

# Regulation of Ethylene Biosynthesis in Avocado Fruit during Ripening<sup>1</sup>

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## ABSTRACT

Preclimacteric avocado (*Persea americana* Mill.) fruits produced very little ethylene and had only a trace amount of 1-aminocyclopropane-1-carboxylic acid (ACC) and a very low activity of ACC synthase. In contrast, a significant amount of 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) was detected during the preclimacteric stage. In harvested fruits, both ACC synthase activity and the level of ACC increased markedly during the climacteric rise reaching a peak shortly before the climacteric peak. The level of MACC also increased at the climacteric stage. Cycloheximide and cordycepin inhibited the synthesis of ACC synthase in discs excised from preclimacteric fruits. A low but measurable ethylene forming enzyme (EFE) activity was detected during the preclimacteric stage. During ripening, EFE activity increased only at the beginning of the climacteric rise. ACC synthase and EFE activities and the ACC level declined rapidly after the climacteric peak. Application of ACC to attached or detached fruits resulted in increased ethylene production and ripening of the fruits. Exogenous ethylene stimulated EFE activity in intact fruits prior to the increase in ethylene production. The data suggest that conversion of *S*-adenosylmethionine to ACC is the major factor limiting ethylene production during the preclimacteric stage. ACC synthase is first synthesized during ripening and this leads to the production of ethylene which in turn induces an additional increase in ACC synthase activity. Only when ethylene reaches a certain level does it induce increased EFE activity.

Climacteric fruits are characterized by a surge of ethylene production at the onset of ripening. Avocado fruit is a typical climacteric fruit; however, it is unique as most avocado varieties lack the ability to ripen as long as the fruit is attached to the tree. This phenomenon is probably due to the fact that avocado fruit attached to the tree produce only trace amounts of ethylene. Some investigators have suggested that some unknown ripening inhibitors move from the leaves or shoots to the fruit where they inhibit ethylene production and hence ripening (5, 18). The question of why preclimacteric fruits do not produce ethylene has been studied by several investigators. It has been suggested that both the conversion of SAM<sup>2</sup> to ACC and the conversion of ACC to ethylene are limiting ethylene production in the precli-

macteric fruit (22). In preclimacteric fruits of several species, including avocado, the ACC content was very low but a massive increase occurred at the climacteric stage (9). Similarly, it was found that ACC level and ACC synthase activity in tomato fruits were almost undetectable before the onset of ripening, but increased following the "breaker" stage (12).

Preclimacteric fruit tissues exhibited only a relatively slight increase in ethylene production following the application of ACC as compared to the marked increase in ethylene production at the climacteric peak (7, 19). These data have led Yang (19, 20) to suggest that preclimacteric fruits also have a limited ability to convert ACC to ethylene. On the other hand, application of ACC to intact preclimacteric fruits significantly enhanced the ripening process (22). Recent data show that in intact preclimacteric cantaloupe and tomato fruits, exogenous ethylene stimulates EFE activity prior to the increase in ACC level and ethylene production (22). Whether or not this is also true during natural ripening of climacteric fruits remains to be clarified. In this study we investigated the regulation of ethylene biosynthesis in avocado fruits during natural ripening.

## MATERIALS AND METHODS

**Plant Material and Treatments.** Avocado (*Persea americana* Mill. cv Fuerte and Hass) fruits were harvested from mature trees grown in the coastal region of Israel. Intact fruits were allowed to ripen naturally at 20°C and 90% RH and assayed daily for ethylene evolution. For treating fruit tissue with metabolic inhibitors and for measuring EFE activity *in vivo*, pericarp plugs, 10 mm in diameter, were excised with a cork borer and then cut into discs, 3 mm thick. Four discs, weighing about 2.5 g, were incubated in 50 ml Erlenmeyer flasks in 3 ml of water containing the desired chemical. The flasks were constantly shaken at 27°C in the dark.

**Ethylene Determination.** Ethylene evolution of fruits attached to the tree was determined by enclosing each fruit in a plastic bag equipped with a small tube sealed with a rubber cap. After sealing, the air was evacuated from the bag and 60 ml of air were injected into each bag. Ethylene was measured in a 2-ml air sample withdrawn from the bag with a hypodermic syringe. It should be noted that plastic bags release ethylene when exposed to sunlight. Therefore, the bags were shaded during measurements. Ethylene evolution of detached fruits was determined by enclosing each fruit in a 750-ml jar and withdrawing an air sample of the gas phase of the jar as above. For measuring ethylene production of discs treated with metabolic inhibitors, four discs were transferred at the end of treatment to a 20-ml syringe sealed with a rubber serum cap. Ethylene in the gas phase of the enclosing syringe was measured in a 2-ml air sample withdrawn with another syringe. Ethylene was assayed by gas

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<sup>2</sup> Abbreviations: SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid; EFE, ethylene forming enzyme; Hepps, 4-(2-(hydroxyethyl)-1-piperazinepropanesulfonic acid; CHI, cycloheximide.

chromatography, using an activated alumina column and a flame ionization detector.

**Determination of ACC and MACC.** Pericarp tissue, about 1 g fresh weight, was homogenized in 8 ml of 80% ethanol by means of an Ultra Turrax homogenizer. The shaft was washed with additional 5 ml of 80% ethanol. The extract and washing were combined, filtered through a glass wool and centrifuged for 10 min at 10,000g. The supernatant was concentrated under reduced pressure at 45°C to remove all ethanol and the extract was brought to a volume of 2 ml with water. Two ml of chloroform were added, the tube was vigorously shaken, and centrifuged as above. ACC in the water phase was assayed by the method of Lizada and Yang (14). For measuring MACC content, an 1.5-ml aliquot was hydrolyzed in 3 N HCl at 100°C for 3 h to liberate ACC. Following neutralization with NaOH the resulting hydrolysate was assayed for ACC as described above. MACC content was calculated as differences in ACC content after and before HCl-hydrolysis. In some experiments, ACC was extracted with 8 ml water instead of ethanol and processed as above except for the concentration step. Comparison of the two extraction methods showed that the water extract increased ACC yield.

**Extraction and Assay of ACC Synthase.** Tissue (2.2 g fresh weight), was frozen in liquid N<sub>2</sub> and ground to a fine powder with a mortar and pestle. The powder was extracted with 20 ml of 50 mM K-phosphate (pH 7.2) containing 5% (w/v) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4 mM DTT, and 5 μM pyridoxal phosphate. Extraction and subsequent steps were carried out at 4°C. The extract was filtered through glass wool and centrifuged for 10 min at 10,000g. ACC synthase was precipitated from the supernatant by slowly adding (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 90% saturation, letting the mixture stand for 1 h and centrifuging as above. The supernatant fraction was discarded and the pellet was dissolved in 4 ml of 10 mM K-phosphate (pH 7) containing 0.1 mM DTT and 2 μM pyridoxal phosphate and dialyzed overnight against the same solution. The dialysate was clarified by centrifugation. ACC synthase activity was determined in a reaction mixture containing 0.5 ml of the enzyme preparation, 40 μmol Hepps buffer (pH 8.2), and 200 nmol SAM in a total volume of 0.6 ml. After incubation for 1.5 h at 30°C, the ACC formed was assayed by the method of Lizada and Yang (14). Enzyme activity is expressed as pmol or nmol ACC formed in 1 h/g fresh weight.

**Assay of EFE Activity.** EFE activity was determined by measuring conversion of administered ACC to ethylene *in vivo*. Pericarp discs were placed in a 50-ml Erlenmeyer flask containing 3 ml of 5 mM ACC. Each flask was sealed with a rubber serum cap and ethylene was determined as above.

## RESULTS

**ACC-Synthase Activity, ACC and MACC Levels, and Ethylene Production.** Avocado fruits are known to possess a very sharp rise and fall in rate of ethylene production during the course of ripening (3). Although individual avocado fruits exhibit the same pattern of ethylene production, the exact time of the climacteric peak may vary between fruits. To overcome this difficulty, the data are presented on a time basis of which the climacteric peak is the zero point (Fig. 1). Negative numbers designate days before the climacteric peak and positive numbers designate days after the peak. Although fruits harvested at various dates differed in rates of ethylene production during the climacteric stage, the patterns of changes in ACC levels and ACC synthase and EFE activities in relation to ethylene production and the response to the various treatments were similar.

Preclimacteric fruits produced very little ethylene and showed only a slight activity of ACC synthase and a trace amount of ACC (Fig. 1). A significant amount of MACC was detected during the preclimacteric phase and it remained constant until the climacteric rise. Both ACC synthase activity and ACC level

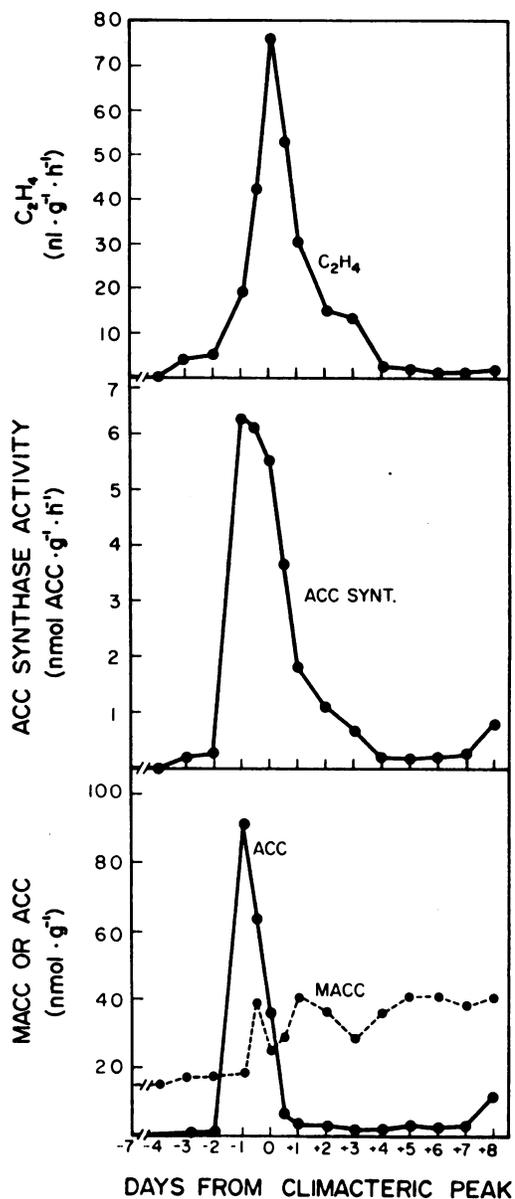


FIG. 1. Changes in ethylene production rate, ACC synthase activity, ACC and MACC levels of avocado fruits during ripening. Ethylene production was individually determined each day for all fruits and then several fruits were sampled for analysis. The ethylene curve was drawn from the means of the ethylene evolution rates of 10 fruits. For each sampling date, 3 fruits with ethylene production rates matching the ethylene production curve at that time were analyzed for ACC synthase activity, ACC and MACC levels.

increased during the climacteric stage reaching a peak shortly before the climacteric peak. Later on, activity of ACC synthase and ACC level decreased rapidly. Level of MACC also increased at the climacteric stage and remained at almost the same level during the postclimacteric period.

**Effect of Metabolic Inhibitors on ACC Synthase Activity.** Activity of ACC synthase during the preclimacteric stage of avocado fruits is very low (Fig. 1). These data can be interpreted to mean that either the enzyme is not synthesized during this stage or that an already existing enzyme is inhibited. Metabolic inhibitors were used to distinguish between these possibilities. Since application of inhibitors to intact fruits caused damage and increased ethylene production, their effect was examined in

pericarp discs, in which wound ethylene is produced after cutting. CHI, an inhibitor of protein synthesis (15), almost completely inhibited the development of ACC synthase activity, ACC accumulation, and ethylene production (Fig. 2). Similarly, cordycepin, an inhibitor of posttranscriptional RNA processing (8), also blocked ACC synthase activity, ACC accumulation, and ethylene production (Fig. 3). The inhibitory effect of cordycepin was lower than that of CHI.

**EFE Activity.** The data presented in Figure 1 indicate that preclimacteric avocado does not possess the ability to convert SAM to ACC. We further studied whether or not the EFE system is active during the preclimacteric period and the time sequence of its development during natural ripening. The ripening stage of each fruit was defined by measuring the ethylene evolution of the intact fruit. It should be noted that different parts of the avocado fruit vary greatly in levels of ethylene production, thus the ethylene production of pericarp discs is not a reliable criterion for defining the ripening stage of the fruits. Preclimacteric fruits showed a low but measurable EFE activity (Fig. 4). During ripening, a significant increase in EFE activity occurred only at the beginning of the climacteric rise. The highest EFE activity was detected at the climacteric peak. Activity at the postclimacteric stage declined sharply and was similar to that of the preclimacteric stage. Similar results were obtained in additional experiments.

The data presented above (Fig. 4) indicate that there is a low EFE activity during the preclimacteric stage. However, due to the possibility that this activity could have resulted from a rapid development of EFE activity after cutting of the discs the following experiment was performed. Preclimacteric discs were pre-treated with CHI, which has been reported to inhibit EFE synthesis (11, 17), and then treated with ACC. Figure 5 shows that both the control and CHI-treated discs had similar EFE activity during the initial period of treatment, suggesting that EFE does exist in the preclimacteric stage. The inhibitory effect of CHI became evident only 3 h after the application of ACC.

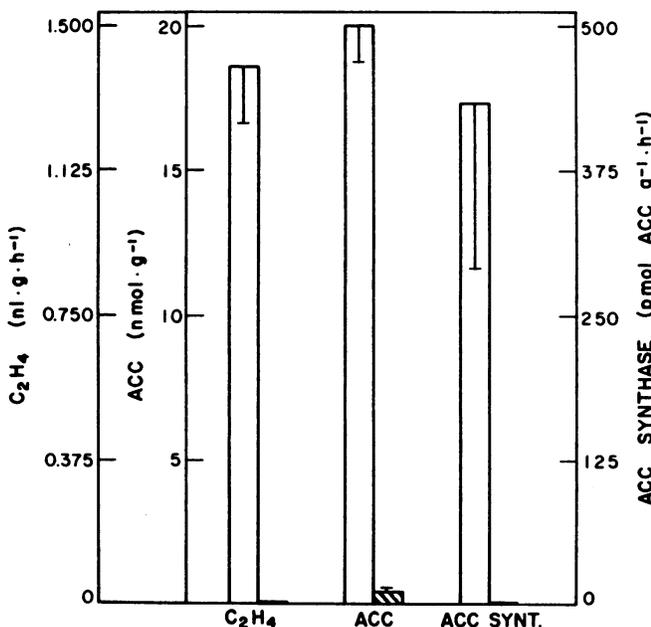


FIG. 2. Effect of CHI (50  $\mu$ M) on ethylene production, ACC synthase activity, and ACC level in pericarp discs of preclimacteric avocado fruits. Ethylene production rate was measured after 8 h of incubation. Immediately after the ethylene measurements had been completed, the same discs were employed for assaying ACC synthase activity and ACC level. (□), H<sub>2</sub>O; (▨), CHI. Vertical bars indicate 1 SE.

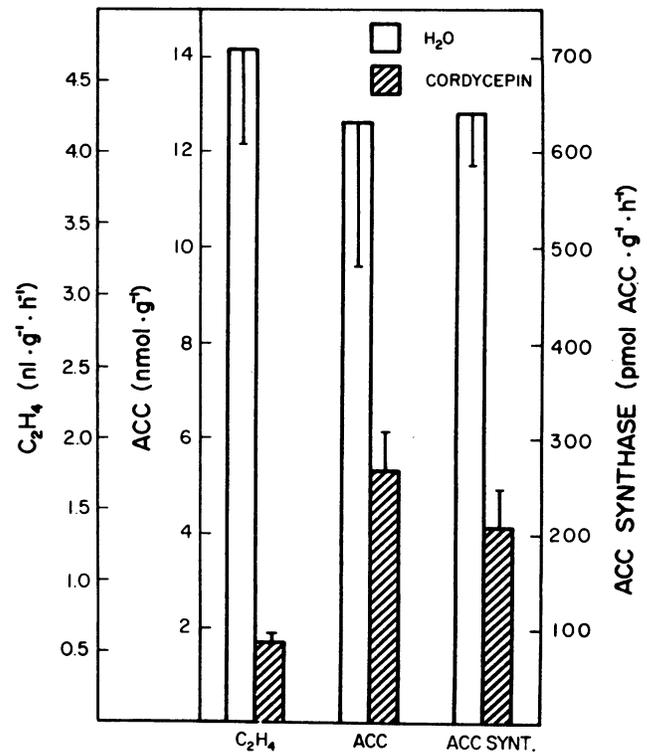


FIG. 3. Effect of cordycepin (100  $\mu$ l ml<sup>-1</sup>) on ethylene production, ACC synthase activity and ACC level in pericarp discs of preclimacteric avocado fruits. Ethylene production rate was measured after 6 h of incubation. Immediately after the ethylene measurements had been completed, the same discs were employed for assaying ACC synthase activity and ACC level. Vertical bars indicate 1 SE.

**Conversion of Applied ACC to Ethylene in Intact Fruits.** Further evidence that the EFE system is already active during the preclimacteric stage is derived from experiments in which intact preclimacteric fruits were treated with ACC. Detached fruits showed two peaks of ethylene production following application of ACC, the first a few h after the beginning of treatment and the second, 3 d later (Fig. 6). The first peak probably resulted from conversion of the applied ACC to ethylene, whereas the second peak represents the natural climacteric peak. A similar experiment was performed with fruits attached to the tree. Again, treated fruits were able to convert ACC to ethylene, showing a typical climacteric curve (Fig. 7). The fruits abscised at the time when ethylene production reached its peak and ripened normally similar to harvested fruits. Unlike detached fruits (Fig. 6), these fruits did not exhibit an initial peak in ethylene production, but rather a slow and gradual increase during the first days. The reason for this is probably the slower transport of ACC into attached fruits.

**Effect of Exogenous Ethylene on EFE Activity.** The fact that EFE activity increased during ripening only after the beginning of the climacteric rise (Fig. 4) suggested that, similar to other climacteric fruits (13, 21), ethylene stimulates EFE activity in avocado fruits. We tested this assumption by treating intact preclimacteric fruits with ethylene for 15 h and then measuring EFE activity. Fruits treated with ethylene showed a marked increase in EFE activity (Fig. 8), confirming that the EFE system can be induced by ethylene. The increased EFE activity in ethylene treated fruits was detected before the fruits evolved a significant amount of ethylene.

## DISCUSSION

Studies on the regulation of ethylene biosynthesis in plants suggest that one or two of the following steps might be rate-

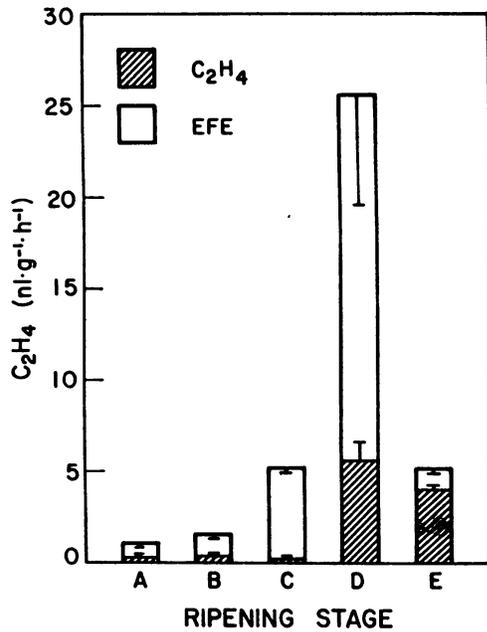


FIG. 4. EFE activity of avocado fruits during ripening. A, Fresh harvested preclimacteric fruit; B, preclimacteric fruit 4 d after harvesting; C, fruit at the early climacteric rise; D, fruit at the climacteric peak; E, postclimacteric fruit 1 d after the climacteric peak. The ripening stage of each fruit was determined by measuring its ethylene evolution and then discs were excised for measuring EFE activity. Shaded bars indicate the ethylene production rate of the discs without ACC. Vertical bars indicate 1 SE.

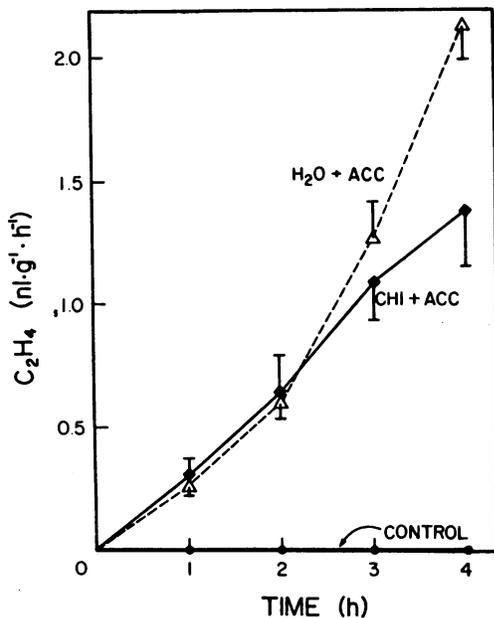


FIG. 5. Development of EFE activity in CHI-pretreated pericarp discs of preclimacteric avocado fruits. Immediately after excision, discs were treated for 0.5 h with H<sub>2</sub>O or CHI (10  $\mu$ M) and then EFE activity was determined by adding ACC. 'Control' refers to the same treatments without ACC. Vertical bars indicate 1 SE.

limiting: the conversion of SAM to ACC and the conversion of ACC to ethylene (22). It has been suggested that both steps restrict ethylene production in preclimacteric fruits. Our results, however, indicate that conversion of ACC to ethylene is not a major limiting factor in preclimacteric avocado. Several types of

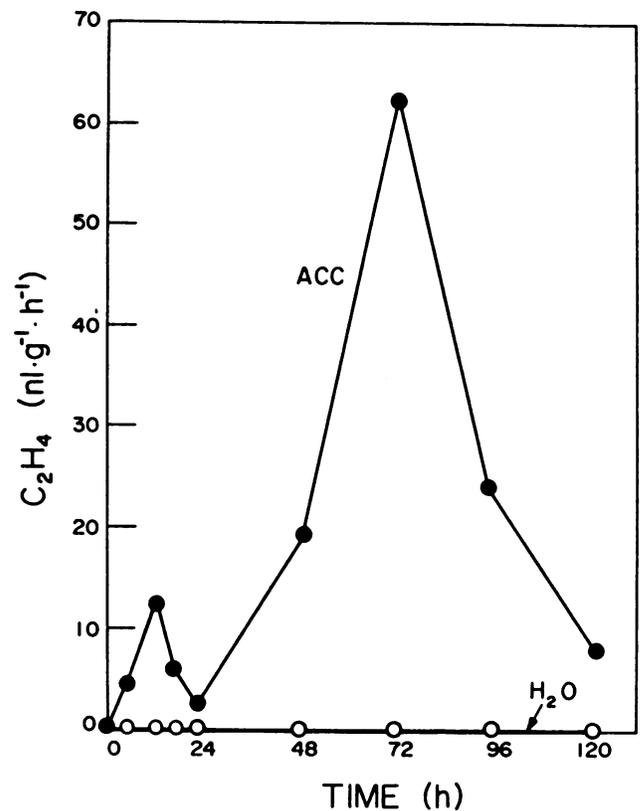


FIG. 6. Ethylene production of detached avocado fruits treated with ACC (5 mM). ACC was applied to the fruits through their cut pedicels for 15 h. Ethylene measurements commenced from the onset of treatment.

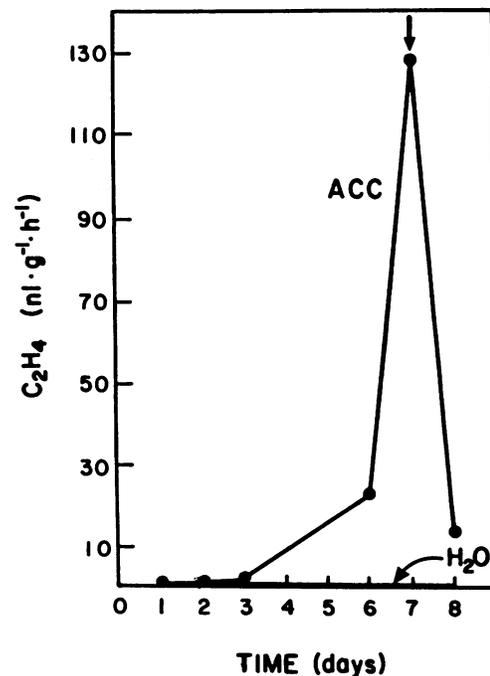


FIG. 7. Ethylene production of attached avocado fruits treated with ACC (10 mM). ACC was applied to the fruits through a cut edge of the branch bearing the fruit during the entire experimental period.

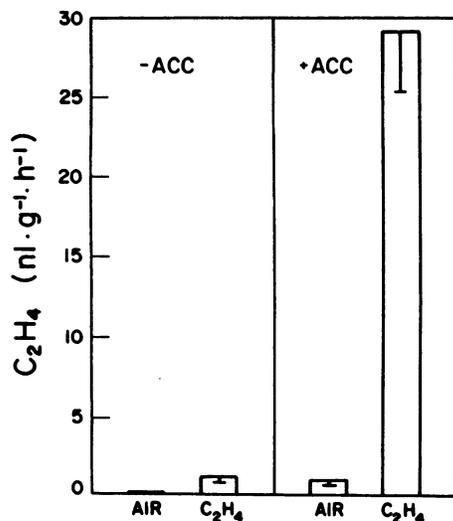


FIG. 8. Effect of ethylene pretreatment ( $10 \mu\text{L}^{-1}$ ) on ethylene production and EFE activity of pericarp discs of preclimacteric avocado. Intact fruits were pretreated with ethylene for 15 h and then discs were cut for measuring ethylene production ( $-ACC$ ) and EFE activity ( $+ACC$ ) during the first 2 h after cutting. Vertical bars indicate 1 SE.

evidence indicate that the EFE system is active during the preclimacteric stage. First, similar levels of EFE activity were measured in discs cut from preclimacteric fruits with or without pretreatment of CHI (Fig. 5). CHI has been shown to inhibit EFE activity and this is attributed to inhibition of the synthesis of the EFE system (11, 17). It was, therefore, expected that if EFE exists in the preclimacteric fruit, CHI would have no effect on the enzyme activity during the initial period of EFE determination. This was indeed the case (Fig. 5). Second, application of ACC to intact but detached preclimacteric fruit, resulted in a rapid increase in ethylene evolution (Fig. 6) and acceleration of the ripening process. Similarly, application of ACC to fruits attached to the tree induced increased ethylene production (Fig. 7) and normal ripening of the fruits. Although EFE activity in preclimacteric avocado fruit is low (Fig. 5), the data in Figures 6 and 7 suggest that it will produce sufficient amount of ethylene to induce autocatalytic production of ethylene when ACC is available. Similar results were obtained with tomato fruits (22), suggesting that also in this fruit EFE activity is not a major limiting factor in the preclimacteric stage.

Our data indicate that the inability of preclimacteric avocado fruit to produce ethylene results solely from lack of ACC due to low activity of ACC synthase (Fig. 1). Others have also reported that activity of ACC synthase and ACC level in the preclimacteric stage of various fruits are very low (9, 12). The question how the activity of ACC synthase is regulated was studied by using metabolic inhibitors. Data in Figures 2 and 3 indicate that development of ACC synthase in preclimacteric avocado discs requires RNA and protein synthesis. Acaster and Kende (2) have also shown by using metabolic inhibitors and density labeling that ACC synthase is *de novo* synthesized when ethylene synthesis is induced by wounding in tomato fruits. Although the effect of metabolic inhibitors on the development of ACC synthase in intact fruits could not be studied, it is likely that a *de novo* synthesis of the enzyme occurs also during natural ripening of avocado fruits.

When exogenous ethylene was applied to preclimacteric tomato and cantaloupe fruits there was no increase in ethylene production rate or ACC level during the initial period (18 h) of treatment, but EFE activity increased markedly (21). Similar results were obtained in the present study when intact avocado fruits were treated with ethylene (Fig. 8). These data suggest that

when preclimacteric fruits are exposed to  $10 \mu\text{L}^{-1}$  of ethylene, the increase in EFE activity precedes that of ACC synthase. However, this sequence of events does not seem to occur during natural ripening of avocado fruits. In several experiments we were unable to detect any increase in EFE activity in harvested fruits during the preclimacteric stage (Fig. 4). EFE activity increased significantly only at the beginning of the climacteric rise when the fruits already showed increased ethylene production. On the other hand, ACC synthase started to increase a few days before the climacteric peak and its activity reached a peak before the climacteric peak (Fig. 1). Similarly, Hoffman and Yang (9) showed that ACC level in ripening avocado fruits reached a peak before the climacteric peak. We suggest that the following sequence of events occurs during natural ripening of avocado fruits: ACC synthase is first synthesized producing ACC which is converted to ethylene by the already existing EFE system. The ethylene produced stimulates additional ACC synthase activity and then, when ethylene production reaches a certain threshold level, it stimulates EFE activity, thereby further increasing ethylene production. Autocatalysis is a common feature of ripening in climacteric fruits (1, 6, 16) and recent data indicate that ethylene enhances the induction of both ACC synthase (4) and EFE (10, 12, 13) in various fruits.

It is well accepted that malonylation of ACC may play a role in the regulation of endogenous ACC level and hence of ethylene production (22). Preclimacteric avocado fruits contain a significant level of MACC (Fig. 1). This indicates that at least part of the low level of ACC which is synthesized in the course of fruit growth is conjugated to MACC. Thus, malonylation of ACC may participate in regulating ethylene production during the preclimacteric stage. In contrast, it does not seem to play a role in regulating ethylene production at the climacteric stage. At this stage, MACC increases only slightly compared to the marked increase in ACC level (Fig. 1). The decrease in ethylene production during the postclimacteric stage results mainly from the decline in the activity of ACC synthase and EFE (Figs. 1 and 5). Hoffman and Yang (9) observed a renewed increase in ACC level during the postclimacteric rise of overripe avocado fruits. In the present study, only a slight renewed increase in ACC level was observed during that stage (Fig. 1), probably because we used fruits harvested at a different maturity stage.

It has been suggested that in preclimacteric fruits ACC synthase is restricted by a repressor which is being destroyed in the course of ripening or ethylene treatment (10). According to this hypothesis ACC synthase will develop only after the destruction of this repressor. This does not seem to be the case with the EFE system, where short exposure to ethylene induces increased activity at a time when there is no synthesis of ACC and no ethylene production (21; Fig. 8). In addition, exogenous ACC induces ethylene production in intact preclimacteric avocado fruits shortly after the beginning of treatment (Fig. 6). In this respect, attached avocado fruits resemble detached fruits. Attached fruits are capable of reaching the climacteric providing ACC is available (Fig. 7). The data suggest that the inability of most avocado varieties to produce ethylene as long as they are attached to the tree results mainly from repression of ACC synthase. This repression is removed after harvesting, resulting in normal ripening of the fruits.

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