

Ethylene Biosynthesis in Fruit Tissues¹

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ABSTRACT

Tracer studies with avocado tissues indicate that methionine is converted to ethylene at stages of the climacteric rise and the climacteric peak, but not at the preclimacteric stage. The results suggest that the control of ethylene biosynthesis is at a step after methionine is synthesized. The endogenous content of methionine was found to be so low that methionine must be actively turned over for ethylene biosynthesis during the stages when the rate of ethylene production is high. Oxygen was found to be essential for this conversion, indicating that at least one of the steps in conversion of methionine to ethylene is oxygen-dependent. The ability of methionine and its keto analogue (α -keto- γ -methylthiobutyric acid) to serve as ethylene precursors by apple tissues was compared. Chemical and kinetic evidence support the view that methionine is a closer precursor of ethylene than its keto analogue.

MATERIALS AND METHODS

Plant Materials and Chemicals. Apples used were Golden Delicious which were bought from the local market. Fuerte avocados were obtained from the south Coastal Field Station of the University of California, Los Angeles. L-Methionine- U -¹⁴C (100 μ C/ μ mole) was a product of Schwarz Biochemicals. KMB- U -¹⁴C was prepared from L-methionine- U -¹⁴C with L-amino acid oxidase according to the procedures described elsewhere (17). Their purity was established by paper radiochromatography (2). Methional- U -¹⁴C was prepared with ninhydrin as described previously (17).

Feeding Experiments. Plugs (1 cm in diameter and 2 cm in length) were cut with a cork borer and razor blade and the radioactive substrates in 2% KCl were introduced into the plug by a vacuum injection technique described previously (2). Except for the O₂-dependent experiments as summarized in Table II, the plugs were sealed in 12-ml syringes. The syringes were flushed with fresh air after each analysis. For the oxygen-dependent experiments, the plugs which had received radioactive substrates were sealed in 25-ml Erlenmeyer flasks with rubber serum caps. The nitrogen atmosphere was prepared by evacuating the flasks with a water aspirator and refilling with nitrogen. These processes were repeated until the oxygen concentration was less than 0.1%.

Gas Analysis. Samples of the gas phase of the reaction syringes or flasks were periodically analyzed for total and radioactive ethylene and carbon dioxide by gas chromatography and gas radiochromatography as previously described (1, 2). In the oxygen-dependent experiments the concentrations of atmospheric nitrogen and oxygen were analyzed by gas chromatography utilizing a thermal conductivity detector and a series of two columns containing silica gel and molecular sieve.

Analysis of Methionine Content. Methionine concentration in avocado tissue was determined by extracting the tissue three times with 80% ethanol. The ethanol was removed, and the crude extract was passed through a Dowex 50 column in the H⁺ form. Amino acids were eluted with 2 M NH₄OH. Since some methionine might have been oxidized to methionine sulfoxide during the procedure, the amino acid extract was reduced with 0.1% (v/v) 2-mercaptoethanol at 40 C overnight. Amino acids were analyzed with a Beckman amino acid analyzer.

Analysis of Reaction Products Isolated from Tissues. The apple plugs which had been fed with KMB- U -¹⁴C were first extracted with 80% ethanol. The crude extract was subjected to paper chromatography using 1-butanol-acetic acid-water (4:1:4, v/v) as developing solvent. The R_F values of KMB, methionine, and β -methylthiopropionic acid were 0.70, 0.50, and 0.85, respectively. These spots, which corresponded to the authentic samples, were eluted; and their identity was further verified with paper electrophoresis at pH 2.5, 6.4, and 11.3.

It is well known that in fruits the dramatic increase in ethylene production is closely associated with the increase in respiration and with ripening (3, 4, 10, 14). Oxygen is essential for this endogenous production of ethylene (4, 10). Evidence from tracer studies in a number of plant tissues has shown that methionine serves as a precursor of ethylene *in vivo* (1, 2, 5, 9, 11, 18). The enzymic conversion of methionine analogues to ethylene catalyzed by peroxidase has been elucidated recently (7, 12, 13, 15, 17, 18); KMB,² the methionine keto analogue, or methional (β -methylthiopropionaldehyde) but not methionine, is the active substrate. A chemical mechanism accounting for such enzymatic reactions has been described (15, 17, 18). On the basis of this information, Yang (16) has proposed the following scheme for the biosynthesis of ethylene in plants: methionine \rightarrow KMB \rightarrow (methional) \rightarrow ethylene. Later, we have shown that in apple tissues methional is not converted to ethylene (2). Although KMB was readily converted to ethylene, the efficiency of its conversion was found to be less than that from methionine (2). These data do not support the biosynthetic scheme proposed above. On the other hand, Mapson *et al.* (12) studied the relative conversion of methionine and KMB into ethylene by cauliflower florets and found that KMB is a more efficient precursor of ethylene than methionine. Therefore, they have supported the view that KMB is an intermediate in the formation of ethylene from methionine.

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² Abbreviation: KMB: α -keto- γ -methylthiobutyric acid.

Chemical identification of methionine was further carried out by oxidizing it to methionine sulfoxide with H_2O_2 followed by cochromatography with authentic methionine sulfoxide (R_f , 0.22).

RESULTS AND DISCUSSION

Conversion of Methionine to Ethylene by Avocado Tissues at Different Ripening Stages. Avocados have a well defined respiratory climacteric accompanied by a large increase in ethylene production (3). When L-methionine- $U-^{14}C$ or D-glucose- $U-^{14}C$ was supplied to avocado tissue at three different stages of ripening, preclimacteric, climacteric rise, and climacteric peak, only the tissues at the climacteric rise and at the climacteric peak converted L-methionine- $U-^{14}C$ to ethylene (Table I). The preclimacteric avocado tissue, which produced no significant amounts of ethylene endogenously, did not convert L-methionine to ethylene. However, all tissues at the three different stages converted methionine and glucose actively to carbon dioxide. In the present experiment D-glucose- $U-^{14}C$ was administered to test the metabolic activity of the avocado plugs to utilize glucose. Thus the ability of avocado tissue to convert methionine to ethylene was parallel to its ability to produce ethylene endogenously. Similarly, when KMB- $U-^{14}C$ was supplied to the avocado tissues in place of methionine- $U-^{14}C$, it was efficiently converted to ethylene by the tissues at stages of climacteric rise and climacteric peak, but not by the tissue at the preclimacteric stage. However, methionine was found to be a superior precursor of ethylene over KMB in tissues at both the climacteric rise and the climacteric peak. Methional- $U-^{14}C$ was found to be an inactive precursor at all stages. As this work was completed, a report by Mapson *et al.* (11) appeared showing that, like avocado, tomato tissue at different ripening stages can convert methionine into ethylene in parallel with its ability to produce ethylene endogenously. In this connection, it is relevant to note the findings of Burg and Clagett (5), who reported that only those pea stem segments which had been pretreated with IAA and had a high rate of ethylene production had the ability to convert exogenously supplied methionine to ethylene. These results, coupled with our finding that the conversion of methionine to ethylene was stereospecific for the L-isomer (2), strongly support the idea that methionine is indeed a biological precursor of ethylene.

In order to examine whether there is any relationship between the rate of ethylene production and the level of ethylene

Table I. Comparison of Conversion of D-Glucose- $U-^{14}C$ and L-Methionine- $U-^{14}C$ into Ethylene and CO_2 by Avocado Tissue at Different Ripening Stages and Its Methionine Content

To each plug of avocado tissue (1×2 cm) which weighed about 1.8 g were injected under vacuum 60 μ l of 2% KCl solution containing either 0.58 μ g of D-glucose- $U-^{14}C$ (4 μ g/ μ mole) or 0.53 μ g of L-methionine- $U-^{14}C$ (100 μ g/ μ mole). Gases evolved between 60- and 180-min incubation were analyzed.

Ripening Stage	Precursor	CO_2		C_2H_4		Endogenous Methionine
		μ moles	μ g	μ moles	μ g	
Preclimacteric	Glucose	24	33	0.0008	0.0	31.5
	Methionine	23	8.5	0.0008	0.0	
Climacteric rise	Glucose	24	23	2.6	0.0	30.4
	Methionine	34	5.3	2.2	4.9	
Climacteric peak	Glucose	31	32	14	0.0	12.5
	Methionine	31	4.5	12	4.1	

Table II. Oxygen Dependence of Ethylene Production from L-Methionine or KMB by Apple Tissue

To each plug of apple tissue (1×2 cm) were injected under vacuum 60 μ l of 2% KCl solution containing 0.43 μ g of L-methionine- $U-^{14}C$ (100 μ g/ μ mole) or 0.65 μ g of KMB- $U-^{14}C$ (100 μ g/ μ mole). Each plug was incubated alternately under air or N_2 atmosphere for 1 hr each.

Experiment	Substrate	Atmosphere	CO_2		C_2H_4	
			μ g	μ moles	μ g	μ moles
1	L-Methionine- $U-^{14}C$	Air	4.3	4.1	8.0	5.9
		N_2	1.9	2.3	1.2	...
		Air	12	3.0	20	...
2	L-Methionine- $U-^{14}C$	N_2	0.0	2.7	0.0	0.006
		Air	7.9	3.0	12	...
		N_2	1.3	2.1	1.1	...
3	KMB- $U-^{14}C$	Air	23	4.3	6.8	6.5
		N_2	4.4	2.3	0.0	...
		Air	7.9	2.8	14	...
4	KMB- $U-^{14}C$	N_2	20	2.8	0.0	0.1
		Air	13	3.0	13	...
		N_2	3.9	1.7	0.0	...

precursor, the concentration of endogenous methionine in avocado tissue at three different ripening stages was analyzed. The level of methionine at the preclimacteric stage and at the climacteric rise was found to be about the same but was lowest at the climacteric peak at which the rate of ethylene production was highest. The present results, that avocado tissue at the preclimacteric stage would not convert methionine to ethylene, but contained the highest endogenous methionine, and that fruit tissue at the climacteric peak was most active in converting methionine to ethylene, but contained the least amount of methionine, indicate that ethylene biosynthesis in fruit tissue is not controlled by the level of methionine but by a step after methionine is synthesized. As shown in Table I, the rate of ethylene production by the avocado tissue at the climacteric peak was about 3.7 μ moles per g per hr while its methionine level was 12.5 μ moles per g; thus, the endogenous level of methionine could sustain ethylene production from methionine for only about 3 hr, assuming that methionine is the sole or the predominant precursor of ethylene (18). Methionine must, therefore, turn over rapidly during the ripening when the rate of ethylene production is high.

Oxygen Requirement for Conversion of Methionine to Ethylene by Apple Tissue. Another characteristic feature of ethylene formation *in vivo* is its oxygen dependence. When air is replaced by nitrogen, ethylene production by plant tissues declines rapidly (4, 6, 10). Table II illustrates the absolute requirement of oxygen for the conversion of methionine or KMB to ethylene by apple tissue. It is important to note that when ^{14}C -methionine was administered to apple tissue there was a 40-min lag phase before a steady rate of ^{14}C -ethylene production occurred (Fig. 1). This explains the data shown in Table II that conversion into ethylene in the air during the 1st hr of incubation was relatively small. When the apple plug was incubated in nitrogen for 1 hr and then switched to air (experiment 2 of Table II), a rapid conversion of methionine to ethylene was observed. The data suggest that the process(es) occurring in the lag phase did not require oxygen. It appears that during the lag period methionine is first converted to an unidentified intermediate, followed by an oxygen-dependent reaction in which the intermediate is degraded into ethylene (6).

Conversion of Methionine or KMB into Ethylene by Apple Tissues. We have previously reported that methionine was a

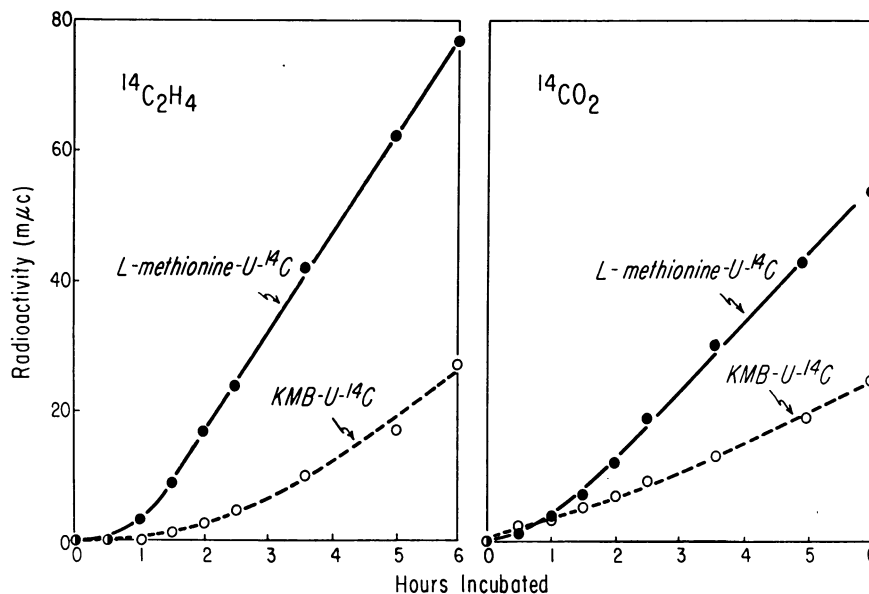


FIG. 1. Conversion of L-methionine- ^{14}C and α -keto- γ -methylthiobutyric acid- ^{14}C to ethylene and carbon dioxide by apple tissues. Each apple plug (1×2 cm, 1.4 g) received $0.5 \mu\text{c}$ ($100 \mu\text{c}/\mu\text{mole}$) of radioactive methionine or KMB. The formation of radioactive ethylene and CO_2 was analyzed with a gas radiochromatograph unit. The rate of endogenous ethylene production from the plugs which received methionine and KMB was 5.8 and $6.4 \text{ m}\mu\text{mole/hr}$, respectively.

Table III. Conversion of α -Keto- γ -methylthiobutyric Acid to Methionine and Other Products by Apple Tissue

To each plug of apple tissue (1×2 cm) were injected under vacuum $60 \mu\text{l}$ of 2% KCl solution containing $0.48 \mu\text{c}$ of KMB- ^{14}C ($100 \mu\text{c}/\mu\text{mole}$). After incubation for 1.5 or 3.0 hr in a sealed flask, the radioactive C_2H_4 and CO_2 produced in the gas phase and the reaction products remaining in the tissue were analyzed as described under "Materials and Methods."

Incubation Time	CO_2	C_2H_4	KMB	Methionine	β -Methylthiopropionic Acid	Un-identified Products
hr	mμc					
1.5	5.6	1.4	20.4	23.2	37.0	346
3.0	23.4	16.7	0.0	17.3	32.5	247

more efficient precursor of ethylene than KMB in apple tissues (2). However, Mapson *et al.* (12) have recently studied the relative incorporation of methionine and KMB into ethylene by cauliflower florets and have found that KMB is a more efficient precursor than methionine. In order to get more insight into this question, we have administered radioactive methionine or KMB to apple plugs and studied their interconversion as related to the efficiency of ethylene formation. When methionine- ^{14}C was fed to the apple tissues, the predominant radioactivity remaining in the tissue after up to 3 hr of incubation was found to be the unchanged methionine, and there was no detectable amount of labeled KMB in the extracts. On the contrary, when KMB- ^{14}C was fed to the tissues, it was very actively converted to a variety of products, including several which were not identified. Table III shows that after 1.5 hr of incubation, during which there was very little radioactive ethylene production, only 5% of the total radioactivity recovered was identified as the unchanged KMB, 6% as methionine, and 9% as β -methylthiopropionic acid. β -Methylthiopropionic acid is an oxidative decarboxylation product of KMB and has been found to be inactive as a precursor of ethylene in apple tissues (S. F. Yang and A. H. Baur, un-

published results). When the incubation time was prolonged to 3 hr, about 5% of the radioactivity recovered was found as methionine, and there was no detectable amount of KMB. During this period, active conversion into ethylene had occurred. It is clear from Table III that there was active conversion of KMB to methionine, and there was essentially no KMB left when the production of ^{14}C -ethylene became substantial. One may argue that, although KMB was actively converted to methionine, the latter was slowly converted back to KMB before its conversion to ethylene. If this were the case, the initial rate of ethylene production from the KMB should be greater than that from methionine. Figure 1 illustrates the kinetics of the conversion of methionine and KMB into ethylene. The rate of ethylene formation from methionine was higher than that from KMB. This difference was more marked at the initial stage, because of the longer lag phase required for KMB (about 90 min) than for methionine (about 50 min). The present results—that KMB was actively converted to methionine while its conversion into ethylene was active, that methionine was more efficiently converted to ethylene than KMB, especially during its early stage of incubation, and that the lag period required for the conversion of KMB to ethylene was longer than that for methionine—strongly suggest that methionine is closer to the immediate precursor of ethylene than KMB. Another approach we have undertaken to answer this question was an isotope experiment. The relative effectiveness of unlabeled methionine or KMB to reduce the incorporation of radioactive methionine or KMB was studied. It was found that unlabeled methionine was more effective than KMB in reducing the incorporation of radioactive methionine to ethylene. Conversely, unlabeled KMB was found to be more effective than methionine in reducing the incorporation of radioactive KMB to ethylene. These experiments failed to establish which substrate was a closer precursor of ethylene.

Our results from apple tissue differ from those of Mapson *et al.* (12), who have studied the conversion of methionine and KMB to ethylene in cauliflower floret tissues. Their conclusion that KMB is a closer precursor of ethylene than methionine was largely based on the following observations: KMB greatly stimulated ethylene production by cauliflower floret tissues,

KMB was incorporated into ethylene more efficiently than methionine, and unlabeled methionine was ineffective in reducing the conversion of radioactive KMB into ethylene, while KMB was effective in reducing the conversion of radioactive methionine to ethylene. However, in the light of the observation that KMB degrades nonenzymatically into ethylene with ease (8, 19), caution must be exercised in interpreting the data. It should be noted that the stimulation of ethylene production in cauliflower floret tissues by the addition of KMB was directly proportional to the KMB concentration over a range from 0 to 6 mM (12). This phenomenon can be due to a high Michaelis constant of the enzymes in question, or perhaps it can also be due to the nonenzymatic decomposition of KMB to ethylene. The possibility that KMB could be degraded nonenzymatically is indicated by their observation that heat-inactivated cauliflower tissue resumes ethylene production from KMB when ascorbic acid is added back (12). Alternatively, it is equally possible that the pathway of ethylene biosynthesis from methionine is different in apple fruits than in cauliflower floret tissues.

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