

CHAPTER 1

Aspects of the Three-Dimensional Intracellular Organization of Mesocarp Cells as Revealed by Scanning Electron Microscopy

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SUMMARY

The osmium-ligand binding technique and scanning electron microscopy have been applied to the study of the three-dimensional organization of mesocarp cells of a mature avocado fruit. Using this approach the mitochondria of the cells appear as elongated, branching structures and the endoplasmic reticulum consists of a complex of tubular strands, vesiculated strands and lamellar sheets. Associations of the endoplasmic reticulum with other organelles are also apparent. It is suggested that this approach provides a valuable means to assess the structural transitions in cell organization that occur during development or with functional changes.

Keywords: Endoplasmic Reticulum; Osmium—ligand binding; Scanning electron microscopy

INTRODUCTION

Ultrastructural studies on the endoplasmic reticulum (ER) have revealed that this membrane system may have a variety of forms: tubular (Jones and Fawcett 1966, Porter 1961), cisternal (Morre et al. 1971), and fenestrated (Morre and Mollenhauer 1974) as well as forms intermediate to these. Additionally, there are several reports of associations of the ER with cellular organelles such as chloroplasts (Whatley 1971), microbodies, mitochondria (Morre et al. 1971), nuclei, vacuoles, and the plasmalemma (see Morre and Mollenhauer 1974). In the virtual two-dimensional views obtained by transmission electron microscopy (TEM), the three-dimensional organizations of the ER and the extent of the interrelationship of this membrane system with other organelles is often missed.

The scanning electron microscope (SEM) provides a means for studying the three-dimensional organization of tissues with a high level of resolution. However, most biological tissues are poor electrical conductors and in the SEM this results in an uneven discharge of secondary electrons from the surface of the specimen which distorts the image and greatly reduces resolution. The necessary conductivity can be

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imparted to the sample by several means. The most common method is vacuum evaporation or cathode sputtering of a conductive metal onto the surface to be examined (Boyde and Wood 1969). Although metal coating has proved valuable for many studies, this method is not without disadvantages. Common problems are uneven or incomplete coating, especially of highly irregular surfaces, heat generation during coating which may cause tissue damage, and the masking of structural detail by the layer of metal. Because of these limitations, alternative techniques have been explored which increase the conductivity of biological tissues sufficiently to eliminate discharge, and, at the same time, allow full utilization of the capabilities of the SEM.

One alternative procedure takes advantage of the conductivity of osmium and the binding capabilities of osmium tetroxide. This technique is based on earlier work by Seligman and co-workers (Hanker et al. 1966, Seligman et al. 1966) in which they cross-linked tissue-bound osmium with the ligand, thiocarbohydrazide (TCH). The technique involves exposing sections of OsO₄-treated tissue to TCH, which binds to the osmium in the tissue and then to additional OsO₄ which binds to the unreacted sites on the bound TCH. This procedure results in an increased contrast of osmiophilic components of cells such as membranes and lipid droplets (Seligman et al. 1966). Subsequently, Kelley et al. (1973) and Malick and Wilson (1975) applied this technique to the preparation of material for the SEM. They found that the additional osmium provided adequate conductivity for successful examination of tissue surfaces without coating them with a metal in an evaporator. This method has also been applied to the study of subcellular organelles in the SEM (Woods and Ledbetter 1976). However, in this case, the tissue was dehydrated, embedded in epoxy resin, frozen, broken, and the surface epoxy removed with acetone. Organelles were visible by this technique, but the epoxy limited the depth of field and obscured important details.

In the present study we have applied the ligand binding technique to the study of subcellular organelles in avocado fruits. We have found that by further increasing the exposure of the tissue to an additional layer(s) of TCH and osmium (OTOTO), and eliminating the use of epoxys by critical point drying the tissue that not only is the surface of the specimen electrically conductive and suitable for direct examination in the SEM but internal cells and cell organelles can be observed and studied. In this paper we describe some aspects of the three-dimensional organization of organelles in mesocarp cells of avocado fruits after being prepared by this procedure and studied with the SEM.

MATERIALS AND METHODS

Three different preparative protocols were performed for this study; one for SEM (OTOTO) and two for TEM (OTO and "conventional"). For all three procedures, the initial fixation was the same. Mesocarp tissue samples were taken from mature avocado fruits (*Persea americana* var. Hass) using a #2 cork borer. The plug of tissue was placed immediately into 1% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) and cut into smaller pieces (1 x 2 mm for TEM and 2 x 4 mm for SEM). These were then transferred into fresh fixative for 2-3 hours. After a brief rinse in buffer, the material was post-fixed overnight in 1% OsO₄ in 50 mM cacodylate buffer, pH 7.2. Samples for SEM and those for the OTO, TEM preparation, were washed 5-6 times in distilled water

over a 20 minute period and then treated with freshly made, filtered, 1% thiocarbohydrazide (TCH) for 20-30 minutes with frequent agitation (Kelley et al. 1973). The tissue was again washed and then treated overnight with 1% OsO₄ in distilled water. At this point, the samples for TEM (OTO) were removed, dehydrated in acetone and embedded in epoxy resin (Spurr 1969). The above procedure (rinsing, 1% TCH for 20-30 minutes, rinsing, and OsO₄, overnight) was repeated the next day for the tissues for SEM. Samples were then dehydrated in acetone and critical point dried according to the method of Anderson (1951) using acetone as the transition fluid, with a Tuisimas, CO₂ critical point dryer. After drying, pieces of tissue were broken in half by the use of two pairs of forceps and mounted on aluminum stubs with a conductive paint containing colloidal silver. They were oriented so that the freshly broken surface, relatively free from contamination, was exposed. Samples were stored in a dessicator until viewing with a Joelco JSM-U3 scanning electron microscope at 10 or 20 KV. After the first overnight OsO₄ fixation, samples for TEM ("conventional") were dehydrated in acetone and embedded in Spurr's resin (Spurr 1969). Sections were stained with aqueous Uranyl acetate for 1 hour and with lead citrate (Reynolds 1963) for 1-2 minutes. Sections from the TEM (OTO) samples were studied without post stain. All sections were examined and photographed on a Philips 400 electron microscope.

RESULTS

Preparation of avocado tissue for transmission electron microscopy by the OTO method resulted in reasonably good preservation of subcellular detail, and sufficient contrast such that post staining of sections was not necessary (Fig. 1). However, some cytoplasmic components and organelles are highly osmiophilic and over-staining sometimes occurs (Fig. 1, white arrows). At higher magnification, some of the overstained membranes are diffuse in appearance, and some components, such as grana, become extremely electron dense, apparently because of excess deposits of osmium (Fig. 3). Nevertheless, this en bloc preparation of material by the OTO technique for TEM serves to illustrate the quality of preservation of subcellular structure for correlation with tissues studied in the SEM.

The use of the OTOTO preparation procedure and critical point drying followed by breaking the tissue to expose internal structures for study by SEM gave striking and interesting results. Although positive identification of specific organelles is often difficult, it is possible by careful study and comparison with transmission electron micrographs (compare Figs. 1 and 2). At low magnification, in both SEM and TEM micrographs, the large oil droplets can be easily recognized (Figs. 1 and 2; O). Their identification in the SEM is based on their size, frequency of occurrence in the cells, as well as by the unique surface indentations (Fig. 2, black arrows) which are also observable in thin section images (Fig. 1, black curved arrows). Reconstructions from micrographs of serial thin sections (unpublished observations) and individual thin sections such as Fig. 4, indicate that the branched, often elongated, worm-like structures observed in SEM micro-graphs are mitochondria (Fig. 2; M). In thin sections the plastids vary somewhat in outline but are considerably larger than the mitochondria (Fig. 1; P). This difference in size permits their tentative identification in scanning electron micrographs (Figs. 2, 6; P). We have identified the many smooth surfaced, balloon-like structures in scanning electron micrographs as probable vesicles or vacuoles of different sizes (Fig. 5, 6; V).

This identification is based on the contour of the vacuoles in thin sections as well as the wide range of sizes that are observed (Fig. 1, V).

At higher magnification in scanning electron micrographs, increased detail is apparent and three-dimensional interrelationships of various organelles are revealed. One of the striking features in these cells is the variation in the endoplasmic reticulum. It varies from sheet-like (Fig. 6; ER) to apparent tubular strands (Figs. 2, 5). Often the ER is closely associated with other structures such as plastids (Fig. 6; ER,) and oil droplets (Fig. 6, ER2). Many of the tubular strands of ER are vesiculated and have the appearance of a string of different sized beads (Fig. 5; ER).

Individual ballooning of the strands is apparent and in some instances apparent continuities between the sheet-like and tubular forms can be seen (Fig. 6; arrow).

DISCUSSION

In the present study using scanning electron microscopy we have observed, in the same cell, three different structural forms of endoplasmic reticulum: tubular strands, vesicular strands and lamellar sheets. Our observations in the SEM also substantiate the TEM serial section studies which show mitochondria to be long, branching structures. In the virtual two-dimensional images provided by transmission electron microscopy, many such important structural features of cell organelles can be missed. Also, the three-dimensional interrelationships of different organelles are not always apparent or are observed only infrequently in TEM. Previously, only by extensive serial section reconstruction could these important three-dimensional aspects of cell organization be studied. The osmium-ligand binding technique, as we have applied it, used in conjunction with scanning electron microscopy, provides an avenue for the assessment of these facets of cell and organelle structure. Also, we suggest that the use of this procedure provides a valuable tool for gaining insight into the structural transitions occurring during developmental and/or functional changes in different plant systems.

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FIGURES AND FIGURE LEGENDS

Fig. 1. Transmission electron micrograph of a thin section of a mature, avocado prepared by the OTO technique. The majority of the cell is occupied by oil droplets (O) which show increased electron density at their periphery, probably due to the osmiophilic nature of lipids. The oil droplets frequently have indentations which are often occupied by organelles (black curved arrows). Some regions of the cytoplasm are excessively osmiophilic (white arrows). Plastids (P) are irregularly shaped and larger than mitochondria (M) which appear either spherical or elongate. V = vesicle X 2000

Fig. 2 Scanning electron micrograph of a cell on the freshly broken surface of avocado fruit tissue prepared by the osmium-ligand binding technique. Oil droplets (O) can be identified by their comparable size, number, and shape when compared to thin sections as in Fig. 1. Black arrows indicate depressions or indentations in the oil droplets. Plastids (P) are irregularly shaped and of larger diameter than mitochondria (M) which are long and of a more consistent diameter. Microbodies and small vesicles (*) are, however, not discernable. Endoplasmic reticulum (ER) of the vesiculating-strand type can be seen. X 2,500

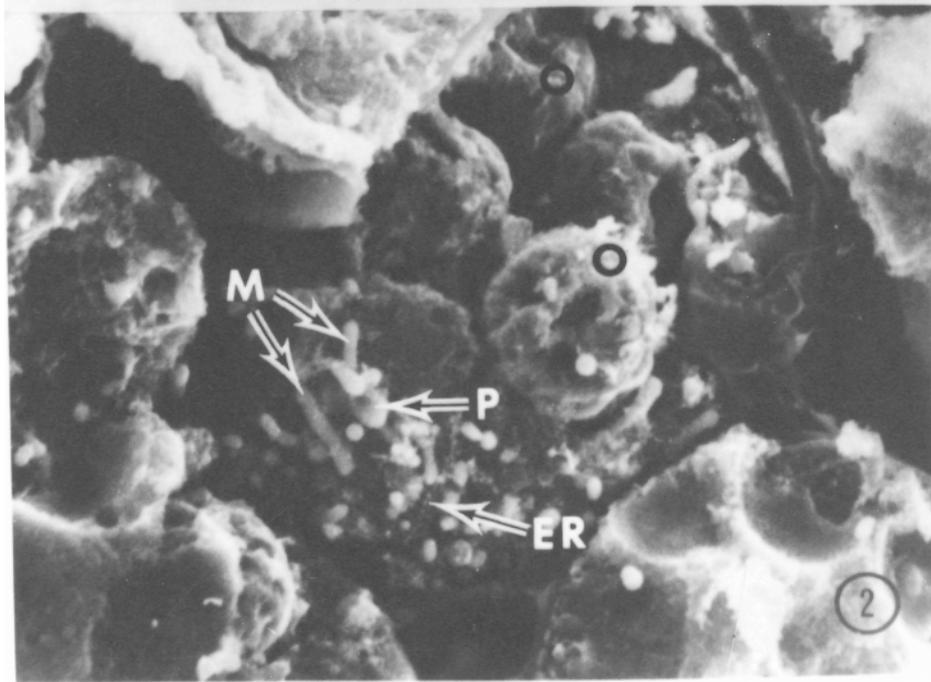
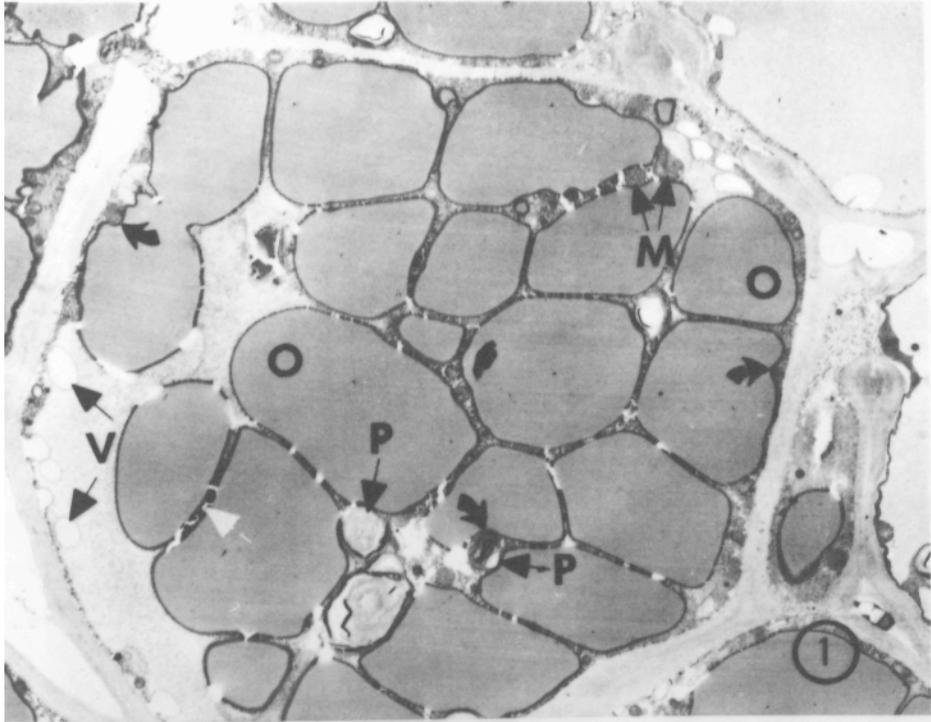


Fig. 3. Transmission electron micrograph of a portion of a mesophyl cell of a mature avocado prepared by the OTO method. The membranes of the chloroplasts (C) and mitochondria (M) show good contrast but are diffuse and granular in appearance. Grana (G) are extremely electron dense. X 15,000

Fig. 4. Transmission electron micrograph of what appears to be several mitochondria (M) in a mesophyl cell of an avocado. Actually, serial sections were taken of this cell and all seven "mitochondria" are part of a single, elongate branching mitochondrion. ER = endoplasmic reticulum, O = oil, N = nucleus. X 25,400

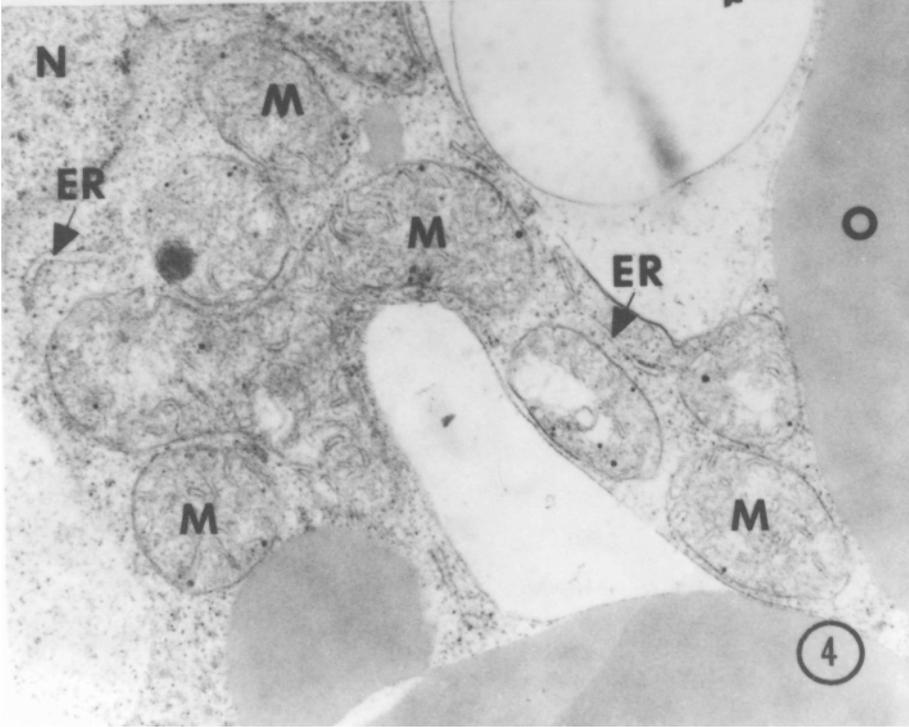
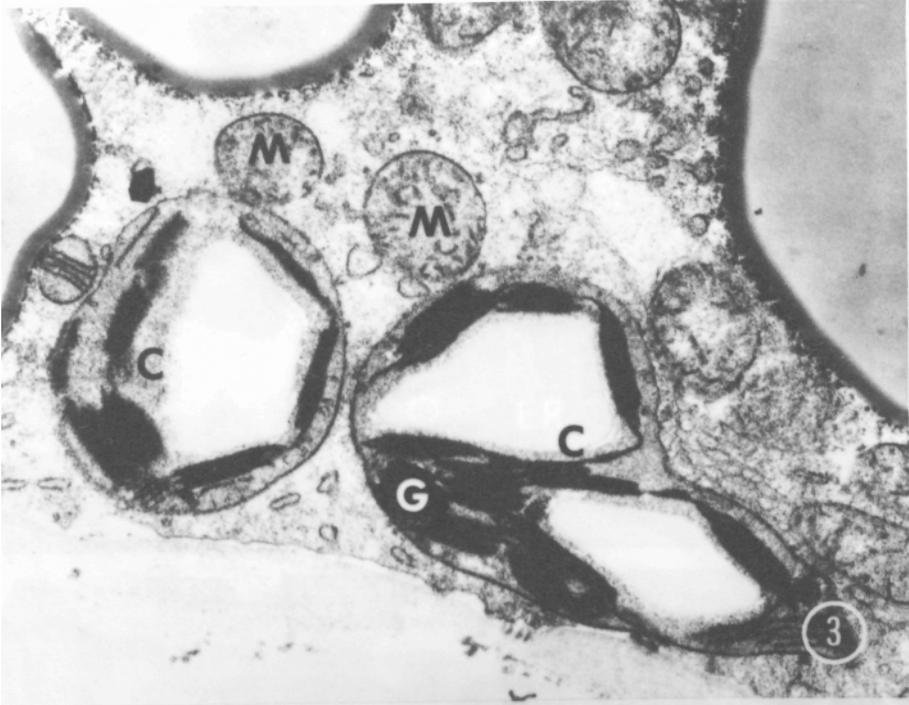


Fig. 5. Portion of a cell of an avocado fruit showing a vesiculating strand of ER (arrow) winding around an oil droplet (O). V = vesicle X 4,600

Fig. 6. Scanning electron micrograph of avocado fruit subcellular organelles. A stellar-shaped lamellar sheet of endoplasmic reticulum (ER-) is in an apparently close association with an underlying plastid (P). Another sheet (ER2) can be seen ensheathing an oil droplet (O). Tubular strands of ER can also be seen in various degrees of vesiculation and in apparent continuity with the sheet forms (arrow). V = vesicle X 8,000

