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Distribution of the anionic sites in the cell wall of apple fruit after calcium treatment

Quantitation and visualization by a cationic colloidal gold probe

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Summary. The ripening and softening of fleshy fruits involves biochemical changes in the cell wall. These changes reduce cell wall strength and lead to cell separation and the formation of intercellular spaces. Calcium, a constituent of the cell wall, plays an important role in interacting with pectic acid polymers to form cross-bridges that influence cell wall strength. In the present study, cationic colloidal gold was used for light and electron microscopic examinations to determine whether the frequency and distribution of anionic binding sites in the walls of parenchyma cells in the apple were influenced by calcium, which was pressure infiltrated into mature fruits. Controls were designed to determine the specificity of this method for in muro labelling of the anionic sites on the pectin polymers. The results indicate that two areas of the cell wall were transformed by the calcium treatment: the primary cell walls on either side of the middle lamella and the middle lamella intersects that delineate the intercellular spaces. The data suggest that calcium ions reduce fruit softening by strengthening the cell walls, thereby preventing cell separation that results in formation of intercellular spaces.

Keywords: Calcium; Cationic gold; Cell wall; Fruit; Pectin; Ripening.

Abbreviations: EDTA ethylenediaminotetraacetic acid; PATAg periodic acid-thiocarbohydrazide-silver proteinate.

Introduction

In fleshy fruits the mechanism of ripening is generally associated with a softening process, i.e., the loss of tissue firmness. Recent research has suggested that, during ripening, depolymerization of the cell wall components may cause or contribute to this softening process (Huber 1983, Brady 1987, Fischer and Bennett 1991). Cell wall autolysis during ripening is no longer thought to be an uncontrolled process but rather one that is regulated temporally and genetically (see the recent review by Brady 1992).

Recent evidence suggests that calcium retards the rate of fruit ripening. However, the physiological reasons for this are not completely understood and the specific role for calcium in this process is a matter of debate (for reviews, see Ferguson 1984, Poovaiah et al. 1988). Calcium is one of the most dynamic elements that is involved as a second messenger and as an intercellular regulator in complex molecular networks (Hepler and Wayne 1985). However, calcium could also be ionically bound to matrix pectic polymers, in which a single calcium ion is attached to two negatively charged groups, e.g., two COO^- groups of pectic polymers (Rees 1977, Rees et al. 1982, Morris et al. 1982, Jarvis 1984). This arrangement could maintain cell wall strength by binding the polymers in the middle lamella and thereby limit cell separation and uphold tissue firmness in the fruit (Bartley and Knee 1982, Cleland et al. 1990). If this hypothesis is correct, increasing the calcium content of fruit tissue might retard not only the rate of fruit softening but also other changes that are related to senescence (see Poovaiah et al. 1988).

Direct demonstration of calcium action requires the measurement and localization of calcium. Cytosolic free calcium can be measured by a range of fluorescent dyes which have a high affinity and selectivity for free

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Ca^{++} (Callaham and Hepler 1991, Read et al. 1992). Conversely, to understand the role of bound calcium in the cell wall, its content, which is much higher than that in the cytoplasm, has been measured by using energy dispersive X-ray analysis (Rigney and Wills 1981, Burns and Pressey 1987), and atomic absorption spectrometry (Poovaiah 1979, Brady et al. 1985). However, comparing results from different techniques are difficult and inconclusive, possibly because of differences in tissue preparation and methodology. To overcome this problem, researchers have suggested that calcium be visualized in situ with either energy-dispersive X-ray analysis (Glenn and Poovaiah 1990), secondary ion mass spectrometry (Jauneau et al. 1992) or by in situ precipitation of calcium with potassium antimonate (Wick and Hepler 1982, Sjolund 1990). These technologies, which are highly sensitive, enable investigators to obtain rapid images of calcium localization. To elucidate the role of calcium in the cell wall architecture, Lin et al. (1991) have chosen to study the thermal properties of the cell wall using differential scanning calorimetry.

Another possible approach would be to localize the anionic sites of the pectin rather than the calcium and to follow the different modifications in the binding sites that may occur after calcium treatments. The locations of several kinds of pectins have been probed recently by using new affinity methods and in muro localization of the matrix polymers. This information has considerably enhanced our knowledge about the structural model of primary cell walls. In situ visualization of pectins has been performed by using an enzyme-gold complex (Vian et al. 1983, Vian 1986, Benhamou and Ouelette 1986, Cabin-Flaman et al. 1993), a lectin-gold complex (Benhamou et al. 1988), and immunogold labelling (Moore 1980, Moore and Staehelin 1988, VandenBosch et al. 1989, Liners et al. 1989, Knox et al. 1990, Zhang and Staehelin 1992). Vreeland et al. (1989) used an ingenious fluorescent probe to demonstrate that pectin distribution is spatially regulated. Finally, Varner and Taylor (1989) have judiciously avoided chemical preparation that could extract some pectin materials by using Ni^{++} and Co^{++} to localize polygalacturonate blocks directly on free-hand sections. The recent development of cationic colloidal gold probe (poly-L-lysine-gold complex) allows the detection of anionic sites in situ (Skutelsky and Roth 1986). This technique is a one-step postembedding procedure in which the anionic sites are labelled by a highly positively charged amino-acid chain that is complexed to colloidal gold. The present study uses this procedure to analyze changes in the distribution of the anionic binding sites, i.e., pectic sites of calcium bridges, in normal and calcium infiltrated apples.

Materials and methods

Plant materials and calcium infiltration

'Golden Delicious' apples (*Malus domestica* Borkh.) were harvested from a commercial orchard in Pennsylvania. Fruits were infiltrated under 103 kPa of pressure for 3 min with either H_2O or 4% $CaCl_2$. After treatment the fruits were allowed to drain for 2 h before storage at 0 °C.

Preparation of specimens

After 2 months of storage, 2–3 mm^3 pieces of pericarp were cut and chemically fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 for 5 h, then washed in cacodylate buffer and postfixed overnight in 1% osmium tetroxide. After dehydration in an alcohol series, the samples were embedded either in LR White methacrylate resin or in Spurr's resin as described by Roland and Vian (1991).

Labelling with cationic colloidal poly-L-lysine gold complex

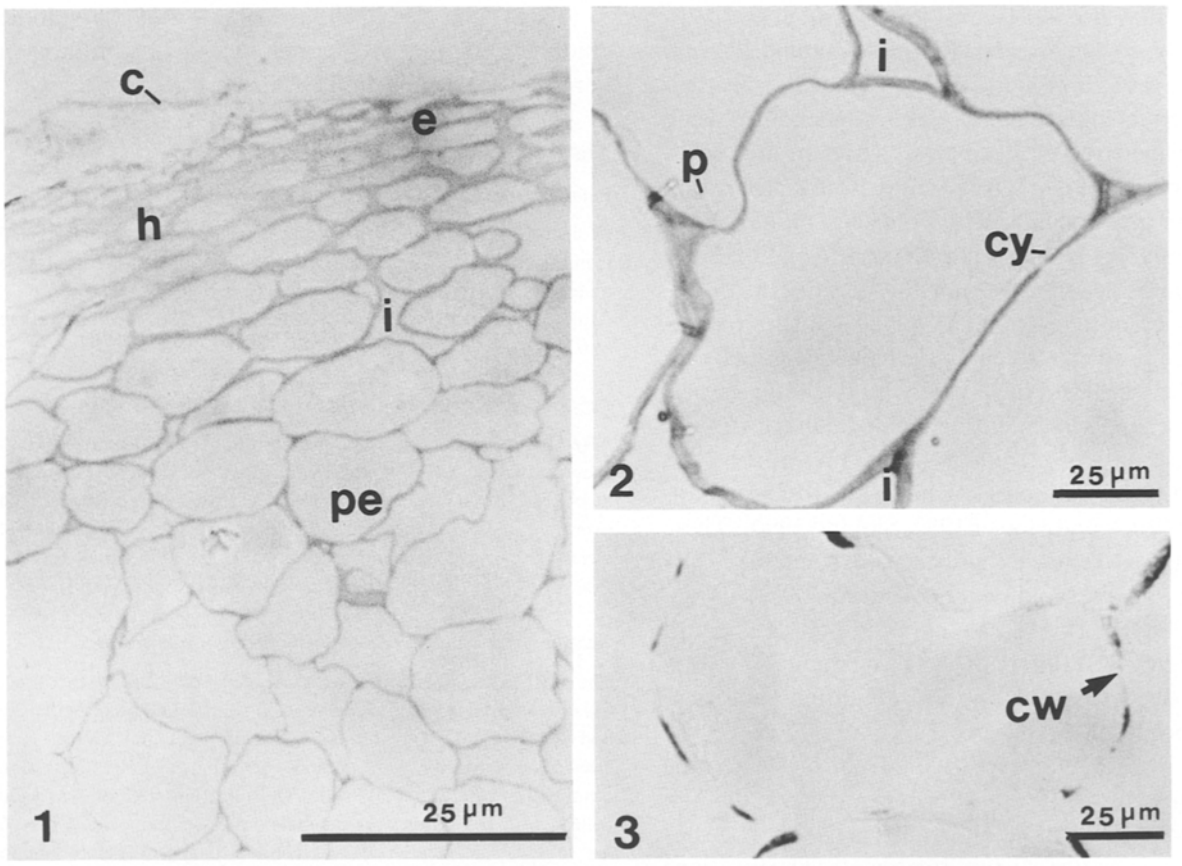
The labelling was performed as a one-step procedure by the direct use of the probe gold complex, according to the method of Skutelsky and Roth (1986). The protocols used for light and transmission electron microscopy were similar. Semithin sections that were mounted on multiwell glass slides and ultrathin sections that were collected on gold grids were incubated in 3% acetic acid at pH 2.6 for 15 min. The incubation was conducted at low pH to obtain maximum specific labelling of the anionic groups (Vian et al. 1992) and to prevent non-specific adherence of cationic gold particles to tissue-free areas of the resin (Vorbrodt 1987). The sections were treated with 10 nm cationic poly-L-lysine colloidal gold complex (CGC10, BioCell Research Laboratories, Cardiff, U.K.) diluted 1/60 (v/v) in 3% acetic acid for 1 h at room temperature. The specimens were then rinsed thoroughly in 3% acetic acid followed by distilled water.

For light microscopy, semithin sections were finally treated with silver enhancer (BioCell kit) for about 10 min at room temperature (while monitoring with a bright field microscope), washed in distilled water, air-dried and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ). The sections were then observed with a bright field microscope (Carl Zeiss Inc., Thornwood, NY).

For transmission electron microscopy, the ultrathin sections were stained with either 1% uranyl acetate for 10 min and 2% lead citrate for 2 min or with the specific PATAg test (periodic acid-thiocarbohydrazide-silver proteinate; Sigma Chemical Co., St. Louis, MO) for polysaccharides in which the detectable groups are the vicinal glycols (Thiéry 1967, Roland and Sandoz 1969). Ultrathin sections were observed with a H-500H transmission electron microscope (Hitachi Scientific Instruments, Mountain View, CA) operating at 75 kV.

Cytochemical controls

Controlled experiments were performed by preincubating the sections with one of the following solutions prior to labelling: (*i*) a 1 mg/ml solution of poly-L-lysine (PM > 350.000; Sigma Chemical); (*ii*) acid hydrolysis in 0.1 M H_2SO_4 at 80 °C for 1 h (Skutelsky and Roth 1986); (*iii*) enzymatic treatment in a proteinase K (protease type IX from *Tritirachium album*; Fisher Scientific) at 1 mg/ml in 0.05 M Tris-



Figs. 1–3. Localization of anionic sites using cationic colloidal gold, followed by silver intensification. Semithin sections of H_2O-treated fruits. Light microscopic observations. Bars: 25 µm

Fig. 1. General view, the enlargement and rounding of cells, which are typical of fruit softening, result in wall separation along the middle lamella and the formation of intercellular spaces. All of the cell walls appear heavily and uniformly labelled. *c* Cuticle, *e* epidermis, *h* hypodermis, *i* intercellular space, *pe* pericarp

Fig. 2. Detail of single parenchyma cell. Labelling occurs throughout the cell wall. Small areas with higher density can be observed. *cy* Cytoplasm, *i* intercellular space, *p* plastid

Fig. 3. Control (same procedure with preincubation carried out in the presence of unlabelled poly-L-lysine prior to the labelling). The extent of labelling is greatly reduced. *cw* Cell wall

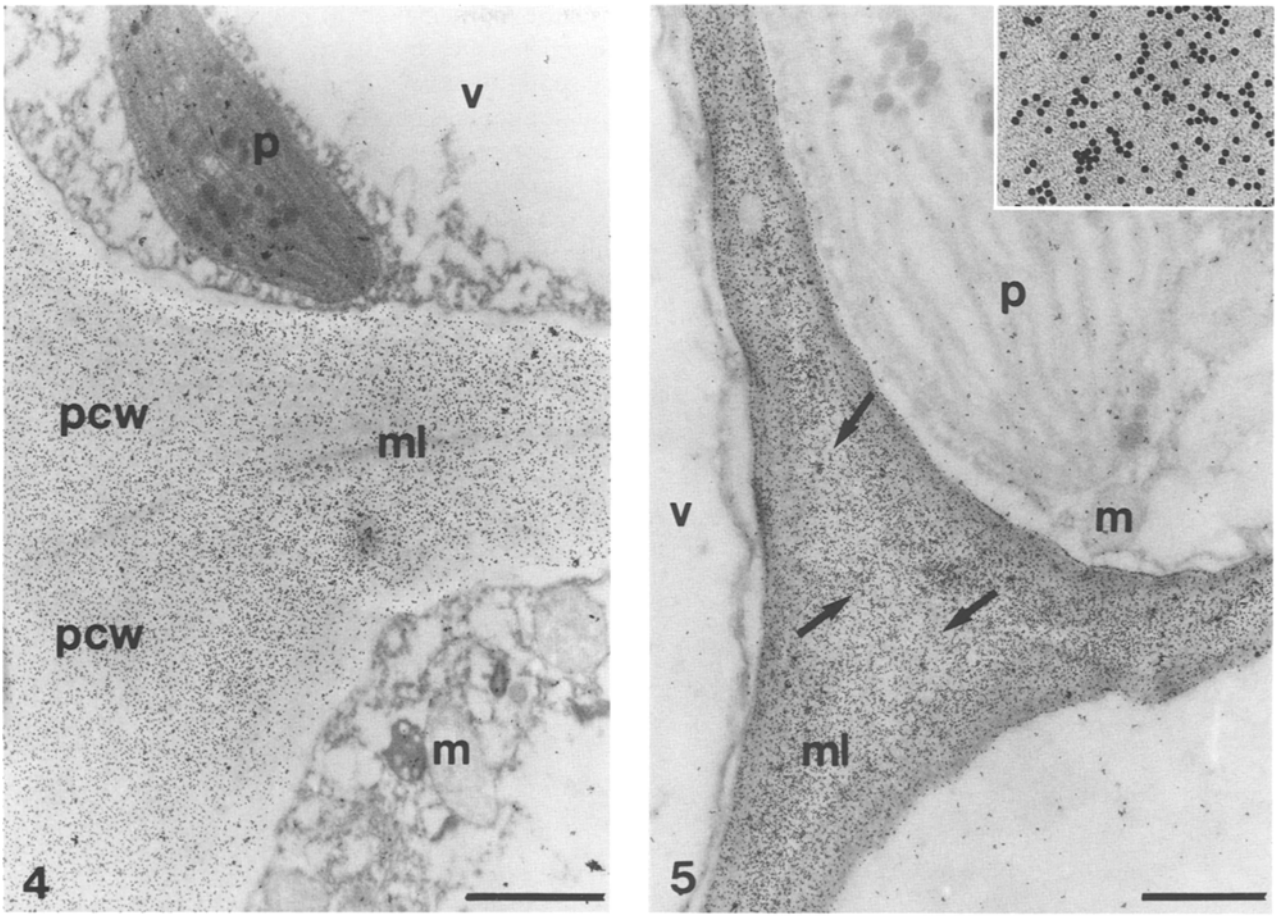
HCl buffer, pH 8.0, at room temperature for 1 h or 6 h (Vreeland et al. 1989); (*iv*) chemical deesterification in 0.1 M Na_2CO_3 at 4 °C for 16 h (Fry 1989); (*v*) chemical methyl-esterification in 1/5 (v/v) acetyl chloride : absolute methanol at 4 °C for 72 h (Vreeland et al. 1989); or (*vi*) chelator treatment in 0.2 M ethylenediaminetetraacetic acid (EDTA), pH 8.0, at 60 °C for 1 h (Wick and Hepler 1980).

Quantitation of the labelling

Quantitative evaluations of the labelling were conducted on all of the tissues that were treated and examined. Briefly, a series of random electron micrographs were recorded on 3 1/4″ × 4″ electron image plates (Eastman Kodak Company, Rochester, NY). After a sufficient number of negatives were obtained (about 15 for each experiment), each negative was placed on a light box. A video camera, which was connected to a Quantimet 970 (Cambridge Instruments, Cambridge, U.K.) image analysis system, was used to digitize the image from the negative. A software program was written (unpublished) that counted the gold particles and correlated their numbers with specific areas in the cell and in the cell wall. For example, the density of labelling was obtained for each negative by correlating the total number of gold particles with each of the different cell wall zones. The results were expressed as the number of particles per µm^2. Comparisons of densities could then be made within or between treatments.

Results

Labelling the anionic sites was only successful with LR White embedded sections. No labelling could be seen on sections from tissue embedded in Spurr's (data not shown), confirming the earlier conclusion that LR White, a hydrophilic resin, is suitable for demonstrating the anionic sites with poly-L-lysine-gold complex (Vorbrodt 1987). Optimal concentrations of the gold-complex were determined by using a dilution series.



Figs. 4 and **5.** In muro visualization of anionic sites. Ultrathin sections of H_2O-treated fruits labelled with poly-L-lysine-gold complexes. *m* Mitochondrion, *ml* middle lamella, *p* plastid, *pcw* primary cell wall, *v* vacuole. Bars: 0.4 µm

Fig. 4. The labelling is distributed throughout the cell wall

Fig. 5. Combination of gold-labelling (poly-L-lysine-gold complex) for anionic sites and the Ag proteinate labelling (PATAg) for polysaccharides. Note the less dense polysaccharide reactivity (electron translucent area) at the separating cell walls (arrows). **Inset** Primary cell wall. The difference between the 10 nm spherical gold particles with high electron opacity and less opaque, fine granular silver proteinate complex is illustrated

Distribution of anionic sites in the H_2O-treated fruit

Intensification of the poly-L-lysine-gold complex with silver enhancement, a procedure that was developed for immunolabelling, was used to accentuate the appearance of the binding sites in sections that were observed with the light microscope. This procedure resulted in the formation of a cloud of reduced silver complex around the gold particles. Differences in the intensity of the brownish precipitate, as observed in the light microscope, indicated differences in labelling intensity.

The pericarp of the fruit was examined and could be differentiated into the following tissues: (a) an epidermis and a thick cuticular covering; (b) three or four layers of small cells that were tightly cemented; and (c) large parenchyma cells that were occasionally separated from one another along a portion of their cell walls thereby forming large intercellular spaces. After silver enhancement, semithin sections exhibited heavy labelling on all cell walls; no apparent localized distribution was observed across the pericarp (Fig. 1). The labelling was restricted to the cell wall; no labelling was observed in the cytoplasm. At higher magnification small areas with higher density could be seen within the cell wall (Fig. 2). Controls performed with poly-L-lysine gold complex after preincubation with unlabelled poly-L-lysine showed greatly reduced labelling (Fig. 3).

The ultrastructural features of the parenchyma cells in apple fruit were similar to those previously described (Ben-Arie et al. 1979). The parenchyma of the fruit was

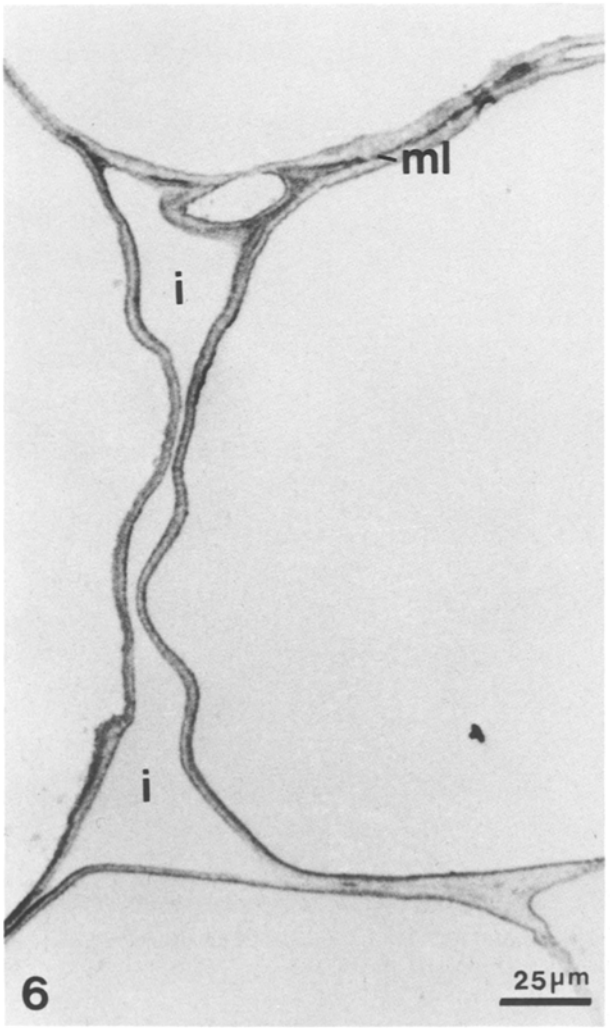


Fig. 6. Localization of anionic sites by the use of cationic colloidal gold. Semithin, silver enhanced-gold sections from calcium-treated fruits. Light microscopic observation shows pericarp cells. A heavy precipitate can be seen along the middle lamella (*ml*) and the region of the primary wall that lies adjacent to the plasmalemma. This precipitate is also found along the wall that opposes the intercellular spaces (*i*). Bar: 25 µm

composed of large isodiametric cells whose cytoplasm was confined to a narrow layer appressed to the cell wall. Most of the volume of the cell was occupied by the large central vacuole. Cross sections of the cell wall showed dense and relatively uniform gold labelling throughout the cell wall (Fig. 4). The cytoplasm was only occasionally labelled, and labelling was only rarely observed in the vacuole. The distribution of labelling in the cell walls that was observed in the electron micrographs was consistent with that found in the photomicrographs.

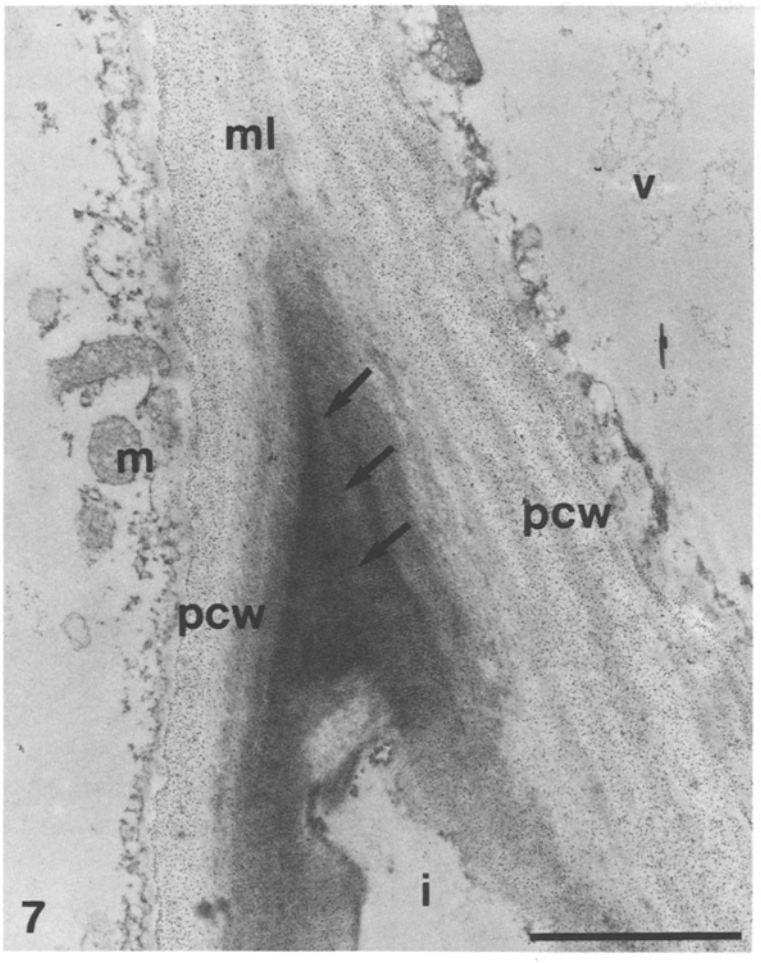
By using cationic colloidal gold labelling to detect the anionic sites in conjunction with the PATAg test to visualize the vicinal-glycol groups of polysaccharides, the anionic sites and the polysaccharides could be visualized in the same sections (Fig. 5, inset). This dual visualization was possible because the 10 nm gold complex appeared as spherical, electron opaque particles; whereas the Ag-proteinate complex formed a very fine granular matrix that appeared less opaque (Roy and Vian 1991). The results indicated that the gold particles were uniformly dispersed across the cell wall. Alternatively the PATAg reaction product was not homogeneously dispersed; at the separating cell walls the middle lamella exhibited less reaction product (Fig. 5). This observation suggests that separation of the middle lamella had begun.

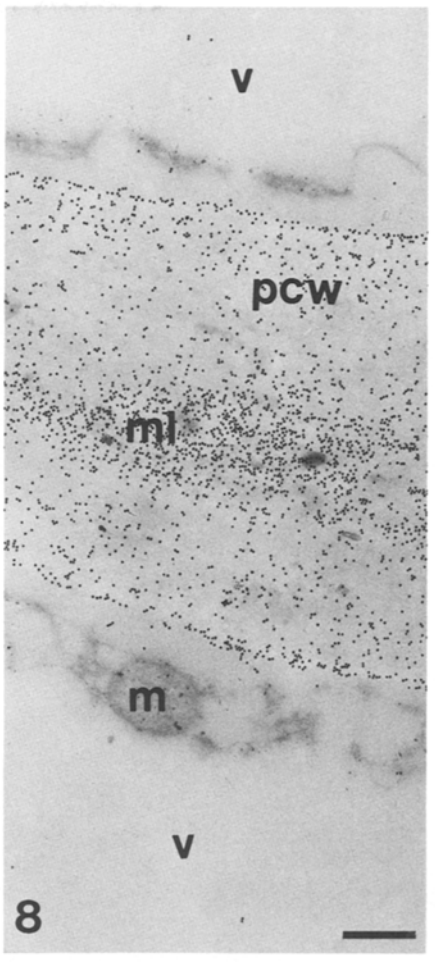
The use of image analysis to quantitate the distribution of gold particles in the parenchyma cells confirmed the electron microscopic observations (Fig. 9). A high concentration of uniformly distributed particles was found in the cell wall (908.33 ± 17.42 gold particles/µm^2); only a low background number was present in the cytoplasm (26.04 ± 1.83 gold particles/µm^2).

Redistribution of the anionic sites in the calcium-treated fruit

After silver intensification of the gold labelled sections, the calcium-treated fruit exhibited heterogeneous labelling in the cell walls. Intense labelling was observed in the layer of the primary cell wall that was adjacent to the plasma membrane and in a layer that corresponded to the middle lamella. When adjacent cells remained cemented, the labelled layer was in the middle lamella; when adjacent cells had separated, the labelled layer, which was the former middle lamella, opposed the intercellular spaces. Only a faint precipitate, which corresponded to the silver intensified gold label, was observed on other parts of the primary cell wall (Fig. 6). These results on the labelling of anionic sites on calcium-treated sections were further confirmed at the ultrastructural level (Figs. 7 and 8). The anionic sites were found along a zone of the cell wall that bordered the plasma membrane and in the intact middle lamella. In addition, a small area of diminished labelling could be seen in the separating walls that form the intercellular spaces (Fig. 7). The associated PATAg test indicated that these regions were rich in polysaccharides (data not shown). In the remaining portions of the primary cell wall, between the middle lamella and that bordering the plasmalemma, the labelling was less dense (Fig. 8).

The impact of calcium infiltration of the apple fruit





Figs. 7 and **8.** In muro visualization of anionic sites. Ultrathin sections of calcium-treated fruits. *i* Intercellular space, *m* mitochondrion, *ml* middle lamella, *pcw* primary cell wall, *v* vacuole. Bars: 0.4 µm

Fig. 7. The arrows denote the decrease in labelling where the middle lamella is separating to form an intercellular space. The intact middle lamella and a layer lining the plasmalemma remain labelled

Fig. 8. Note less dense labelling of the primary cell wall on either side of the middle lamella

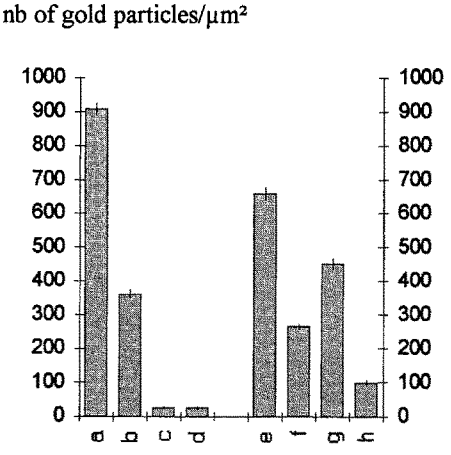
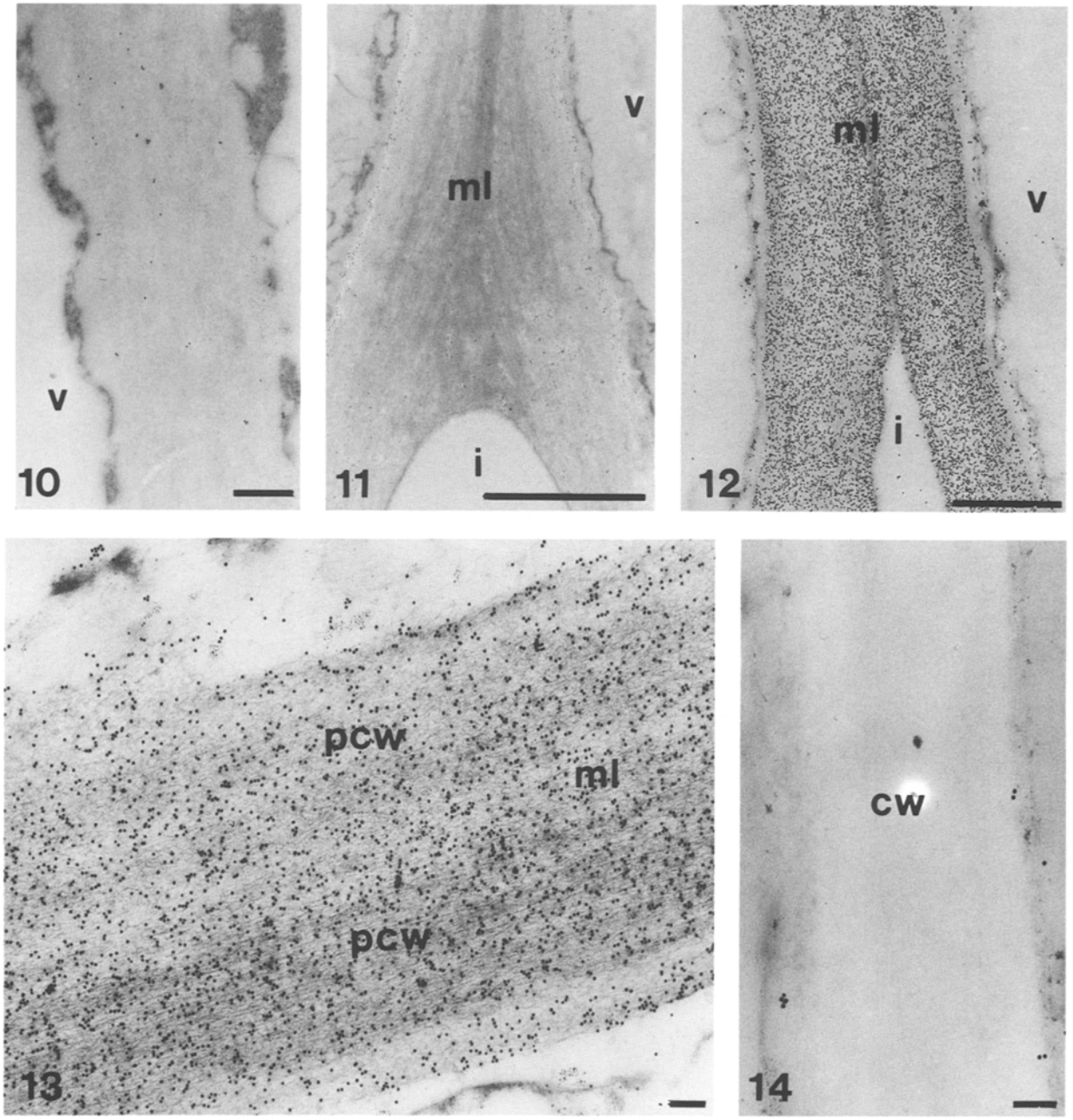


Fig. 9. Quantitative analysis of labelling. Density of labelling obtained with the poly-L-lysine-gold complex over ultrathin sections. *a* Cell wall of H_2O-treated fruit; *b* cell wall of calcium-treated fruit; *c* cytoplasm of H_2O-treated fruit; *d* cytoplasm of calcium-treated fruit. Density of labelling over sections of calcium-treated fruit are shown in *e–h*: *e* middle lamella; *f* primary cell wall; *g* border of the primary cell wall along the plasmalemma; and *h* corner of the intercellular space



Figs. 10–14. Effect of preincubation prior to labelling with cationic colloidal gold probe. Except for Fig. 13, which illustrates the labelling of ultrathin sections of calcium-treated fruits, all the other figures illustrate labelling on ultrathin sections of H_2O-treated fruit. *cw* Cell wall, *i* intercellular space, *ml* middle lamella, *pcw* primary cell wall, *v* vacuole. Bars: 0.4 μm

Fig. 10. Preincubation with unlabelled poly-L-lysine. Labelling is clearly reduced

Fig. 11. Acid hydrolysis results in loss of labelling

Fig. 12. Enzyme treatment with Proteinase K has no effect on labelling

Fig. 13. Chemical deesterification spreads even labelling throughout the primary cell wall

Fig. 14. Chemical methyl-esterification greatly reduces the labelling

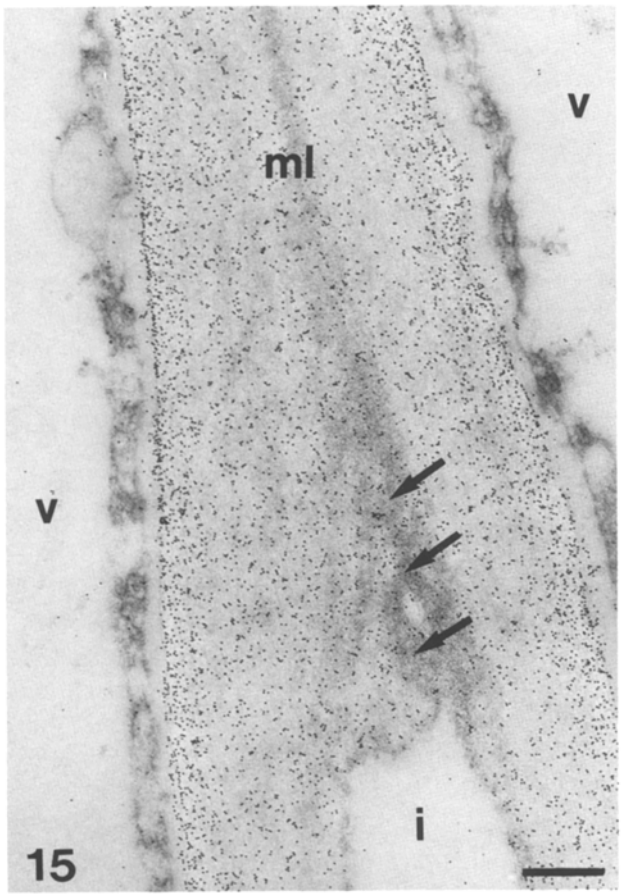


Fig. 15. Effect of calcium-chelator treatment prior to labelling with cationic colloidal gold probe. Ultrathin sections of calcium-treated fruit. Removal of calcium surface of sections results in even labelling throughout the primary cell wall. Arrows denote the restoration of labelling in the middle lamella (*ml*) that is separating to form an intercellular space (*i*). *v* Vacuole. Bar: 0.4 μm

was also illustrated in the image analysis data. Quantitation showed an average of 361.30 ± 14.82 gold particles/μm^2 for the entire cell wall of calcium-treated fruit (Fig. 9), which was less than half of that in the water control. However, within the cell wall, the predominant labelling was along the middle lamella and the narrow band along the plasmalemma. Significantly less labelling was found in the remainder of the primary wall and those small areas of the wall that were separating to form the intercellular spaces.

Influence of preincubation prior to colloidal-gold labelling

To assess labelling specificity, control sections were preincubated with a solution of unlabelled poly-L-lysine. Labelling in these sections was rare (Fig. 10). After acid hydrolysis the labelling was also rare (Fig. 11). To eliminate the possibility that the labelling resulted from gold-complex binding with cell wall proteins, H_2O-treated fruit sections were pretreated with proteinase K; no reduction in the density of labelling was observed in these sections (Fig. 12). When calcium-treated fruit sections were preincubated with sodium carbonate to chemically deesterify the sections, the cationic-gold-complex labelling increased throughout the cell wall (compare Fig. 13 with Fig. 8). Conversely, when sections were treated with acetyl chloride : methanol to chemically methyl-esterify the anionic groups, the labelling was greatly reduced (Fig. 14). This observation confirmed that methyl esterification effectively blocked anionic binding sites in the cell wall. When calcium-treated sections were preincubated with EDTA, a calcium-chelator, the labelling was restored throughout the cell wall (Fig. 15).

Discussion

Application of the technique to plant cell wall

Skutelsky and Roth (1986) introduced the cationic gold probe to demonstrate that anionic binding sites could be visualized in thin sections. Since this technique was described, anionic sites have been demonstrated in the endothelial cells of blood vessels (Vorbrodt 1987, Bush and Allt 1990, Klein et al. 1993), in the large intestine and salivary gland cells (Kashio et al. 1992), and in the glomerula capillary (Goode et al. 1992, Russo et al. 1993). In addition, this technique has been used to characterize glucuronic acid residues of the xylan matrix in the mucilage produced by epithelial cells of quince seeds (Reis et al. 1991), and charged polymers on dissociated cell wall fractions (Reis et al. 1992, Vian et al. 1992).

The major negatively charged groups in cell wall polymers are the COO^- groups of galacturonic acid in non-esterified pectins and glucuronic acid in xylans (Fry 1988). These groups will bind cationic colloidal gold particles. Fry (1988, 1989) and Showalter (1993) showed that glycoproteins such as arabino-galactan proteins could be negatively charged because they had an isoelectric point in the range of pH 2 to 5. The lack of protease action on labelling observed here suggests that the poly-L-lysine gold-complex is specific for the polysaccharide anionic sites. The loss of labelling after chemical methyl-esterification, acid hydrolysis, and preincubation with unlabelled poly-L-lysine, and the extension of labelling after chemical deesterification and after removal of calcium from the surface of the section with an EDTA treatment, confirm that cationic

colloidal gold is a reliable marker for pectic anionic binding sites in the cell wall.

Effective use of this technique to correctly interpret the effect of the calcium on the cell wall depends on the care taken to prevent ion movement during tissue preparation. Our samples were prepared by classical chemical fixation; therefore, loss or relocation of certain mineral ions probably occurred during preparation. However, previous studies have shown that calcium ions in the cell wall are tightly bound and are not easily mobilized. Campbell et al. (1979) showed that calcium ions were left in situ during glutaraldehyde fixation. Labelling of anionic sites appears to be a valid method to visualize the effects of calcium binding in the wall, because no strong chelator is used in the preparation of the sample.

Our intent was to quantitatively evaluate the effects of the calcium treatment. Image analysis was used to quantitate gold labelling. However, Goode et al. (1992) have shown that sensitivity of the gold complex could be altered during storage, mainly due to conformational changes of the poly-L-lysine coating. Therefore, for our comparative studies, all tissues received identical fixation and embedding conditions and the incubations of the different sections were performed simultaneously.

Effect of calcium treatment on the cell wall

The distribution of cationic gold labelling in the cell wall is consistent with the distribution pattern of unesterified pectin that was localized by immunocytochemistry in the cell wall of other tissues (VandenBosch et al. 1989, Bonfante-Fasolo et al. 1990, Knox et al. 1990, Vian and Roland 1991, Roy et al. 1992). The observed differences in the distribution of anionic sites in the water and calcium infiltrated fruits provided evidence for the structural effects of calcium infiltration in the fruit. Because compositional changes in the cell walls of calcium-treated fruits were smaller than those in non-treated fruits (Tobias et al. 1992), the disappearance of labelling did not indicate hydrolysis of pectins but rather a real change in the distribution of anionic sites. Calcium bridging of polysaccharides is thought to be a non-enzymatic process (Fry 1989). The foregoing data are used to visualize the formation of calcium bridges. Mainly two domains appear to be transformed by the calcium: (*i*) the primary cell wall on either side of the middle lamella and (*ii*) small areas of separating wall adjacent to the forming intercellular spaces. Jarvis (1984) suggested that with excess calcium, primary units of two galacturonic acid chains link to form large, possibly sheet-like aggregates. In this arrangement, the additional calcium ions are more weakly bound. The consistent labelling observed in the middle lamella of calcium-treated fruit does not mean that no transformation occurred because the high concentration of anionic sites in the middle lamella could be responsible for steric hindrance. In that case, steric hindrance would indicate that the presence of one gold-complex physically obstructs others from binding to closely located sites (Hayat 1992). However, calcium treatment causes an increase in the strength of the cell wall by the formation of a gel network that limits the formation of intercellular spaces. This phenomenon could be related to the natural development and formation of intercellular spaces. The degree of cell separation is indeed precisely controlled by and characteristic of tissue types (Roberts 1990). In the structural model of the pectin matrix, Carpita and Gibeaut (1993) proposed that if sufficient Ca^{++} is present in the calcium junction zone, some interruption of esterified galacturonic acid can be tolerated. Moreover, Rees et al. (1982) have shown that methyl esterified segments have no effect on gel strength. Methyl esterified pectin segments might also participate in the junction of pectic chains by H-bonding and by hydrophobic binding between methyl groups, creating a mixed-type of gel (Gidley et al. 1980). Using experimental cleavage of ester bonds, we have shown that the labelling can be extended throughout the cell wall, principally in the area where labelling was formerly scarce, i.e., the primary cell wall that is rich in new calcium bridges. These data indicate that the pectic polymers could self-associate even if esterified areas are still present. The observations also confirm the fact that the state of esterification of pectins is spatially regulated (Knox et al. 1990, Roy et al. 1992, Zhang and Staehelin 1992). The relative ease with which the cell wall forms calcium bridges could be explained by the low Ca^{++} concentration usually found in the flesh of apple fruit (Perring and Wilkinson 1965, Gormley 1981). Rapid cell expansion into the pericarp must involve flexibility in the cohesive properties of the middle lamella; therefore, a low extracellular Ca^{++} concentration is maintained (Ferguson 1984). Moreover, this cooperative interaction is possible if free Ca^{++} is relatively high (Braudo et al. 1992).

Effect of calcium treatment on ripening

The time of storage was too short to document differences in the degree of cell wall dissolution in the H_2O

vs. the calcium-treated fruits. Poovaiah et al. (1988) and Glenn and Poovaiah (1990) have shown that after 7 months of storage, calcium treatment had a significant effect on cell wall structure. Fruit softening has been associated with changes in cell wall polymers and could lead to cell separation or to changes in wall elasticity (Brady 1992). A greater degree of calcium binding in pectic polymers may reduce the rate of pectin solubilization (Ferguson 1984). The increase in cell wall strength and the limitations in the formation of intercellular spaces, effects that are attributed to calcium, contribute to the firmness of the fruit. Nevertheless, the secondary aggregation caused by excess calcium ions adds only limited strength to the polygalacturonate gels (Powell et al. 1982), but can restrict access to hydrolytic enzymes (Buescher and Hobson 1982, Conway and Sams 1984). Cleland et al. (1990) have suggested that free calcium may modify the apoplastic pH by movement of protons. The resulting increase in pH could inhibit activity of wall loosening enzymes which possess acidic pH optima.

In the cell wall, the presence of microdomains illustrates the diversity of polymer structure. Changes in the structure of the cell wall occur during autolysis, but subtle modifications in this process may prevent loosening of cell wall microfibrils. Further studies will focus on the location of calcium in muro to help understand how calcium, junction zones and anionic sites of pectin affect the stages of autolysis in the cell wall.

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