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Biosynthesis of 1-aminocyclopropane-1-carboxylic acid and ethylene from [delta]-aminolevulinic acid in ripening tomato fruits

Diaa El-Deen Ahmed El-Rayes
Iowa State University

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El-Rayes, Diaa El-Deen Ahmed, Ph.D.

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Biosynthesis of 1-aminocyclopropane-1-carboxylic acid and ethylene from δ-aminolevulinic acid in ripening tomato fruits

by

Diaa El-Deen Ahmed El-Rayes

A Dissertation Submitted to the
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GENERAL INTRODUCTION

Ethylene (C_2H_4), the ripening hormone of plants, is the simplest unsaturated carbon compound. It exerts a major influence on many aspects of plant growth, development, and senescence, apparently at regulatory levels of metabolism. It affects seed germination (33), seedling growth (10), root growth (14), leaf growth (27), many kinds of stress phenomena (19), and it regulates ripening, aging, and senescence (18). Ethylene is, therefore, an important component in the array of hormonal regulatory factors that control growth, development, and senescence. Depending upon when C_2H_4 production occurs, it may be beneficial or harmful to harvested horticultural crops. Efficient postharvest technology therefore requires the ability to control the effects of C_2H_4 to suit our practical needs. Before C_2H_4 can exert such responses, C_2H_4 has to be biosynthesized by the plants, or it must be supplied from external sources.

The ripening of fruit showing a climacteric rise in respiration is triggered by endogenously produced C_2H_4. It also can be advanced by exogenously applied ethylene. If the endogenous, autocatalytic C_2H_4 production is chemically or physiologically inhibited, ripening and senescence processes including the climacteric are prevented unless C_2H_4 is added.

The number of biochemical and physiological studies of C_2H_4 biosynthesis in both higher plants and microorganisms have increased significantly in recent years. Several model systems by which C_2H_4

could be synthesized have been described (1, 25, 37). Current research in the area of C_2H_4 biochemistry and physiology has, as its aim, the establishment of the pathway of biosynthesis of this hormone in plants.

GENERAL LITERATURE REVIEW

Precursors of C_2H_4

The task of unravelling the precursors of C_2H_4 has not been easy. Because of the simple chemical structure of C_2H_4, there are many compounds that could be converted to C_2H_4 through various chemical and physiological reactions. Consequently, a number of compounds have been proposed as precursors of C_2H_4 such as linolenic acid, propanal, ethionine, ethanol, acetic acid, and methionine. All of these compounds are of biological origin and are converted to C_2H_4 via various model systems or cell-free extracts. Research on these compounds indicated that methionine is the most likely C_2H_4 precursor (36). Subsequent research on C_2H_4 biosynthesis has led to the following pathway: methionine ⟶ S-adenosylmethionine ⟶ ACC ⟶ C_2H_4 (37).

Evidence for C_2H_4 precursors other than methionine

Although some data indicate that methionine is the sole precursor of C_2H_4 in plant tissues (36, 37), there are suggestions that additional precursors and pathways may occur in some ripening fruits (3, 23). Research on C_2H_4 production from fruit tissues has shown that the concentration of free methionine was two to three times higher in unripe fruits than in those fully ripe (5). However, the same researchers also showed that the concentration of free methionine could not account for the quantity of C_2H_4 produced during the climacteric rise and that this concentration could sustain C_2H_4 production for only 3 hr. These data have been confirmed by others who reported that free methionine in

tomato fruits at the green stage is relatively low (13.5 μmol/100 g fresh weight) and that it decreases as the fruits ripen (6.5 μmol/100 g fresh weight at the climacteric rise, and 7.5 μmol/100 g fresh weight at the climacteric peak) (38). These results allow the interpretation that either methionine is turned over rapidly or there are important precursors of C_2H_4 other than methionine.

Ethylene production in green tomato fruits is inhibited significantly by aminoethoxyvinylglycine (AVG), but as the fruit ripens, sensitivity to AVG declines considerably (4). These same researchers also reported that rhizobitoxine analog, which inhibits the conversion of methionine into C_2H_4 by 50 to 70% in green tomato fruit slices, showed only slight effects when tomato fruits were tested at the climacteric peak. The incorporation of ^{14}C from (^{14}C)methionine into C_2H_4 in green and pink tomato tissues was inhibited by rhizobitoxine analog to about the same extent as the inhibition of total C_2H_4 production. Therefore, the ability of tomato fruits to convert methionine into C_2H_4 does not parallel its ability to produce C_2H_4 naturally (4). AVG also showed no effect on C_2H_4 production by tomato fruits during the first 8 hr of incubation (24). Avocado tissue slices also are relatively insensitive to AVG (4), and they did not convert (^{14}C)methionine into $^{14}C_2H_4$ in preclimacteric tissues (5).

It has been reported that methionine had little effect upon C_2H_4 production in flower tissue of Ipomoea tricolor Cav. (23), and there are some precursors that are converted more efficiently to C_2H_4 than methionine. Similar results were found with sorghum stem tissues, pea

homogenates, and red tomato (11, 22). In these same studies, methionine had no appreciable effect, but there was a large response to ACC in all cases. In other research, it was found that methionine either had no effect or slightly inhibited C_2H_4 production in apple tissues (2) and that, in pea stem sections, C_2H_4 production was promoted only slightly by treatment with methionine (29).

Baker et al. suggested that, during ripening, the tomato fruit switches from methionine to an unknown compound as the major precursor of C_2H_4, but it retains its ability to utilize methionine as a precursor of C_2H_4. Two pathways seem to be involved in C_2H_4 production by tomato, and the same researchers concluded that some fruits utilize precursors other than methionine for C_2H_4 production (3).

Ethylene production by microorganisms

Although C_2H_4 production from methionine has been reported in some microorganisms (28), there are some doubts about the physiological or metabolic nature of C_2H_4 production by these microorganisms, especially because they required light and were stimulated greatly by reduced iron, conditions that convert methionine to C_2H_4 nonenzymatically (26). However, with Penicillium digitatum, there is some disagreement whether or not methionine can be the precursor of C_2H_4 (20, 28). With Penicillium digitatum, methionine inhibited C_2H_4 production by 30 to 65% (15), and uniformly labeled methionine was not converted into labeled C_2H_4 (21). Furthermore, Chou and Yang concluded that, in the case of P. digitatum, glutamate is a more efficient precursor of C_2H_4 than any of

the other precursors (16).

Precursors of ALA

δ-aminolevulinic acid was found to be formed first by the condensation of glycine and succinyl-CoA (31). However, subsequent research indicated that, in higher plants and algae, most, and possibly all, ALA is synthesized via a 5-carbon pathway that utilizes the intact carbon skeleton of glutamate (8, 9) and α-ketoglutarate (12, 35), and, due to the likelihood of rapid interconversion of glutamate and α-ketoglutarate to ALA in vivo, it has been impossible to deduce which of these compounds is the more direct precursor of ALA (8).

It is interesting that glutamate, the precursor of C_2H_4 in Penicillium digitatum (16), is the precursor of ALA in higher plants (6). Moreover, carbon atoms 3 and 4 of glutamate or α-ketoglutarate are metabolized into either C_2H_4, in P. digitatum (16), or ALA (carbon atoms 2 and 3 in ALA), in plants (9).

δ-aminolevulinic acid metabolism

ALA is the first committed intermediate during the biosynthesis of all naturally occurring tetrapyrroles like heme, chlorophyll, corrins, vitamin B_{12}, and bile pigments (8, 30). The role of this compound as a rate-limiting precursor in chlorophyll synthesis in angiosperm leaves is well documented (7, 13). However, there now is a growing body of evidence that indicates that ALA may be metabolized via nonporphyrin(s) in a number of organisms (7, 12, 17, 32, 34).

SECTION I. METABOLISM OF δ-AMINOLEVULINIC ACID TO ETHYLENE BY TOMATO FRUIT

ABSTRACT

The influence of δ-aminolevulinic acid (ALA), methionine, 1-aminocyclopropane-1-carboxylic acid (ACC), glutamine, glutamate, and α-ketoglutarate on ethylene (C_2H_4) and ACC production in 'Heinz 1350' tomato pericarp discs was determined. ALA increased C_2H_4 to 232% and ACC to 410% of the control production rates, whereas methionine increased C_2H_4 to 152% and ACC to 167% of the production rates of the control. ACC enhanced C_2H_4 production to 242% of the control, while glutamine, glutamate, and α-ketoglutarate were not different from the control or from each other. The C_2H_4 production rate varied with the ALA concentration and the stage of fruit development. As the ALA concentration increased from zero to 40 mM, the C_2H_4 production rate increased. Both treated and untreated pericarp discs from fruits at the pink stage of development yielded the largest C_2H_4 production rate. Application of (2,3-^{3}H)ALA to pericarp discs caused the accumulation of radioactivity in both ACC and C_2H_4 but not in methionine. These data suggest that, during ripening of tomato fruits, C_2H_4 is formed from ALA via ACC.

INTRODUCTION

Ethylene (C_2H_4) has a profound effect on plants, and its effects have been the subject of many reports (1, 16, 26). Several model systems by which C_2H_4 could be synthesized have been described (1, 16, 26). Methionine was suggested first as a precursor of C_2H_4 by Lieberman and Mapson (17), and subsequent research on C_2H_4 biosynthesis has led to the following pathway: methionine ⟶ S-adenosylmethionine (SAM) ⟶ ACC ⟶ C_2H_4 (26). In addition, several other compounds may be precursors under certain conditions (26).

Research on C_2H_4 production from fruit tissues has shown that the concentration of free methionine was two to three times higher in unripe fruits than in those fully ripe (4). However, the same researchers also showed that the concentration of free methionine could not account for the quantity of C_2H_4 produced during the climacteric rise and that this concentration could sustain C_2H_4 production for only 3 hr. These data have been confirmed by others who reported that free methionine in tomato fruits at the green stage was relatively low (13.5 μmol/100 g fresh weight) and that it decreased as the fruits ripened (6.5 μmol/100 g fresh weight at the climacteric rise, and 7.5 μmol/100 g fresh weight at the climacteric peak) (27).

It has been reported that methionine had little effect upon C_2H_4 production in flower tissue of Ipomoea tricolor Cav. (14), and similar results were found with sorghum stem tissues, pea homogenates, and red tomato (6, 13). In these same studies, methionine had no appreciable

effect, but there was a large response to ACC in all cases. In other research, it was found that methionine either had no effect or slightly inhibited C_2H_4 production in apple tissues (2), and that, in pea stem sections, C_2H_4 production was promoted only slightly by treatment with methionine (22). Furthermore, with Penicillium digitatum, there is some disagreement whether or not methionine can be the precursor of C_2H_4 (11, 21). With Penicillium digitatum, methionine inhibited C_2H_4 production by 30 to 65% (8), and uniformly labeled methionine was not converted into labeled C_2H_4 (12).

As tomato fruits ripen, their sensitivity to aminoethoxyvinylglycine (AVG) declines considerably, and it also was found that avocado slices are relatively insensitive to AVG (3). These same researchers also reported that rhizobitoxine analog, which inhibits the conversion of methionine into C_2H_4 by 50 to 70% in green tomato fruit slices, showed only slight effects when tomato fruits were tested at the climacteric peak. The incorporation of ^{14}C from (^{14}C)methionine into C_2H_4 in green and pink tomato tissues was inhibited by rhizobitoxine analog to about the same extent as the inhibition of total C_2H_4 production. Therefore, the ability of tomato fruits to convert methionine into C_2H_4 does not parallel its ability to produce C_2H_4 naturally (3). AVG also showed no effect on C_2H_4 production by tomato fruits during the first 8 hr of incubation (15). These results allow the interpretation that either methionine is turned over rapidly or there are important precursors of C_2H_4 other than methionine.

The precursors of ALA can be glutamate, glutamine, and α-keto-glutarate, but the major precursor is glutamate (5). The precursor of porphyrins in green plants is ALA, and chlorophyll is one of the major products of these tetrapyrrole biosynthetic pathways (7). However, there now is a growing body of evidence that indicates that ALA may be metabolized via nonporphyrin pathways in a number of organisms, and it was found that ALA could be catabolized to CO_2 and other metabolites (9, 23). In previous studies, it was found that both porphobilinogen deaminase, one of the enzymes that converts ALA into porphobilinogen, and chlorophyll content increased to a maximum in 35-day tomato fruits and then decreased to near zero in ripe fruits (20). The objective of this research was to determine whether ALA is converted into ACC and ultimately into C_2H_4 during tomato fruit ripening.

MATERIALS AND METHODS

Plant materials

Lycopersicon esculentum Mill. 'Heinz 1350' tomato plants were trained to a single stem and grown in an environmentally controlled greenhouse under standard cultural practices. Tomato fruits were harvested as needed, graded according to USDA standards for grades of fresh tomatoes, and rinsed with tap water and then with deionized water several times.

Fruit disc preparation and incubation

Discs of tomato pericarp tissue (1 cm diam, 2.5 mm thick, and 265±15 mg fresh weight per disc), with the epidermis intact, were cut from tomato fruits at the pink stage unless otherwise stated. Four discs were incubated for 48 hr at 25 C in a 125-ml Erlenmeyer flask sealed with a rubber serum stopper. Each flask contained one layer of Whatman No. 1 filter paper wetted with 1.5 ml of one of the following compounds: methionine, ALA, ACC, glutamine, glutamate, or α-ketoglutarate, all at 40 mM, or water (control).

Ethylene and ACC analysis

Two-milliliter samples of the gas phase were withdrawn every 6 hr with a gas-tight syringe and analyzed for C_2H_4 on a Varian 3700 gas chromatograph by using methods described previously (24). At the end of the incubation period, immediately after the analysis for C_2H_4, the

discs were analyzed for ACC by using the procedure of Lizada and Yang (18).

Radioisotope studies

For radiotracer studies, discs were incubated in 1.5 ml of solution containing 10 μCi of (2,3-^{3}H)ALA as described above in 'fruit disc preparation and incubation'. Flasks were sealed with rubber serum stoppers, and labeled C_2H_4 was trapped with $Hg(ClO_4)_2$ by venting the headspace every 6 hr with a vacuum pump through gas-absorbing traps. At the end of each venting period, the absorption apparatus was disconnected from the incubation flask, and the contents were prepared for analysis of radioactivity in a Tracor Delta 300 liquid scintillation counter. The incubating flasks then were flushed with fresh air and resealed for subsequent gas sample analyses.

Purification and determination of labeled methionine and labeled ACC

Two-dimensional thin-layer chromatography procedures published previously were used to separate labeled materials (19, 25), and a liquid scintillation counter was used for measuring the radioactivity.

Chemicals and statistical analysis

ALA was purchased from Aldrich, and methionine and ACC were purchased from Sigma. (2,3-^{3}H)ALA was purchased from RPI. All experiments were done in triplicate, and the data were pooled for statistical analysis.

RESULTS

Effect of ALA concentration on C_2H_4 production

Previous research has shown that the C_2H_4 production rate is greatest in fruit at the pink stage (10). Therefore, discs at the pink stage of development were used to determine the effect of the ALA concentration on the C_2H_4 production rate. As the ALA concentration was increased from zero to 40 mM, the C_2H_4 production rate increased to almost three times that of the control (Fig. 1). However, at 50 mM, the C_2H_4 production rate declined slightly. The rates of C_2H_4 production were significantly larger for each increment of ALA concentration up to 40 mM; therefore, 40 mM was chosen as the ALA concentration for all subsequent experiments.

Stage of development and conversion of ALA into C_2H_4

For discs incubated in water, the C_2H_4 production rate was the greatest at the pink and light red stages of development at 6 and 12 hr (Fig. 2). When 40 mM ALA was supplied to the discs, again the pink and light red stages of development had the greatest rate of C_2H_4 production at 6 and 12 hr. All other stages, except mature green, produced significantly more C_2H_4 when supplied ALA as compared with those receiving only water. Both the amount of C_2H_4 produced and the length of time during which the discs produced C_2H_4 were larger for discs treated with ALA as compared with water. Neither the timing of the C_2H_4 production pattern nor the amount of C_2H_4 produced by green tomato discs

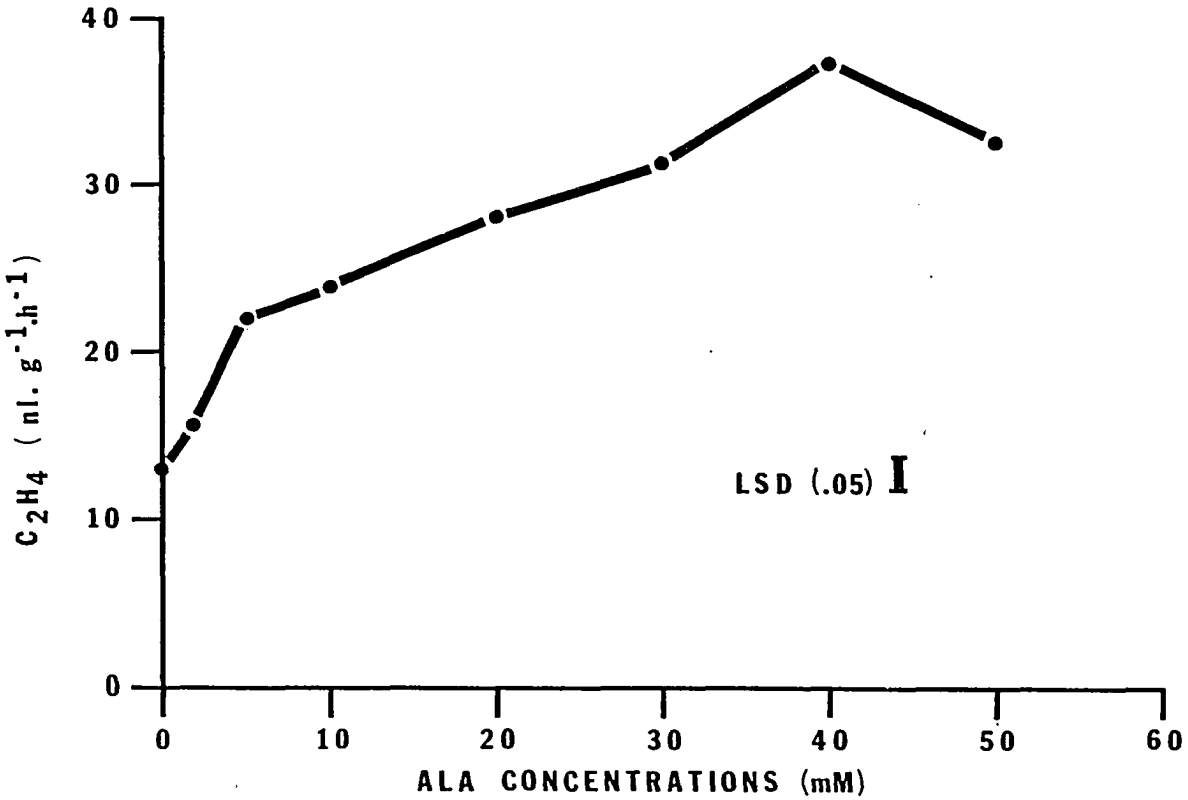

Figure 1. Ethylene production rate of tomato pericarp tissue at the pink stage as a function of ALA concentration

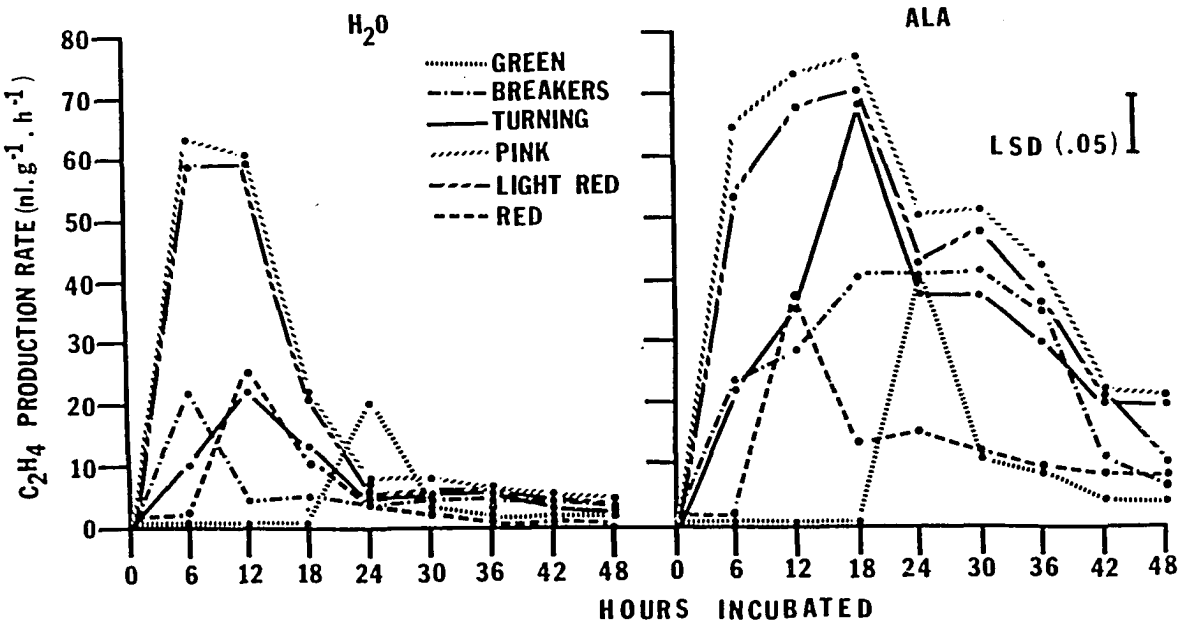

Figure 2. Timecourse of C_2H_4 production from control and ALA-treated (40 mM) tomato pericarp discs at 6 stages of development

was affected by ALA as compared with water. Therefore, discs relatively close to either the pink or light red stages of development produced more C_2H_4 when treated with ALA than when they were treated with water.

Influence of ALA, ALA precursors, and C_2H_4 precursors on C_2H_4 production

Tomato fruit discs treated with the ALA precursors glutamate, glutamine, and α-ketoglutarate did not show any significant increase in C_2H_4 production from the control during incubation (Fig. 3). At 12 hr, however, glutamine and α-ketoglutarate showed a decreased rate of C_2H_4 production. Tomato fruit discs treated with the C_2H_4 precursor methionine, however, showed a slight increase in C_2H_4 production over both the control and the ALA precursors at 24, 30, and 36 hr. On the other hand, fruit discs treated with either ALA or ACC showed a significant increase in C_2H_4 production over all other treatments after 12 hr of incubation, and the C_2H_4 production rate of ALA- or ACC-treated discs remained greater than all other treatments throughout the incubation period (Fig. 3).

Influence of ALA, ALA precursors, and C_2H_4 precursors on C_2H_4 and ACC production

Subsequent experiments showed that discs treated with ALA and ACC had the greatest C_2H_4 production rate, and there was no significant difference between the discs treated with ACC or ALA (Fig. 4). Discs treated with ALA or ACC produced significantly more C_2H_4 than those treated with methionine or any of the ALA precursors. In addition to

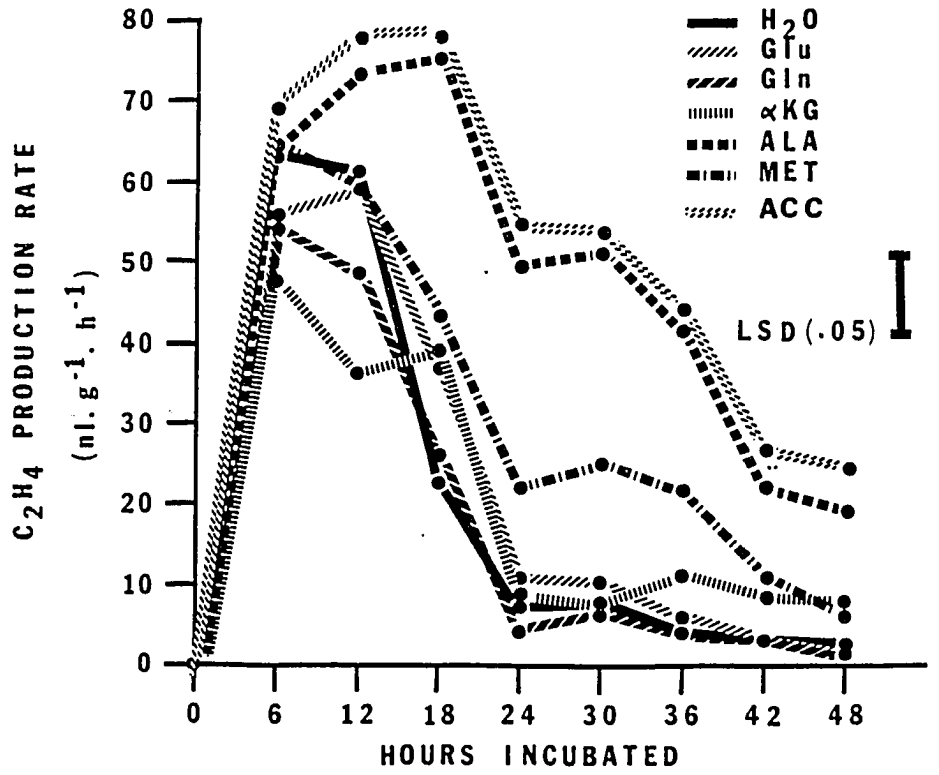

Figure 3. Effect of glutamate, glutamine, α-ketoglutarate, ALA, methionine, and ACC on C_2H_4 production by tomato pericarp tissue at the pink stage

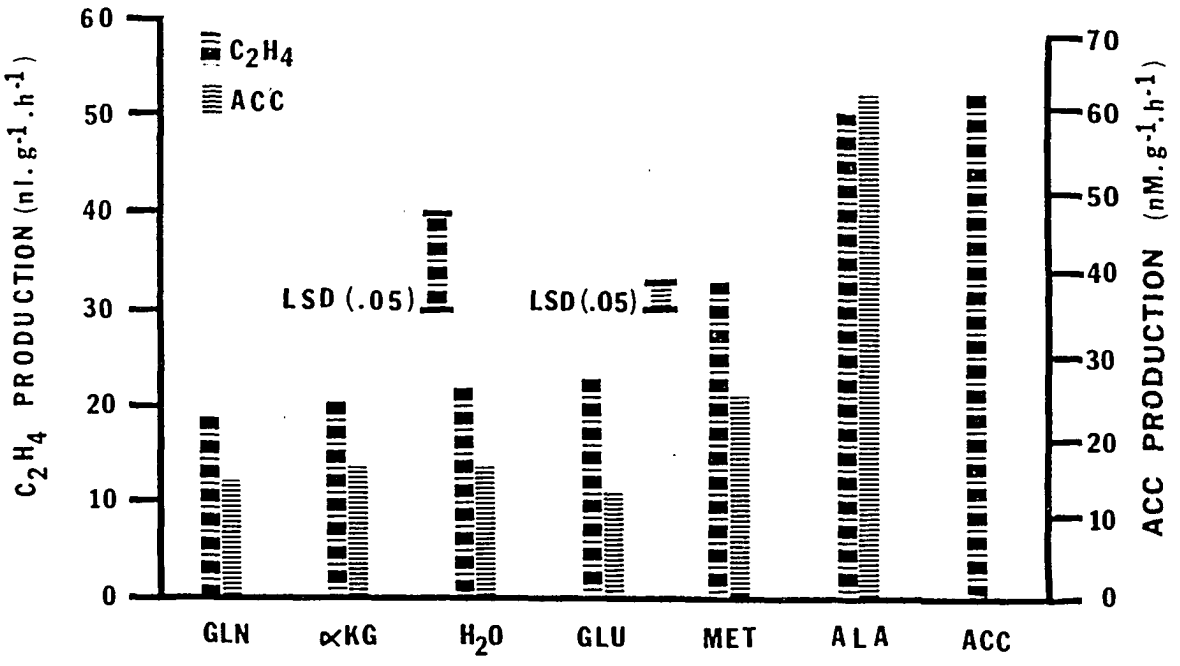

Figure 4. Effect of glutamine, α-ketoglutarate, water (as control), glutamate, methionine, ALA, and ACC on C_2H_4 and ACC production by tomato pericarp tissue at the pink stage

stimulating C_2H_4 production, ALA caused an even more dramatic increase in ACC content of the discs (Fig. 4). The ACC produced by treatment with methionine exceeded that of the water control and that of the ALA precursor, and the ACC produced by treatment with ALA was significantly greater than the ACC produced by treatment with methionine.

Conversion of (2,3-^{3}H)ALA into methionine, ACC, and C_2H_4

Tomato pericarp discs incubated in (2,3-^{3}H)ALA produced labeled methionine, C_2H_4, and ACC (Table 1). Radioactivity in C_2H_4 must be derived from ^{3}H-ALA, and this provides direct evidence that tomato pericarp tissue converted ALA into C_2H_4. After incubation, the pericarp tissue was analyzed for ACC and methionine to determine whether ALA was converted to methionine or ACC before it was converted to C_2H_4. After purification and chromatography of the tissue extracts, two of the radioactive metabolites were identified as methionine and ACC (Table 1). However, the amount of radioactive methionine detected was insignificant as compared with the amount of radioactivity found in C_2H_4 and ACC, and other metabolites yielded insignificant amounts of radioactivity (data not presented).

Table 1. Conversion of (2,3-^{3}H)ALA into methionine (MET), C_2H_4, and ACC by tomato fruit discs

Metabolite	Radioactivity (DPM x 10^3)
MET	24.3
C_2H_4	1266.4
ACC	4830.0

DISCUSSION

δ-aminolevulinic acid was supplied to tomato pericarp discs at six stages of fruit development, and discs at the pink and light red stages showed the greatest ability to convert ALA into ACC and C_2H_4 (Figs. 2, 3, and 4). As the pericarp disc tissue stage of development approached the climacteric peak, its ability to convert ALA into C_2H_4 increased in a manner corresponding to its ability to produce C_2H_4 endogenously (Fig. 2). Previously, it was reported that 1 mM ACC stimulated C_2H_4 production to a greater extent in climacteric fruit than in early climacteric rise fruit (2) and that the stimulatory effect of methionine on C_2H_4 production declined as the tomato fruits ripened (6). The data in this report and those of the previous research indicate a similarity between ALA and ACC in their ability to affect C_2H_4 production. Because ALA and ACC are similar to one another but different from methionine in their ability to produce C_2H_4 (Figs. 3 and 4), it is clear that ALA enters the pathway of C_2H_4 biosynthesis at a point closer to C_2H_4 than methionine.

This research presents information on another compound, ALA, that increased not only C_2H_4 production but also ACC production. The molecular structure of ALA is such that it could be converted into either methionine or ACC directly. These data lead to the conclusion that ALA does not enter the C_2H_4 biosynthetic pathway at methionine (from conversion of ALA into methionine) but that it enters the pathway after methionine and before or at ACC. If ALA entered the pathway at

methionine, both ACC accumulation and C_2H_4 production from ALA would be similar to, or slightly less than, that of methionine, but these data show that methionine produces significantly less ACC and C_2H_4 than does ALA (Figs. 3 and 4). In addition, ALA treatment produced the same amount of C_2H_4 as did treatment with ACC (Figs. 3 and 4). Therefore, ALA probably is not converted into methionine, but it enters the C_2H_4 biosynthetic pathway at a point closer than methionine to C_2H_4. The only plausible way in which the pathway ALA $\longrightarrow$ methionine $\longrightarrow$ ACC $\longrightarrow$ C_2H_4 could be operable would be if there was an extremely rapid turnover of methionine into ACC, but these data do not support this hypothesis (Fig. 4 and Table 1).

At first, the fact that ALA did not increase the C_2H_4 production rate appreciably in tomato fruit discs at the green stage of development during incubation was difficult to explain (Fig. 2). However, it is well known now that porphobilinogen deaminase is still active at this stage of development (20) and that the ALA probably was used for chlorophyll production rather than C_2H_4 production.

There may be two separate and independent pathways by which C_2H_4 could be synthesized during tomato fruit development. One pathway is that of methionine $\longrightarrow$ SAM $\longrightarrow$ ACC $\longrightarrow$ C_2H_4, and this pathway probably is operable at all times, but at a relatively low rate. The other pathway utilizes ALA directly for the production of ACC, which subsequently is metabolized to C_2H_4. This latter pathway becomes operable only when the activity of the enzyme porphobilinogen deaminase decreases to zero or nearly zero and ALA is no longer used for

chlorophyll biosynthesis. This change from the methionine-based pathway of C_2H_4 biosynthesis into the ALA pathway must be researched in more detail before explicit hypotheses about its relationship to ripening in fruit tissue can be advanced.

Also, further research must be done on the pathway by which ALA is metabolized to C_2H_4. Experiments that again verify the conversion of ALA to ACC must be conducted, as well as experiments that provide evidence for the mechanism by which each portion of the ALA molecule becomes converted into the ACC molecule. Finally, all the results of these experiments must be developed into a hypothesis that accounts for the decreased chlorophyll and porphobilinogen deaminase activity during the time in which ALA becomes converted to ACC and subsequently to C_2H_4.

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SECTION II. δ-AMINOLEVULINIC ACID: A NEW PRECURSOR OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID AND ETHYLENE IN RIPENING TOMATO FRUITS

ABSTRACT

The fate of δ-aminolevulinic acid (ALA) applied to 'Heinz 1350' tomato fruits at the pink stage of development has been studied. Discs of pericarp tissue were incubated in individual treatments of 10 μCi (2,3-^{3}H)ALA, 1 μCi (4-^{14}C)ALA, or 1 μCi (5-^{14}C)ALA for 48 hr, and radioactive ethylene (C_2H_4), carbon dioxide (CO_2), and 1-aminocyclopropane-1-carboxylic acid (ACC) were measured. Radioactivity from ^{3}H was detected in both ACC and C_2H_4. Radioactivity from ^{14}C-4 was detected in both ACC and CO_2, but not in C_2H_4. Radioactivity from ^{14}C-5 was detected in CO_2, and its amount was greater than that obtained from ^{14}C-4. Neither ACC nor C_2H_4 showed any radioactivity when (5-^{14}C)ALA was supplied to the fruit discs, and this implies that carbon atom 5 of ALA is lost as CO_2 during the formation of ACC from ALA. When (2,3-^{3}H)ALA or (4-^{14}C)ALA was supplied to the fruit discs, radioactivity was detected in other metabolites such as fumarate, succinate, malate, glutamate, glutamine, α-ketoglutarate, and methionine, but the amount of radioactivity was insignificant compared with the amount of radioactivity found in C_2H_4 and ACC. These results suggest that ALA is metabolized to ACC and ultimately to C_2H_4 in ripening tomato fruits. One route by which ALA can be converted into ACC is transamination followed by decarboxylation of carbon atom 5 and then cyclization to ACC. A new pathway for C_2H_4 biosynthesis is presented, and this pathway may have an important relationship to chlorophyll biosynthesis and the associated degreening processes in ripening tomato fruit.

INTRODUCTION

Ethylene exerts a major influence on many aspects of plant growth, development, and senescence. Several model systems by which C_2H_4 could be synthesized have been described (1, 8, 16). Methionine was suggested first as a precursor of C_2H_4 (9), and subsequent research on C_2H_4 biosynthesis has led to the following pathway: methionine $\longrightarrow$ S-adenosylmethionine (SAM) $\longrightarrow$ ACC $\longrightarrow$ C_2H_4 (16). However, there are additional precursors and pathways that occur in some ripening fruits (2, 3, 7).

Ethylene production in green tomato fruits is significantly inhibited by aminoethoxyvinylglycine (AVG), but as the fruit ripens, sensitivity to AVG declines considerably (2, 3). The conversion of methionine into C_2H_4 was inhibited by AVG by 50 to 70% in green tomato fruit slices, and it affected the C_2H_4 production by tomato fruits only slightly when they were tested at the climacteric peak (3). The incorporation of ^{14}C from (^{14}C)methionine into C_2H_4 in green and pink tomato tissues was inhibited by rhizobitoxine analog to about the same extent as the inhibition of total C_2H_4 production, and therefore, the ability of tomato fruits to convert methionine into C_2H_4 does not parallel its ability to produce C_2H_4 naturally (3). Baker et al. suggested that, during ripening, the tomato fruit switches from methionine to an unknown compound as the major precursor of C_2H_4, but retains its ability to utilize methionine as a precursor of C_2H_4 (2). Two pathways seem to be involved in C_2H_4 production by tomato (3). The

same researchers concluded that some fruits utilize precursors other than methionine for C_2H_4 production (3).

δ-aminolevulinic acid is the first committed intermediate during the biosynthesis of all naturally occurring tetrapyrroles like heme, chlorophyll, corrins, or bile pigment (4, 12). The role of this compound as a rate-limiting precursor of chlorophyll in leaves is well documented (4, 5). However, now there is a growing body of evidence that indicates that ALA may be metabolized via nonporphyrin(s) in a number of organisms (4, 5, 6, 13, 15).

MATERIALS AND METHODS

Plant materials

Lycopersicon esculentum Mill. 'Heinz 1350' tomato plants were trained to a single stem and grown in an environmentally controlled greenhouse under standard cultural practices. Tomato fruits were harvested as needed, graded according to USDA standards for grades of fresh tomatoes, rinsed with tap water, and then with deionized water several times.

Fruit disc preparation and incubation

Discs of tomato pericarp tissue (1 cm diam, 2.5 mm thick, and 265±15 mg fresh weight per disc), with the epidermis intact, were cut from tomato fruits at the pink stage. Four discs were incubated for 48 hr at 25 C in a 125-ml Erlenmeyer flask sealed with a rubber serum stopper. Each flask contained one layer of Whatman No. 1 filter paper wetted with 1.5 ml of one of the following compounds: 10 μCi of (2,3-^{3}H)ALA, 1 μCi of (4-^{14}C)ALA, or 1 μCi of (5-^{14}C)ALA.

Measurement of (^{3}H)C_2H_4 and $^{14}CO_2$ production

(^{3}H) C_2H_4 and $^{14}CO_2$ produced by tomato fruit discs were trapped with $Hg(ClO_4)_2$ and NaOH respectively. A gas stream was pulled every 6 hr with a vacuum pump from the incubating-flask headspace through a gas-absorbing apparatus. The gas-absorbing apparatus consisted of 2 gas-scrubbing flasks for trapping $^{14}CO_2$ and 2 gas-scrubbing flasks for trapping (^{3}H)C_2H_4, in series. At the end of each venting period, the

absorption apparatus was disconnected from the incubation flask, and the contents were prepared for analysis of radioactivity in a Tracor Delta 300 liquid scintillation counter. The incubating flasks then were flushed with fresh air and resealed for subsequent gas analyses.

Purification and determination of ALA-labeled metabolites

Two-dimensional thin-layer chromatography procedures published previously were used to separate labeled materials (10, 14), and a liquid scintillation counter was used for measuring the radioactivity.

Chemicals and statistical analysis

(4-^{14}C)ALA and (2,3-^{3}H)ALA were purchased from Research Products International Corp., Mount Prospect, Illinois, and (5-^{14}C)ALA was purchased from New England Nuclear. All chemicals used were of reagent grade. ALA was purchased from Aldrich, and methionine, ACC, malic acid, fumaric acid, glutamine, glutamate, and α-ketoglutarate were purchased from Sigma. Succinic acid was purchased from Fisher. Within each experiment, each treatment was replicated three times, and all experiments were conducted independently three times, and the data were pooled for statistical analysis.

RESULTS

Timecourse of $^{14}CO_2$ and $C_2{}^3H_4$ production from (^{14}C)ALA and (2,3-^{3}H)ALA

The $^{14}CO_2$ produced by tomato pericarp tissues at the pink stage of development treated with either 1 μCi (4-^{14}C)ALA or 1 μCi (5-^{14}C)ALA separately has been examined (Fig. 1). $^{14}CO_2$ production from both (4-^{14}C)ALA and (5-^{14}C)ALA increased as a function of time. $^{14}CO_2$ derived from (5-^{14}C)ALA was significantly greater than the $^{14}CO_2$ derived from (4-^{14}C)ALA beginning 18 hr after the start of the incubation period. The production rate of $^{14}CO_2$ from (5-^{14}C)ALA was almost twice the amount of $^{14}CO_2$ produced from (4-^{14}C)ALA throughout the remainder of the incubation period. The production of $C_2{}^3H_4$ from tomato discs treated of incubation increased (Fig. 1).

Catabolism of ALA molecule

Tomato pericarp tissue at the pink stage was incubated separately with 1 μCi (4-^{14}C)ALA, 1 μCi (5-^{14}C)ALA, or 10 μCi (2,3-^{3}H)ALA for 48 hr. Both ^{14}C-4 and ^{14}C-5 activities were found in $^{14}CO_2$ but not in C_2H_4. Radioactivity from (2,3-^{3}H) was detected in C_2H_4. After the incubation period, the pericarp tissues were analyzed and the activity of ^{14}C from (5-^{14}C)ALA was found in glutamate, α-ketoglutarate, and glutamine. There was no detectable radioactivity in C_2H_4, ACC, fumarate, succinate, malate, or methionine (Table 1). Radioactivity from ^{14}C-4 was detected in ACC, fumarate, succinate, malate, glutamate, α-ketoglutarate, and traces in both glutamine and methionine (Table 1).

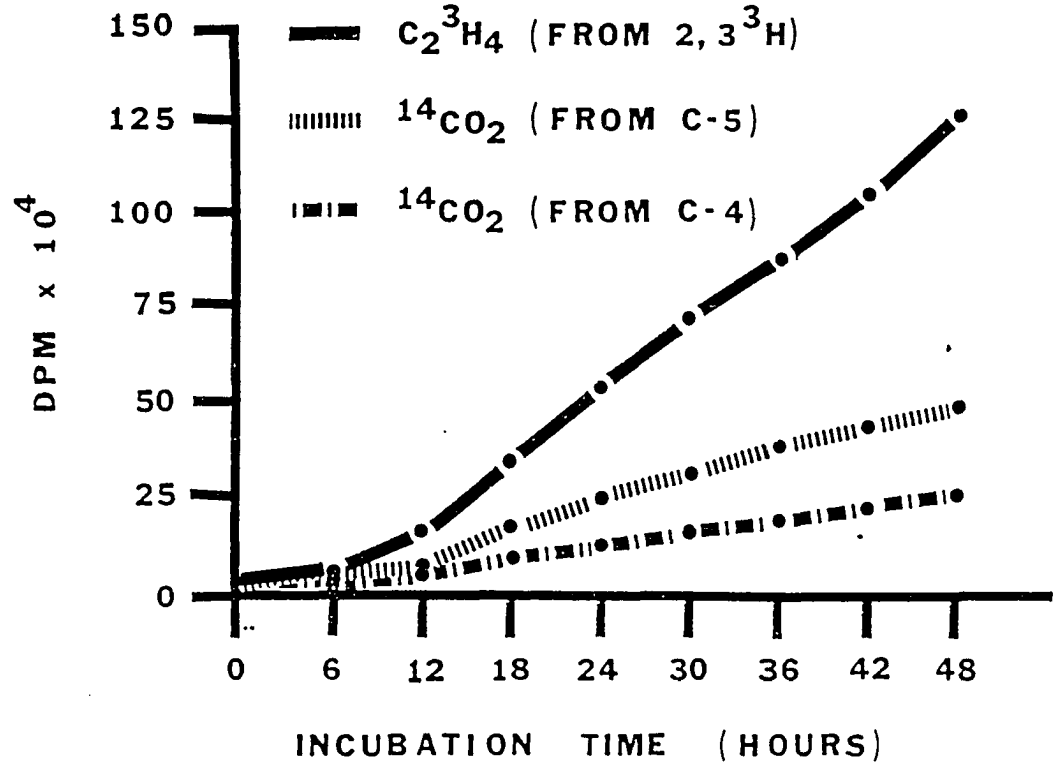

Figure 1. Timecourse of (^{3}H)C_2H_4 and $^{14}CO_2$ production from 10 μCi (2,3-^{3}H)ALA, 1 μCi (4-^{14}C)ALA, or 1 μCi (5-^{14}C) ALA, separately, by tomato fruit discs at the pink stage

Table 1. Distribution of radioactivities in different compounds synthesized *in vivo* from 10 μCi (2,3-^{3}H)ALA, 1 μCi (4-^{14}C)ALA, or 1 μCi (5-^{14}C)ALA by tomato fruit discs at pink stage of development (after 48 hr of incubation)

Compound[z]	2,3-^{3}H (DPM x 10^{3})	4-^{14}C (DPM x 10^{3})	5-^{14}C (DPM x 10^{3})
ACC	4,830	167	0
C_2H_4	1,266	0	0
Fumarate	191	18	0
Succinate	101	10	0
Malate	61	6	0
Glutamate	55	12	18
α-Ketoglutarate	35	11	15
Glutamine	25	6	9
Methionine	24	3	0
CO_2	---[y]	258	568

[z]SAM is not reported here because there was an overlap between SAM and some other compounds.

[y]Radioactivity from ^{3}H is not expected in CO_2. Therefore, the radioactivity has not been measured.

The activities of ^{3}H-2 and ^{3}H-3 were detected in ACC, fumarate, succinate, malate, glutamate, α-ketoglutarate, and traces in both glutamine and methionine (Table 1). Both ACC and, ultimately, C_2H_4 are the major end products of C-2 and C-3 of ALA, and both ACC and CO_2 are the end products of C-4 ALA. However, the major end product of C-5 ALA was CO_2.

DISCUSSION

These experiments dealt mainly with the effect of ALA on ACC and C_2H_4 synthesis in tomato pericarp tissues at the pink stage. Tomato pericarp discs at the pink stage of development treated with either 10 μCi (2,3-^{3}H)ALA or 1 μCi (4-^{14}C)ALA metabolized (2,3-^{3}H)ALA into (^{3}H)ACC and $C_2{}^3H_4$, and (4-^{14}C)ALA into (^{14}C)ACC (Table 1). These results confirm earlier studies that show that ALA can be catabolized to ACC and ultimately into C_2H_4 (section I). Furthermore, tomato pericarp discs metabolized both (4-^{14}C)ALA and (5-^{14}C)ALA into $^{14}CO_2$ (Fig. 1 and Table 1), and these results have been confirmed by other researchers (6). In addition, evidence has been provided that the metabolic fate of the C-5 of ALA differs from that of the C-4 (Table 1), and C-5 activity is less sensitive to inhibition by anaerobiosis or by malonate than is C-4 activity (6).

In previous research, it has been found that the ability of the tomato fruit discs to convert ALA into C_2H_4 varied according to the stage of fruit development (section I). In addition, it was reported that the changes in porphobilinogen deaminase activity were parallel and related directly to the changes in chlorophyll content with respect to fruit age (11). These findings, as well as data in Fig. 1 and Table 1, provide evidence that the metabolic fate of ALA in tomato fruits varies according to the stage of development of the fruit.

Because ^{14}C-5 was detected as $^{14}CO_2$ and did not appear in either ACC or C_2H_4 (Table 1), we believe that the cleavage of ^{14}C-5 from ALA into $^{14}CO_2$ occurred before the conversion of the ALA molecule into ACC

(Fig. 2). ^{14}C-4 was detected not only in $^{14}CO_2$ but also in ACC (Table 1 and Fig. 1). Accordingly, it is believed that the release of ^{14}C-4 as $^{14}CO_2$ occurred during the catabolism of the ACC molecule into C_2H_4 and $^{14}CO_2$.

The presence of carbon atoms 2, 3, and 4 of the ALA molecule in ACC (Table 1), and the presence of carbon atoms 2 and 3 of the ALA molecule in the C_2H_4 molecule permits the conclusion that ALA is metabolized to ACC via a new biosynthetic pathway (Fig. 2). Carbons 1 to 4 would become ACC, carbons 2 and 3 would become C_2H_4, and carbon 5 would become CO_2. The first step in the catabolism of ALA involves deamination or transamination, and this already has been confirmed (6). In the second step, the C_4-C_5 bond is cleaved, and C_5 is oxidized concomitantly to CO_2 via both O_2-sensitive and O_2-insensitive reactions that have been demonstrated by using (5-^{14}C)ALA (6). The remaining 4-carbon compound is cyclized to ACC, and, ultimately, it is converted to C_2H_4 with C_2 and C_3 forming the C_2H_4 molecule.

These data support the idea that there are two separate and independent pathways by which C_2H_4 is synthesized during the ripening of tomato fruits (2, 3, 8). Tomato fruits utilize methionine as the major precursor of C_2H_4 during the green stage of fruit development (2, 3) while the activity of PBG deaminase and its concomitant use of ALA is high (11). However, as the activity of PBG deaminase declines during the later stage of growth and development, ALA becomes the major precursor of C_2H_4 and the role of methionine as a precursor of C_2H_4 becomes minimal (2, 3).

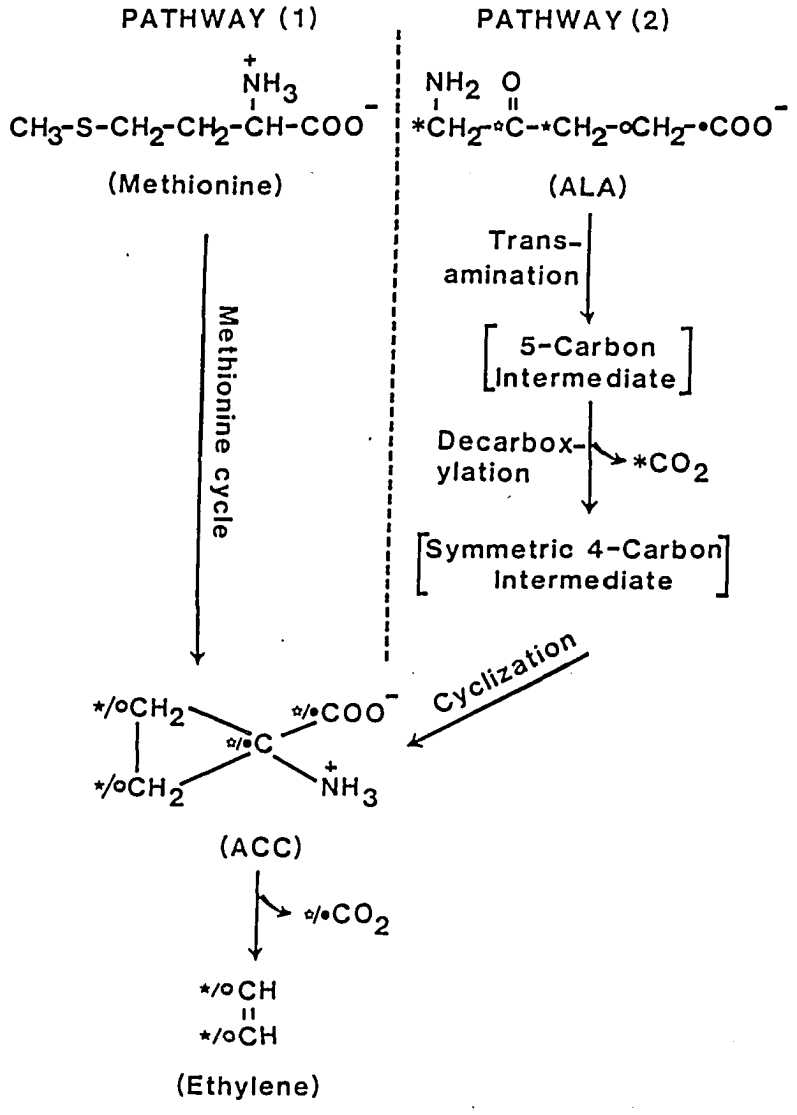

Figure 2. Proposed pathway for the conversion of ALA into ACC and ultimately into C_2H_4 in ripening tomato fruit discs

The studies reported here further establish that the metabolic fate of ALA is not exclusively associated with porphyrin biosynthesis and that this amino acid can be catabolized, at certain stage of tomato fruit development, to ACC, C_2H_4, and CO_2.

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GENERAL SUMMARY AND CONCLUSIONS

The influence of ALA, methionine, ACC, glutamine, glutamate, and α-ketoglutarate on C_2H_4 and ACC production in 'Heinz 1350' tomato pericarp discs was determined. ALA increased C_2H_4 to 232% and ACC to 410% of the control production rates, whereas methionine increased C_2H_4 to 152% and ACC to 167% of the production rates of the control. ACC enhanced C_2H_4 production to 242% of the control, and glutamine, glutamate, and α-ketoglutarate were not different from the control or from one another. The C_2H_4 production rate varied with ALA concentration and the stage of fruit development. As the ALA concentration increased from zero to 40 mM, the C_2H_4 production rate increased. Both treated and untreated pericarp discs from fruits at the pink stage of development yielded the largest C_2H_4 production rate.

Discs of pericarp tissue at the pink stage were incubated in individual treatments of 10 μCi (2,3-^{3}H)ALA, 1 μCi (4-^{14}C)ALA, or 1 μCi (5-^{14}C)ALA for 48 hr, and radioactive C_2H_4, CO_2, and ACC were measured. Radioactivity from ^{3}H was detected in both ACC and C_2H_4. However, radioactivity from ^{14}C-4 was detected in both ACC and CO_2, but not in C_2H_4. Radioactivity from ^{14}C-5 was detected in CO_2, and its amount was much greater than that obtained from ^{14}C-4. Neither ACC nor C_2H_4 showed any radioactivity when (5-^{14}C)ALA was supplied to the fruit discs, implying that carbon atom 5 of ALA is lost as CO_2 during ACC formation. In addition, when (2,3-^{3}H)ALA or (4-^{14}C)ALA was supplied to the fruit discs, radioactivity was detected in other metabolites such as fumarate,

succinate, malate, glutamate, glutamine, α-ketoglutarate, and methionine, but the amount of radioactivity was insignificant as compared with the amount of radioactivity found in C_2H_4 and ACC.

These results suggest that ALA is metabolized to ACC and ultimately to C_2H_4 in ripening tomato fruits. One route by which ALA can be converted into ACC is transamination followed by decarboxylation (of carbon atom 5) and then cyclization to ACC. A new pathway for C_2H_4 biosynthesis is presented, and this pathway may have an important relationship to chlorophyll biosynthesis and the associated degreening processes in ripening tomato fruit.

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