Ethylene-Independent and Ethylene-Dependent Biochemical Changes in Ripening Tomatoes

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ABSTRACT

Fruits of Lycopersicon esculentum Mill cv Sonatine stored in 6% CO2, 6% O2, and 88% N2 for 14 weeks at 12°C, exhibited a temporal separation of certain biochemical events associated with ripening.

The specific activity of two citric acid cycle enzymes, citrate synthase and malate dehydrogenase, fell substantially during the first 2 weeks of storage when changes in organic acid concentration also occurred. During this period, lycopene, polygalacturonase, and ethylene were undetectable.

When fruit were removed from store, ethylene was evolved and polygalacturonase and invertase activity were rapidly initiated as was synthesis of lycopene.

To determine whether the changes in organic acid metabolism were affected by ethylene, fruit was kept at 22°C in either a normal atmosphere or a normal atmosphere supplemented with 27 microliters per liter of ethylene, and it was shown that in both atmospheres similar quantitative changes to those described above occurred in the citric acid cycle enzymes specific activities before any detectable increase in the specific activities of invertase and polygalacturonase. These latter changes, together with pigment changes, occurred between 2 and 3 days earlier in fruit exposed to ethylene, compared with those kept in a normal atmosphere.

Tomato fruit development is characterized by a wide range of biochemical changes at the onset of ripening. There is a large respiratory increase accompanied by radical alteration in the concentration of two organic acids of the citric acid cycle: citric and malic acid. It is not known whether these two events are interconnected, or whether they are associated with the conversion of starch to monosaccharides.

At present, little is known about the control of enzymes of the citric acid cycle in tomatoes, except some general information concerning changes in enzyme multiple forms (13). In contrast, much is known about the structure and site of action of PG2 (polygalacturonide glycanohydrolase, EC 3.2.1.15) which is located in the cell wall. Rapid synthesis of this enzyme occurs at the onset of ripening (12). The increase in PG seems to be due to net synthesis of protein as shown by radioimmunoassay (28, 29). Degradation of isolated tomato cell walls by this enzyme has been established (26). In addition to enzyme synthesis, there is also formation of new mRNA in ripe fruit (22), although it has not been directly established that this codes for polygalacturonase. PG is present as two multiple forms which can be separated on an ion-exchange column. PG I has double the relative molecular mass of PG II (Mr = 1 x 105 for I and 4.6 x 104 for II). PG I does not reach a very high concentration during ripening, whereas PG II increases rapidly (30). PG II can be converted to PG I by a heat stable, nondialysable factor isolated from tomato fruit. The amount of this factor increases during ripening, while the concentration of PG I remains constant (30).

Tomato fruit contain an acid invertase (3β-fructofuranoside fructohydrolase, EC 3.2.1.26). This enzyme is present in mature-green fruit and its activity increases as ripening progresses (11). It has been suggested that the increase in activity of this enzyme is also due to de novo synthesis (15). There is disagreement, however, as to whether senescent fruit contain more or less invertase than ripe fruit (17, 20). There is evidence that a neutral protease selectively degrades inactive invertase molecules in senescent fruit (21).

Enzyme synthesis in ripening tomato fruit is always associated with loss of Chl, increase in lycopene, and evolution of ethylene. It has often been reported that the phytohormone ethylene is capable of coordinating ripening events in many fruits, including the tomato (23). In all cases, ethylene is supposed to increase the synthesis of 'new' enzymes, control organic acid concentrations based in the mitochondria, and regulate the synthesis of carotenoids in the chloroplast. However, there have been reports that the respiratory rise in the banana can occur separately from other ripening events (18). The inhibition of respiration invariably inhibited the other facets of banana ripening (particularly color change).

In the tomato, Dostal and Leopold (5) were able to delay color change with gibberellic acid but were not able to retard climacteric respiration. It has therefore been proposed that the respiratory climacteric based in the mitochondria operates relatively independently of other ripening changes (24). During the experiments mentioned above, no biochemical changes were measured. Thus, Dostal and Leopold made no attempt to investigate the changes in the concentration of citrate, malate, and monosaccharides nor to seek any correlation between such changes and the increase in respiration. There may well be a role for phosphofructokinase (EC 2.7.1.56) in the control of glycolysis and respiration, and the enzyme may well have a bearing on the respiration rate of ripening tomato fruit (3).

It is well known that the storage of fruit in 6% O2, 6% CO2, and 88% N2 prevents ripening, but recent results in the Bristol laboratories have shown a clear separation of color changes from changes in organic acids and starch degradation to monosaccha-

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2 Abbreviations: PG, polygalacturonase; MOPS, 3-(N-morpholino)propanesulphonic acid; BICINE, N,N-bis(2-hydroxyethyl)glycine; DTNB, 5,5'-dithiobis(2-nitrobenzoate); FID, flame ionization detection.
Treatments of Fruit in the Storage Experiment. Four hundred and fifty kg of fruit of *Lycopersicon esculentum* Mill cv. Sonatine were picked at the mature-green stage (fully expanded fruit with mature seeds but no loss of green color), washed at 46°C for 2 min to help prevent fungal attack (Stead and Goodenough, unpublished data), and dried.

The fruit were placed in a sealed storage chamber (1.35 x 2.10 x 2.5 m) monitored at 6% O₂, 6% CO₂, and 88% N₂. RH 98%, and temperature 12°C. Gas composition was monitored by IR gas analysis for CO₂ and by paramagnetic oxygen analysis for O₂. These monitoring devices operated solenoids switches connected to O₂ and N₂ reservoirs which counteracted a slight continuous air leak so that the gas concentration was therefore maintained automatically. Breathing apparatus was worn during sampling so that perturbation to the composition of the gas atmosphere was minimized.

Treatment of Fruit in Experiments with or without Ethylene. Ten kg of mature-green fruit were placed in an unsealed tank (200 x 200 x 100 cm). The fruit were placed on two metal racks which gave a single layer of fruit on each rack and allowed CO₂ to fall to the bottom of the tank and escape through vents. The tank was connected to an air flow of 50 ml h⁻¹ to provide a slight positive pressure and maintain an ambient atmosphere. O₂ and CO₂ were monitored daily. Ten kg were placed in a sealed glass tank containing concentrated KOH (situated below the fruit) to remove CO₂; the atmosphere in this tank was supplemented with ethylene to give a final concentration of 27 µl 1⁻¹. This tank was opened daily and fresh ethylene was added after flushing with air. Both tanks were kept at ambient temperature 22°C and stored in separate rooms.

Enzyme Extraction. Citrate synthase, malate dehydrogenase, and malic enzyme were extracted as follows: Weighed samples of fruit were quartered and homogenized at full speed in a Waring Blender for 15 s in a buffer comprising a final concentration of 100 mM MOPS, 100 mM BICINE, and 3 mM EDTA, adjusted to pH 8.2 with NaOH. The buffer temperature was 4°C. After homogenization, 1% PVP (w/v) was added. For the assay of malic enzyme, the extraction buffer also contained 5 mM 2-mercaptoethanol (v/v). The homogenate was strained through four layers of muslin and spun at 20,000 g for 15 min in a Beckman J21B centrifuge at 4°C. The supernatant was used immediately for enzyme assay.

PG and invertebrate were extracted by the method described by Tucker et al. (29). Tomato pericarp was homogenized in a Polytron homogenizer at speed setting 6 for 50 s. The homogenate was centrifuged at 17,000 g for 10 min and the pellet was resuspended in 1 M NaCl. The pH was adjusted to 6.0 using NaOH, and the extract was left for 4 h at 4°C. The suspension was centrifuged at 17,000 g for 10 min and the supernatant was removed, filtered, and made to 75% saturation by the addition of solid (NH₄)₂SO₄. Proteins were precipitated overnight. The precipitate was centrifuged at 17,000 g for 10 min, and the pellet was taken up in a small volume of 0.15 M NaCl. This was then dialyzed against 0.15 M NaCl overnight. All operations were at 4°C.

**Enzyme Assays.** Citrate synthase was assayed using DTNB by the method of Srere et al. (25). The assay mixture contained the following final concentrations: 100 mM MOPS and 100 mM BICINE at pH 8.0, 0.2 mM acetyl-CoA, 0.1 mM DTNB, and 50 µM of extract. The reaction was initiated by the addition of oxaloacetate to give a concentration of 0.2 mM, making a total volume of 1 ml. The molar absorbance of the thionitrobenzoate ion is 13.6 x 10³ 1 mol⁻¹ cm⁻¹ at 412 nm, and 1 unit of activity is that amount of enzyme which catalyzes the formation of 1 µmol of CoA-SH min⁻¹ under assay condition.

The reaction mixture for malate dehydrogenase contained the following final concentrations: 100 mM MOPS and 100 mM BICINE at pH 8.0, 0.4 mM NADH and 5 µl of extract in a total volume of 1 ml. The reaction was started by the addition of oxaloacetate to give a final concentration of 0.2 mM. The oxidation of NADH was recorded at 340 nm and a molar absorbance of 6.22 x 10³ 1 mol⁻¹ cm⁻¹ was used to calculate the unit of activity (the amount of enzyme catalyzing the oxidation of 1 µmol of NADH min⁻¹ under assay condition).

Malic enzyme was estimated in 3 ml of a reaction mixture containing final concentrations of 100 mM MOPS and 100 mM BICINE at pH 7.0, with 5 mM l-malate, 0.5 mM NADP⁺, and 5 mM Mn²⁺. The reaction was started by the addition of 200 µl of extract, and the absorbance was measured continuously at 340 nm. The unit of activity is that amount of enzyme catalyzing the reduction of 1 µmol of NADP⁺ min⁻¹ under assay condition.

Polygalacturonase activity was assayed in 1 ml of a mixture with final concentrations of 150 mM NaCl, 50 mM sodium acetate, 0.5% polygalacturonic acid (pH 3.8). Invertase activity was assayed in 1 ml of a mixture giving a final concentration of 150 mM NaCl, 50 mM sodium acetate, 200 mM sucrose adjusted to pH 4.6. Both assays were at 25°C. Reducing groups formed were measured by the arsenomolybdate method of Nelson (28).

The unit of activity is that amount of enzyme catalyzing the hydrolysis of 1 µmol of galacturonic acid or sucrose min⁻¹ under assay conditions. A more detailed version of these assays is in Appendix A.

**Citrate and Malate.** These were extracted and estimated as described previously (8).

**Protein.** This was estimated by the method of Bradford (2) and Lowry (16) using BSA as a standard.

**Pigment Extraction.** Pigments were extracted according to Tomes (27). Three grams of tomato pericarp were homogenized in a 6:4 mixture of hexane and acetone using a Polytron homogenizer at speed setting 6 for 1 min. The extract was centrifuged at 10,000 g to clear debris and air bubbles and was then scanned in a spectrophotometer using a 1-cm cell. Lycopene was calculated assuming 320 optical density (A) units at 502 nm = 1 mg ml⁻¹ solution. Total Chl concentration in µg ml⁻¹ was calculated as 6.45 x A₆₆₅ + 17.72 x A₇₅₃.

**Sugar Determination.** Fruit were weighed and samples cut into segments and placed in boiling 80% ethanol. These segments were later continuously extracted for 20 h in a Soxhlet extractor in 80% ethanol. The ethanolic residue was evaporated to 100 ml and used for monosaccharide determination.

Monosaccharides and disaccharides were detected and quantified by formation of the trimethylsilyl derivatives (8). These were separated by gas chromatography using a 1-m × 0.32-cm column packed with 3% E51 on 80/100 mesh Suprasorb WHP. Temperature programming was as given earlier (8); detection was by FID using N₂ as carrier gas.

**Ethylene Determination.** Ethylene evolution was measured by sealing fruit in a vessel of known volume and leaving for 1 h. Two-ml samples from the gas phase were injected into a 1-m ×...
0.37-cm column of Poropak T in a gas chromatograph. The carrier gas was N₂, detection was by FID, and the oven temperature was 150°C.

RESULTS

Results of Storage Experiments. When citrate synthase was extracted and assayed at approximately 7-d intervals for 14 weeks, a characteristic trend was apparent. A fall in specific activity of citrate synthase was noted soon after the fruit were picked and placed in a modified gas atmosphere (within 6 h of removal from the vine) (Fig. 1). After 3 weeks, citrate synthase remained constant, except for an apparent decrease between weeks 7 and 8 followed by a return to the initial value. After 12 weeks of storage, the fruit were removed and allowed to stand at ambient conditions for 1 week. After this time, citrate synthase had fallen slightly in specific activity.

During the initial 2 weeks, the citrate content of the fruit rose by 25% and remained at this level for 3 weeks before rising further and then falling to a concentration lower than that at the beginning of the experiment. This concentration of citrate accumulated in the first 5 weeks was not equivalent to the concentration of citrate lost from the tissue in this time. As soon as the fruit were placed in a modified gas atmosphere, a decrease in malate was recorded (Fig. 2); after 1 week, 73% of the original malate remained, and, after 2 weeks, 58% of the malate was still extractable. However, the concentration did not fall any further even after the fruit were returned to normal atmospheres for several days. Figure 2 indicates that the specific activity of NADP-linked malic enzyme started to rise as soon as the fruit were removed from the vine. After reaching a maximum representing 250% of the original activity, the level of malic enzyme steadily declined, except for an apparent rise between the 4th and 5th weeks. When the fruit were returned to air and allowed to ripen, the enzyme activity fell to zero. The activity of malate dehydrogenase exhibited a slight rise after 7 d, but then fell rapidly to 55% of its original activity (Fig. 3).

In contrast to the rapid changes in specific activity that occurred to citrate synthase (Fig. 1), malic enzyme (Fig. 2), and malate dehydrogenase (Fig. 3), the specific activity of cell wall-bound acid invertase was maintained at a low level throughout the storage period and that of PG was undetectable until week 8 (Fig. 4). However, the specific activities of both enzymes increased dramatically within 24 h of removing the fruit from store.
FIG. 4. Changes in specific activity of polygalacturonase and invertase in tomato fruit held at 12°C in 6% CO₂, 6% O₂, and 88% N₂, and after removal to ambient atmosphere and temperature. Specific activity of polygalacturonase during storage (Δ—Δ) or after removal to ambient conditions (Δ−−Δ). Specific activity of invertase during storage (O—O) or after removal to ambient conditions (O−−−O). Bars, SE.

FIG. 5. The concentration of pigments in tomato fruit held at 12°C in 6% CO₂, 6% O₂, and 88% N₂, and after removal to ambient temperature and atmosphere. Concentration of Chl during storage (O—O) or after removal to ambient conditions (O−−−O). Concentration of lycopene during storage (Δ—Δ) or after removal to ambient conditions (Δ−−−Δ). Bars, SE.

FIG. 6. Ethylene evolution from tomato fruit held at 12°C in 6% CO₂, 6% O₂, and 88% N₂, and after removal to ambient temperature and atmosphere. Ethylene evolution during storage (O—O) or after removal to ambient conditions (O−−−O). Bars, SE.

FIG. 7. Changes in specific activity of citrate synthase during aging of tomato fruit at ambient atmosphere and in an atmosphere containing 27 μl/l of ethylene, both at 22°C. (O—O), Ambient atmosphere. (Δ—Δ), Atmosphere containing 27 μl/l of ethylene. Bars, SE.

The store, minute quantities of ethylene were detectable. However, after 1 week in storage, ethylene was indiscernible and it was not until 6.5 weeks into the storage period that ethylene production became detectable, and this rose to a peak at 7 weeks and then remained constant (Fig. 6). It has been reported that complete anoxia prevents the formation of ethylene from 1-amino-1-carboxycyclopropane (1), and it is possible that ethylene synthesis is retarded by low O₂ tension. When fruits were removed from storage to a normal atmosphere, the evolution of ethylene immediately increased to a high level and then decreased.

The data for concentration of monosaccharides is not displayed but it rose within the 1st week of storage from 407.81 mg/g dry weight to reach a peak after 2 weeks of 454.1 mg/g dry weight. There was a second peak in the concentration of fructose.
immediately after the fruit were placed in the chambers, and there was no significant difference between the two treatments. The data is not displayed, but malic acid concentration finally fell to 50% of the starting concentration in both cases. Citric acid concentration rose slightly in both cases, but was not affected by ethylene treatment.

Both invertase and polygalacturonase started to increase in specific activity after 5 d in a normal atmosphere, whereas in the ethylene-supplemented atmosphere, invertase began to increase in specific activity after 3 d and polygalacturonase after 4 d (Fig. 9). A similar apparent ethylene stimulus occurs with Chl degradation and lycopene synthesis (Fig. 10).

RESULTS

Results from the storage experiment indicate a clear temporal separation between those biochemical processes that appear to be stimulated by ethylene from those that are not, and the data from the second set of experiments corroborates these results. The fall in specific activity of malate dehydrogenase and citrate synthase in the storage experiment was quantitatively similar in the ethylene-stimulated experiment, and ethylene did not significantly enhance the rate of fall of either enzyme.

Malate dehydrogenase is known to be both cytosolic and mitochondrial, and the decrease in specific activity of this enzyme stops at a particular value and declines no further even when the fruit are removed from store. This could represent a loss of the cytosolic enzyme, since work in the Bristol laboratory indicates that less than 10% of the total malate dehydrogenase activity is mitochondrial (Jeffery, unpublished results). Hobson (13) examined malate dehydrogenase isoenzymes during normal ripening but found no evidence of one isoenzyme decreasing more than another. This does not preclude the loss of activity from the cytosolic enzyme, since staining for activity on PAGE gels is a purely qualitative measurement.

There appears to be evidence to suggest a switch from malate dehydrogenation to decarboxylation during ripening, since malic enzyme more than doubles in specific activity during the first 2 weeks of storage. The 'malate effect' is well documented in Malus and Pyrus species (7) and there is one report of this in tomato fruit (14) where a single value for cv Ailsa Craig is quoted at the green and red stage. The loss of malate is consistently about one-half of the amount found in fruit before the start of storage (9) and parallels the loss found during normal ripening. An NADP+—
linked malic enzyme is situated in the cytosol, and it would seem that the malate utilized must either be transported from the vacuole to the cytosol or be present already in the cytosol. The unaltered pool of malate may be irreversibly compartmentalized in the vacuole, and this is supported by the fact that, even after removal from storage condition, the malate concentration remained unaltered, although other ripening changes occurred.

The 70% fall in specific activity of citrate synthase during the first 2 weeks of storage, compares almost exactly with the loss of activity during normal and ethylene-stimulated ripening. It is possible that citrate synthase is compartmentalized within the cell, the fall in activity representing the loss of enzyme from one compartment. It has been suggested that the glyoxylate cycle is present in tomato fruit (6), but the evidence obtained so far is that citrate synthase is only present in tomato fruit mitochondria (Jeffery, unpublished results).

Previous results have indicated that radiolabeled citrate is metabolized more slowly by ripe fruit than mature-green fruit (4) and this may indicate a reduction in the pathway of citrate metabolism. There is evidence at present for a slightly lowered activity of NADP⁺-dependent isocitrate dehydrogenase, but the activity of the NAD⁺-dependent enzyme has proved difficult to assay. It is noticeable both in this and previous work (9) that the increase in concentration of citrate is followed by a slow decline back to the original value. The citrate in the cytosol may initially accumulate and then be utilized by the mitochondria, whereas the bulk of the acid is retained in the vacuoles.

Whatever the mechanism of acid gain and loss, we have shown that ethylene is not evolved until 4 weeks of storage have passed. It has been reported that very small amounts of ethylene can induce the respiratory climacteric in tomato fruit (19). However, our results show an immediate change in organic acids and associated enzymes immediately after the fruit are removed from the vine. As it was impossible to measure a true respiration rate during the storage experiment, the changes in organic acids cannot be correlated with a respiratory rise.

In direct contrast to the changes described above, which are associated with an early stage of ripening and are not affected by ethylene, are those biochemical events that occur later and appear to be ethylene stimulated, namely the changes in pigment, appearance of polygalacturonase, and the increase in invertase activity. It is assumed that lack of polygalacturonase activity until 50 d of storage indicates that no enzyme protein is present, although this has not been proved by radioimmunoassay (28). Ethylene certainly stimulates polygalacturonase appearance and it is possible to say that it stimulates either transcription of mRNA or subsequent translation. Invertase concentration, although measurable throughout fruit growth, responds rapidly to ethylene addition and is unchanged during storage until normal gas atmospheres are restored.

The synthesis of lycopene also appears to be regulated by ethylene, since lycopene does not appear during storage or when the fruit are removed to a normal gas atmosphere, until ethylene synthesis begins. When tomato fruit are stimulated by ethylene, lycopene appears in response. Although loss of Chl occurs even in the absence of ethylene, the rate of loss is increased in the presence of the gas. The act of lowering the O₂ concentration and temperature has the result of preventing ethylene formation by the fruit and enables us to link ethylene, polygalacturonase activity, invertase activity, and lycopene production. The data presented here with regard to accumulation of the monosaccharides glucose and fructose from starch indicates that invertase has no role to play in monosaccharide appearance during starch breakdown. It is obvious that during storage the monosaccharide increase is not controlled by ethylene and falls into the same category as the changes in organic acids. As the increase in invertase is temporally separated from starch breakdown, the increased amount of enzyme must be used for another type of action in the cell wall, possibly breakdown of cell wall linkages other than glucose or galacturonic acid.

From the data in these experiments, three classes of biochemical change can be formulated:

1. Changes in the specific activities of citrate synthase, malate dehydrogenase, and malic enzyme, with the consequent changes in citrate and malate metabolism. Metabolism of starch to equal quantities of glucose and fructose. All of these appear to occur immediately after the fruit are removed from the vine at the mature-green stage and also occur in fruit ripening on the vine. They are not stimulated by ethylene and, as discussed in References 9 and 28, they do not rely on cell wall breakdown by polygalacturonase.

2. Loss of Chl, which occurs slowly when fruit are removed from the vine but without the changes mentioned in class 3. Enhanced by ethylene to a large extent.

3. Formation of lycopene, polygalacturonase, and increase in invertase. Seemingly intimately dependent on ethylene for initi-
ation and continuance of the response.

Changes in class 1 seem to require regulation of pre-existing enzymes, post-translational modification. Class 3 changes seem to require transcription and translation for the change to occur and seem to be the main target for ethylene stimulation. Further work will try to identify whether the formation of the enzymes and lycopene is transcriptional or translational control by ethylene.

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APPENDIX A

Enzyme Assays. Citrate synthase was assayed using DTNB by the method of Sere et al. (25). The assay mixture contained 900 μl of 110 mM MOPS and BICINE at pH 8.0, 20 μl of 10 mm acetyl-CoA, 10 μl of 10 mM DTNB, and 50 μl of extract. The reaction was initiated by addition of 20 μl of 10 mM oxaloacetate. This gave a final concentration of 100 mM MOPS and BICINE, 0.2 μM acetyl-CoA, 0.1 mM DTNB, and 0.2 mM oxaloacetate. The molar absorbance of the thionitrobenzoate ion is 13.6 × 10^3 M^\(-1^) cm^\(-1^) at 412 nm, and 1 unit of activity is that amount of enzyme which catalyzes the formation of 1 μmol of CoA-SH min\(^{-1}\) under assay conditions.

The assay mixture for malate dehydrogenase contained 930 μl of 107.5 mM MOPS and BICINE at pH 8.0, 45 μl of 10 mM NADH, and 5 μl of extract. The reaction was initiated by addition of 20 μl of 10 mM oxaloacetate. The molar absorbance of NADH at 340 nm is 6.22 × 10^3 M^\(-1^) cm^\(-1^) and the decrease in absorbance was used to give the unit of activity. This latter figure is that amount of enzyme catalyzing the oxidation of 1 μmol of NADH min\(^{-1}\) under assay conditions.

Malic enzyme was estimated in reaction mixture consisting of 1.25 ml of 100 mM malate, 250 μl of 50 mM NADP, and 125 μl of 1 mM MnSO\(_4\) made up to 25 ml with 100 mM MOPS and BICINE buffer at pH 7.0. All constituent compounds were dissolved in 100 mM MOPS and BICINE at pH 7.0, except NADP which was dissolved in distilled water at pH 6.0. The reaction was started by addition of 200 μl of extract to 3 ml of the reaction mixture. The final concentration of the constituents was 100 mM MOPS and BICINE pH 7.0, 5 mM malate, 0.5 mM NADP, and 5 mM Mn^2+\(^{2+}\). The unit of activity is that amount of enzyme catalyzing the reduction of 1 μmol of NADP+ min\(^{-1}\) under assay conditions.

Polygalacturonase activity was measured by adding 50 μl of extract to 1 ml of a solution of 50 mM sodium acetate containing 150 mM NaCl and 0.5% polygalacturonic acid at pH 3.8. The NaCl and polygalacturonic acid were dissolved in sodium acetate solution and adjusted to pH 3.6 before the solution was adjusted to the final volume to give the concentrations stated.

Similarly, the activity of invertase was measured in a solution with a final concentration of 150 mM NaCl, 50 mM sodium acetate, and 200 mM sucrose adjusted to pH 4.6. Reducing groups formed were measured by the arsenomolybdate method of Nelson (28). The units of activity are: that amount of enzyme catalyzing the release of 1 μmol of galacturonic acid min\(^{-1}\); that amount of enzyme catalyzing the hydrolysis of 1 μmol of sucrose under assay conditions. All assays were at 25°C.