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Biochemistry

Ethylene biosynthesis: Identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene

(*S*-adenosylmethionine/aminoethoxyvinylglycine/fruit ripening/apple)

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ABSTRACT **L-[*U*-^{14}C]Methionine fed to apple tissue was efficiently converted to ethylene when the tissue was incubated in air. In nitrogen, however, it was not metabolized to ethylene but was instead converted to 1-aminocyclopropane-1-carboxylic acid (ACC). When apple tissues were fed with L-[*methyl*-^{14}C]methionine or L-[^{35}S]methionine and incubated in nitrogen, radioactivity was found subsequently in methylthioribose. This suggests that methionine is first converted to *S*-adenosylmethionine which is in turn fragmented to ACC and methylthioadenosine. Methylthioadenosine is then hydrolyzed to methylthioribose. The conclusion that ACC is an intermediate in the conversion of methionine to ethylene is based on the following observations: Labeled ACC was efficiently converted to ethylene by apple tissue incubated in air; the conversion of labeled methionine to ethylene was greatly decreased in the presence of unlabeled ACC, but the conversion of labeled ACC to ethylene was little affected by the presence of unlabeled methionine; and 2-amino-4-(2′-aminoethoxy)*trans*-3-butenoic acid, a potent inhibitor of pyridoxal phosphate-mediated enzyme reactions, greatly inhibited the conversion of methionine to ethylene but did not inhibit conversion of ACC to ethylene. These data indicate the following sequence for the pathway of ethylene biosynthesis in apple tissue: methionine → *S*-adenosylmethionine → ACC → ethylene. A possible mechanism accounting for these reactions is presented.**

Ethylene is a plant hormone that initiates fruit ripening and regulates many aspects of plant growth and development (1). It is generally thought that methionine is the common precursor of ethylene throughout diverse higher plant tissues in which the hormone occurs and exerts its many effects (2–4). In apple tissue the conversion of methionine to ethylene represents the major metabolism of methionine (5). In this conversion, C-1 of methionine is converted to CO_2, C-2 to formic acid, and C-3,4 to ethylene. The sulfur atom, however, is retained in the tissue (2). Because the conversion of methionine to ethylene is greatly inhibited by uncouplers of oxidative phosphorylation, Burg (6) and Murr and Yang (7) proposed that *S*-adenosylmethionine (SAdoMet), formed from methionine and ATP, is an intermediate between methionine and ethylene. Adams and Yang (8) have presented evidence showing that 5′-methylthioadenosine (MeSAdo) and its hydrolysis product, 5-methylthioribose (MeSRib), are derived from the CH_3S group of methionine during its conversion to ethylene and have substantiated the role of SAdoMet as an intermediate in the biosynthesis of ethylene from methionine.

It has been known for some time that endogenous ethylene production, as well as the conversion of methionine to ethylene, ceases in plant tissues placed in an anaerobic atmosphere and that a surge of ethylene production occurs upon exposure of the tissue to air. These observations are interpreted to indicate that an intermediate accumulates during anaerobic incubation and is subsequently converted to ethylene upon exposure to oxygen (2, 9). We therefore examined the metabolism of methionine under these conditions. We now report that 1-aminocyclopropane-1-carboxylic acid (ACC) is the intermediate in the conversion of methionine to ethylene. The conversion of methionine to ACC is strongly inhibited by aminoethoxyvinylglycine (AVG), an inhibitor of pyridoxal phosphate-mediated reactions, and the conversion of ACC to ethylene requires oxygen.

MATERIALS AND METHODS

Plant Material. Apples (*Malus sylvestris* Mill, var. Golden Delicious) were picked near Camino, CA, during commerical harvest. Fruit were stored at 0°C and were transferred to room temperature 24–48 hr prior to the experiment.

Chemicals. L-[*U*-^{14}C]Methionine and DL-[4-^{14}C]homoserine were purchased from Schwarz/Mann, L-[^{35}S]methionine and L-[*methyl*-^{14}C]methionine from Amersham/Searle, and DL-α-amino[3-^{14}C]butyric acid from Calbiochem. AVG was a gift from J. P. Scannell (Hoffmann–LaRoche). Some authentic samples of ACC were kindly provided by L. F. Burroughs and W. C. J. Ross, and others were purchased from Calbiochem. DL-Vinylglycine was a product of Calbiochem. Homoserine lactone was synthesized from homoserine (10).

Feeding Experiments. Plugs, 1 cm in diameter and 2 cm long, were cut from apple fruit with a cork borer and scalpel. They were quickly rinsed in 2% (wt/vol) KCl and blotted with a paper towel. Indicated substrates were infused by a vacuum infiltration technique as described (11). For incubation in a nitrogen or air atmosphere, a tissue plug was placed in a 12-ml plastic syringe fitted with a three-way stopcock. Two holes (3 mm diameter) were made near the end of the syringe to allow flushing with a stream of nitrogen or air from the stopcock inlet. After flushing, the syringe was sealed for incubation. Gas samples were withdrawn periodically from the incubation syringe with a sampling syringe and assayed for total and radioactive ethylene. Total ethylene was determined by gas chromatography of a 1-ml sample. Radioactive ethylene was determined by injecting a 5-ml sample into a scintillation vial sealed with a rubber serum cap and containing 0.2 ml of 0.25 M mercuric perchlorate as absorbant. After 90 min, radioactivity was assayed by scintillation counting.

Characterization of Radioactive Metabolites. After incubation, ice-cold ethanol was introduced into the syringe which was then placed in a dry ice/acetone bath. The plug of tissue was later homogenized and extracted with 80% ethanol.

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Abbreviation: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine [2-amino-4-(2′-aminoethoxy)*trans*-3-butenoic acid]; MeSAdo, 5′-methylthioadenosine; MeSRib, 5-methylthioribose; SAdoMet, *S*-adenosylmethionine.

The extracts were concentrated *in vacuo* at 38°C and chromatographed on Whatman 3 MM paper with butanol/acetic acid/water, 4:1:5 (vol/vol), as developing solvent. Paper electrophoresis was performed at pH 2.2 (10% acetic acid), pH 6.8 (25 mM phosphate buffer), pH 10.8 (20 mM Na_2CO_3), or pH 11 (50 mM sodium borate). Radioactivity on the paper was detected by a radioscanner. Amino acids on paper were visualized by spraying with ninhydrin and heating briefly at 80–90°C; MeSRib was detected by spraying with an aniline/phosphoric acid solution and heating briefly at 105°C (12).

Preparation of [^{14}C]ACC. Radioactive ACC was obtained by injecting L-[*U*-^{14}C]methionine into apple plugs that were then incubated for 6 hr in a nitrogen atmosphere. After extraction with ethanol as described above, radioactive amino acids were first separated from the other components by adsorption on an ion exchange resin (Dowex 50, H^+) column followed by elution with 2 M NH_4OH. After concentration, the cationic fraction was chromatographed on Whatman 3 MM paper as described above. The radioactive region corresponding to ACC was cut from the chromatogram and eluted with 70% ethanol. The eluate was concentrated under a stream of nitrogen and subjected to electrophoresis on Whatman 3 MM paper at pH 2.2. The region corresponding to ACC was again eluted and purified on an ion exchange resin (Dowex 50, H^+) column. The purified radioactive ACC was dissolved in 2% KCl for feeding experiments or dissolved in 50% methanol for hydrogenation.

Hydrogenation. Hydrogenation of compound X was performed in 50% aqueous methanol at 25°C with 2.7 atmospheres (1 atmosphere = 1.013×10^5 Pa) of hydrogen and palladium on charcoal as a catalyst. After hydrogenation the products were separated and characterized by paper electrophoresis at pH 2.2.

Cocrystallization. Compound X recovered and purified from feeding experiments and its hydrogenation product were further characterized by cocrystallization with the corresponding authentic compounds. The radioactive samples containing a known amount of radioactivity were mixed with 50 mg of the authentic compound. The mixture was dissolved in 70% ethanol and recrystallized four times from ethanol/water. After each recyrstallization the specific radioactivity of the material was determined. For amino acid determination the ninhydrin method of Yemm and Cocking (13) was used, except that for ACC the boiling time was increased to 1 hr to obtain a suitable color reaction. Radioactivity was determined by liquid scintillation counting.

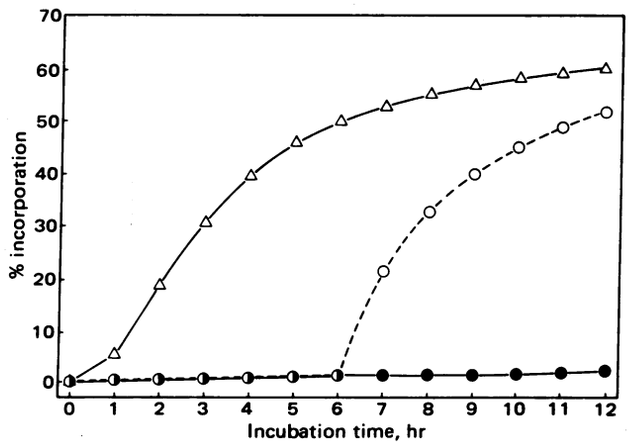

FIG. 1. Incorporation of L-[*U*-^{14}C]methionine into ethylene by apple plugs. The plugs were infiltrated with 0.5 μCi of L-[*U*-^{14}C]-methionine (100 μCi/μmol) and incubated in air (△), in nitrogen (●), or in nitrogen for 6 hr and then in air (○).

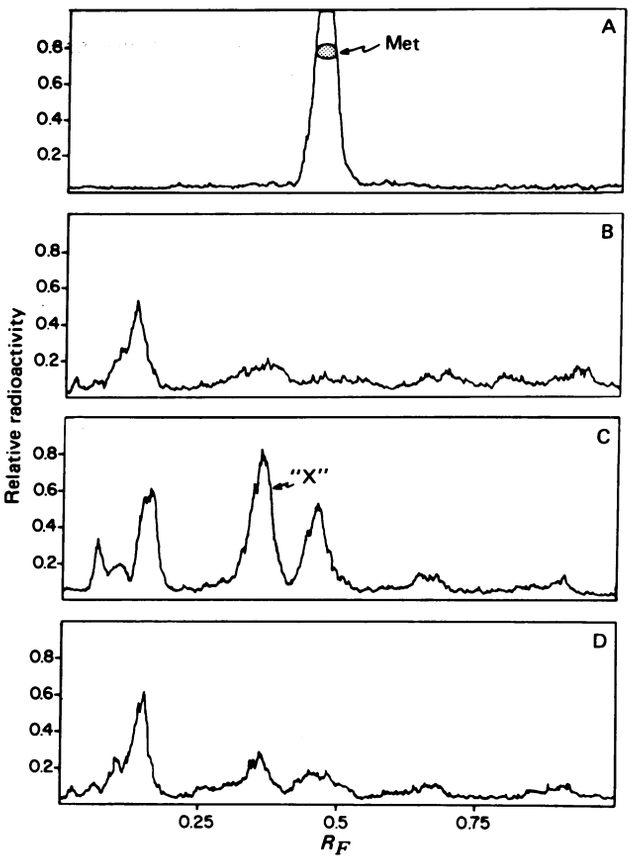

FIG. 2. Radiochromatogram scans of ethanol extracts prepared from apple plugs described in Fig. 1. (*A*) L-[*U*-^{14}C]Methionine used. (*B*) Extract prepared from the plug incubated in air for 12 hr. (*C*) Extract prepared from the plug incubated in nitrogen for 6 hr. (*D*) Extract prepared from the plug incubated for 6 hr in nitrogen and then transferred to air for 6 hr.

RESULTS

Characteristics of the Conversion of Methionine into an Unknown Metabolite. Fig. 1 shows the production of radioactive ethylene from plugs of apple tissue fed with [*U*-^{14}C]-methionine. One plug was incubated in air continuously, another under nitrogen for 6 hr without exposure to air, and the third was incubated under nitrogen for 6 hr and subsequently transferred to air for 6 hr. The plug incubated in air converted about half of the radioactivity to ethylene whereas the plug incubated in nitrogen produced very little radioactive ethylene. The plug that was incubated in nitrogen and subsequently transferred to air produced very little ethylene while in nitrogen. Upon transfer to air, however, it produced ethylene at a rate higher than did the plug that was incubated continuously in air.

When the plugs were homogenized and the extracts analyzed by paper radiochromatography, it was found that only a small amount of methionine (R_F 0.45) remained in plugs incubated in air (Fig. 2*B*). In plugs incubated in nitrogen, however, an unknown radioactive metabolite (designated "compound X") with an R_F of 0.37 accumulated (Fig. 2*C*). In plugs returned to air after incubation in nitrogen, compound X disappeared (Fig. 2*D*) with the concomitant production of ethylene (Fig. 1).

In further study of the formation of compound X, we compared the metabolism of uniformly labeled and methyl-labeled methionine in plugs incubated under nitrogen. Plugs fed [*U*-

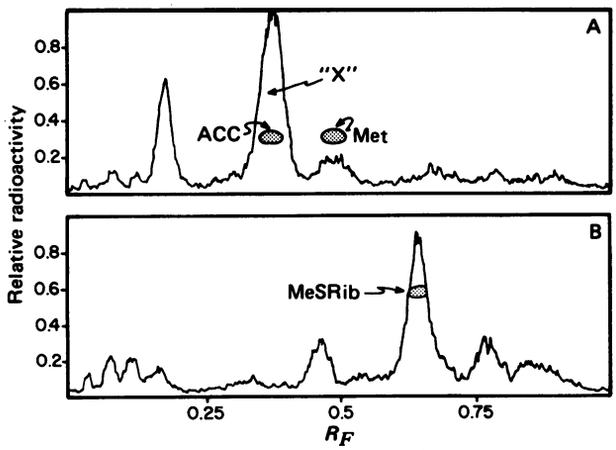

FIG. 3. Radiochromatogram scans of ethanol extracts prepared from apple plugs infiltrated with 0.5 μCi of L-[*U*-^{14}C]methionine (100 μCi/μmol) (*A*) or 0.5 μCi of L-[*methyl*-^{14}C]methionine (49 μCi/μmol) (*B*). After infiltration the plugs were incubated for 6 hr in a nitrogen atmosphere.

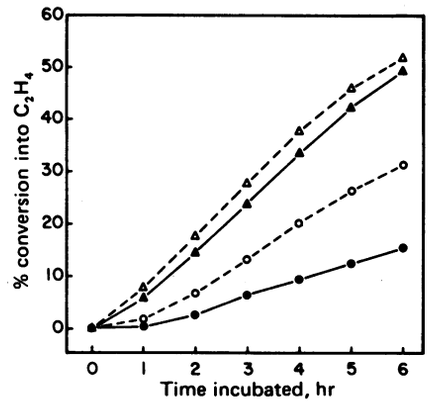

FIG. 4. Conversion of radioactivity into ethylene by apple plugs infiltrated with 0.6 μCi and 6 nmol of L-[*U*-^{14}C]methionine (○), 0.6 μCi and 6 nmol of L-[*U*-^{14}C]methionine plus 0.1 μmol of unlabeled ACC (●), 0.3 μCi of [^{14}C]ACC (△), or 0.3 μCi of [^{14}C]ACC plus 0.1 μmol of unlabeled methionine (▲). All plugs were incubated in air.

^{14}C]methionine contained compound X but plugs fed [*methyl*-^{14}C]methionine did not (Fig. 3). In both situations, methionine was metabolized extensively. Plugs fed [*methyl*-^{14}C]methionine contained a metabolite (R_F, 0.68) that was later identified as MeSRib by paper cochromatography and coelectrophoresis with authentic material, as described (8). In plugs fed [^{35}S]methionine and incubated under nitrogen, labeled MeSRib was observed but not compound X; this was also the result in the experiment using [*methyl*-^{14}C]methionine. These observations indicate that compound X possesses neither the sulfur atom nor the methyl group of methionine. Furthermore, because the metabolism of methionine to compound X is accompanied by transfer of the CH_3S group of methionine to MeSRib, the present results are in agreement with the conclusion that methionine is converted to compound X via SAdoMet as an intermediate (8). In this conversion, MeSAdo is assumed to be the primary product because it has been established that MeSAdo is rapidly hydrolyzed to MeSRib in apple tissue.

Chemical Identification of Compound X. The unknown radioactive material was cationic on electrophoresis at pH 2.2, neutral at pH 6.8, and anionic at pH 11. It reacted with 2,4-dinitrofluorobenzene and gave a derivative that migrated as an anion at pH 6.8. These results suggest that the metabolite could be an amino acid. Upon hydrogenation it gave a radioactive product (40% yield) that comigrated with α-aminobutyric acid in both paper chromatography and paper electrophoresis at pH 2.2 and 10.8. The identity of the hydrogenation product as α-aminobutyric acid was confirmed by cocrystallization of the radioactive material with authentic DL-α-aminobutyric acid to constant specific activity (Table 1). The mobility of compound X was compared with that of authentic samples of ACC, azetidine-2-carboxylic acid, 2-amino-3-butenoic acid (vinylglycine), and homoserine lactone in paper chromatography and in paper electrophoresis, because these compounds are the dehydrogenated forms of α-aminobutyric acid. Only ACC was found to comigrate with compound X in both paper chromatography and paper electrophoresis at pH 2.2 and 10.8. When authentic ACC was hydrogenated, it also yielded α-aminobutyric acid. Like ACC, compound X reacted very slowly with ninhydrin as revealed by paper chromatography, suggesting that it contained no α-hydrogen. Identification of radioactive compound X as ACC was further confirmed by cocrystallization of the radioactive material with authentic ACC to constant specific radioactivity (Table 1). To ensure reliability of the method, DL-α-amino[3-^{14}C]butyric acid was cocrystallized with authentic ACC or DL-α-aminobutyric acid. As expected, activity was not retained with ACC but was retained with DL-α-aminobutyric acid. The initial decrease in specific radioactivity when compound X was cocrystallized with authentic ACC may be due to contamination by some radioactivity other than ACC in the purified sample of compound X.

Conversion of ACC to Ethylene in Apple Tissue. To provide direct evidence that the tissue is capable of converting ACC into ethylene, ACC was prepared and isolated from plugs that had been fed [*U*-^{14}C]methionine and incubated under nitrogen. It can be seen from Fig. 4 that ACC was efficiently converted to ethylene. If ACC is an intermediate in the conversion of methionine to ethylene, the addition of unlabeled ACC should significantly dilute the incorporation of radioactivity from methionine into ethylene, but the incorporation of radioactivity from ACC into ethylene should be less affected by the administration of unlabeled methionine. The results of the dilution experiments shown in Fig. 4 are in close agreement with this prediction. It has been shown that the conversion of methionine to ethylene and MeSAdo is greatly inhibited by AVG (8). In the present study we have presented further evidence that methionine is converted into MeSRib and ACC presumably via SAdoMet, and that this conversion is sensitive

Table 1. Cocrystallization of the radioactive metabolite and its hydrogenated product with authentic samples

^{14}C sample	Unlabeled authentic compound	Specific radioactivity at each crystallization, cpm/μmol				
		Initial	1	2	3	4
Compound X	ACC	804	691	725	718	680
DL-α-Amino[3-^{14}C]butyric acid	ACC	681	447	231	92	25
Hydrogenated X	DL-α-Aminobutyric acid	135	109	103	99	112
DL-[4-^{14}C]Homoserine	DL-α-Aminobutyric acid	634	271	110	40	9
DL-α-Amino[3-^{14}C]butyric acid	DL-α-Aminobutyric acid	505	460	498	488	464

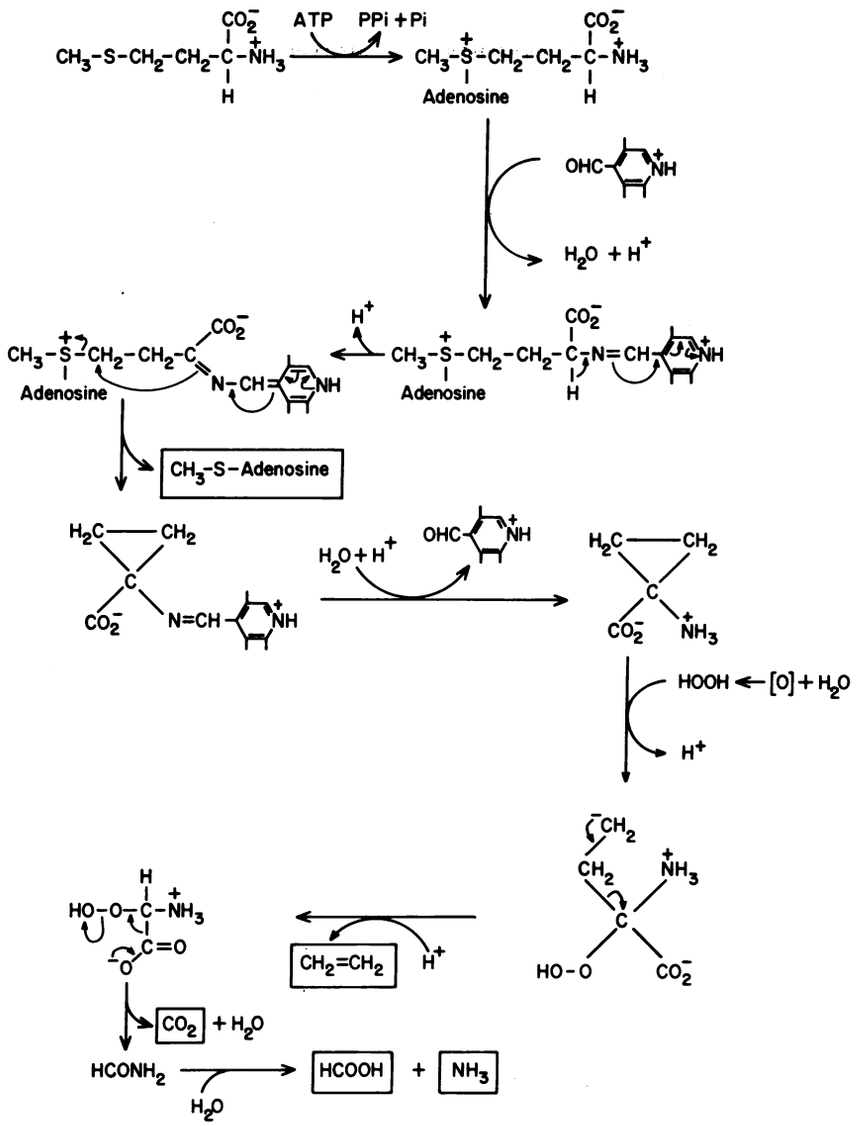

FIG. 5. Postulated mechanism for the biosynthesis of ethylene from methionine. The substituted pyridinecarboxaldehyde stands for pyridoxal phosphate.

to AVG inhibition; the conversion of ACC to ethylene, however, is uninhibited by AVG. Table 2 illustrates the effect of AVG on the conversion of methionine or ACC to ethylene. The conversion of methionine to ethylene was greatly inhibited by AVG as reported (8), but the conversion of ACC to ethylene was stimulated by AVG. A possible explanation of this stimulatory effect of AVG is that it inhibits the conversion of endogenous methionine to ACC, resulting in a smaller endogenous ACC pool and consequently less dilution of the labeled ACC. Our results predicate the following sequence for the conversion of methionine to ethylene in apple tissue: Methionine → ACC → ethylene. The first step is sensitive to AVG inhibition; the last step requires oxygen.

Table 2. Effect of AVG on the conversion of methionine and ACC to ethylene by apple tissue

Compound infiltrated	$^{14}C_2H_4$ nCi	%
Exp. 1:		
L-[U-^{14}C]Methionine	91	100
L-[U-^{14}C]Methionine + AVG	3.9	4
Exp. 2:		
[U-^{14}C]ACC	50	100
[U-^{14}C]ACC + AVG	76	153

Each plug of apple tissue was infiltrated with 0.1 ml of 2% KCl solution containing 0.7 μCi of L-[U-^{14}C]methionine (100 μCi/μmol) or 0.24 μCi of [U-^{14}C]ACC and 2.5 nmol of AVG as indicated. The plugs were incubated for 6 hr in experiment 1 and 7 hr in experiment 2. Percentage incorporation was calculated relative to corresponding plugs that received no AVG.

DISCUSSION

Endogenous ethylene production and the conversion of methionine to ethylene are greatly inhibited by rhizobitoxine and its ethoxy analog (AVG) in a number of plant tissues (3, 4, 14). These compounds are known to inhibit pyridoxal phosphate-mediated reactions and a mechanism for their action has been discussed by Rando (15). In a previous study we found evidence that implicated SAdoMet as an intermediate in the conversion of methionine to ethylene (8). This was based on the observation that MeSAdo and MeSRib are the predominant products derived from the methylthio group of methionine in apple tissue while it is producing ethylene. In the present study we have

demonstrated an accumulation of labeled ACC formed from the methionine administered to tissue incubated in nitrogen, an atmosphere that inhibited the conversion of methionine to ethylene (Figs. 1 and 2). The formation of ACC from methionine was accompanied by the production of MeSRib (Fig. 3), and the formation of ACC and MeSRib from methionine was concomitantly inhibited by AVG. It is reasonable to assume that ACC and MeSRib are formed via fragmentation of SAdoMet. However, the present data do not establish whether AVG inhibits the conversion of methionine to SAdoMet or of SAdoMet to ACC. We have examined the effect of AVG on SAdoMet formation from labeled methionine in apple tissue and have found that the amount of labeled SAdoMet increases severalfold in the presence of AVG as compared to control tissue without the inhibitor (unpublished observations). It is suggested therefore that AVG does not inhibit the conversion of methionine to SAdoMet but inhibits the conversion of SAdoMet to ACC. If the AVG inhibition is a result of its interference with pyridoxal phosphate-mediated enzyme reactions, and if the conversion of SAdoMet to ACC is indeed the step at which AVG inhibits ethylene production, then it is probable that this step is mediated by pyridoxal phosphate.

A postulated mechanism to account for these reactions is shown in Fig. 5. The first step is the activation of methionine by ATP to produce SAdoMet, which then reacts with pyridoxal phosphate to form a Schiff base. It is known that pyridoxal phosphate is capable of catalyzing γ-elimination reactions (16). This is a novel example, however, in that the α-hydrogen is eliminated along with the γ-substituent, resulting in the formation of a cyclopropane ring. The carboxyl, C-1, C-2, and C-3 of ACC would be derived from C-1, C-2, C-3, and C-4, respectively, of methionine. It would therefore be expected that the end products of ACC metabolism to ethylene in apple tissue would be CO_2, HCOOH, and ethylene. The fate of each carbon has been fairly well established, but there is little information about the mechanism of ACC conversion to ethylene except that the process requires oxygen. The involvement of hydrogen peroxide in the cleavage of cyclopropane ring, as shown in Fig. 5, awaits experimental evidence. Hydrogen peroxide is a likely candidate because it is the product of certain oxidases, has been implicated in the onset of fruit ripening and ethylene production (17), and reacts with ACC at elevated temperature to produce ethylene chemically (unpublished observation). The formation of ethylene, CO_2, formic acid, and ammonia is in keeping with the results obtained with tracer studies in apple tissue (2). That ACC is indeed a precursor of ethylene is further supported by unpublished observations in this laboratory (A. Cameron, Y. Yu, and L. Fenton): upon treatment with ACC, a remarkable increase in ethylene production occurs in various plant tissues that normally produce little ethylene. These results and others suggest that the conversion of SAdoMet to ACC is the rate-limiting step in these plant tissues.

ACC was first isolated and characterized from cider apples and perry pears by Burroughs (18) in 1957. Subsequently, it was identified in ripe cowberries (*Vaccinium vitis-idaea*) (19) but could not be detected in unripe cowberries. This observation was later confirmed by Burroughs (20), who also observed that this amino acid increased considerably during storage of pears after harvest. Burroughs (20) suggested that it might be related in some way to the process of fruit ripening, although he was unable to assign a metabolic role for this unusual amino acid. We have now demonstrated the role of ACC in ethylene biosynthesis and thus established that it has, indeed, a most significant role in fruit ripening.

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