation, and a discussion of its significance and application to other studies.

A. IDIOBLASTS

The specialized oil cells, or idioblasts, are larger than the parenchyma cells and are scattered throughout the mesocarp of the avocado fruit (fig. 1). Cummings and Schroeder (1942) noted that these cells occupy approximately 2% of the volume of the tissue. They are almost completely filled with oil which varies in electron density and usually stains with a different density than the oil of the parenchyma cells. Although histochemical, cytochemical or biochemical analysis of the oil in these cells were not performed in the present study, the staining characteristics of the oil after normal preparative techniques (OsO_4, postfixation, and uranyl acetate and lead citrate post staining) indicate some difference in composition between the oil of the idioblasts and that found in the cytoplasm of the parenchyma cells (figs. 1, 2). In light microscope studies of avocado, Scott et al. (1963) also noted a difference in staining of the oils of the two cell types. The idioblasts are apparently not metabolically functional. The electron dense cytoplasm at the periphery of the cell contains vesicles, small lipid droplets, and what appear to be remnants of various organelles and membranes (fig. 2). The probable reason for this cytoplasmic degradation can be seen by studying the walls of these cells. There are three distinct structural layers in the walls of the idioblasts. The outermost layer appears to be a normal cellulosic wall, continuous with the walls of the surrounding parenchyma cells. Internal to this is a layer which occasionally has a striated appearance similar to that described and identified as being suberin by Kolattukudy et al., in potato tubers, and other tissues (Dean et al., 1977; Kolattukudy, 1980). Additionally, previous reports on the structure of idioblasts in avocado mesocarp (Scott et al., 1963) as well as those of other tissues (Gross, 1978; Amelunxen and Gronau, 1969) also describe a suberin layer in the wall surrounding the oil cell.

The third layer, internal to the suberin, is similar to the tertiary wall described in idioblasts of a woody ranalean (Gross, 1978) and in *Acorus* (Amelunxen and Gronau, 1969). The staining characteristics of this tertiary wall range from light and fibrillar to quite electron dense with granular deposits (figs. 2, 3). A somewhat ordered, layered construction is often apparent (fig. 3), and esterified pectins are present as indicated by the positive reaction with hydroxamic acid-$FeCl_3$ (fig. 4).

Plasmodesmata are only occasionally seen in the primary wall of parenchyma cells adjacent to idioblasts, and serial sections have shown that when they do occur, they do not penetrate the suberin layer (fig. 5). A similar blockage of the plasmodesmata has also been reported in guard cells of *Phaseolus* (Wilmer and Sexton, 1979) and in the basal region of the trichomes of *Larrea* (Thomson et al., 1979), apparently as a result of secondary wall formation. It is therefore apparent that the contents of the mature idioblasts are isolated from both the symplast and the apoplast of the rest of the fruit. The necrotic appearance of the cytoplasm of these cells could therefore be real. However, it could also be the result of an inability of aqueous fixatives to penetrate the suberized layer which apparently completely surrounds the cell. A cellolosic peg with a considerable number of plasmodesmata, as reported by Scott et al. (1963), was not seen in this study. However extensive serial sections of idioblasts were not performed.

B. PLASTIDS

As discussed in Chapter 3, the plastids of avocado mesocarp occur in a variety of forms, depending on their location in the tissue. The presence of a membrane bound inclusion with frequent associations with the membranes of both grana and prolamellar bodies was noted only in the light green tissue, and not in the dark green or yellow parts of the fruit. Membrane bound bodies have been described in young tissues which contain developing chloroplasts. They have been shown to decrease in size and content as the granal system develops and have therefore been implicated to have a role in the storage or production of granal precursors (Stetler and Laetsch, 1969; Platt-Aloia and Thomson, 1977, 1979). Therefore, the presence of membrane bound bodies in plastids of interior cells of the avocado fruit where granal development is apparently limited by lack of light, and not in the chloroplasts of the outer, dark green tissues, might be further evidence of a developmental relationship between the contents of the membrane bound bodies and the photosynthetic membranes. The results of freeze fracture studies of these plastids appears to further corroborate this suggestion by the presence of large, 130-150Å, intramembranous particles (IMPs) in the membrane of what appears to be a membrane bound body (fig. 1). These large particles have been reported to occur only in the photosynthetic membranes of chloroplasts (Sprey and Laetsch, 1976; Miller et al., 1977), and have been correlated with the presence of light reaction centers. The presence of these large IMPs in the membrane of the membrane bound body might be evidence of a partial incorporation of components of the photosynthetic apparatus into this membrane.

When the envelope of plastids is freeze fractured, the fracture plane frequently skips from one envelope to the other (fig. 8). The EF face of the outer envelope membrane has a low density of particles (650 IMPs/μm^2) and the PF face of the inner envelope is particle-rich (2900 IMPs/μm^2). Very few studies of freeze fractured chloroplasts have

included density counts of the inner and outer envelope membranes. Sprey and Laetsch (1976) reported densities of 133±23 particles/ μm^2 on the EF face of the outer envelope and 18201167 particles/ μm^2 on the PF face of the inner envelope of isolated spinach chloroplasts (Table 1). Bisalputra and Bailey (1973) reported 170 IMPs/ μm^2 for the EF face of the outer envelope and 240 IMPs/ μm^2 for the PF face of the inner envelope of the chloroplasts of the red alga *Banzia.* Although my data are only preliminary, the figures from both of the above studies are lower than we have found for the particle densities of avocado plastid envelopes. However, Milkonian and Robenik (1979) have recently studied particle densities in the outer chloroplast envelope of the flagellate *Tetraselmis* and their result of 567±82 IMPs/ μm^2 for the EF face is similar to those found for avocado plastids. The differences in particle densities, especially in the outer envelope might reflect either preparative and/or functional differences and therefore further investigation is required.

C. PLASMALEMMA CHANGES DURING RIPENING

As discussed in Chapter 3, the significance of the changes in particle density of the EF face of the plasmamembrane of avocado mesocarp parenchyma cells during ripening is unknown, and interpretations are speculative.

In order to quantify the increase in particle density observed in this study, histograms were made of the frequency distribution of particles in the 0.01 μm^2 subdivisions of the grid used for counts (Table 2). During the climacteric rise (Table 2B), there is a possible indication of a biphasic distribution of particle density, with peaks at 13 and 19 particles/0.01 μm^2. These peaks coincide with the peaks observed in preclimacteric and climacteric peak membranes, respectively, and therefore most likely correspond to counts made from membranes at early and late stages of the rise. This conclusion is supported by the decrease in frequency of 13 IMPs/0.01 μm^2 counts of particle frequency of the climacteric peak membranes.

The observed increase in particle number coincides with several prominent physiological phenomena which might be correlated with an increase in intramembranous particles in the plasmamembrane. These phenomena include: 1) the peak of C_2H_2 production; 2) apparent fusion of ER vesicles with the plasmamembrane, and 3) maximum activity of wall hydrolytic enzymes.

1) The evolution and release of ethylene has been shown to have an apparent requirement for the presence of the plasmamembrane (Matoo and Lieberman, 1977; Anderson, Lieberman and Stewart, 1979). Additionally, Laties (Lieberman, 1979) has evidence from $^{13}C/^{12}C$ ratios that the ethylene produced during the climacteric may be, at least partially, derived from fatty acids of the membrane lipids. Therefore, the interaction of a lipophilic substance, such as ethylene, with the membrane, and/or the utilization of membrane fatty acids in the production of ethylene might result in perturbations of the membrane components. However, there is no evidence to support an ethylene-induced incorporation of IMPs, even the induction of non-protein IMPs, in the form of inverted micelles, as shown by De Kruijff et al. (1979, 1980). Therefore, it does not appear that ethylene production is the physiological correlate of the increased number of IMPs in the climacteric peak plasmamembranes.

2) There also appears to be a vesiculation of the endoplasmic reticulum during ripening of avocados, as discussed in Chapter 3. These vesicles apparently fuse with the plasmamembrane, and are possibly involved in the secretion of wall hydrolytic enzymes, or some other aspect of ripening. Freeze fracture studies of membrane fusion events frequently report rearrangement of IMPs into rosettes, or aggregation of particles resulting in particle-depleted areas (see Satir, 1980, for review). However, these or other rearrangements of IMPs were not seen at any stage of ripening in avocados. Consequently, membrane fusion does not appear to be the physiological correlate of increased particle density seen during the climacteric, unless, increased numbers of IMPs are required for the fusion process to occur in avocados.

3) Another aspect of ripening which is at a maximum at the climacteric peak is the activity of the wall hydrolytic enzymes cellulase and polygalacturonase (Awad and Young, 1979). It is possible that transport of these enzymes across the plasmamembrane may be occurring, and this activity could result in a transient increase in the particle density of the membrane. Cellulase has been shown to be associated with the plasmalemma in kidney bean abscission zones (Koehler et al., 1976). It is possible that this is also true in avocados, and the increased particle density reflects an increase in membrane-associated cellulase or other enzymes.

Definitive conclusions concerning the particle density changes of the plasmamembrane during ripening cannot be made at this time. Additional studies must be made to determine whether there is a complementary decrease in the density of particles in the PF face. This would indicate a change in partitioning of the particles rather than a total increase in density. Isolation of the plasmamembrane and labeling of this fraction with anticellulase-ferritin conjugates may be informative in determining the possibility that the particles represent cellulase. The ultimate significance of this aspect of the study, and that of the chloroplast envelope freeze fracture, will depend not only on future results on these tissues, but on the findings of other experiments in determining the true nature of the intramembranous particles visualized by the freeze fracture technique.

D. SOME COMMENTS ON TECHNIQUES

Probably the most significant result of this study was the accomplishment of satisfactory preservation of the delicate tissues of the soft, ripe fruits. A combination of several factors was found to be necessary. The first was the use of low osmolarity buffer (50 mM) for the initial glutaraldehyde fixation, as well as in the subsequent washes and post fixation. Sexton et al. (1974, 1977, and personal communication) also found that low osmotic strength buffers allowed good preservation of the delicate cells of the abscission zone.

The second factor which I found to improve fixation was an overnight exposure to OsO_4. Although long osmium postfixation has, in our experience, not been found necessary in most circumstances, certain plant tissues apparently require this treatment. These tissues have the common characteristic of a large amount of very osmiophilic substances, such as the oils of the avocado, or the resinous secretions of *Larrea* (Thomson et al., 1979). It was found that overnight osmium treatment allowed sufficient time for adequate infiltration and reaction of the osmium with the tissue.

Another factor in the preparation of avocado mesocarp which seemed to improve preservation of the delicate cells was a very slow initial infiltration of the epoxy embedding medium. The tissue pieces were placed in 1 ml. of 100% acetone, and the epoxy was added dropwise over several (5-6) hours until it was approximately 50%, at which time the rate of addition was increased, and finally the tissue was placed in 100% resin and left at room temperature overnight, before final polimerization at 70°C. The rationale for the slow infiltration is that the "front" of resin moving through the tissue presents a certain amount of stress which would be reduced by a smaller gradient of change in viscosity between the fluid already in the cell and that moving into the cell.

The final procedure used in the present study which allowed optimum subcellular preservation was found necessary because of the large size of the cells in avocado mesocarp. It was found that by trimming deeper than usual into the block for sectioning, fewer cells exhibited degradative changes. This has been found to be a problem with many tissues. Evidently, more than just the outermost layer of cells is damaged or shows incipient degradation due to cutting of the tissue in the initial preparation for microscopy. For the present study, large samples taken from the fruit were placed immediately into 2.5%, buffered glutaraldehyde and were then cut into smaller pieces while submerged in the fixative. After embedding, samples were trimmed deep enough to remove the outermost 2-3 cell layers. Usually, even then, several cells in a section exhibited some form of damage, but the presence of well preserved cells in the same sections indicated that this occasional degradation was due to damage during preparation, rather than incipient senescence.

Freeze fracture of whole plant cells presents technical problems not encountered with membrane fractions or most animal tissues. The primary concern is the removal of the cuticle from the replica. Although cuticle is not present, and therefore not a problem, in avocado mesocarp the technique devised for dissolving cuticle from other plants was useful in removing the excess oil of avocados, thus allow-ing a good, clean replica. The technique calls for a several hour wash in each of sulfuric-chromic acid, and 12% KOH in 95% EtOH. Since the alcohol evaporates, it cannot be left overnight, as the acid wash. However, several hours seems to be sufficient for the removal of both oils and cuticles from the replicas.

E. CONCLUSIONS AND SIGNIFICANCE OF THE DISSERTATION

In summary, when studying a delicate or apparently senescing tissue accurate conclusions can only be drawn if the cells are prepared in such a way that any artifacts due to damage are completely eliminated. When this study was begun, it was felt that by combining the three different preparative and examining techniques of thin section, freeze fracture, and scanning electron microscopy, natural degradation could more easily be differentiated from damaging artifacts. This would allow an evaluation of the actual degree of deterioration of cells and organelles which occur during ripening, and provide a means for a more accurate assessment of whether ripening should be considered a senescence phenomenon. Additionally, the use of the SEM in combination with the osmium-ligand binding preparative technique allows a much more detailed study of the three-dimensional structure and interrelationships of the various organelles. This ability has not been previously available, and should provide valuable information

concerning ultrastructural organization in many different tissues. It will also provide a means for assessing changes in subcellular organization which may occur during several other developmental or functional transitions.

The primary conclusion of the dissertation is that, in the avocado, ripening and senescence are two distinct and separate processes. This finding at the ultrastructural level, provides support for previous biochemical and physiological studies which indicate continued biosyn-thesis and cellular functioning during ripening. Further, this study indicates the need for a similar, multi-faceted approach to studies on the ultrastructure of the ripening process of other fruits, as well as other systems thought to be undergoing senescence. The possibility that tissue degradation as revealed by ultrastructural or leakage studies is due to artifactual damage during preparation rather than due to senescence, was verified by Sexton et al. (1974, 1977) in their studies of abscission zones. They showed the zone to be composed of intact, apparently functional cells, even after abscission had occurred. This was contrary to previous studies of Bornman (1967) and Webster (1973), which led to the conclusion that abscission was a senescence phenomenon.

The need to differentiate ripening and senescence, as pointed out in the Introduction, is not merely a matter of semantics, but if, as this study indicated, they are separate and distinct phenomena, then biochemical and physiological, as well as ultrastructural studies of each process will only be significant or useful if ripening and senescence are treated as different processes.

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TABLE 1

Particle Densities of Chloroplast Envelope Membranes

as Cited in the Literature and as Reported

in the Present Study

Tissue	Number of Intramembranous Particles/μm^2	
	EF (outer)	PF (inner)
Spinach[1]	133 ± 23	1820 ± 167
Banzia[2]	170	240
Tetracaselmis[3]	567 ± 82	————
Avocado[4]	650	2900

[1] Stetler and Laetsch, 1976
[2] Bisalputra and Bailey, 1973
[3] Milkonian and Robenik, 1979
[4] Present study

Table 2. Histogram of the frequency distribution of intramembranous particles (IMPs) in the 0.01µ subdivisions of the quadrants used for particle density counts of the EF fracture face of the plasma membranes of avocado mesocarp cells at various stages of ripening. The pre- (A) and post- (D) climacteric membranes have 2 fairly definite peaks at 13 to 16 particles/0.01µ, whereas the predominant density of IMPs of the membranes from the climacteric peak membranes (C) is 16-24, much higher than the others. The membranes from fruit during the climacteric rise (B) in respiration exhibit a biphasic distribution of particle counts with 2 peaks at both the lower, 12-14 count and at 18-20 counts/0.01µ. This figure further illustrates the apparent gradual increase in particle density in the EF face of the plasmamembrane of avocado mesocarp cells during the climacteric rise in respiration, with a peak in density at the climacteric peak, and a subsequent decrease in density in the post climacteric fruit.
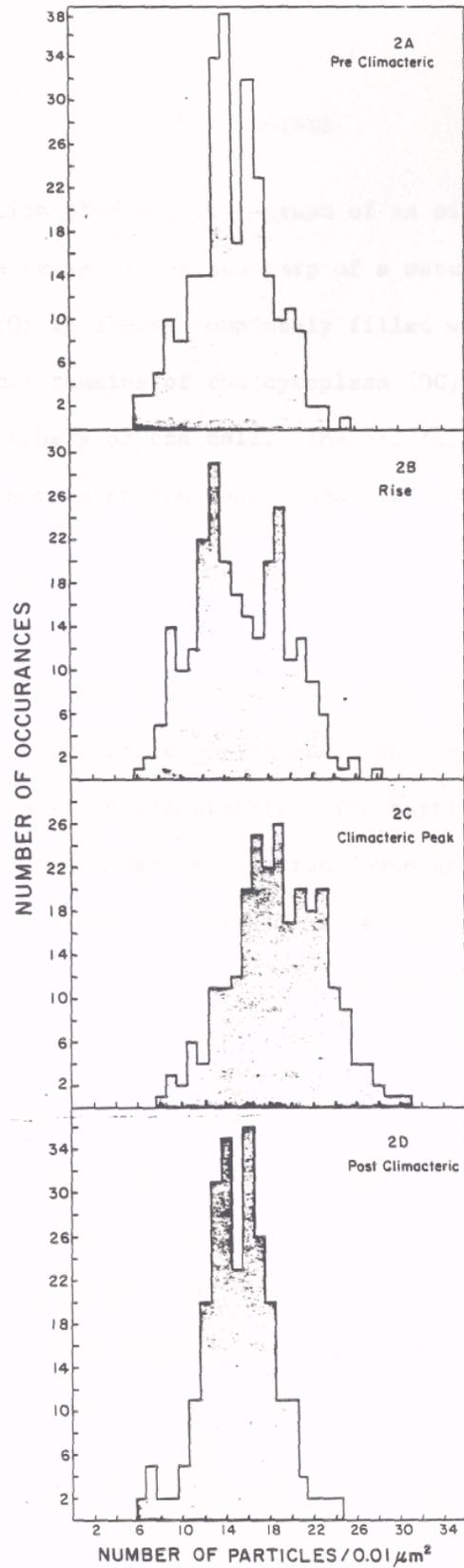
FIGURES AND FIGURE LEGENDS

1. Low magnification electron micrograph of an oil cell and surround- ing parenchyma cells in the mesocarp of a mature, unripe avocado, The oil cell (0) is almost completely filled with a light-stain- ing oil, and the remains of the cytoplasm (OC) are barely discernable at the periphery of the cell. The oil (L) of the surrounding parenchyma is more electron dense than that in the oil cell. X 750.

2. Electron micrograph of a portion of an oil cell and an adjoining parenchyma cell. The oil in the idioblast (O) is less electron dense than that in the parenchyma cell (L).   The cytoplasmic remains of the oil cell (OC) are electron dense and degenerate; few organelles or membranes are discernable. The tertiary cell wall (T) adjacent to the cytoplasm is electron dense and fibrous in consistency. A suberin layer (S) is visible at the outer edge of the tertiary wall. The primary wall (PW) of the oil cell is continuous with the walls of the adjacent parenchyma cells (PC). X 7,400.

3. Higher magnification electron micrograph of the wall of an oil cell.   The fibrous nature of the tertiary wall (T) is evident, as is the layered appearance of the suberin layer (S) between the tertiary and primary (PW) walls. The oil (O) of the oil cell is fairly electron dense in this case, as is the cytoplasm of the oil cell (OC). X 30,600.

4. Electron micrograph of portions of an oil cell (0) and adjoining parenchyma cells (PC) which have been treated with hydroxamic acid and FeCl as a stain for pectins. Both the primary and tertiary walls stain positively for pectin esters. The suberin layer appears electron transparent and somewhat wider than normal after this treatment. X 7,500.
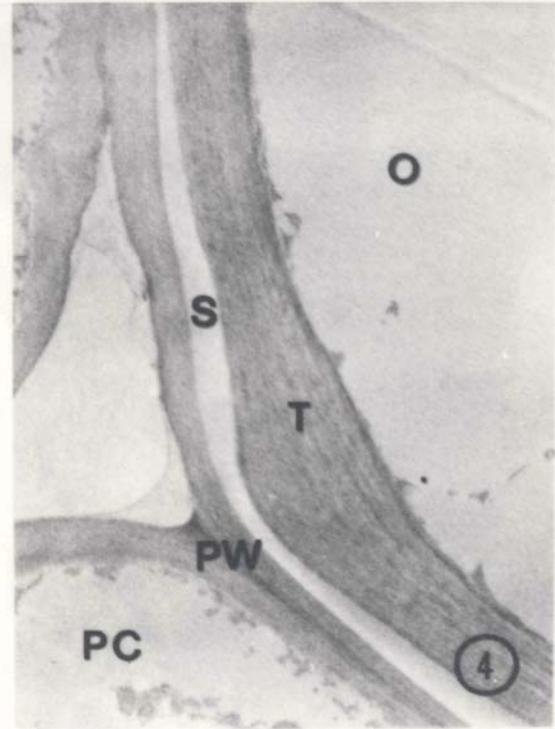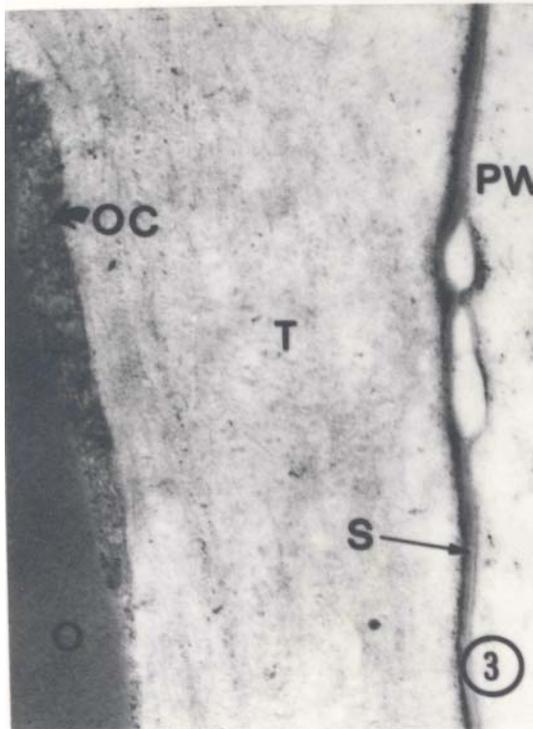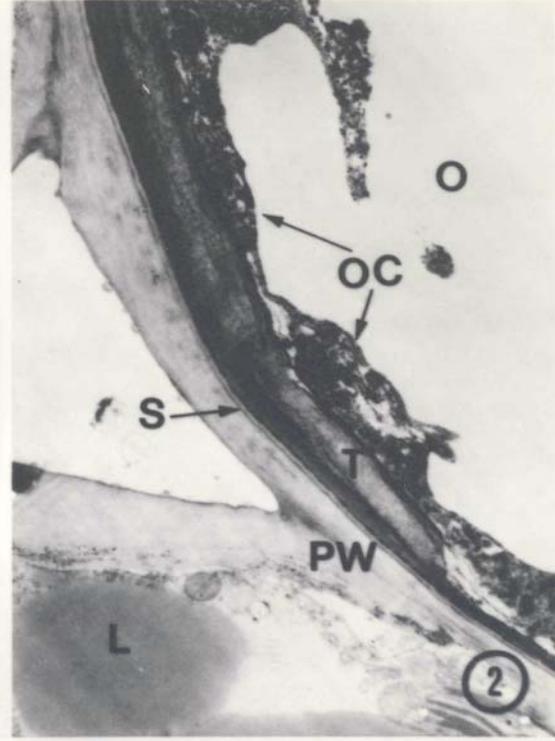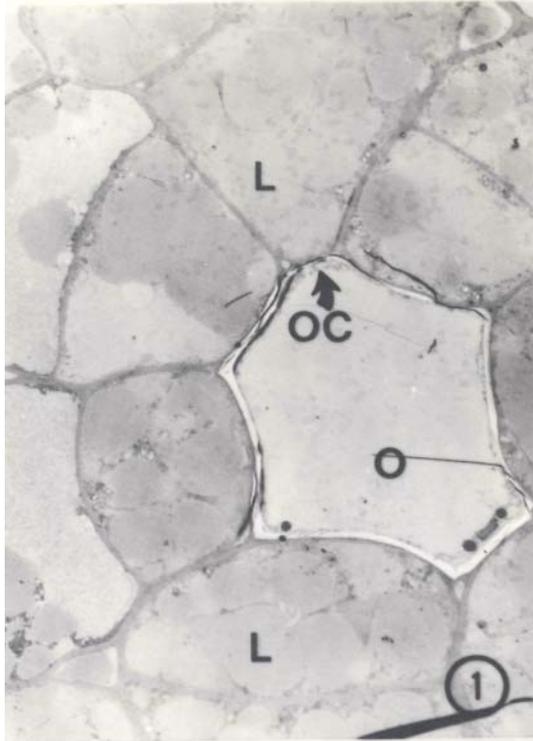
5. Electron micrograph of the cell wall of *a* parenchyma cell adjacent to an oil cell illustrating the apparent blockage of plasmodesmata (Pd) at the suberin layer(S). This is one of several serial sections through this region and this micrograph illustrates the deepest penetration of this group of plasmodesmata into the wall. This fruit was at the climacteric peak, and illustrates a loosening of the tertiary wall (T) of the oil cell as well as of the primary wall (PW) of the parenchyma cell. The endoplasmic reticulum (ER) of the parenchyma cell is vesiculated, as is normal for this stage of ripening. X 27,800.

6. Plastid from the light green portion of the mesocarp of the mesophyll of an avocado fruit. The membrane bound body (MB) shows apparent continuity with the membranes of the grana (G). A small prolamellar body (PB) is present. X 36,100.

7. Freeze fracture replica of a plastid from the light green tissue of the mesocarp of an avocado. A prolamellar body (PB) in the upper half of the plastid is apparently continuous with membranes which contain intramembranous particles with a diameter of ISO- ISO A. In the lower part of the plastid what appears to be a membrane bound body (MB) also contains large (130-150 A) particles (IMPs). These IMPs of the internal membranes of the plastid appear larger than the IMPs of the plasmamembrane shown in the upper right hand corner of the figure (PM). The arrowhead in the lower left corner indicates the direction of shadow. X 29,000.

8. Freeze fracture electron micrograph of a plastid from the mesocarp of a mature, unripe avocado fruit. The PF face of the inner envelope membrane (PI) is particle rich, and the EF face of the outer envelope (EO) is particle poor. The arrowhead in the lower left corner indicates the direction of shadow. X 28,000.