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INHIBITION OF THE METHIONINE CYCLE ENZYMES*

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Key Word Index—1-Aminocyclopropane-1-carboxylic acid; ethylene biosynthesis; inhibition; methionine; 5'-methylthioadenosine; 5-methylthioribose.

Abstract—Inhibition of the enzymes involved in the production of 1-aminocyclopropane-1-carboxylic acid (ACC) and the subsequent salvage of methionine from 5'-methylthioadenosine (MTA) was studied. Possible product inhibition of ACC synthase, which converts *S*-adenosylmethionine (SAM) to ACC and MTA, and MTA nucleosidase, which hydrolyses MTA to 5-methylthioribose (MTR) and adenine, was investigated. ACC synthase was weakly inhibited by MTA (K_i = 0.2 mM). MTA nucleosidase was inhibited by adenine competitively (K_i = 40 μM), but not by MTR. Some analogues of the enzymes' substrates were inhibitory. ACC synthase was strongly and competitively inhibited by sinefungin, a SAM analogue (K_i = 2 μM); MTA nucleosidase was inhibited by various MTA analogues, including 5'-chloroformycin, 5'-chloroadenosine, and 5'-ethylthioadenosine. The conversion of MTR to methionine in avocado extract was inhibited by the MTR analogues 5-chlororibose and 5-ethylthioribose, which exert their inhibitory effects by inhibiting MTR kinase. The capacity to convert MTR to methionine in ripening apple tissue appears to be ample; thus, this conversion does not appear to be a limiting factor of ethylene production.

INTRODUCTION

The sulphur-containing amino acid methionine serves as a precursor of the plant hormone ethylene. The biosynthetic pathway of ethylene has been established as follows: Methionine → *S*-adenosyl methionine (SAM) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene [1]. When ACC is formed from SAM by the enzyme ACC synthase (E.C. 4.4.1.14), 5'-methylthioadenosine (MTA) is released as the co-product. MTA is cycled back to re-form methionine by way of the methionine cycle through which the methionine supply is maintained for continued ethylene production (for review, see [2]). The sequence of the pathway is as follows: MTA → 5-methylthioribose (MTR) → MTR-1-phosphate (MTR-1-P) → 2-oxo-4-methylthiobutanoic acid → methionine. MTA nucleosidase (E.C. 3.2.2.16) catalyses the hydrolysis of MTA to MTR, and MTR kinase (E.C. 2.7.1.100) catalyses the phosphorylation of MTR to MTR-1-P.

It is generally accepted that the development of ACC synthase is the major factor controlling the rate of ethylene biosynthesis [2, 3]. However, the possible regulation of the ethylene biosynthesis by those enzymes in the methionine cycle which recycle MTA to SAM, and thus sustain the SAM supply for continued ACC synthesis, also should be considered. Indeed, Kushad *et al.* [4] have reported that the specific activity of MTR kinase increased during tomato ripening, and suggested that this increased activity may play a role in increasing ethylene production during ripening. Aside from changes in the amount of enzyme, changes in enzymatic activity can be brought about by effector molecules. One common form of such enzymatic regulation is product inhibition, where an enzyme's product at sufficient concentrations inhibits its own synthesis. The possibility that methioinine cycle enzymes were subject to product inhibition was suggested by Yu *et al.* [5], who found that MTA could inhibit ACC synthase, and by Guranowski *et al.* [6], who found that MTA nucleosidase from lupine seeds was inhibited by its product adenine with a K_i of 11 μM. It is possible that adenine may inhibit MTA nucleosidase *in vivo*, thereby blocking the methionine cycle. In addition, inhibition of MTA nucleosidase may lead to an accumulation of MTA, which in turn could inhibit ACC synthase, thus inhibiting ethylene biosynthesis.

This study was carried out to characterize further the regulation of those enzymes involved in the methionine cycle by the use of various inhibitors.

RESULTS AND DISCUSSION

ACC synthase inhibition

We examined various compounds for inhibitory activity on ACC synthase (Table 1). Miura and Chiang [7] have reported that sinefungin is a powerful inhibitor of ACC synthase, although they did not determine the mode of inhibition. Indeed, sinefungin was the most potent ACC synthase inhibitor we tested, with an $[I]_{0.5}$ of about 10 μM. By using double reciprocal plots, we have determined that sinefungin inhibits competitively with a K_i

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; DTT, dithiothreitol; ETA, 5'-ethylthioadenosine; ETR, 5-ethylthioribose; ETR-1-P, 5-ethylthioribose-1-phosphate; HEPPS, *N*-2-hydroxyethylpiperazine-*N*-2-propanesulphonic acid; MTA, 5'-methylthioadenosine; MTR, 5-methylthioribose; MTR-1-P, 5-methylthioribose-1-phosphate; SAM, *S*-adenosylmethionine.

Table 1. Effect of various compounds on tomato ACC synthase activity

Inhibitor	Concentration (μM)	Activity (% of control)	Approximate $[I]_{0.5}$
Sinefungin	1	65	10 μM
	10	33	
	100	4	($K_i = 2\ \mu$M)
	1000	2	
ETA	1	79	0.2 mM
	10	82	
	100	59	
	1000	24	
MTA	1	81	0.2 mM
	10	89	
	100	66	($K_i = 0.2$ mM)
	1000	15	
5'-Chloroadenosine	1	87	0.1 mM
	10	92	
	100	51	
	1000	17	
5'-Chloroformycin	1	92	>1 mM
	10	90	
	100	75	
	1000	69	
Putrescine	10	104	1 mM
	100	91	
	1000	65	
Spermidine	10	101	1 mM
	100	82	
	1000	43	
Spermine	10	98	1 mM
	100	90	
	1000	52	

The reaction mixtures contained 50 mM HEPPS–KOH (pH 8.5), 50 μM SAM (this concentration is equivalent to 3 K_m), various concentrations of inhibitors, and 0.4 ml of tomato extract, in a total volume of 0.6 ml. After incubation for 1 hr at 30°, ACC was determined by the method of ref. [8].

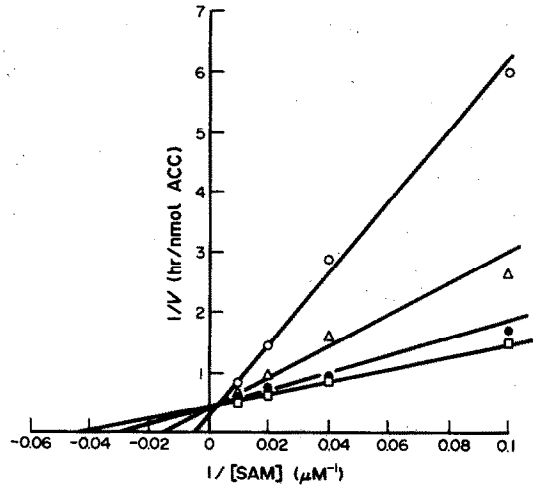

Fig. 1. Double reciprocal plot of ACC synthase vs SAM concentration in the presence of 0 (□), 1 (●), 5 (△), or 10 (○) μM sinefungin. Velocity is expressed as nmol ACC formed per hr in the reaction mixture, as described in Experimental.

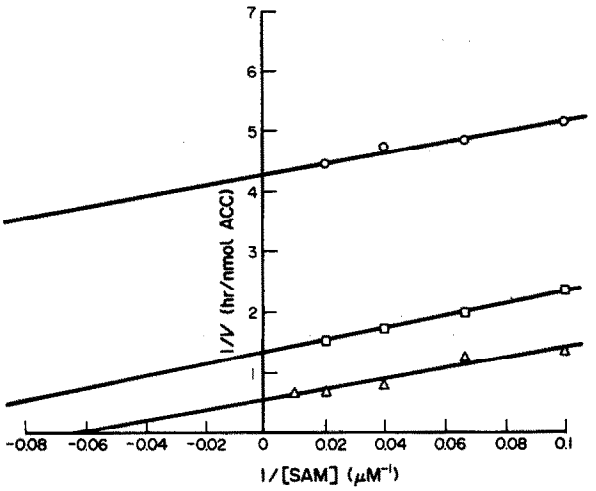

Fig. 2. Double reciprocal plot of ACC synthase activity vs. SAM concentration in the presence of 0 (△), 0.2 (□), or 1.0 (○) mM MTA. Velocity is expressed as nmol ACC formed per hr in the reaction mixture, as described in Experimental.

of 2 μM (Fig. 1). That sinefungin is a competitive inhibitor is not surprising, since sinefungin is an analogue of SAM, the substrate of ACC synthase. MTA, one of the products of ACC synthase, also inhibited ACC synthase ($[I]_{0.5}$ for MTA = 0.2 mM), but to a lesser extent than sinefungin. The kinetics of MTA inhibition as revealed by double reciprocal plots indicate that this inhibition is uncompetitive or mixed type noncompetitive–uncompetitive (Fig. 2), with a K_i of 0.2 mM. During the completion of this work, it was also reported that MTA uncompetitively inhibited ACC synthase prepared from winter squash [8].

Yu *et al.* [5] were the first to observe that MTA inhibited ACC synthase, but they were uncertain of the inhibitory MTA concentrations, because the high MTA nucleosidase activity in their ACC synthase preparation rapidly hydrolysed the MTA. In the present study, we inhibited MTA hydrolysis by adding 5'-chloroformycin, which effectively inhibited the MTA nucleosidase without affecting the ACC synthase activity. Although we observed that MTA inhibits the enzyme, its $[I]_{0.5}$ concentration (0.2 mM) and K_i (0.2 mM) were too high to be physiologically significant. Such high MTA levels probably are never present *in vivo*, for MTA nucleosidase rapidly hydrolyses MTA to MTR [5, 9]. The other product of ACC synthase, ACC, has been shown not to product-inhibit this enzyme [5]. Thus, it appears that product inhibition does not play a significant role in regulating ACC synthase.

5'-Chloroadenosine, an analogue of MTA, inhibited ACC synthase with an $[I]_{0.5}$ close to that of MTA. Since 5'-chloroadenosine contains a chloro- moiety, we investigated the possibility that the compound exerts its inhibitory effect by covalently attaching to and inactivating the enzyme. Preincubation of the enzyme extract for one hr with 5'-chloroadenosine before the ACC synthase assay did not appreciably increase inhibition over samples

which were not preincubated, suggesting that this inhibitor did not inactivate the enzyme covalently.

We also examined the effect of polyamines on tomato ACC synthase (Table 1). Polyamines were suspected as possible inhibitors of ACC synthase, since ethylene and polyamine biosynthesis compete for SAM as a common precursor, and polyamines have been shown to decrease ethylene production and ACC levels significantly in IAA-treated soybean hypocotyls [10]. We found that while putrescine, spermidine, and spermine all caused ACC synthase inhibition, with spermidine the most effective of the three, the concentrations required to obtain 50% inhibition were all high (about 1 mM). It was recently reported that these polyamines inhibited winter squash ACC synthase noncompetitively [8]. The K_i's for putrescine, spermidine, and spermine were calculated to be 4.9 mM, 2.9 mM, and 1.6 mM, respectively, it was suggested that such inhibition may play a role in regulating ACC synthase. Although polyamines have been shown to accumulate in plant tissues under stress conditions [11], the levels were still 10 times below those of the K_i values. Thus, polyamines are weak inhibitors of ACC synthase, and thier role in regulating ACC synthase activity *in vivo* may be rather limited.

Inhibition of MTA nucleosidase in tomato extract

Various compounds were examined for inhibitory activity on tomato MTA nucleosidase. Key among them was adenine, a product of MTA nucleosidase, which was reported in ref. [6] to inhibit purified lupine seed MTA nucleosidase competitively with a K_i of 11 μM. Although we found that 1–5 mM adenine was necessary to cause 50% inhibition of the MTA nucleosidase activity in our tomato extract assay system, the relatively high substrate concentration (50 μM) we used led to the rather high $[I]_{0.5}$ value. When the substrate concentration was lowered to 5 μM, the observed $[I]_{0.5}$ was approximately 150 μM. We calculated the K_i for adenine to be 40 μM, given the K_m of 2 μM that we calculated for MTA. Although adenine inhibits MTA nucleosidase *in vitro* with a low K_i value, it is not known whether adenine regulates the enzyme *in vivo*; adenine may not accumulate to inhibitory levels, since it is readily salvaged into ATP and ADP [12].

In agreement with the observations in ref. [6], MTA nucleosidase was not inhibited by MTR, a product of the enzyme. Similarly, MTR analogues such as 5-chlororibose and 5ethylthioribose (ETR) were ineffective in inhibiting the enzyme (Table 2).

Additionally, we examined the ability of various MTA analogues to inhibit MTA nucleosidase (Table 2). Consistent with the results of ref. [4], 5′-chloroadenosine, 5′-ethylthioadenosine (ETA), and 5′-chloroformycin inhibited the nucleosidase to various degrees. While the $[I]_{0.5}$ for chloroadenosine was approximately 0.3 mM, ETA and 5′-chloroformycin were more potent with $[I]_{0.5}$'s of about 40 μM. Thus, MTA nucleosidase was inhibited by analogues of its substrate. We found that 5′-chloroadenosine, ETA, and 5′-chloroformycin all caused competitive inhibition with K_i's of 30 μM, 4 μM, and 6 μM, respectively. (It should be noted that the K_i values were lower than the $[I]_{0.5}$ values under our assay conditions, where relatively high concentrations of MTA were used.) Thus, these compounds are better MTA nucleosidase inhibitors than adenine. Some of the inhibitors actually are also substrates of the enzyme, for Guranowski *et al.* [6] have reported that ETA is a substrate for the enzyme. It is not known whether 5′-chloroadenosine also is a substrate. It is unlikely that 5′-chloroformycin is a substrate, for it contains a non-hydrolysable C–C bond in place of MTA's C–N glycosidic bond. Since the enzyme was not inhibited by 5-chlororibose and ETR, the deadenylated counterparts of 5′-

Table 2. Effect of various compounds on tomato MTA nucleosidase activity

Inhibitor	Concentration (μM)	Activity (% of control)	Approximate $[I]_{0.5}$
Sinefungin	1	91	not
	10	100	inhibitory
	100	108	
	1000	98	
ETA	1	93	80 μM
	10	100	
	100	47	(K_i = 4 μM)
	1000	9	
ETR	1	91	not
	10	98	inhibitory
	100	96	
	1000	89	
5′-Chloroadenosine	1	96	0.35 mM
	10	102	
	100	78	(K_i = 30 μM)
	1000	31	
5-Chlororibose	1	92	not
	10	92	inhibitory
	100	98	
	1000	100	
5′-Chloroformycin	1	107	45 μM
	10	84	
	100	33	(K_i = 6 μM)
	1000	9	
MTR	1	102	not
	10	94	innibitory
	100	96	
	1000	90	
Adenine	1	95	1 mM
	10	94	
	100	86	(K_i = 40 μM)
	1000	49	
Adenosine	1	97	not
	10	95	inhibitory
	100	97	
	1000	96	

The reaction mixtures contained 50 mM HEPPS–KOH (pH 8.5), 50 μM [Me-^{14}C]MTA (48 uCi/umol) (this concentration represents 100 K_m), various concentrations of inhibitors, 75 μl of tomato extract, in a total volume of 0.15 ml. After incubation for 1 hr at 30°, the unreacted [Me-^{14}C]MTA was removed by ion exchange chromatography on Dowex 50 (H^+), and the effluent containing the [^{14}C]MTR was quantitated by liquid scintillation counting.

chloroadenosine and ETA, respectively, the enzyme must recognize the adenine moiety.

Preincubation of the enzyme extract for one hr with 5'-chloroformycin and 5'-chloroadenosine did not increase inhibition over the non-preincubated samples, suggesting that the inhibition was not caused by the inhibitors covalently attaching to the enzyme.

Effect of inhibitors on the conversion of MTR to methionine

The compounds which were tested for inhibitory effects on ACC synthase and MTA nucleosidase also were examined for their effects on the metabolism of MTR and MTR-1-P to methionine in avocado extract. [Me-^{14}C]MTR or [Me-^{14}C]MTR-1-P was incubated with avocado extract in the presence of the inhibitors, as described in Experimental, and the [^{14}C]methionine produced was measured. 5'-Chloroformycin, sinefungin, and 5'-chloroadenosine showed no effect on the conversion of MTR to methionine. With MTR as the substrate, ETR and 5-chlororibose were the most potent inhibitors tested, while ETA inhibited moderately (Table 3). However, ETA, ETR, and 5-chlororibose had little effect on the conversion of MTR-1-P to methionine, indicating that these compounds exerted their inhibitory influence on MTR kinase, which catalyses the conversion of MTR to MTR-1-P (Table 4). Thus, MTR kinase is inhibited by analogues of its substrate MTR. It is possible that ETR inhibits MTR kinase activity by serving as a substrate of MTR kinase and thereby competing with MTR for the same enzyme. Such a mechanism would yield ETR-1-P from ETR. In addition, ETR-1-P may be further metabolized to ethionine, in a manner similar to that of MTR-1-P to methionine. Our observation that ETR did not inhibit the conversion of MTR-1-P to methionine does not rule out the possibility that ETR is converted to ethionine, for the amount of ETR-1-P formed may have been too little to affect the metabolism of MTR-1-P. To test such hypotheses, we incubated [Et-^{14}C]ETR with avocado extract in the presence of ATP, and analysed the radioactive metabolites. No [^{14}C]ethionine was detected. However, we observed a radioactive compound, the production of which was ATP-dependent, and which migrated at R_f 0.18 in the paper chromatographic system used. The compound was anionic in paper electrophoresis at pH 2; upon treatment with 1 M HCl at 100° for 10 min, the radioactive compound became neutral in paper electrophoresis at pH 2 and co-migrated with ETR in paper chromatography. These data suggest that the unknown compound may be the anionic ETR-1-P, a compound which readily yields ETR and phosphate upon acidic hydrolysis.

These observations indicate that ETR is not appreciably converted to ethionine by avocado extract, which is capable of efficiently converting MTR to methionine. However, other data indicate that plant systems are capable of converting ETR to ethionine. We have observed that mungbean segments can convert [^{14}C]ETR to [^{14}C]ethionine, which we identified by paper co-chromatography and co-electrophoresis with the authentic compound (data not shown). In addition, Schlenk and Ehninger [13] reported that yeast cells converted 5'-ethylthioadenosine to *S*-adenosylethionine, presumably by a mechanism similar to that of MTA to SAM via methionine.

Table 3. Effects of various compounds on metabolism of MTR to methionine by avocado extract

Inhibitor	Concentration (μM)	Conversion to methionine (% of control)
None		100
ETA	10	98
	100	61
	1000	0
ETR	10	100
	100	0
	1000	0
5'-Chloroadenosine	10	84
	100	93
	1000	68
5-Chlororibose	10	95
	100	43
	1000	0
5'-Chloroformycin	10	104
	100	91
	1000	72
MTA	10	73
	100	0
	1000	7

The reaction mixtures contained 50 mM potassium phosphate (pH 7), 0.5 mM ATP, 5 mM $MgSO_4$, 1 mM L-glutamine, 10 μM [Me-^{14}C]MTR, 50 μl of avocado extract, and various concentrations of inhibitors, in a total volume of 0.1 ml. After incubation for 5 hr, the radioactive [^{14}C]methionine formed was separated by ion exchange chromatography on Dowex 50 (H^+), and quantitated by scintillation counting.

Table 4. Effects of various compounds on metabolism of MTR-1-P to methionine by avocado extract

Inhibitor	Concentration (μM)	Conversion to methionine (% of control)
None		100
ETA	10	80
	100	64
	1000	83
ETR	10	72
	100	88
	1000	96
5-Chlororibose	10	88
	100	91
	1000	101

The reaction mixtures contained 50 mM potassium phosphate (pH 7), 0.5 mM ATP, 5 mM $MgSO_4$, 1 mM L-glutamine, 10 μM [Me-^{14}C]MTR-1-P, 50 μl of avocado extract, and various concentrations of inhibitors, in a total volume of 0.1 ml. After incubation for 5 hr, the radiactive [^{14}C]methionine formed was separated by ion exchange chromatography on Dowex 50 (H^+), and quantitated by scintillation counting.

Relationship between changes in the ethylene production rate and the rate of methionine salvage from MTR in apple tissue

In climacteric fruits such as apples and avocados, the rate of ethylene production rises several hundred-fold during ripening. Thus, it is pertinent to ask whether the capacity to salvage methionine may limit the rate of ethylene biosynthesis. Adams and Yang [9] previously have found that apple tissues in both preclimacteric and climacteric stages were capable of converting MTA to methionine. In the present study, we compared quantitatively the ability to salvage methionine from MTR in relation to ethylene production rates in apple fruits at various ripening stages. We fed [Me-^{14}C]MTR to apple fruit plugs and quantitated the [^{14}C]methionine produced. Although ethylene production rates differed markedly among different fruits, we observed no significant difference in their ability to convert MTR to methionine (Table 5). Since the ethylene production rates increased dramatically without a corresponding increase in the capacity to salvage methionine from MTR, it appears that fruit tissues at any ripening stage have such an ample capacity to recycle methionine that it does not limit the ethylene biosynthesis rate. In Table 5, we compare the amounts of administered MTR converted to methioinine. The highest ethylene production rate measured was 8.5 nmol/(g fresh wt)/hr, whereas the corresponding conversion rate of exogenous MTR to methionine was calculated to be 0.8 nmol/(g fresh wt)/hr. However, this represents an underestimate, because the amount of the endogenous MTR present in the tissue was not taken into account, and because the ethylene production rate represents that produced from the whole apple plug, whereas the conversion of exogenously administered MTR to methionine was limited to those portions of the apple plugs that received labelled MTR.

EXPERIMENTAL

Materials. [Me-^{14}C]SAM was purchased from Research Products International, Mt. Prospect, IL. [Et-^{14}C]ethionine was obtained from New England Nuclear, Boston, MA. *S*-Adenosyl[Et-^{14}C]ethionine was prepared by incubating yeast cells in the presence of [Et-^{14}C]ethionine [14]. [Me-^{14}C]MTA and [Me-^{14}C]MTR were prepared from [Me-^{14}C]SAM as described previously [13]. Similarly, [Et-^{14}C]ETR was prepared from *S*-adenosyl[Et-^{14}C]ethionine. [Me-^{14}C]MTR-1-P was kindly provided by Dr Adolph Ferro, Oregon State University, Corvallis, OR. ACC was obtained from Calbiochem, while polyamines and sinefungin were from Sigma. 5'-Chloroformycin was prepared by Dr Michael Pirrung, Stanford University, Stanford, CA. ETA and 5'-chloroadenosine were a generous gift from Dr Douglas Adams, Stauffer Chemical Co., Richmond, CA. ETR and 5-chlororibose were prepared from the corresponding adenosine derivatives by acid hydrolysis [13].

Table 5. Conversion of MTR to methionine by apple tissue in different stages of ripening

Ethylene produced (nl/g/hr)	MTR converted to methionine (%)
0.1	26
5.4	29
43	37
75	32
116	37
190	30

Apple plugs were vacuum infiltrated with 0.1 ml of a solution containing 3% (w/v) KCl, 0.1 μmol L-methionine, and 10 nmol [Me-^{14}C]MTR (11 nCi/nmol). After 5 hr, the plugs were extracted with 80% (v/v) ethanol, and the [^{14}C]methionine was quantitated by scintillation counting after separation by ion exchange chromatography on Dowex 50 (H^+).

Enzyme extract preparation. Tomato fruit (*Lycopersicon esculentum*) extract was prepared by a modified version of ref. [5]. Tomato fruit tissue was sliced into pieces and incubated for 8 hr on moist paper towels at 25° to increase the level of ACC synthase. All subsequent steps were carried out at 4°. The tissue was homogenized in a Waring blender with buffer containing 100 mM HEPPS–KOH (pH 8.5), 4 mM DTT, 2 μM pyridoxal phosphate, and 0.2% (v/v) Triton X-100. For each gram of tissue, 1 ml of buffer was used. The homogenate was centrifuged at 25 000 *g* for 20 min, and the supernatant was dialysed overnight against buffer containing 2 mM HEPPS–KOH (pH 8.5), 0.1 mM DTT and 1 μM pyridoxal phosphate. Avocado tissue (*Persea americana*) was homogenized in buffer (1 g tissue to 1 ml buffer) containing 50 mM KPi (pH 7.0), 3 mM DTT, and 0.3% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 20 000 *g* for 20 min at 4°. The supernatant was used as a source of enzyme.

ACC synthase assay. The reaction mixtures contained 50 mM HEPPS–KOH (pH 8.5), 10–100 μM SAM, various concentrations of suspected inhibitors, and 0.4 ml tomato extract, in a total vol. of 0.6 ml [5]. After incubation at 30° for one hr, the reaction was stopped by boiling the reaction mixtures for five min. ACC formed was assayed by the method of ref. [15].

MTA nucleosidase assay. Reaction mixtures with tomato extract typically contained 50 mM HEPPS–KOH (pH 8.5), 50 μM [Me-^{14}C]MTA (48 μCi/μmol), various concentrations of potential inhibitors, and 75 μl of tomato extract, in a total vol. of 0.15 ml. After incubation for one hr at 30°, the reaction mixtures were boiled and then centrifuged to remove any precipitate. Fifty μl of the resulting soln was passed through a small (0.1 ml) column of Dowex 50 cation exhange resin (H^+ form, 200–400 mesh), and washed with 0.5 ml H_2O. Radioactivity in the effluent, which contained the hydrolysed product MTR, was determined by liquid scintillation counting. The difference in radioactivity between that loaded on the Dowex 50 and that in the effluent was taken to be the amount of unhydrolysed MTA. The identities of the compounds present in the fractions were confirmed by paper co-chromatography and co-electrophoresis with authentic standards.

Recycling of MTR and MTR-1-P to methionine. Reaction mixtures typically contained 50 mM KPi (pH 7), 3 mM DTT, 0.5 mM ATP, 5 mM $MgSO_4$, 1 mM L-glutamine, various concentrations of inhibitors, 10 μM [Me-^{14}C]MTR (48 μCi/μmol) or [Me-^{14}C]MTR-1-P (45 μCi/μmol), and 50 μl crude avocado extract, in a total vol. of 0.1 ml. After incubation for 5 hr at 25° the reaction mixtures were boiled and centrifuged. The supernatant was analysed for the proportion of cationic radioacity relative to the total radioactivity, as is described above for MTA hydrolysis. The cationic counts were attributed to methionine, the identity of which was confirmed paper chromatography and electrophoresis.

Metabolsim of ETR by avocado extract. [Et-^{14}C]ETR (25 μM) was incubated with avocado extract in a reaction mixture similar

to that described above, except that ETR was used in place of MTR. The reaction mixture was incubated 5 hr at 25°, boiled to stop the reaction, and centrifuged. The supernatant was chromatographed on Whatman 1 paper with *n*-BuOH–HOAc–H_2O (4:1:1) as the developing solvent. Radioactive metabolites were detected with a Packard radioscanner. The metabolites were eluted from the chromatogram for further characterization.

Apple feeding experiment. Preclimacteric apples (*Malus sylvestris* cv Golden Delicious) grown locally were harvested and stored at 20° until used. C_2H_4 production was measured by GC. Plugs were cut from the fruit with a cork borer, rinsed in 3% (w/v) KCl, and blotted dry on paper towels. Plugs were vacuum infiltrated with 0.1 ml of a soln containing 0.1 mM [Me-^{14}C]MTR (11 μCi/umol), 1 mM L-methionine, and 3% (w/v) KCl. (The methionine was added to cause accumulation of [^{14}C]methionine, which we assayed.) After incubation at 25° for 5 hr, the plugs were homogenized in 80% EtOH and extracted overnight. The supernantant was concentrated under vacuum, and the cationic fraction containing the [^{14}C]methionine was adsorbed on Dowex 50 (H^+) and subsequently eluted with 2 M NH_4OH. Radioactivity was quantitated by scintillation counting. Identities of metabolities were verified by PC and electrophoresis.

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