Plant Physiol. (1979) 64, 197-202
0032-0889/79/64/0197/06/$00.50/0

Ultrastructural Changes in the Cell Walls of Ripening Apple and Pear Fruit[1]

Received for publication September 6, 1978 and in revised form March 26, 1979

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ABSTRACT

Ultrastructural changes in the cell walls of "Calville de San Sauveur" apples (*Malus sylvestris* Mill) and "Spadona" pear (*Pyrus communis* L.) fruit were followed during ripening. In apple, structural alterations in cell walls became apparent at advanced stages of softening and showed predominantly dissolution of the middle lamella. In pears softening was also associated with the dissolution of the middle lamella, and in addition a gradual disintegration of fibrillar material throughout the cell wall. In fully ripe fruit almost all of the fibrillar arrangement in the cell wall was lost. Application of enzyme solutions containing polygalacturonase and cellulase to tissue discs from firm pear fruit led to ultrastructural changes observed in naturally ripening pears. In apple polygalacturonase alone was sufficient to dissolve the middle lamella region of the cell walls, as was also found to occur in naturally ripening fruit. In both apple and pear the cell wall areas containing plasmodesmata maintained their structural integrity throughout the ripening process. At advanced stages of ripening vesicles appeared in the vicinity of plasmodesmata.

A characteristic of the ripening process common to most fruits is a decrease in fruit firmness. Loss in firmness is shown to be associated with the activity of cell wall-degrading enzymes (9, 16) and it is a reasonable hypothesis that this activity could lead to the chemical and structural alterations in cell walls. In an early microscopic study of cell walls from developing apple fruits, no differences were observed in thickness or structure during storage and softening of the fruit (14). Other studies in pear showed that although in lignified walls of stone cells there was no change during fruit maturation, in overripe fruit the parenchymatous cell walls became extremely thin and tenuous (18). Recent studies in avocado (15) also suggested that in softening fruit cell walls lose their structure. In the present work we extend these studies aimed at: (*a*) using ultrastructural techniques to observe the detailed microscopic changes occurring in the walls of apple and pear, representing slowly, and rapidly softening pome fruit; and (*b*) to ascertain the relationship between the changes in cell walls as occurring naturally in softening fruit to those resulting from the activity of applied cell wall-degrading enzymes.

[1] This research was supported by a grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel. Contributions of the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, 1977 series, No. 225-E, and the Journal Series, New Jersey Agricultural Experiment Station, Cook College, Rutgers—The State University, New Brunswick, New Jersey 08903.

MATERIALS AND METHODS

Plant Material. "Calville de San Sauveur" apples (*Malus sylvestris*, Mill) and "Spadona" pears (*Pyrus communis* L.) of uniform size and color were harvested at the mature stage and allowed to ripen at 20 C. Tissue samples from mature and soft apples and pears were then used for the EM[2] study. Prior to sampling for EM, the firmness of the fruit was determined with a "Hunter" penetrometer, equipped with an 11.1-mm tip for apples or a 7.9-mm tip for pears. The tissue firmness of the mature hard apples was from 6.5 to 5 kg and of soft fruit 3.3 kg. In pears the firmness at the mature hard stage was 7 kg and in soft fruit 2.5 kg.

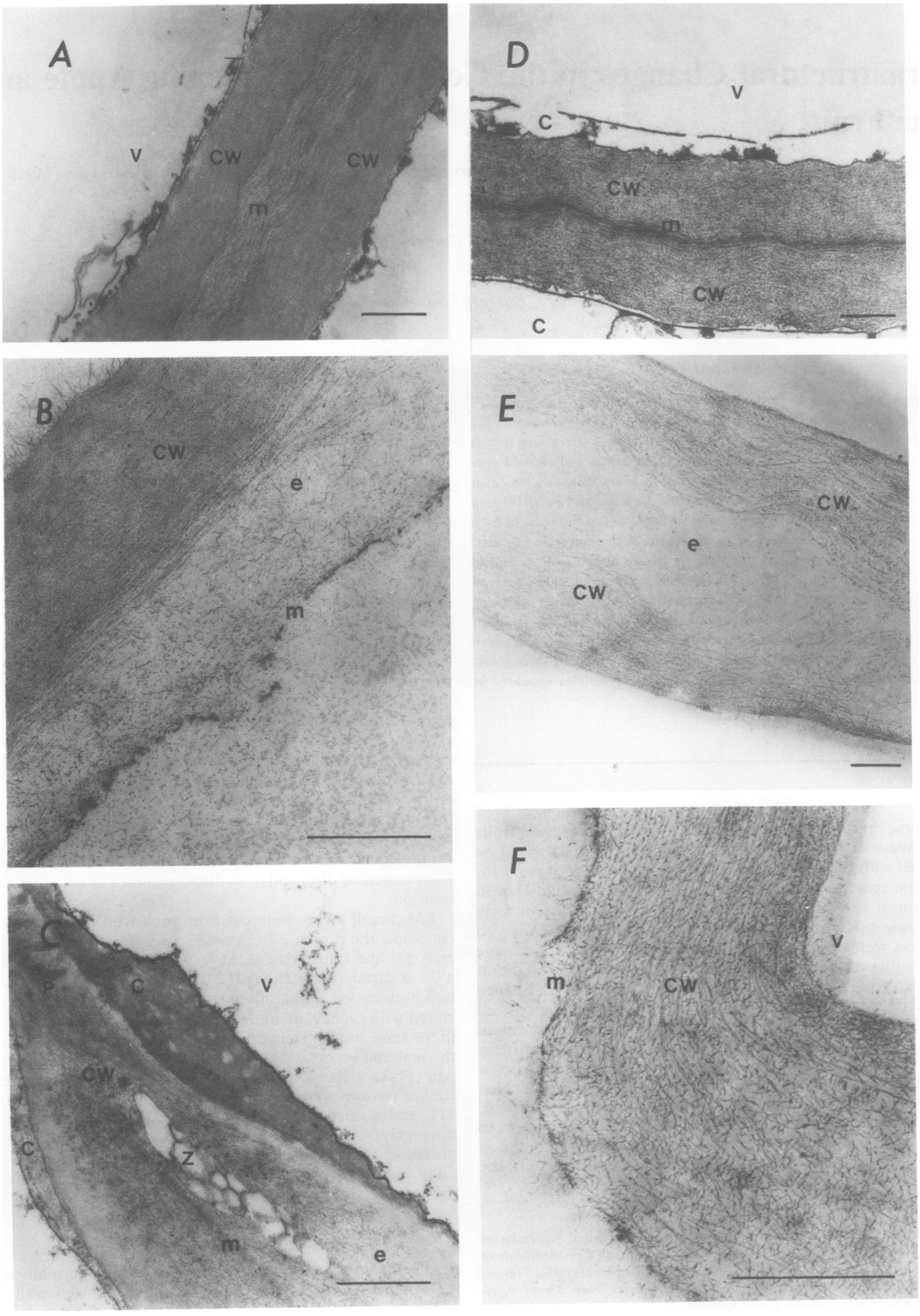
Ultrastructural studies were also made on fruit discs treated with applied enzymes. Small discs, 1-cm diameter, 3 mm thick, from mature hard fruit were incubated for 1 to 2 h in acetate buffer solutions containing commercial enzyme preparations. The enzyme solutions consisted of 25 mg/ml tomato PG (NBC), 16 mg/ml fungal cellulase (Worthington CSE11), or 23 mg/ml pronase (Calbiochem). (The tomato PG had predominantly endo-activity. Since this work was done, Bartley [5] has shown that native PG of apples has exo-activity.) After tomato PG was found to contain a high cellulase activity, it was purified by chromatography using a DEAE-cellulose column, and 0.1 M acetate buffer (pH 5.0) as eluting solution. With this procedure 90% of the cellulase activity was removed. The fungal cellulase preparation contained no PG activity. For control, samples were prepared from untreated discs and from discs incubated in the acetate buffer solution.

EM. Small pieces were cut from each fruit approximately 0.5 cm below the peel and from tissue discs treated with the applied enzymes and transferred to 3.5% (v/v) glutaraldehyde fixative in a 0.1 M cacodylate buffer (pH 7.0). Fixation was carried out for 2 to 3 h under vacuum at room temperature. The fixed pieces were rinsed with cacodylate buffer and postfixed for 2 h with 2% OsO_4 in the same buffer. Dehydration was carried out with ethanol and the material was embedded in either Epon-812 or Epon-Duracapan (Fluka Laboratories, Switzerland) embedding mixtures. Ultrathin sections were cut with glass knives on an LKB Ultratome 111, and stained with uranyl acetate and lead citrate. Electron micrographs were taken with JEM-7A and Phillips-300 electron microscopes. To avoid variation due to tissue processing or handling, fruits of different firmness were treated simultaneously by the same fixation and staining procedures. Three to seven blocks were sectioned from each sample for observation.

RESULTS

The ultrastructural changes in the cell walls of normally softening apples and pears are shown in Figure 1. Cell walls from

[2] Abbreviations: EM: electron microscopy; PG: polygalacturonase.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immature and fully mature apples (Fig. 1A) were similar in structure and both displayed darkly stained tightly packed fibrillar material in the walls (cw) of adjacent cells. The middle lamella (m) region appeared also to contain fibrillar structures. In soft mealy fruit (Fig. 1B) some of the fibrillar material from the outer part of the wall had undergone dissolution and appeared to be dispersed, although the inner part maintained its previous arrangement. The most evident destruction was in the middle lamella area as seen by the disintegration of the remaining middle lamella, and the appearance of empty (e) regions. In these fruit, vesicles of different sizes were often found in walls, especially in the area of the middle lamella of walls near intercellular air spaces (Fig. 1C). Intermediate stages of softening generally displayed a progressive dissolution of the cell wall and the middle lamella (data not shown).

Our observations on pears were mainly on isodiametric parenchymatous and elongated neighboring stone cells. Extreme structural differences in the walls were seen between soft and hard fruit. In mature hard fruit (Fig. 1D) cell walls consisted of tightly packed and darkly stained fibrillar material and a conspicuous middle lamella in between. No differences were observed between cell walls from immature and mature hard fruits. Soft fruits (Fig. 1, E and F) showed sharply reduced staining of walls in most cells and sparse appearance of fibrils. The amount of fibrillar material between neighboring cell walls, the region previously occupied by the middle lamella, was also reduced and appeared empty (Fig. 1E). Between these outlined extremes intermediate phases could be found in softening fruit with reduced stainability and fewer fibrils (data not shown).

The ultrastructural changes in cell walls of tissue discs following incubation with applied cell wall-degrading enzyme solutions are shown in Figure 2. Tissue discs from mature hard fruit incubated in acetate buffer (control) had the same appearance as untreated tissue taken from fruits of the same firmness as shown in Figure 1, A and D. Pronase digestion did not change this appearance (data not shown). Incubation with PG resulted in slight dissolution chiefly of the areas between adjacent cell walls in both apples and pears (Fig. 2, A, and B, respectively). In pears (Fig. 2B) some of the middle lamella was still visible, but had undergone degradation and formation of empty regions in the remainder of the wall. The changes were comparable to those occurring naturally (Fig. 1, B and E), and appeared to be similar in both types of tissues. The results of cellulase digestion differed, however, from the pectinase action. In apple tissue, applied cellulase markedly reduced the amount of the fibrillar material between neighboring cell walls and also in the outer parts of walls (Fig. 2C). Some of the remaining fibrils in walls appeared as long dissociated threads, yet the inner part of the wall, next to the cell content, was unaffected and appeared tightly packed and darkly stained. More drastic destruction was often seen with complete dissolution of wall material even in the inner part of the wall, and with no clearly defined structure remaining. Mature hard pear tissue, after incubation with applied cellulase (Fig. 2D) also displayed fibrils in a state of dispersal in the outer region of adjoining cell walls, and as in apples, tightly arranged and darkly stained material next to the inner part of the walls. Other samples (Fig. 2E) showed that as in apple the cell wall digestion could be quite progressive with only thin areas, in the inner walls appearing intact. In both apple (Fig. 2C) and pear (Fig. 2, D and E) the action of the cellulase appeared to dissolve the middle lamella only in part, although the region of the latter appeared to have been largely dissolved. When cellulase and PG were applied jointly to pear (Fig. 2F) there was dissolution of both the middle lamella itself, and the wall material next to it. In addition, walls lost their intense stainability and fibrillar material appeared to be dissolving. These walls had an appearance of the combined enzyme action (Fig. 2, B and D), and resembled those in naturally softening pears (Fig. 1E).

A plasmodesmata-wall complex was often observed in the cell walls of immature hard apples and pears, showing a darker staining and a denser fibrillar structure than in the surrounding cell walls (Fig. 3A). The difference between the plasmodesmata-wall complex and the surrounding cell wall became more obvious when the fruit softened, as the former did not appear to undergo any significant structural changes, whereas the middle lamella and the primary cell wall underwent degradation as shown by reduced stainability and appearance of empty regions (Fig. 3B). Fairly large vesicles (z) which occurred generally in ripe fruit (Fig. 1C) were often observed in the vicinity of plasmodesmata. The cross-section of the plasmodesmata complex (Fig. 3C) shows the vesicles in close proximity and suggests that vesicles are part of this complex. Application of PG and cellulase to mature hard pear tissue (Fig. 3D) led to the dissolution of the middle lamella and of fibrillar material in the cell wall, but in spite of the extensive degradation no structural changes were observed in the plasmodesmata-wall complex, as was also observed in the walls of naturally softening fruit (3B). It appeared as if the plasmodesmata remained the point of connection between the cells, holding them together as cell walls lost their structure and disintegrated, either naturally or as a result of the digestion by the applied enzymes.

DISCUSSION

Albersheim *et al.* (2) presented evidence that pectic substances are the main component of the middle lamella, but can also be found in the primary walls and along the interface of young cell walls and the plasma membranes. Fibrillar material of the cell wall is generally considered to be cellulose (1) but pectic substances have also been found to have a fibrillar structure in the region of the middle lamella (8, 11).

The EM examination cannot readily discern between the chemical composition of the various cell wall polysaccharides and it is difficult to ascertain which of the wall polymers undergo degradation in normally softening fruit. For that reason we applied enzymes to dissolve polysaccharides in the cell walls of excised tissues selectively and to compare the changes with those occurring in normally softening fruit. Use of externally applied PG and cellulase had a widely different effect on the cell walls of the two tissues. In apples, PG treatment, without cellulase activity (Fig. 2A) resulted in similar, though somewhat more extensive dissolution of the middle lamella than that occurring in soft, mealy fruit (Fig. 1B). Treatment with both enzyme activities caused cell wall disintegration far in excess of that occurring naturally even in overripe fruit (data not shown). A similar effect has been described by Reis and Roland (17) with cellulase and pectinase

FIG. 1. Ultrastructure of cell wall of apple (A, B, C), and pear fruit (D, E, F), at different stages of ripening. A: walls (cw) of adjacent cells from mature hard apple showing tightly packed and darkly stained material, and fibrillar middle lamella (m). B: cell wall (cw) from soft apple showing breakdown and dispersal of fibrils, formation of empty (e) regions, and the middle lamella (m) as it disintegrates. C: cell wall (cw) from fruit in B showing darkly stained plasmodesmata (p) region, and appearance of vesicles (z). Other areas (e) are lightly stained and appear to undergo breakdown. D: walls of adjacent cells from mature hard pear showing darkly stained and tightly packed fibrils in the cell walls (cw) and the middle lamella (m). E: walls (cw) from adjacent cell in soft pear showing loss of stainability, fibrils in a state of dispersal, and formation of empty (e) areas in the middle lamella region. F: detail of wall from fruit in E, showing lightly stained cell walls (cw) and disintegrating middle lamella (m). Fibrillar material is in a state of disorganization and short fibrils appear to be attached to long ones. Also shown: c: cytoplasm; v: vacuole. A (× 22,000); B (× 44,000); C (× 33,000); D (× 22,000); E (× 20,000); F (× 67,500); bar = 0.5 μm.

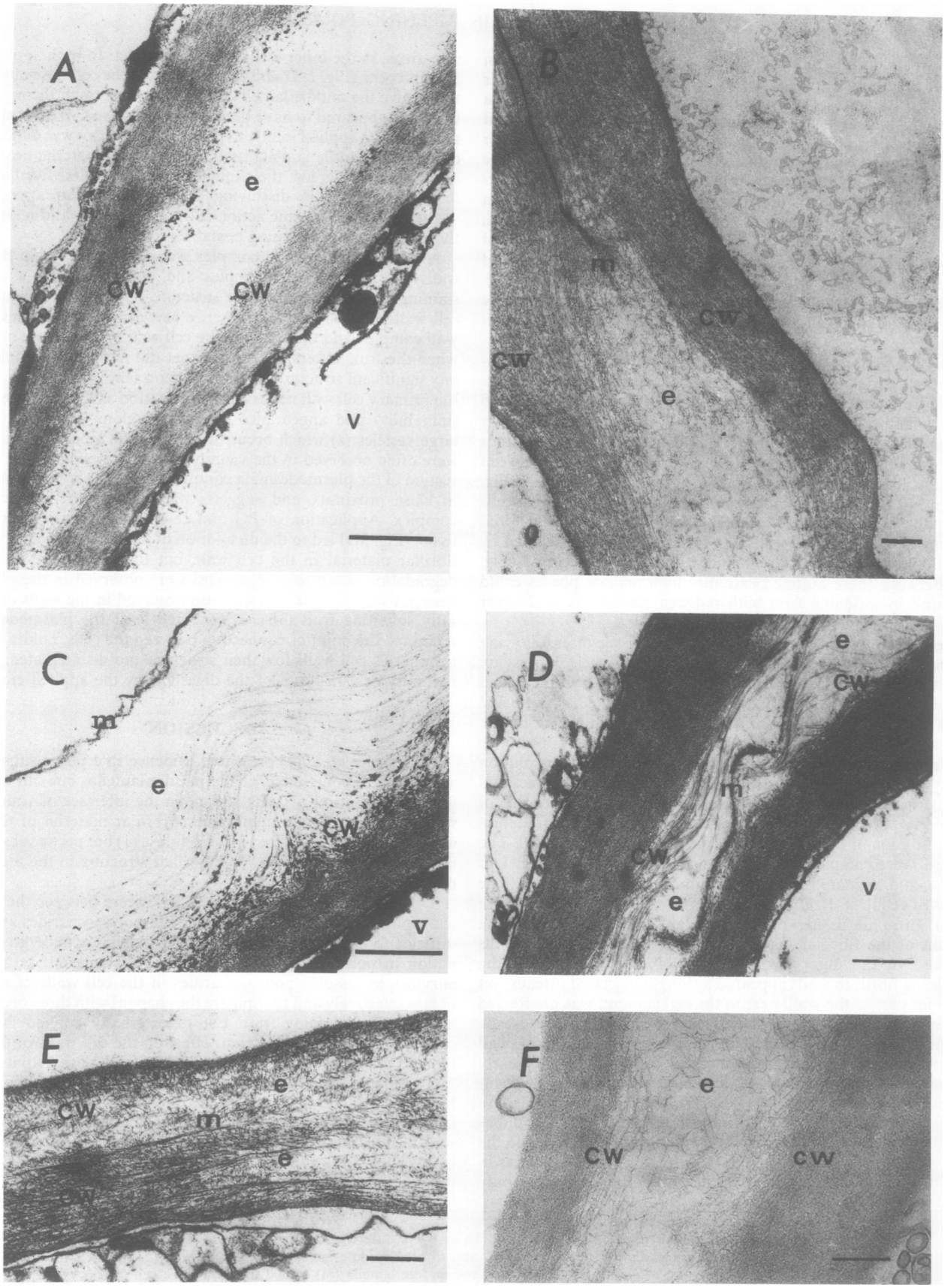

FIG. 2. Effect of applied PG and cellulase on cell walls in tissue discs from mature apple and pear fruit. A (apple), B (pear): in both tissues applied PG led to the destruction of the middle lamella (m) and appearance of empty (e) areas. In pears some of the fibrillar material in the outer cell walls (cw) was also digested. C (apple), D, E (pear): in apple applied cellulase caused extensive dissolution of fibrillar material (e) on the outer side of the cell wall (cw). Similar, but lesser effect is shown in pear as fibrillar material is seen dispersing (e) along the outer side of adjoining cell walls in D or throughout the cell wall in E. Middle lamella (m) is still visible. F: adjacent cell walls (cw) in pear after incubation with PG and cellulase. Middle lamella region appears empty and fibrillar material from cell walls (cw) is dispersing (e). A (× 30,000); B (× 15,000); C (× 40,000); D (× 22,000); E, F (× 20,000); bar = 0.5 μm.

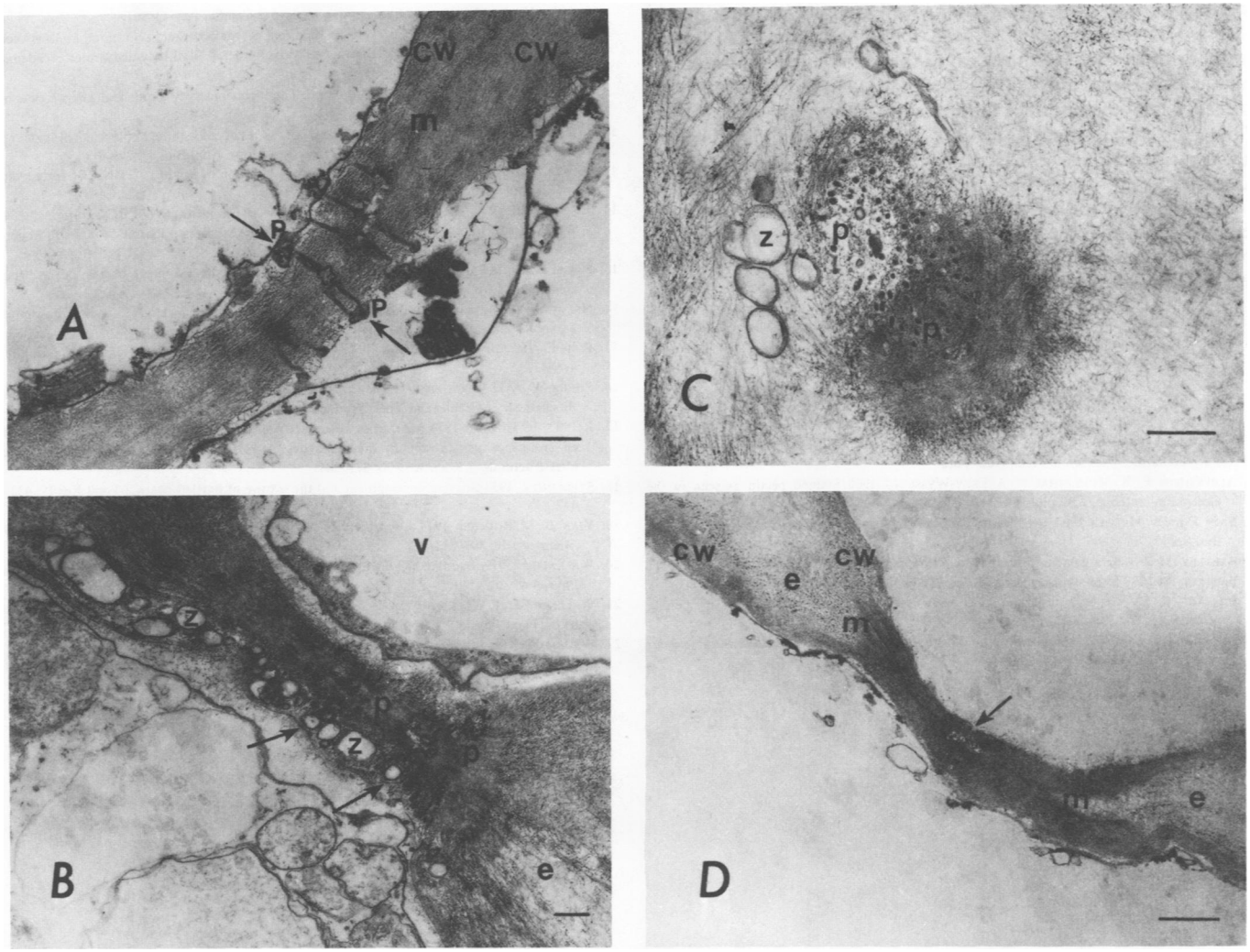

FIG. 3. Plasmodesmata area in cell walls of softening pear tissue. A: mature hard pears. Plasmodesmata (p) appears in darkly stained areas (arrows). Cell wall (cw), and middle lamella (m) appear to be intact. B, C: detailed cross-section through plasmodesmata area in softening fruit. Plasmodesmata (p), represented by small dark dots, is surrounded by a darkly stained area containing tightly packed fibrils. Vesicles (z) are adjacent to the plasmodesmata and appear to be connected to membranes (arrows) in B. D: plasmodesmata complex following digestion of the cell wall with cellulase. Disappearance of middle lamella (m) and cell wall (cw) is seen as "empty" (e) areas, whereas the plasmodesmata region (arrow) appears to have resisted the digestion of the cell wall. A (× 20,000); B (× 10,000); C (× 20,000); D (× 16,500); bar = 0.5 μm.

treatment of growing vegetative tissues. In pears, however, both the fibrils in the middle lamella and the cell walls became sparse as the fruit softened (Fig. 1, E and F) presumably due to the combined action of pectolitic enzymes and cellulase. This view is supported by the results showing the similarity in the ultrastructure of cell walls from normally softening pears (Fig. 1E) and cell walls from firm tissue discs treated with PG and cellulase (Fig. 2F). In apples the loss in lamellation and density of cell wall fibrils during softening was much less obvious than in pear tissues, but it was observed frequently in the walls from soft fruit. Generally, dispersal of cell wall material was encountered in the middle lamella region of ripe apples. It is suggested that whereas PG is likely to be involved in cell wall degradation in both apple and pear, cellulase might have a role in cell wall degradation only in softening pears. Although cellulase activity has been found in a number of ripening fruits (9) including pears (6) its presence in apples has not yet been demonstrated. PG activity has been previously detected in ripe pears (13, 22) and was shown to increase during pear ripening (6). Recently, the presence of an exo-PG in apples has been established by us (unpublished data) and other workers (5).

Although the action of PG and cellulase appears to be involved in cell wall degradation in some ripening fruits, the probable involvement of other hydrolytic enzymes cannot be excluded. Charvat and Esau (7) showed the presence of acid phosphatase in the middle lamella, and in xylem elements along the primary cell wall undergoing lignification. Likewise, the increase in β-galactosidase activity in ripening tomatoes (21) and apples (4) is a further indication of their possible contribution to the cell wall modifications in softening fruit. The clearly disorganized and sparse structure throughout the cell wall of soft pear fruit indicates that other polysaccharides are likely to be degraded simultaneously as was also suggested by Wallner and Bloom (20).

Characteristic of middle lamella dissolution in ripe fruit was the frequent occurrence of vesicles appearing usually in the area close to intercellular spaces. Vesicles were usually observed in areas adjacent to the plasmodesmata-wall complex (Fig. 3). A similar "reticulate structure," but in larger quantities, was described in apples by Fuller (10), who concluded that this was a symptom of cell wall breakdown as they seem to appear after the cell wall digestion by PG. They might, therefore, be extensions of the protoplast into space vacated by degraded cell wall material. Their role is a matter of speculation.

The dark stainability of the cell wall regions surrounding plasmodesmata and their persistence in cell walls undergoing degradation in normally softening fruit, or tissues treated with cell wall-

degrading enzymes, suggest that the wall material surrounding the plasmodesmata is resistant to the action of pectinase and cellulase. Similar observations with regard to the persistence of plasmodesmata in abscission layers (12, 17, 19) has led to the suggestion that the cell wall at the interface is apparently different in composition. The persistence of the cell wall-plasmodesmata complex throughout the ripening process is similar to that of other organelles, notably the mitochondria which also persist in senescing fruit tissue (3). As in the latter the cell wall-plasmodesmata complex remains functional throughout the senescence process, apparently to support the extensive metabolic processes which occur in the cell walls of senescing fruit tissues.

Acknowledgments—We thank Mrs. M. Zeidman for assistance in preparations for EM. The provision for EM facilities by Dr. V. Ben-Gayat is appreciated.

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