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Some unusual staining properties of tannic acid in plants

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Summary. Maize root tips were fixed in glutaraldehyde fixatives containing tannic acid and then processed for electron microscopy. Under these conditions, tannic acid selectively stained the contents of the Golgi apparatus secretory vesicles of some outer root cap cells, the cell walls of all cells, and substances in, and adjacent to, intercellular connections of mature primary walls and of secondary walls. Intercellular connections of the young primary walls were not stained. Plasma membranes, and substances associated with the outer leaflets of the plasma membranes, were also stained. Tannic acid-positive material was associated with the cell plate vesicles of forming walls but very little, or none, was associated with the Golgi apparatus vesicles of dividing cells.

Introduction

Tannic acid has been used as a fixative, mordant, and/or stain for clarifying membrane structure (Mizuhira and Futaesaku 1971; Simionescu and Simionescu 1976a, b; Wagner 1976; Saito et al. 1978), microtubules (Ledbetter and Porter 1963; Futaesaku et al. 1972), microfilaments (La Fountain et al. 1976; Mizuhira et al. 1976), cell junctions (Futaesaku et al. 1972; Campbell 1980), mucins (Pizzolato and Lillie 1973), "thin" membranes (Mollenhauer et al. 1976, 1977; Mollenhauer and Morré 1986), and for preserving phospholipids (Kalina and Pease 1977a, b). Tannic acid may react with proteins, alkaloids, mucins, and mineral ions (Futaesaku et al. 1972; Pizzolato and Lillie 1973) and with some phospholipids (Kalina and Pease 1977a, b). It is normally used as a component of the glutaraldehyde fixative solution at a concentration of 0.5%–4.0%.

In this report we note that tannic acid, when used to stain maize root tips, selectively contrasts some Golgi apparatus vesicles in the root cap, components of the plasma membrane, and the wall region of intercellular connections.

Materials and methods

Maize (var Golden Bantam) was germinated in paper rolls in the dark, and at room temperature. The tips of primary roots 2–8 cm long were sliced 2× longitudinally after which the center section was excised and placed immediately into a fixative containing 2% glutaraldehyde (buffered with PIPES, cacodylate, or phosphate to pH 7.2–7.4), 0.05 *M* sucrose, and 0.5%–2.0% tannic acid (Mallinkrodt ÷ 1764, Polysciences Inc. ÷ 4459 or Electron Microscopy Sciences ÷ 61210). Alternatively, the tissues were prefixed in 2% glutaraldehyde for 30 min before exposure to the tannic acid. The glutaraldehyde fixations were at room temperature (about 23° C) for 1–72 h after which the tissues were postfixed for 1–2 h in 1% osmium tetroxide (buffered as above) at icebath temperature. In a few instances, the tissues were block-stained after the osmium tetroxide fixation for 1–2 h with 0.5% aqueous uranyl acetate. After fixation, the tissues were washed in water, dehydrated in ethanol and acetone, and embedded in a Spurr (1969) resin mixture containing lecithin (Mollenhauer 1986).

Except as noted in the figure captions, all illustrations are from roots prefixed in 2% glutaraldehyde/PIPES for 30 min, and then in 2% glutaraldehyde/tannic acid/PIPES for 90 min. After the glutaraldehyde fixation, tissues were transferred to osmium tetroxide and processed as described above.

The preparations of Fig. 7 were fixed 2 h at 23° C in 2% aqueous potassium permanganate, rinsed very briefly in water, and then dehydrated and embedded as for the tannic acid-fixed tissues.

Tissue blocks were sectioned with diamond knives and the sectioned tissues stained by submersion into a lead citrate solution for 1–5 min (Mollenhauer 1975). Sections were examined with a Philips EM 300 electron microscope.

Results

Golgi apparatus secretory vesicles

Approximately the same results were obtained with all of the tannic acids tested.

Specific staining of Golgi apparatus secretory vesicles was observed in some of the outer 2–3 layers of mucilage-secreting cells that cover the root cap (Figs. 1 and 2). Most stain was in the secretory vesicles and almost none was found in the flattened parts of the dictyosome cisternae. The density of the secretory vesicles attached to the dictyosomes decreased in the direction of vesicle maturation, i.e., from the cis to the trans faces of the dictyosomes (Figs. 1 and 2). However, detached secretory vesicles were often more intensely stained than those still attached to the dictyosomes (arrowheads, Fig. 1). The selectively-stained secretory vesicles were limited to a small band of cells around the side of the root cap. Stain was present also in remnants of the sloughed cisternae and the small vesicles associated with the sloughed cisternae (arrowheads, Fig. 2). No stain was found in endoplasmic reticulum (ER, Fig. 2).

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Cell walls and intercellular connections

A generalized staining of wall material was apparent throughout the root tip (Figs. 1, 3–6), and densely-stained lamellar figures (and/or tubule profiles) were common along the cell surfaces of root cap cells and root cells (Fig. 3; also see Figs. 5, 6, 9). The surfaces of most plasma membranes precipitated stain (Figs. 4–6) and this could be used to identify projections of plasma membrane and wall material into the cell (Fig. 6).

A dense, relatively-amorphous annulus was present around the ends of most intercellular connections (Fig. 4). The diameters of the intercellular connections were sometimes less at the annuli than at the mid-regions of the walls (Fig. 4; also see Fig. 7). However, in newly-formed walls, this pattern of stain deposition was absent or illdefined, and the diameters of the intercellular connections at their endpoints were not constricted (Fig. 5). A core structure within the intercellular connections such as that observed with other fixatives (e.g., Dolzmann 1965; Lopez-Saez et al. 1966; Burgess 1971; Robards 1971; Fig. 7) was absent or only occasionally visible in the tannic acid-fixed tissues (Figs. 4, 8). Staining of wall material and intercellular connections was very consistent and seemed to be relatively independent of both tissue size and fixation time.

The cell plate

Tannic acid reaction product was present in the forming walls of some cell plate vesicles even though there was no apparent connections between these vesicles and the established cell walls (Fig. 9). Additionally, the newly-formed plasma membrane of the cell plate was often stained as in mature walls (Fig. 9). However, vesicles adjacent to the cell plate and (presumably) containing wall precursor material, were seldom stained or only lightly stained (arrowheads, Fig. 9). These vesicles were structurally the same as the dictyosome vesicles (not illustrated).

General observations

Block staining tissue with uranyl acetate after tannic acid fixation was not advantageous in that tannic acid reaction products in secretory vesicles and cell walls were reduced or eliminated (not illustrated). Tannic acid added to the initial glutaraldehyde fixative increased membrane contrast but caused aggregation and distortion of cellular constituents, and the differential staining of secretory vesicles and cell wall constituents was not observed (Fig. 10). Results were similar in the glutaraldehyde prefixed tissue if the subsequent tannic acid fixation times exceeded about 2 h (not illustrated).

Discussion

Tannic acid has been used effectively for the preservation and/or identification of selected tissue elements (see introduction for refs.) and the observations reported here further confirm its usefulness in this capacity.

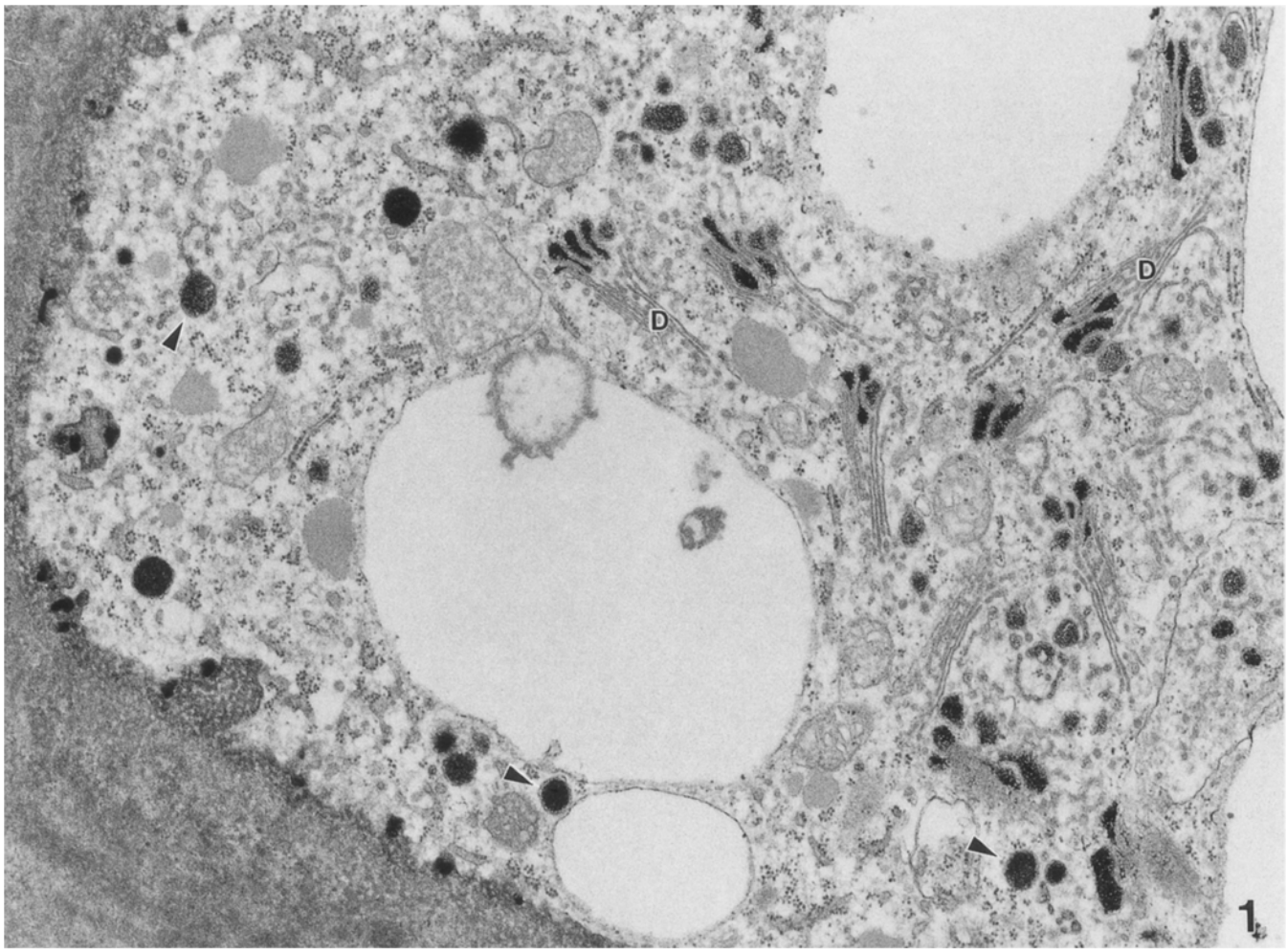
A number of unusual characteristics in the staining patterns were observed. One, for example, was that tannic acid, which normally does not penetrate tissues very well (Futaesaku and Mizuhira 1974; Aoki et al. 1976; Simionescu and Simionescu 1976a, b), stained all cell walls equally after only 1/2 h of exposure to tannic acid, and even stained cell plate components not yet continuous with the cell wall. Yet, the rather course image sometimes associated with tannic acid (Mollenhauer et al. 1976; Fig. 10) was not apparent. Thus, there appear to be at least two staining reactions that differ in their selectivity to tissue elements and reflect different rates of tannic acid penetration. Commercially available tannic acids are most often derived by extraction from the oriental nutgall or similar plant source, and consist of complex mixtures of polyphenolic material, all containing a galloylated glucose structure (Simionescu and Simionescu 1976a, b). Reactions with tissue elements undoubtedly reflect differences in tannic acid composition and could account for the marked differences in tissue appearance reported here and by others. Moreover, molecular weights of tannic acid vary widely indicating the possibility for marked differences in the rate and/or depth of tannic acid penetration into tissue.

In animal cells, tannic acid effectively characterizes a (presumed) lysosomal system which appears to be associated (or contiguous) with the trans Golgi network (Mollenhauer and Morré 1986). The membranes of this lysosomal system appear "thin" after tannic acid fixation as contrasted with other membranes of the cell which retain the double track or "unit" configuration. In this study, it was hoped that a similar membrane-tannic acid reaction would aid in the identification of a trans Golgi network in plants. However, the membrane thinning observed in animal cells was not apparent in plants.

In plants, the most trans cisternae of dictyosomes appear to be sloughed periodically as evidenced in the secretory cells of the maize root cap (Mollenhauer 1971). Upon release from the stack, the secretory vesicles separate from the cisternae, which then break down into small vesicles, at least some of which are coated. Whether these cisternae are part of a trans Golgi apparatus network (Griffiths and Simons 1986), has not been determined. When fixed with tannic acid, these sloughed cisternae were stained by tannic acid in the same manner as the secretion vesicles attached to them (see Fig. 2) suggesting that they, too, contained residues of secretory product. Whether these small vesicles fuse with the plasma membrane in the same manner as the secretory vesicles, or incorporate product from endocytic vesicles, could not be determined from this study.

The selective staining of root cap secretory vesicles presumably reflects differences in the composition of the mucosubstances within the vesicles. The mechanisms of tannic acid binding to mucopolysaccharides may be complex and multiple, including van der Waal's forces and adsorption and attachment to hydroxyl, amino, carboxyl and sulfuric acid radicals (Pizzolato and Lillie 1973). Thus, these data do not shed much light on the kind of stain reaction that takes place or what constituents of the secretory vesicles are actually contrasted by the tannic acid.

The annular structures near the ends of the intercellular connections (Fig. 4) apparently correspond to the sphincter structures described by Olesen (1979). Olesen suggested that sphincters limit the opening of the intercellular connections and possibly act as a valve for controlling the rates and directions of symplastic transport of solutes through the intercellular connections. Our data also confirm that the diameters of the intercellular connections at the annuli are often slightly constricted as compared with their diameters near the mid-regions of the walls (see Figs. 4, 7), and that



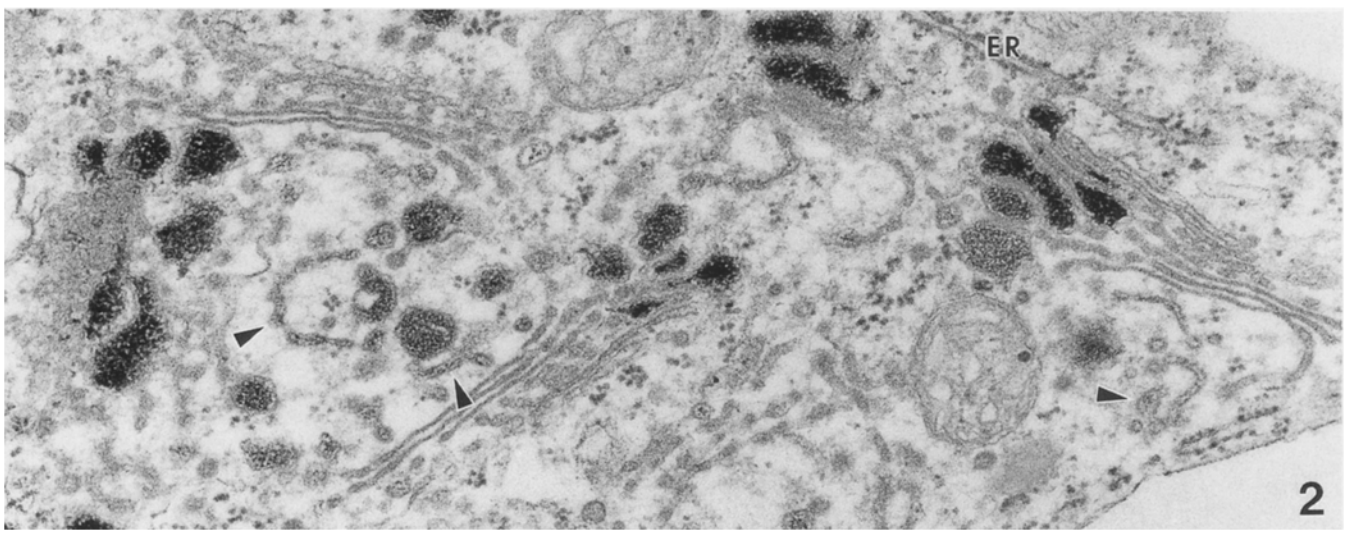
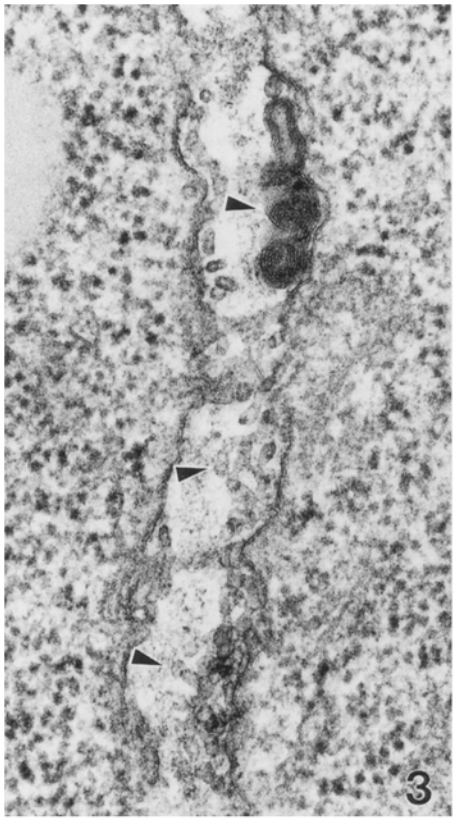


Fig. 1. Outer root cap cell showing distribution of tannic acid-reactive product along the cell surface, in detached secretory vesicles (*arrowheads*), and in secretory vesicles still attached to the dictyosomes (*D*). Only a small band of cells around the mid part of the root cap had dictyosomes that stained in this manner

Fig. 2. Similar to Fig. 1 except at higher magnification to show distribution of tannic acid-reactive product in the components (dictyosomes) of the Golgi apparatus. Most product was associated with the secretory vesicles and with the sloughed cisternae and attached vesicles near the trans poles of the dictyosomes (*arrowheads*). The sloughed cisternae appear positionally equivalent to the trans Golgi network of animal cells (Griffiths and Simon 1986). No tannic acid-reactive product was identified in endoplasmic reticulum (*ER*)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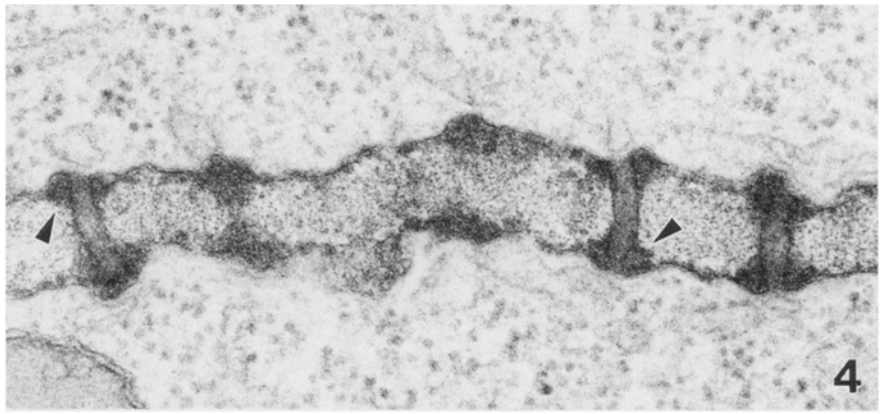
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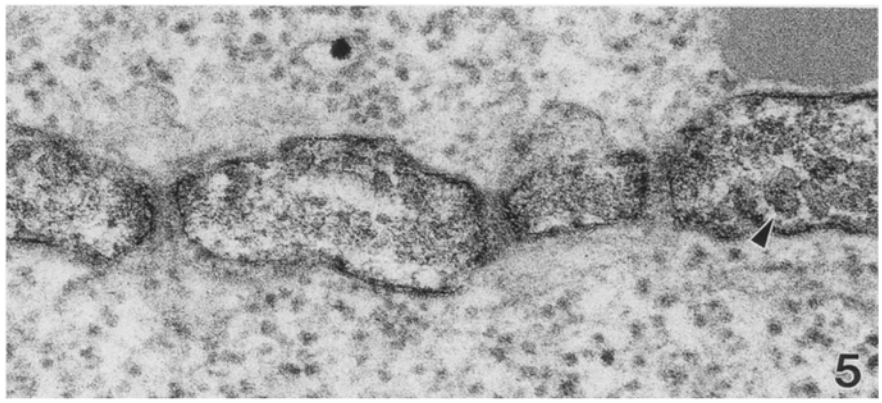
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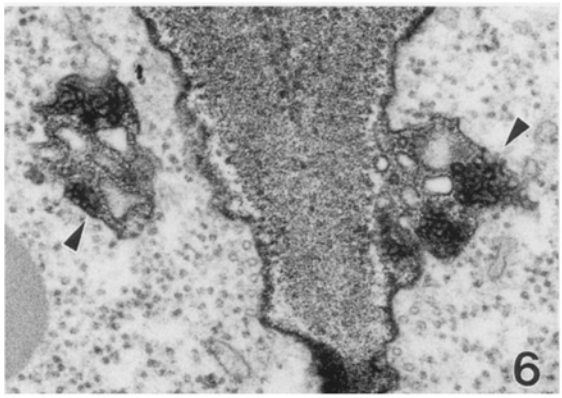
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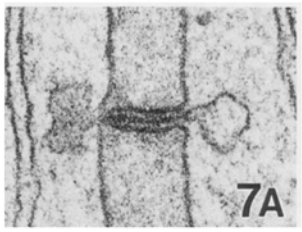
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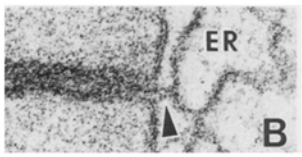
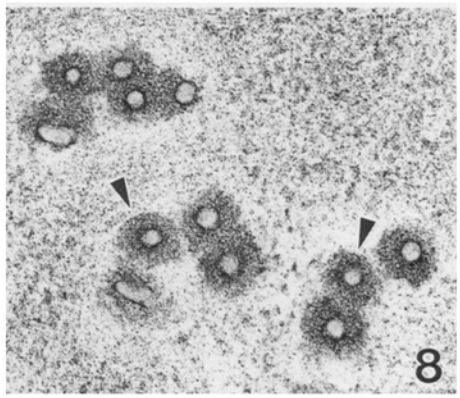

ER
B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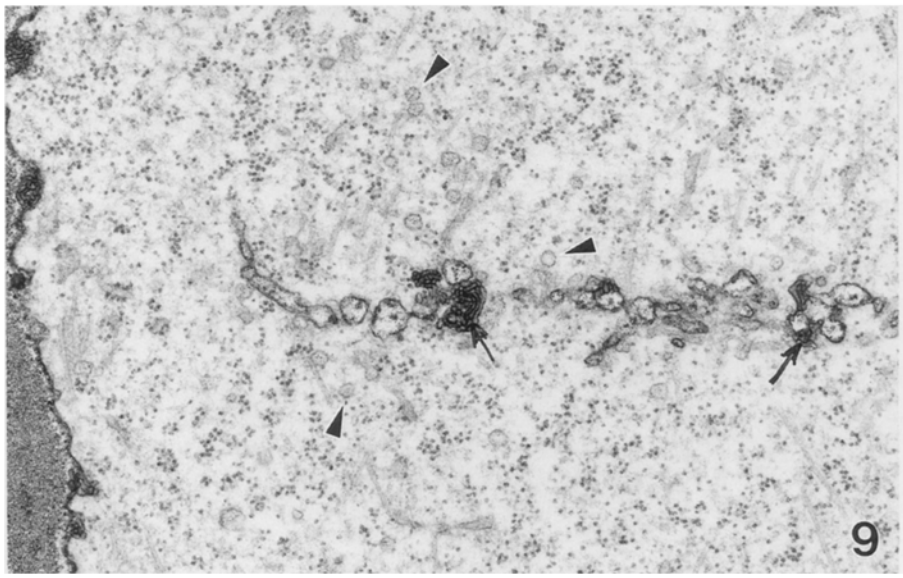
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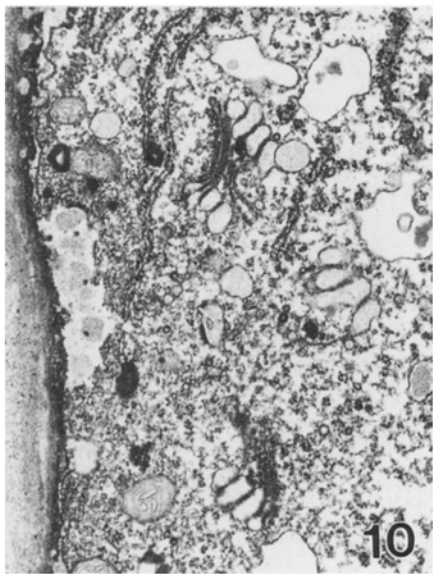
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Fig. 3. Both lamellar figures and/or tubular profiles (*arrowheads*) were present throughout the root in tannic acid-fixed tissues but were especially conspicuous in cell plates and newly-formed walls

Fig. 4. Cell wall between two cortical cells. Intercellular connections were intensely stained and almost all had an annulus of stained material around each end (*arrowheads*). The matrix within the intercellular connections was usually of uniform density and a distinct central core (see Fig. 7) was seldom visible. The diameters of the intercellular connections at the annuli were sometimes smaller than at the mid-regions of the walls. Plasma membranes were heavily contrasted, and a granular tannic acid positive substance was distributed in the wall matrix

Fig. 5. Same as Fig. 4 except that the wall appeared to be more recently formed. Though wall substances stained, no distinct annuli were present around the ends of the intercellular connections nor were the intercellular connections constricted at their endpoints. Apparently, the annular substances form later in wall development. Vesicle profiles (*arrowhead*), as well as lamellar structures (Fig. 3), were common in all tannic acid-fixed roots (also see Fig. 9)

Fig. 6. Same as Fig. 4 except showing some invaginations of wall material into the cytoplasm (*arrowheads*). The dense staining of plasma membrane allowed easy identification of these structures

Fig. 7A and B. Longitudinal section through intercellular connections from tissue fixed in potassium permanganate demonstrating the presence of a central core which, in potassium permanganate-fixed tissues, appears as a small tubule continuous with the lumina of endoplasmic reticulum (*arrowhead in B*)

Fig. 8. Intercellular connection equivalent to that of Fig. 7 except that the tissue was fixed in tannic acid and the plane of section was transverse to the intercellular connection (see Figs. 4, 5 for longitudinal planes). Central cores of intercellular connections were either absent or indistinctly defined. Tannic acid positive annuli are indicated by the *arrowheads*

Fig. 9. A cell plate (from a cortical cell) showing tannic acid positive plasma membrane and some granular material like that in the mature wall. Small vesicles adjacent to the forming plate (*arrowheads*) were not heavily contrasted by the tannic acid. Vesicular or tubular membrane profiles in the plate (*arrows*) and lamellar figures (not illustrated) were common in tannic acid-fixed tissues. Dictyosome vesicles were mostly unstained except for a few near the trans poles which were sparsely stained in the same manner as those indicated at the *arrowheads* of this figure (not illustrated)

Fig. 10. Root cap cell equivalent to that of Figs. 1, 2. When tannic acid was a constituent of the glutaraldehyde prefixative, or when tannic acid exposures were very long (e.g., >2 h in these tissues) following glutaraldehyde prefixation, all cell constituents became heavily contrasted and much of the strong differential staining of secretory vesicles was lost. Additionally, it appeared as though some cellular constituents were aggregated and/or extracted

constrictions are not apparent in young walls which have no annuli (compare Figs. 4, 5). Thus, the annuli seem to play some role in the structural configuration of intercellular connections.

It was of some interest that the vesicles of phragmoplast dictyosomes had very little tannic acid-positive material within them even though the forming plate, itself, was often heavily contrasted (Fig. 9). These data suggest that the tannic acid-positive components are only minimally present in the Golgi apparatus prior to, or during, wall formation and that most of the tannic acid-positive materials are either added at, or near, the forming wall as the vesicles become incorporated into the cell plate. Alternatively, the bulk of the tannic acid-positive materials may be derived from sources other than Golgi apparatus though this seems unlikely in light of the well-established role of Golgi apparatus in wall formation (Whaley and Mollenhauer 1963; Whaley et al. 1966).

References

Aoki A, Einstein J, Fawcett DW (1976) Tannic acid as electron-dense probe in the testis. Am J Anat 146:449–453

Burgess J (1971) Observations on structure and differentiation in plasmodesmata. Protoplasma 73:83–95

Campbell KR (1980) Gap junctions between cells of bone marrow: An ultrastructural study using tannic acid. Anat Rec 196:1–7

Dolzmann P (1965) Sekundärer Verschluss von Plasmodesmen bei der Bildung von Interzellularräumen. Planta 63:99–102

Futaesaku Y, Mizuhira V (1974) Fixation of mechanism of tannic acid and d-catecin. Application I: A detection of oxidase activity by the use of d-catecin. 8th Int Congress Electron Microsc Canberra, Vol II, pp 142–143

Futaesaku Y, Mizuhira V, Nakamura H (1972) The new fixation method using tannic acid for electron microscopy and some observations of biological specimens. Proc Int Congr Histochem Cytochem 4:155–156

Griffiths G, Simons K (1986) The trans Golgi network: sorting at exit site of the Golgi complex. Science 234:438–443

Kalina M, Pease DC (1977a) The preservation of ultrastructure in saturated phosphatidyl cholines by tannic acid in model systems and type II pneumocytes. J Cell Biol 74:726–741

Kalina M, Pease DC (1977b) The probable role of phosphatidyl cholines in the tannic acid enhancement of cytomembrane electron contrast. J Cell Biol 74:742–746

LaFountain JR jr, Zobel CR, Thomas HR, Galbreath C (1976) Negative staining of F-actin and actin-like microfilaments using tannic acid. Proc Electron Microsc Soc Am 34:324–325

Ledbetter MC, Porter KR (1963) A "microtubule" in plant cell fine structure. J Cell Biol 19:239–250

Lopez-Saez JF, Gimenez-Martin G, Risueno MC (1966) Fine structure of the plasmodesm. Protoplasma 61:81–84

Mizuhira V, Futaesaku Y (1971) On the new approach of tannic acid and digitonin to the biological fixatives. Proc Electron Microsc Soc Am 29:494–495

Mizuhira V, Futaesaku Y, Nakamura H (1976) Fine structure of the elastic fibers and the elastin crystalloids by means of tannic acid fixation method. Proc Electron Microsc Soc Am 34:318–319

Mollenhauer HH (1971) Fragmentation of mature dictyosome cisternae. J Cell Biol 49:212–214

Mollenhauer HH (1975) Poststaining sections for electron microscopy: an alternate procedure. Stain Technol 50:292

Mollenhauer HH (1986) Surfactants as resin modifiers and their effect on sectioning. J Electron Microsc Tech 3:217–222

Mollenhauer HH, Morré DJ (1986) Tannic acid characterized membranes of animal cells. Protoplasma 134:43–52

Mollenhauer HH, Hass BS, Morré DJ (1976) Membrane transformations in Golgi apparatus of rat spermatids. A role for thick cisternae and two classes of coated vesicles in acrosome formation. J Microsc Biol Cell (Paris) 27:33–36

Mollenhauer HH, Morré DJ, Hass BS (1977) Plasma membrane transformations in spermatogenesis revealed by aldehyde fixatives containing tannic acid. J Ultrastruct Res 61:166–171

Olesen P (1979) The neck constriction in plasmodesmata. Planta 144:349–358

Pizzolato P, Lillie RD (1973) Mayer's tannic acid-ferric chloride stain for mucins. J Histochem Cytochem 21:56–64

Robards AW (1971) The ultrastructure of plasmodesmata. Protoplasma 72:315–323

Saito A, Wang C-T, Fleischer S (1978) Membrane asymmetry and enhanced ultrastructural detail of sarcoplasmic reticulum revealed with use of tannic acid. J Cell Biol 79:601–616

Simionescu N, Simionescu M (1976a) Galloylglucoses of low molecular weight as mordant in electron microscopy. I. Procedure, and evidence for mordanting effect. J Cell Biol 70:608–621

Simionescu N, Simionescu M (1976b) Galloylglucoses of low molecular weight as mordant in electron microscopy. II. The moiety and functional groups possibly involved in the mordanting effect. J Cell Biol 70:622–633

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43

Wagner RC (1976) Tannic acid as a mordant for heavy metal stains. Proc Electron Microsc Soc Am 34:316–317

Whaley WG, Mollenhauer HH (1963) The Golgi apparatus and cell plate formation-a postulate. J Cell Biol 17:216–221

Whaley WG, Dauwalder M, Kephart JE (1966) The Golgi apparatus and an early stage in cell plate formation. J Ultrastruct Res 15:169–180