Plant Physiol. (1982) 69, 93–97
0032-0889/82/69/0093/05/$00.50/0

Biosynthesis of Ethylene from Methionine in Aminoethoxyvinylglycine-Resistant Avocado Tissue

Received for publication April 27, 1981 and in revised form August 26, 1981

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ABSTRACT

This study was conducted to determine if aminoethoxyvinylglycine (AVG) insensitivity in avocado (*Persea americana* Mill., Lula, Haas, and Bacon) tissue was due to an alternate pathway of ethylene biosynthesis from methionine. AVG, at 0.1 millimolar, had little or no inhibitory effect on either total ethylene production or [^{14}C] ethylene production from [^{14}C]methionine in avocado tissue at various stages of ripening. However, aminooxyacetic acid (AOA), which inhibits 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (the AVG-sensitive enzyme of ethylene biosynthesis), inhibited ethylene production in avocado tissue. Total ethylene production was stimulated, and [^{14}C]ethylene production from [^{14}C]methionine was lowered by treating avocado tissue with 1 millimolar ACC. An inhibitor of methionine adenosyltransferase (EC 2.5.1.6), L-2-amino-4-hexynoic acid (AHA), at 1.5 millimolar, effectively inhibited [^{14}C]ethylene production from [^{14}C]methionine in avocado tissue but had no effect on total ethylene production during a 2-hour incubation. Rates of [^{14}C]AVG uptake by avocado and apple (*Malus domestica* Borkh., Golden Delicious) tissues were similar, and [^{14}C]AVG was the only radioactive compound in alcohol-soluble fractions of the tissues. Hence, AVG-insensitivity in avocado tissue does not appear to be due to lack of uptake or to metabolism of AVG by avocado tissue. ACC synthase activity in extracts of avocado tissue was strongly inhibited (about 60%) by 10 micromolar AVG. Insensitivity of ethylene production in avocado tissue to AVG may be due to inaccessibility of ACC synthase to AVG. AVG-resistance in the avocado system is, therefore, different from that of early climacteric apple tissue, in which AVG-insensitivity of total ethylene production appears to be due to a high level of endogenous ACC relative to its rate of conversion to ethylene. However, the sensitivity of the avocado system to AOA and AHA, dilution of labeled ethylene production by ACC, and stimulation of total ethylene production by ACC provide evidence for the methionine → SAM → ACC → ethylene pathway in avocado and do not suggest the operation of an alternate pathway.

The bacterial phytotoxins, rhizobitoxine, AVG[2], and methoxyvinylglycine, were shown to be potent inhibitors of ethylene production in apple fruit and in a variety of plant tissues (8, 13). The last two compounds were more readily available than was rhizobitoxine, and AVG found widespread use by a number of investigators probing ethylene biosynthesis. This inhibitor was important in establishing SAM and ACC as intermediates in the biosynthetic pathway of ethylene from methionine (1). We reported earlier that AVG was relatively ineffective in inhibiting ethylene production in pink or ripe tomato tissues and in avocado tissue (3, 4). This finding had practical, as well as theoretical, implications. Did the lack of AVG inhibition in these tissues indicate an alternate pathway for ethylene biosynthesis that bypassed the AVG sensitive step? The study we now report was directed toward answering this question.

MATERIALS AND METHODS

Plant Material and Procedure. Avocado (*Persea americana* Mill., Lula, Haas, and Bacon) fruit were obtained from a local wholesale market and stored at 6 to 7°C, then allowed to ripen to the desired stage at 14°C. Peeled fruit were cut into 3-mm-thick slices with a meat slicer, and 1-cm discs were cut with a cork borer. Apple (*Malus domestica* Borkh., Golden Delicious) fruit were grown at the Beltsville Agriculture Research Center. Apple discs were about 0.5 cm in thickness and 1.0 cm in diameter. The discs (2 g) were incubated at 25°C in stoppered 25-ml Erlenmeyer flasks containing 3 ml of solution. The mixture contained 0.4 M sucrose; 27 mM citrate; 47 mM dibasic sodium phosphate (pH 4.6). In experiments on the AVG-insensitive, early climacteric, Golden Delicious apple tissue, a buffer system containing 0.6 M sorbitol and 10 mM K-phosphate buffer (pH 7.0) was used. In certain experiments testing the effect of phosphate on magnitude of AVG-inhibition, sodium phosphate was omitted, and pH of the sucrose-citrate solution was adjusted to pH 4.6 with KOH. Also, in these experiments, a second buffer system containing 50 mM Mes (pH 6.7) and 0.4 M sucrose with or without 50 mM sodium phosphate, was used. AVG and other inhibitors were made up in those buffers. Gas samples were removed by hypodermic syringe for ethylene analysis by the method of Meigh *et al.* (12). Measurement of [^{14}C]ethylene, derived from L-[3,4-^{14}C]methionine, was carried out according to the method described by Aharoni *et al.* (2). The procedure involved incubating avocado tissue as above, except that 1.5 μCi L-[3,4-^{14}C]methionine (specific activity 49 or 53 mCi/mmol) were added to the incubation medium. To determine possible effects of certain inhibitors on uptake of methionine or protein synthesis, tissue was removed from the buffer after incubation, washed three times with 6 ml fresh buffer containing no label, and frozen. The total time of washing was 45 min. The discs were later ground in 10% TCA, and total cpm and TCA-precipitable cpm were determined. The former is a measure of [^{14}C]methionine uptake, while the latter indicates protein synthesis.

[^{14}C]AVG Uptake. Discs (2 g, 3 mm thick and about 10 mm diameter) from postclimacteric apple and climacteric-rise avocado

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[2] Abbreviations: AVG, aminoethoxyvinylglycine; AOA, aminooxyacetic acid; AHA, L-2-amino-4-hexynoic acid; SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; EPPS, *N*-2-hydroxyethylpiperazine propane sulfonic acid.

fruits, were incubated for 4 h at 25°C in 3 ml of the standard sucrose-citrate-phosphate buffer, which contained 126 nmol [4-^{14}C]AVG (specific activity 2.4 mCi/mmol). The discs were removed from the incubation medium, washed three times with unlabeled medium, and extracted three times with hot 70% ethanol (final concentration). The combined ethanol extracts (30 ml) were taken to dryness and redissolved in 5 ml H_2O. Insoluble material was centrifuged, and the extract was passed through a Dowex 50 (H^+ form) column of 1.5-ml bed volume. Compounds retained by the column were eluted with three bed volumes of 3 M NH_4OH. The eluate was taken to dryness and dissolved in 1 ml H_2O. Part of this solution was measured for radioactivity, and part was spotted on Whatman 3 MM paper strips and chromatographed using 1-butanol:acetic acid:H_2O (4:1:5 v/v) as the solvent system. Unlabeled AVG was added to low AVG samples containing only [^{14}C]AVG, so that AVG could be located with ninhydrin (0.2% in 95% ethanol). The paper strips were dried and scanned with a Baird Atomic[3] Model 901363 radiochromatogram scanner.

ACC Synthase Activity. Activity of the enzyme ACC synthase that catalyzes ACC formation from SAM was measured in extracts of avocado prepared by a method similar to that used by Boller *et al.* (5) for extracting the enzyme from tomato tissue. Tissue (1:2 w/v) was ground in a buffer solution containing 0.1 M EPPS, 4 mM DTT, and 0.4 μM pyridoxal phosphate (pH 8.0). The extract was dialyzed overnight at 4°C against at least 20 volumes of a buffer containing 2 mM EPPS, 1 mM DTT, and 0.2 μM pyridoxal phosphate (pH 8.0). For the enzyme assay, avocado extract (1.3–1.5 mg protein) was incubated in 0.1 M EPPS (pH 8.0), containing 1.5 μCi *S*-adenosyl-L-[3,4-^{14}C]methionine (38 mCi/mmol) in a total volume of 0.5 ml. After 6-h incubation at 25°C, 100 nmol ACC was added to the reaction mixture as an internal standard, and the mixture was then passed through a small column (1 ml tuberculin syringe) containing 50 to 100 mesh Bio Rex 70 (0.5-ml bed volume). This weak cation-exchange resin in the H^+ form will retain sulfonium ions and certain other compounds. Unreacted SAM was retained by the column, while ACC passed through the column. The column was washed three times with 0.5 ml H_2O. The effluent from the column was collected in test tubes, which were then capped with serum stoppers, and ACC was degraded by the hypochlorite method (10). The gas phase from the test tube was transferred via an argyle extension tube into an evacuated scintillation vial capped with a serum stopper. A gas sample was taken for total ethylene determination, and 1 ml methanolic mercuric acetate was then added to the vial. After 3 h at 4°C, 10 ml Aquasol was added to the vial, and radioactivity was determined. Total ethylene production reflected conversion efficiency of ACC to ethylene in the hypochlorite assay (10). Correction factors were computed for each sample and applied to the dpm values. The corrected dpm values indirectly measure the amount of [^{14}C]ACC formed in the reaction.

Chemicals. AVG was provided by A. Stempel, Research Division, Hoffmann LaRoche, Inc., Nutley, NJ. AHA was a gift from Dr. Paul Talalay, Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, MD. EPPS, DTT, Mes, and pyridoxal phosphate were products of Sigma Chemical Co. L-[3,4-^{14}C]Methionine (specific activity 49 or 53 mCi/mmol) and *S*-adenosyl-L-[3,4-^{14}C]methionine (specific activity 38 mCi/mmol) were obtained from Research Products International. L-[4-^{14}C]AVG (specific activity 2.4 mCi/mmol) was provided by David L. Preuss, Hoffman LaRoche. Methanol for mercuric acetate solutions was from Burdick and Jackson Laboratories. Bio-Rex 70 ion-exchange resin was obtained from Bio-Rad Laboratories. Other chemicals were reagent grade from various major suppliers.

Replication of Experiments. The data presented are means of three samples, unless otherwise indicated, and are typical of at least three experiments, with two exceptions. The experiments on effects of AHA on ethylene production, and [C^{14}]AVG uptake by tissue slices were repeated once.

RESULTS AND DISCUSSION

AVG-Sensitivity. AVG at 0.1 mM, a concentration which inhibits ethylene production in climacteric apple tissue, is much less effective at inhibiting ethylene production in avocado tissue (3) and sometimes shows no inhibitory effect (Fig. 1). Methoxyvinylglycine also is ineffective as an inhibitor of ethylene production in this tissue (data not shown). To understand AVG-insensitivity in this tissue, it is important to examine the effect of AVG on [^{14}C]ethylene production from [^{14}C]methionine. It is evident in Figure 2 that the effect of AVG on [^{14}C]ethylene production was no greater than its effect on total ethylene production. It was suggested (9) that AVG-insensitivity in certain plant tissues could be due to the presence of a pool of ACC that is large in relation to the rate of ethylene production. If this were so, [^{14}C]ethylene production from [^{14}C]methionine still would be inhibited by AVG. Inasmuch as this was not the case for avocado, its insensitivity to AVG cannot be attributed to the presence of a large ACC pool. However, the size of the ACC pool varies between 0.06 nmol/g fresh weight in preclimacteric avocado fruit and 45 nmol/g fresh weight at the later stage of climacteric rise; then, it declines (9).

During the first hours of incubation, 0.1 mM AVG did not

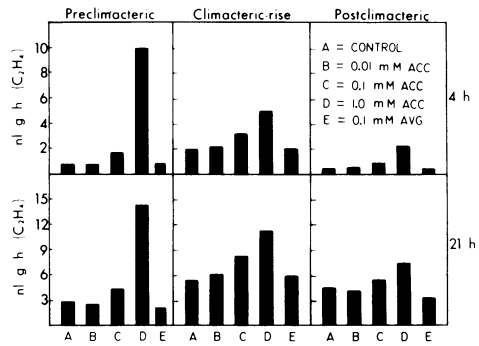

FIG. 1. Influence of ACC and AVG on total ethylene production of avocado discs. Measurements were made at 4- and 21-h incubation.

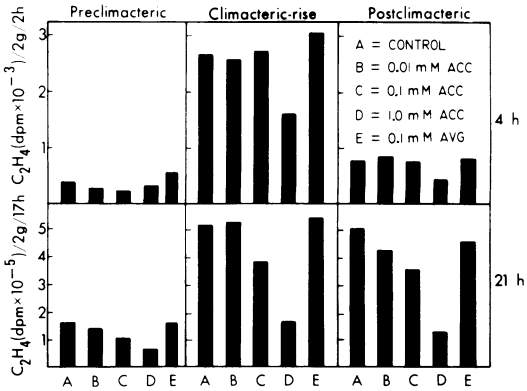

FIG. 2. Influence of ACC and AVG on [^{14}C]ethylene production from L-[3,4-^{14}C]methionine in avocado discs. The values for the 4-h incubation represent dpm in ethylene produced between 2- and 4-h incubation, while the values at 21 h represent dpm in ethylene produced between 4 and 21 h. The incubation medium (3 ml) contained 1.5 mCi L-[3,4-^{14}C]methionine (specific activity 53 μCi/mmol).

[3] The mention of specific instruments, trade names, or manufacturers is for the purpose of identification and does not imply any endorsement by the United States Government.

inhibit total ethylene production in tissue of Golden Delicious apples at the early climacteric stage, but it strongly inhibited its production in tissue from climacteric apples (Fig. 3). [^{14}C]Ethylene production from [^{14}C]methionine was inhibited effectively (over 90%) by AVG at both stages tested in these tissues (Fig. 3, legend). The ACC content was higher in early climacteric (15 nmol/g fresh weight) than in climacteric (10 nmol/g fresh weight) tissue, but the rate of ethylene production was much lower in early climacteric tissue than it was in climacteric tissue, (0.7 *versus* 5.1 nmol/g·h). ACC pools and rates of ethylene production indicate that, in the presence of AVG, early climacteric tissue can produce ethylene for an extended period but that climacteric tissue would deplete the ACC pool within 2 h. It appears, therefore, that AVG-insensitivity in early climacteric apple tissue can be adequately explained on the basis of an ACC pool that is large relative to its rate of conversion to ethylene and that apple and avocado tissues must differ basically with respect to factors involved in AVG-insensitivity.

ACC Effects. The question of whether or not the pathway from methionine to ethylene in avocado involves SAM and ACC was investigated by studying the effects of added ACC on total ethylene production and [^{14}C]ethylene production from [^{14}C]methionine. Incubation of tissue in 1 mM ACC causes an increased ethylene production, particularly in preclimacteric tissue (Fig. 1). On the other hand, 1 mM ACC lowered the amount of [^{14}C]-ethylene produced from [^{14}C]methionine (Fig. 2; Table I), suggesting a dilution of a labeled ACC pool by added ACC. It was determined that ACC did not inhibit [^{14}C]methionine uptake or protein synthesis (data not shown). These results are consistent with the methionine → SAM → ACC → ethylene pathway.

AHA-Sensitivity. AHA is an inhibitor of the methionine adenosyltransferase reaction (11), and, at 1.5 mM, it effectively inhibited [^{14}C]-labeled ethylene production from [^{14}C]methionine in avocado discs (Table I). The concentration of AHA required for 50% inhibition of methionine adenosyltransferase isolated from three sources ranged from 1.8 to 3.5 mM (11). The somewhat larger inhibition (82%) of [^{14}C]ethylene production in avocado tissue may indicate secondary effects of AHA on processes or systems other than methionine adenosyltransferase. It was determined that AHA did not affect uptake of [^{14}C]methionine by avocado tissue (data not shown). Total ethylene production was not inhibited by AHA after 2-h incubation but was lower after 21-h incubation (data not shown). These results are consistent with AHA inhibition of the synthesis of SAM via methionine adenosyltransferase, thereby lowering the rate of [^{14}C]ethylene production from [^{14}C]methionine while, at the same time, allowing the SAM and ACC pools to continue fueling total ethylene synthesis unabated until ACC concentration becomes limiting.

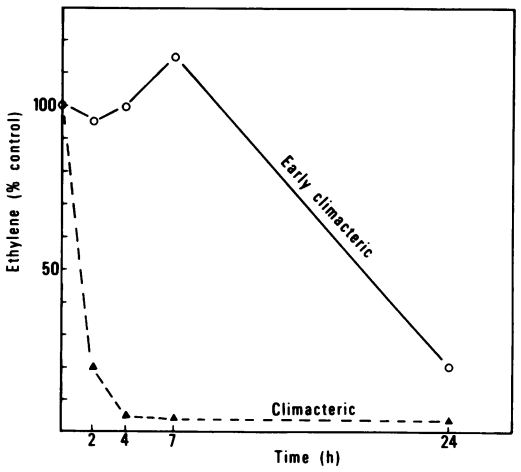

FIG. 3. Influence of 0.1 mM AVG on ethylene production by Golden Delicious apple tissue slices. Curves show the time course for AVG inhibition of total ethylene production. ACC contents of early climacteric and climacteric tissues, at the start of incubation, were 15 and 10 nmol/g, respectively. Rates of ethylene production for early climacteric and climacteric tissues were 0.7 and 5.1 nmol/g·h, respectively, after 5 h. The incubation medium (3 ml) contained 5 μCi L-[3,4-^{14}C]methionine (specific activity 49 mCi/mmol). The radioactivity in ethylene after a 5-h incubation was 401 dpm (control) and 20 dpm (0.1 mM AVG) for early climacteric tissue and 8,215 dpm (control) and 25 dpm (0.1 mM AVG) for climacteric tissue.

Table I. *Effects of AHA, AVG, and ACC on Ethylene Production of Early Climacteric-Rise Avocado Tissue*

Ethylene was measured after a 2-h incubation period at 25°C.

Treatment	Radioactivity in Ethylene	Inhibition (Dilution)	Total Ethylene	Inhibition (Increase)
	dpm/g·h ± SE	%	*nl/g·h ± SE*	%
Control	353 ± 7		4.9 ± 0.23	
AHA, 1.5 mM	62 ± 2	82.4	4.9 ± 0.06	0
AVG, 0.1 mM	340 ± 22	3.7	4.1 ± 0.2	16.3
AVG, 1 mM	295 ± 44	17.6	3.8 ± 0.4	22.4
ACC, 1 mM	217 ± 3	(38.5)	8.4 ± 0.23	(71.4)

Table II. *Inhibition of Ethylene Production in Avocado Slices by Aminooxyacetate*

Ethylene was measured after a 4-h incubation period at 25°C.

Aminooxyacetate Concentration	Ethylene	Inhibition
mM	*nl/g·h ± SE*	%
0	8.3 ± 0.4	
1	3.4 ± 0.1	59.0
0.1	6.2 ± 0.3	25.3
0.01	7.0 ± 0.2	15.7

AOA-Sensitivity. AOA, an inhibitor of pyridoxal phosphate-linked enzymes, has also been shown to inhibit ACC synthase, the AVG-sensitive enzyme in ethylene biosynthesis (14). At 1 mM, AOA inhibited ethylene synthesis in avocado discs by 59% after a 4-h incubation (Table II). This result is again consistent with the methionine → SAM → ACC → ethylene pathway, but the exact mechanism whereby AOA inhibited ethylene production is not known, inasmuch as the ACC concentration in the tissue was not determined, and experiments to ascertain the influence of AOA on labeled ACC synthesis from labeled methionine were not performed.

Effect of Phosphate on AVG-Sensitivity. It has been reported that phosphate inhibits ethylene production in *Penicillium digitatum* (6) and, to a lesser extent, in higher plant tissues (7). In the case of ripe tomato tissue, phosphate obscures an inhibitory effect of AVG on ethylene production (7). Inasmuch as the incubation medium used in this and an earlier study included 47 mM sodium phosphate, we investigated the possibility of a phosphate effect obscuring an effect of AVG on ethylene production. Data in Table III show that ethylene production in avocado discs by AVG was inhibited about the same in sucrose-citrate buffer at pH 4.6, with or without phosphate, 18 and 22%, respectively. The control (minus AVG) rate of ethylene production was somewhat lower in the presence than it was in the absence of 50 mM phosphate. The inhibition of ethylene production by AVG was somewhat greater in sucrose-Mes buffer (pH 6.7) in the absence, rather than in the presence, of phosphate (22% and 12%, respectively). The control (minus AVG) rate was lower in the presence than it was in the absence of phosphate. Phosphate (50 mM) had no significant effect on the magnitude of AVG inhibition in apple tissue incubated in

Table III. *Effects of AVG on Ethylene Production by Avocado and Apple Tissue in the Presence and Absence of Inorganic Phosphate*

Values for ethylene were for 2 g tissue after 2-h incubation. Concentration of buffer components were: sucrose, 0.4 M; citrate, 30 mM; sodium phosphate, 50 mM; and Mes, 50 mM. The concentration of AVG was 0.1 mM.

Tissue	Experiment	Buffer		Ethylene	Inhibition
				nl ± SE	%
Avocado	1	Sucrose-citrate + phosphate (pH 4.6)	Control	100 ± 2.7	
			Plus AVG	82 ± 4.0	18.0
		Sucrose-citrate (pH 4.6)	Control	132 ± 3.4	
			Plus AVG	103 ± 3.6	22.0
	2	Sucrose-Mes + phosphate (pH 6.7)	Control	62.7 ± 2.2	
			Plus AVG	55.3 ± 2.3	11.8
		Sucrose-Mes (pH 6.7)	Control	78 ± 5.2	
			Plus AVG	61 ± 3.6	21.8
Apple	1	Sucrose-citrate + phosphate (pH 4.6)	Control	132.3 ± 8	
			Plus AVG	52.5 ± 1.3	60.4
		Sucrose-citrate (pH 4.6)	Control	148.9 ± 10.6	
			Plus AVG	60.7 ± 3.6	59.2

Table IV. *Uptake of [^{14}C]AVG by Avocado and Apple Slices*

System	AVG Concentration	Ethylene[a]	Inhibition	Radioactivity[b] in Tissue and Media		
				Alcohol-insoluble	Alcohol-soluble	Left in media + wash
	μM	*nmol/g·h*	%	*dpm × 10^{-3}*		
Avocado						
A. Control	0	0.53				
B. [^{14}C]AVG	42	0.53	0	12.0	52.9	543.1
C. [^{14}C]AVG	100	0.46	13.2	13.2	53.9	567.8
Apple						
A. Control	0	0.21				
B. [^{14}C]AVG	42	0.05	76.2	4.9	44.6	593.5
C. [^{14}C]AVG	100	0.03	85.7	4.5	44.8	590.0

[a] Values are rates of ethylene production between 3- and 4-h incubation at 25°C and are means of two samples.

[b] Measurements were made after a 4-h incubation. Total radioactivity present at the start of incubation was 670,000 dpm. The same amount of radioactive AVG was present in B and C, but unlabeled AVG was added to C to adjust AVG concentration to 100 μM. Values are means of two samples.

sucrose-citrate buffer (pH 4.6). Hence, there is no indication from these experiments that AVG-insensitivity is related to the presence of phosphate in the sucrose-citrate buffer. However, the presence of 50 mM phosphate in each buffer system depressed the control rate of ethylene production slightly.

[^{14}C]AVG Uptake. The amounts of radioactivity in the alcohol-soluble fractions of avocado tissue after incubation with [^{14}C]AVG were about 19% higher than they were in the corresponding apple fraction (Table IV). Approximately the same amount of radioactivity was taken up in the presence of unlabeled AVG as that in its absence in both apple and avocado discs. Inasmuch as the AVG concentration was approximately 2.5 times greater with unlabeled AVG than it was with [^{14}C]AVG alone, it may be assumed that mass uptake was correspondingly higher. Therefore, the concentration of AVG necessary for maximum rate of uptake was not exceeded in this experiment. The quantities of AVG present in the apple discs produced an inhibition of ethylene production of 76 to 80%, while like quantities in avocado discs produced little or no inhibition. The radioactivity in alcohol-soluble fractions cochromatographed with AVG, as determined by comparing the location of a single radioactivity peak with the location of the ninhydrin spot for AVG (data not shown). There were no other radioactive peaks in this fraction. In both avocado and apple discs, there was radioactivity in the alcohol-insoluble residue after extraction. The possibility of AVG incorporation into proteins is being investigated.

Table V. *Inhibition by AVG of ACC Formation from [^{14}C]SAM in a Cell-Free Extract of Avocado Tissue*

ACC in effluents from Bio-Rex 70 columns was degraded to ethylene by the hypochlorite procedure (10).

Concentration of AVG	Radioactivity in Ethylene	Inhibition
mM	*dpm ± SE*	%
0	22,087 ± 283	
1.0	990 ± 151	95.5
0.1	1,734 ± 187	92.1
0.01	8,307 ± 686	62.4

AVG-Inhibition of ACC-Synthase Activity. Activity of ACC synthase in crude extracts of avocado tissue was very low and could not be measured accurately by degrading ACC produced from unlabeled SAM. ACC synthase was detected and estimated indirectly by measuring [^{14}C]ethylene produced in the hypochlorite degradation of [^{14}C]ACC produced from [^{14}C]SAM. From Table V, it is evident that the conversion of [^{14}C]SAM to a product yielding [^{14}C]ethylene in the hypochlorite degradation reaction (10) was inhibited by AVG at 0.01 mM. This generally agrees with the finding of Boller *et al.* (5), who showed that AVG inhibits the ACC synthase of pink tomato tissue, which, like avocado, is AVG-resistant (3).

The evidence presented herein; including sensitivity of the ethylene-forming system to AHA (an inhibitor of methionine adenosyltransferase), dilution of labeled ethylene production from methionine by added ACC, and stimulation of total ethylene production by ACC; indicates that the methionine → SAM → ACC → ethylene pathway functions in avocado tissue. This is an example that ineffectiveness of a known inhibitor does not necessarily preclude the operation of a pathway. ACC synthase, the AVG-sensitive enzyme in this pathway, is inhibited by AVG in cell-free extracts. This latter observation suggests that, in avocado tissue, some factor prevents AVG from reaching the active site of ACC synthase.

Acknowledgments—We thank Mr. Irving Newman for technical assistance. We also thank Drs. David L. Preuss and A. Stempel, Hoffman LaRoche, Inc., for gifts of 4-[^{14}C]AVG and unlabeled AVG, respectively.

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