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Localization of the Ethylene-synthesizing System in Apple Tissue[1]

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ABSTRACT

Apple (*Malus sp.*) slices gradually lost the ability to synthesize ethylene when incubated with a mixture of enzymes that digest cell walls. The released protoplasts did not produce ethylene. The release of protoplasts was faster from climacteric fruit slices than from preclimacteric tissue. In protoplast suspension culture, as new cell wall was deposited (as judged by the intensity of fluorescence of regenerating protoplasts stained with Calcofluor White and the incorporation of labeled *myo*-inositol into their ethanol-insoluble residue), ethylene synthesis was gradually regained. Restored ethylene synthesis reached a maximum after 80 hours in protoplasts from preclimacteric fruit and in 120 hours in those from climacteric tissue. Addition of methionine (1 mM) to the culture medium was essential for appreciable synthesis of ethylene; and this synthesis was inhibited by the aminoethoxy analogue of rhizobitoxine and by propyl gallate, inhibitors of ethylene synthesis in higher plants. We suggest that the ethylene-synthesizing enzyme system is highly structured in the apple cell and is localized in a cell wall-cell membrane complex.

The role of ethylene as a plant hormone regulating many aspects of growth and development has attracted considerable attention in recent years (16). With the discovery of methionine as the immediate precursor of ethylene in higher plants (17), it was hoped that an enzyme system converting methionine to ethylene would be isolated. So far, however, no such system has been demonstrated *in vitro*. Apparently this is due to something unique about the ethylene-synthesizing system *in vivo*, perhaps its structure, location, or mode of action. We recently reported (21) that the ethylene-synthesizing system in higher plants is influenced by its lipid environment and appears to be localized in the plasma membrane.

Since the enzymic ethylene-synthesizing system could not survive the destruction of the cell, we thought that intact protoplasts might be useful for the study of ethylene biosynthesis. We therefore undertook to study the relation of the ethylene-synthesizing system of apple tissue to cell wall degradation and regeneration. From data presented we suggest that the enzymic system converting methionine to ethylene is highly structured and located in the cell membrane-cell wall complex of a higher plant cell.

MATERIALS AND METHODS

Apple Slices. Preclimacteric or climacteric apple (*Malus sp.* cv. Delicious) fruits were surface-sterilized with 70% ethanol and then washed with sterile water. Slices (9 mm in diameter, 2 mm thick) cut with a meat-slicing machine and a corkborer were preincubated in a sterile solution of 0.6 M sorbitol (pH 5.8), chloramphenicol (100 μg/ml), and fungizone (0.5 μg/ml; Flow Labs., Rockville, Md.) for 30 min at 25 C. The presence of chloramphenicol and fungizone at the concentrations indicated did not alter ethylene production by fruit slices.

Enzymes. Pectinase (Calbiochem) and rhozyme HP-150 concentrate (Rohm and Haas, Philadelpha, Pa.) were desalted (10) on a Sephadex G-25 column (5 × 60 cm) equilibrated with deionized H_2O containing an antiprotease inhibitor, trasylol (200 KIU[3]/ml, Mobay Chemical Co., New York), and chloramphenicol (10 μg/ml). Cellulase (Calbiochem) was used without any pretreatment. Enzyme mixtures were sterilized by Millipore filtration.

Radioactive Compound. 2-^{3}H-*myo*-Inositol, nominally labeled, was obtained from New England Nuclear.

Preparation of Protoplasts and Their Regeneration. The solutions used were sterilized either by autoclaving at 15 p.s.i. for 15 min or by filtration through 0.45-μm Millipore filters. Glassware was autoclaved at 21 p.s.i. for 20 min. Preincubated apple slices were immersed in a lytic enzyme solution (5 ml/g of fresh tissue) of sorbitol (0.6 M), pectinase (0.2%), cellulase (0.5%), rhozyme HP-150 concentrate (0.5%), chloramphenicol (50 μg/ml), and fungizone (0.5 μg/ml) adjusted to pH 5.8. Control slices were immersed in a solution of sorbitol-chloramphenicol-fungizone only. Twenty-five-ml Erlenmeyer flasks containing the slices were incubated at 30 C on a shaker (shaking speed 42 strokes/min) until microscopic examination indicated the presence of protoplasts in the solution. The enzyme-protoplast mixture was passed through a sterile filter screen of stainless steel (516 μm), and the filtrate passed through another filter screen (146 μm). The second filtrate was saved (protoplast suspension A). Moist unfiltered protoplasts from the second step were gently transferred with a Pasteur pipette to the bottom of a graduated 100-ml cylinder containing the wash medium (80 ml), which consisted of 0.6 M mannitol, 0.06 M sucrose, 9 mM Ca^{2+}, chloramphenicol (100 μg/ml), and fungizone (0.5 μg/ml). The protoplasts floated to the surface of the solution and were collected and added to the protoplast suspension A. The suspension was centrifuged at 500*g* for 3 min. The supernatant was decanted, and the residual protoplasts were washed four times with 15-ml portions of wash medium and once with the culture medium (Table I).

For regeneration, the washed protoplasts were resuspended

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[3] Abbreviations: KIU: kallikrein inactivator units; $EDTANa_2$: disodium ethylenediaminetetraacetate; EIS: ethanol-insoluble.

Table I. Composition of the medium for culturing apple protoplasts

The culture medium is a modification of various media described in Ref. 10.

Compound	mg/l
B 5 - Mineral salts plus	(reference 9,10)
Inositol	100.0
Nicotinic acid	1.0
Pyridoxine·HCl	1.0
Thiamine·HCl	10.0
2,4-D	0.1
NAA	1.0
Benzyladenine	0.5
N-Z amine[a]	250.0
Glucose	30000.0
Mannitol	100000.0
pH	5.8

[a] Sheffield Chemical, Norwich, N.Y.

in a known volume of the culture medium, and 1-ml aliquots distributed into a series of 10-ml Erlenmeyer flasks plugged with cotton. The flasks were incubated at 28 C. At 60-hr intervals during incubation, fresh liquid culture medium (0.5 ml) was added to the flasks.

Ethylene Determination. The flasks were flushed with air and capped with serum caps for various incubation periods; then the atmosphere above the liquid was sampled by gas-tight hypodermic syringes. Ethylene was determined by gas chromatography (17). Triplicate samples of each treatment were analyzed.

Isotope Incorporation Studies. The incorporation of ^{3}H-*myo*-inositol into cell wall components was studied (30) by two types of experiments. In one type, 2 μCi (13.1 Ci/mmol) of the labeled compound was added to each ml of protoplast suspension at the start of culture. Daily 500-μl aliquots were withdrawn for assay of radioactivity in the ethanol-insoluble (EIS) fraction. In the second, or pulse type of experiment (results in Fig. 4 signified by triangles), the labeled compound was added (2 μCi/ml) to cultures that were incubating for different periods, from 24 to 150 hr. Six hr after the addition of the label on each day, aliquots were removed for assay. Each aliquot was ground in 80% (v/v) ethanol in a glass homogenizer and filtered on Whatman glass fiber filters (2.4 cm). To remove all traces of soluble ^{3}H, the EIS residue was washed several times on the filter with fresh portions of 80% ethanol, with several portions of 100% ethanol, and with ether; then it was dried in a hot air oven at 75 C. The dried residue along with the glass fiber filter was then placed in glass scintillation vial with 5 ml of Liquifluor (New England Nuclear). Radioactivity was determined in a Packard liquid scintillation spectrometer.

Radioactivity incorporated into pectic constituents was released by incubating the dried EIS material with 0.2% (w/v) pectinase and 0.1% (w/v) disodium EDTA (30) in 2(-N-morpholino)ethanesulfonic acid buffer (pH 5) at 30 C. After 16 hr, 3 volumes of 100% ethanol were added, and the residue washed twice with 100% ethanol. The supernatants were combined in a scintillation vial and evaporated to dryness. After addition of 5 ml of Liquifluor, radioactivity was determined in the Packard liquid scintillation spectrometer.

Fluorescence Microscopy and Spectrometry of Regenerated Cell Wall. Regenerated cell walls on protoplasts were visualized according to Nagata and Takebe (26). The cultured protoplasts were stained with 0.5% Calcofluor White M2R New (American Cyanamid Co., N.J.) for 5 min, washed five times with deionized water, centrifuged at 500*g* for 10 min, and the pellet taken up in 0.25 M sucrose. Aliquots were mounted on glass slides, and fluorescence was monitored under a Leitz microscope fitted with a UV source. Fluorescence was quantitated in an Aminco fluoro-colorimeter connected to a voltage frequency converted (Vido 260) with a digital counter (Data Precision). Corning No. 7.51 was used as primary filter and Wratton No. 4 as secondary filter.

Measurement of Protein Content and Dry Weight. Protein precipitated from protoplast samples with 10% trichloroacetic acid was determined fluorimetrically with fluorescamine (5). For dry weight determination, protoplasts were extracted with 70% ethanol (v/v), and the EIS residue was washed with 100% ethanol, with ether and then dried to a constant weight.

Experiments were repeated at least three times, and results presented are typical representatives. The trends or relative differences between treatments were consistently the same from experiment to experiment but the absolute values varied since apple fruits change markedly in their ability to produce ethylene on aging.

RESULTS

Loss of Ethylene-synthesizing Capability in Relation to Release of Protoplasts from Apple Tissue. Upon incubation of fruit slices with the lytic enzyme solution, apple cells gradually lost their cell walls, and protoplasts were released (Fig. 1). The rate of cell wall digestion depended upon the stage of ripeness of the fruit. Cell walls of preclimacteric fruit required 7 to 8 hr and those from climacteric and postclimacteric fruits only 4 to 5 hr for complete digestion. The dimensions of the released apple cells ranged from (long side × middle) 210 × 168 to 308 × 168 μm and of isolated protoplasts (oblong to spherical) from 140 × 105 to 168 × 118 μm. On staining with Calcofluor White, the dye that stains polysaccharides (26), apple cells fluoresced intensely whereas no fluorescence was detected in freshly isolated protoplasts lacking cell walls.

As apple slices were converted into protoplasts, with increased time in the cell wall-digesting enzyme solution, a considerable decrease occurred in the rate of ethylene production (Fig. 2). Ethylene-forming ability was lost at a faster rate from climacteric than from preclimacteric fruit slices; and these rates were likely related to those at which the protoplasts were released from the cells. Inclusion of methionine in the protoplast-releasing enzyme mixture delayed but did not prevent the decrease in the rate of ethylene production (Fig. 2). Apple cells and cell aggregates incubated in cellulase alone retained their ethylene-synthesizing capability.

Restoration of Ethylene Synthesis in Regenerating Protoplasts as Related to Cell Wall Formation. In osmotically stabilized culture medium (Table I), protoplasts which ranged in size from 176 to 224 μm by 20 hr, increased to 250 to 300 μm by 120 hr. In a 72-hr growth period deposition of cell wall material on the surface of protoplasts increased (Fig. 3). The intensity of fluorescence, after staining with Calcofluor White, increased markedly between 48 and 72 hr, reaching a maximum at 120 hr (Fig. 4a). Fluorescence was greater in protoplasts incubated with methionine than without.

The fluorescence data (Fig. 4a) corresponded closely with the data on incorporation of labeled *myo*-inositol into the EIS residue of the regenerating protoplasts (Fig. 4b). From 50 to 60% of the radioactivity in this residue was released into solution by treatment with pectinase. The pulse experiment showed that the incorporation of label from *myo*-inositol into cell wall polysaccharides increased linearly with time (Fig. 4b).

As new cell wall material was deposited on the surface of the cultured protoplast, ethylene synthesis was gradually regained; it reached a maximum after an incubation period of 80 hr in protoplasts from preclimacteric fruit (Fig. 5a) and of 120 hr in those from climacteric fruit (Fig. 5b). In protoplasts from climacteric tissue methionine was essential for ethylene synthesis; its effect, however, was not noticeable until after at least 48 hr of protoplast regeneration (Fig. 5b). In protoplasts from preclimacteric tissue (without methionine) ethylene production increased slightly after 72 hr but was considerably enhanced by exogenously added methionine (Fig. 5a).

Inhibition of Methionine-dependent Ethylene Synthesis in

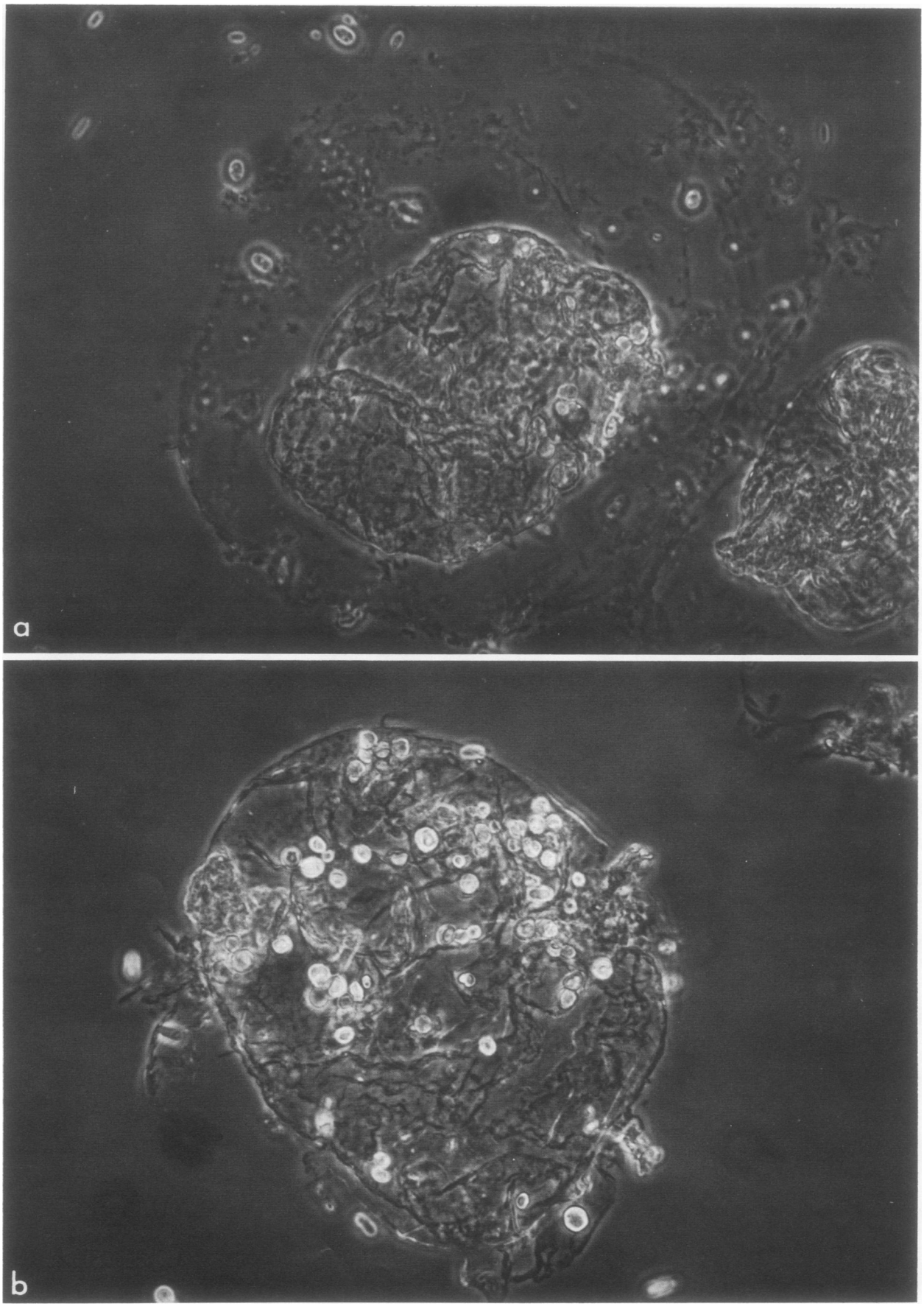

FIG. 1. a: Light micrograph (× 80) of a preclimacteric apple cell after 3 hr of incubation with the lytic enzyme mixture; b: light micrograph (× 80) of isolated protoplast from preclimacteric apple cell.

Regenerated Protoplasts by Pronase and Specific Inhibitors. The newly regained ethylene-synthesizing ability in cultured protoplasts was inhibited strongly by the aminoethoxy analogue of rhizobitoxine (3, 18) and by propyl gallate (3), inhibitors of ethylene synthesis in higher plants, and was sensitive to proteolytic digestion (Table II).

Influence of Changing Osmolarity of Incubating Medium on Ethylene Production by Apple Slices. Rate of ethylene production decreased markedly when apple slices were incubated in either water or tris-buffer (Table III). Between 0.6 and 0.9 M, sorbitol prevented the decline in ethylene production and instead, the rate of production continued linearly. At concentrations below 0.6 M and above 0.9 M sorbitol, the rate of decline was not as drastic as in either water or tris buffer (Table III). The damage caused by incubation of the apple slices for 3 hr in either water or tris-buffer or 0.2 to 0.4 M sorbitol was found irreversible (data not shown). Microscopic examination of tissue slices so incubated showed swelling of many cells and quite an appreciable number had burst open.

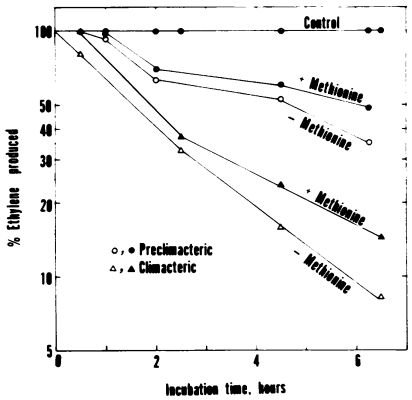

FIG. 2. Loss of ethylene synthesis in apple slices from preclimacteric and climacteric fruits incubated in a mixture of cell wall-digesting enzymes. Ethylene synthesis of controls incubated without the lytic enzyme mixture was taken as 100%. When indicated, methionine concentration was 1 mM.

DISCUSSION

Our study showed (*a*) the loss of ethylene production in apple slices incubated in an enzyme mixture that specifically hydrolyzes cell walls; (*b*) the recovery of ethylene production in protoplasts regenerating cell wall in the presence of methionine; and brought to light the possibility of a highly structured ethylene-synthesizing enzyme system localized in a cell wall-cell membrane complex of the apple cell.

The susceptiblity of the ethylene-synthesizing machinery to cell wall digestion suggests that this enzyme system is linked to "essential" polygalacturonic (and perhaps hemicellulosic) units of the cell wall and that the cell wall as a whole may provide a "specific" structural matrix for the enzyme complex. Additionally, the lipid matrix of the cell membrane seems essential for maintaining the structural integrity of the ethylene-synthesizing enzyme in higher plants, since detergents like Triton X-100 influence markedly the temperature-activity relationship for the enzyme system (21). We believe that one major cause of the failure to isolate an active, *in vitro* enzymic system that can

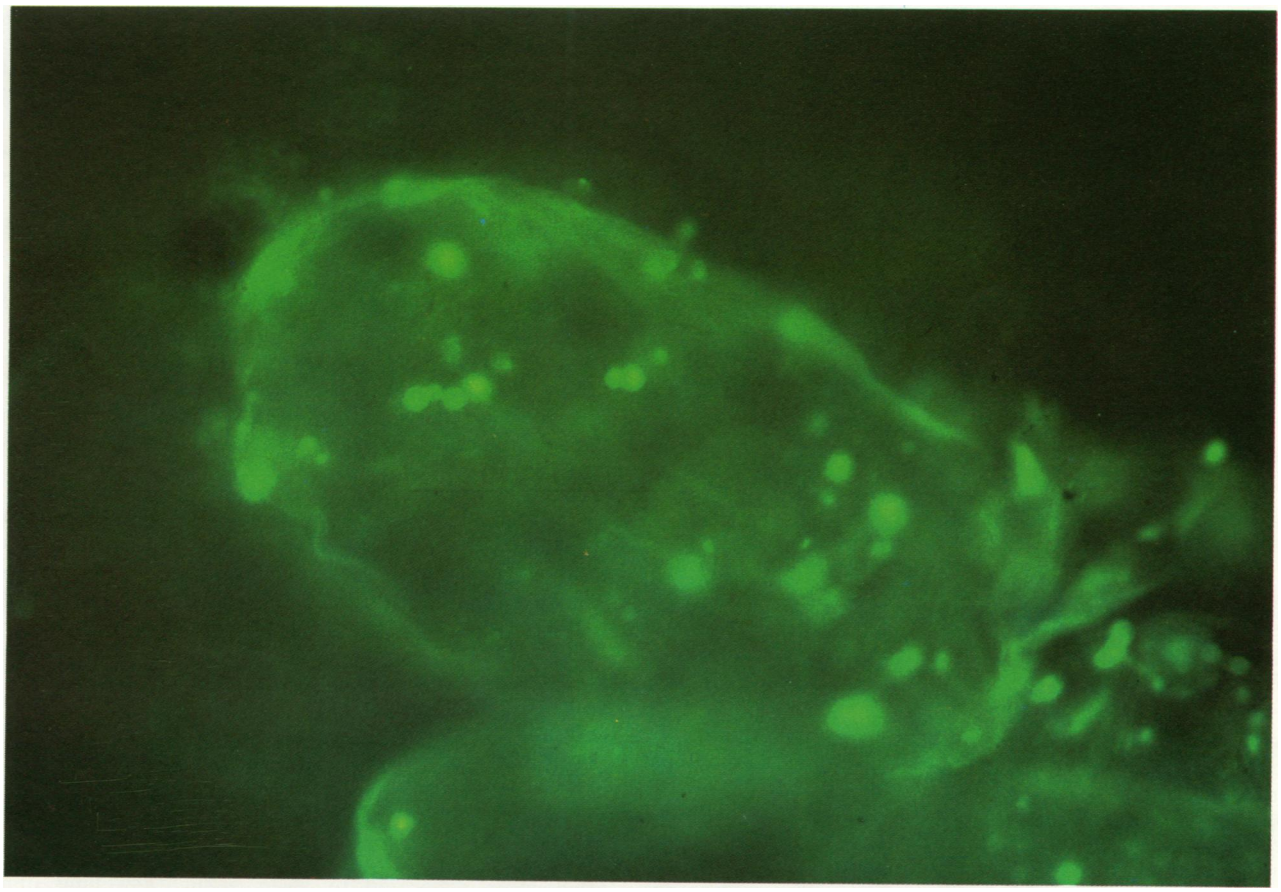

FIG. 3. Polysaccharide fluorescence due to Calcofluor White staining of the cell wall (× 80) of regenerating protoplast (72 hr in culture medium).

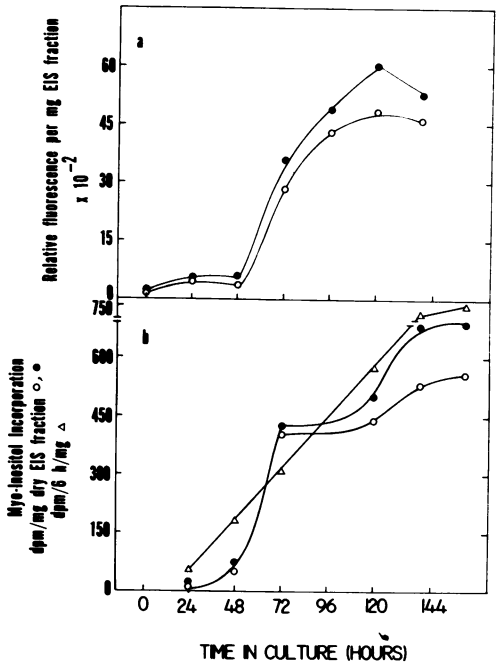

FIG. 4. a: Increase in intensity of fluorescence in regenerating protoplasts due to cell wall formation. ○: culture medium; ●: culture medium + methionine (1 mM); b: incorporation of ^{3}H-*myo*-inositol into ethanol-insoluble (EIS) fraction of regenerating protoplasts in culture medium without (○) and with (●) methionine (1 mM). The label was given at zero hr of cultivation and samples were removed at different hr of growth. Data points (△) were also obtained when labeled *myo*-inositol was added to growing cultures, on different days, and the incorporation of the label for 6 hr into EIS fraction was determined.

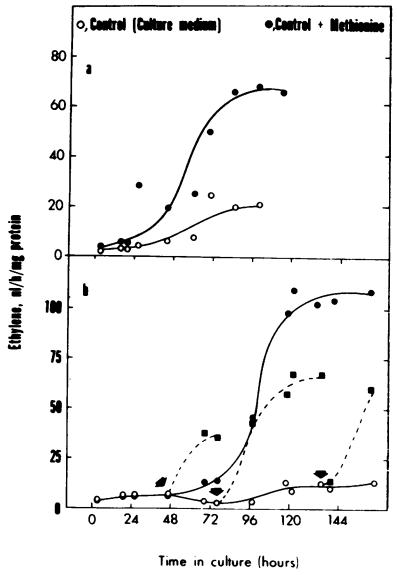

FIG. 5. Restoration of ethylene synthesis in regenerating protoplasts. Source: a, preclimacteric fruit; b, climacteric fruit. ■ indicates ethylene synthesis in those control flasks which received methionine (1 mM) at times indicated by arrow.

convert methionine to ethylene may be the breakdown of special, labile, lipid-protein and polysaccharide-protein links upon homogenization of the tissue.

Differences in the rate of loss of ethylene production between preclimacteric and climacteric (and postclimacteric) fruit slices upon incubation with the lytic enzyme mixture possibly reflect differences that have been shown to occur in plant membranes

Table II. Ethylene synthesis in regenerated protoplasts: dependence on methionine and inhibition by pronase and specific inhibitors

	Ethylene formation nl/hr
1. Control (6 day Culture)	0.48
2. 1 + Methionine (1 mM)	6.53
3. 2 + Rho[a] (1 mM)	2.16
4. 2 + Propylgallate (1 mM)	2.21
5. 2 + Pronase[b] (50 µg/ml)	2.35

[a] L-2-amino-4-(2-aminoethoxy)-trans-3-butenoic acid (ethoxy analog of rhizobitoxine).

[b] incubated for 1 hr at 25 C.

Table III. Effect of various concentrations(changing osmolarity) of sorbitol on the rate of ethylene production of apple tissue slices

Postclimacteric fruit. Apple slices were preincubated in a solution of 0.6M sorbitol and 10 mM tris-HCl, pH 7.5 for 15 min at 30 C and then transferred to separate flasks containing either buffer or different molar solutions of sorbitol (3 ml/g tissue) in 10 mM tris-HCl buffer, pH 7.5 The rates given for ethylene production were obtained for the same batches at 1 and 2 hr after transfer.

Medium	Ethylene formation nl/g·hr	
	Incubation time, hr	
	1	2
10 mM tris-HCl, pH 7.5	26.2	9.7
Sorbitol (in tris-HCl) M		
0.12	30.1	12.3
0.20	39.1	22.8
0.24	42.1	22.7
0.40	56.2	43.9
0.50	61.6	62.7
0.60	74.1	75.5
0.90	80.1	79.0
1.20	70.1	59.0

on aging (2, 24). Faster recovery of ethylene synthesis in regenerating protoplasts from preclimacteric than from climacteric fruit (Fig. 5) suggests greater regenerating capability (viability) of the former. The need for the constant presence of methionine in the culture medium to increase (*a*) ethylene production by regenerated protoplasts (Fig. 5) and (*b*) incorporation of labeled *myo*-inositol into cell wall components (Fig. 4b) suggests additional roles for methionine in the regeneration of protoplasts, apart from the role of substrate for ethylene synthesis. Addition of methionine to protoplasts cultured without methionine induced ethylene production, but the amount produced was not as high as that from protoplasts cultured throughout with methionine (Fig. 5b). Methionine may play an additional role as a feedback signal for the recovery of the ethylene enzyme system and also for the deposition of cell walls on the surface of the protoplasts. When supplied exogenously to higher plants, methionine is a source of methyl groups in phenolic substances (7, 11) and of methyl ester in pectic substances (31, 32). Roberts *et al.* (30) showed that the methyl group of L-methionine-methyl-^{14}C was recovered as 4-O-methyl-^{14}C-D-glucuronic acid in the polysaccharides of *Zea mays* root tips. Methionine may also be needed for protein-synthesizing systems in which it may not be available at saturating concentrations.

Albersheim (1) and Loewus and co-workers (19, 30) have shown that *myo*-inositol is incorporated into cell wall polysaccharides. The pattern of *myo*-inositol incorporation into EIS residue of protoplasts (Fig. 4b) during culture coincided closely with that of the relative increase in fluorescence of the cell wall polysaccharides on staining with Calcofluor White M2R New (Fig. 4a). This suggests the possibility of using labeled *myo*-inositol for studying cell wall synthesis by cultured fruit protoplasts, especially since pectin is an essential component of fruit cell walls (23). Our data further show that during culture protoplasts synthesize polygalacturonic unit in addition to cellulose.

Data in Table III support the earlier finding of Burg and Thimann (6) on the sensitivity of an ethylene-producing system to the solute concentration and show that the system producing ethylene is dislocated by even a slight swelling or drastic

shrinking of the structure. We interpret these data to support our suggestion that the ethylene-synthesizing system, located in the plasma membrane-cell wall complex, is disrupted when the structural relationship between cell wall and cell membrane is disturbed. Attempts to isolate this complex *in vitro* probably have not been successful to date because, among other factors, when the tissue is homogenized the specific details of structural integrity of the "enzyme complex" are not maintained in their native state. We are continuing to work on this problem.

The suggestion that the ethylene-synthesizing enzyme system is localized in the cell membrane-cell wall complex may have important implications. It is now apparent that plasma membrane and cell wall may share proteins and glycoproteins (13, 25, 27, 29, 33, 34) and hormone-binding receptors or components (4, 12, 20, 28); also the involvement of plant hormones, *viz.* IAA, gibberellins, cytokinins, and ABA with ethylene production has been pointed out (8, 14, 15). Thus, the mechanism of action of plant hormones, in particular ethylene, may be intimately connected with receptors or recognition sites on plant cell membranes-cell wall complexes.

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