

Intermediates in the Recycling of 5-Methylthioribose to Methionine in Fruits¹

Received for publication March 8, 1983 and in revised form May 23, 1983

MOSBAH M. KUSHAD, DARYL G. RICHARDSON, AND ADOLPH J. FERRO

Department of Horticulture (M. M. K., D. G. R.) and Department of Microbiology (A. J. F.), Oregon State University, Corvallis, Oregon 97331-3804

ABSTRACT

The recycling of 5-methylthioribose (MTR) to methionine in avocado (*Persea americana* Mill, cv Hass) and tomato (*Lycopersicon esculentum* Mill, cv unknown) was examined. [¹⁴CH₃]MTR was not metabolized in cell free extract from avocado fruit. Either [¹⁴CH₃]MTR plus ATP or [¹⁴CH₃]5-methylthioribose-1-phosphate (MTR-1-P) alone, however, were metabolized to two new products by these extracts. MTR kinase activity has previously been detected in these fruit extracts. These data indicate that MTR must be converted to MTR-1-P by MTR kinase before further metabolism can occur. The products of MTR-1-P metabolism were tentatively identified as α -keto- γ -methylthiobutyric acid (α -KMB) and α -hydroxy- γ -methylthiobutyric acid (α -HMB) by chromatography in several solvent systems. [³⁵S] α -KMB was found to be further metabolized to methionine and α -HMB by these extracts, whereas α -HMB was not. However, α -HMB inhibited the conversion of α -KMB to methionine. Both [U-¹⁴C] α -KMB and [U-¹⁴C]methionine, but not [U-¹⁴C] α -HMB, were converted to ethylene in tomato pericarp tissue. In addition, aminoethoxyvinylglycine inhibited the conversion of α -KMB to ethylene. These data suggest that the recycling pathway leading to ethylene is MTR \rightarrow MTR-1-P \rightarrow α -KMB \rightarrow methionine \rightarrow S-adenosylmethionine \rightarrow 1-aminocyclopropane-1-carboxylic acid \rightarrow ethylene.

Methionine serves as a precursor of ethylene (15), SAM² and ACC being intermediates (6, 22). ACC synthase catalyzes the conversion of SAM to ACC and MTA (2). This reaction has been reported to be the rate-limiting step of ethylene biosynthesis in both vegetative and fruit tissues (6, 7, 12, 13, 22, 24).

In plants, MTA is degraded by a nucleosidic cleavage via MTA nucleosidase to MTR and adenine (11). Various moieties of MTA have been shown to be recycled into methionine in higher plant tissue. Adams and Yang (1) found that the methylthio group of MTA was effectively recycled as a unit into methionine. Yung *et al.* (25) subsequently showed that, in apple tissue, the ribose portion of MTR was also incorporated into methionine. Recently, Wang *et al.* (20) demonstrated in tomato tissue that the ribose moiety of MTA was metabolized to form the four-

carbon unit (2-aminobutyrate) of methionine and suggested that ethylene was formed from the ribose portion of MTA via MTR, methionine, and ACC.

Kushad *et al.* (14) found that cell-free extracts from several fruit tissues contain MTR kinase activity. This enzyme catalyzes the ATP-dependent phosphorylation of MTR to MTR-1-P (9). The presence of this enzyme suggests that MTR-1-P also may be an essential intermediate in the recycling of MTA to methionine. The present study was undertaken to examine the fate of MTR-1-P.

MATERIALS AND METHODS

Plant Material. Unripe mature avocado fruits (*Persea americana* Mill, cv Hass) and tomato (*Lycopersicon esculentum* Mill, cv unknown) in the breaker stage were purchased from a local store.

Chemicals. [³⁵S]Methionine (1,000 Ci/mmol), [U-¹⁴C]methionine (285 mCi/mmol), and [¹⁴CH₃]SAM (58 mCi/mmol) were purchased from Amersham. [¹⁴CH₃]MTA was prepared from [¹⁴CH₃]SAM (16), [¹⁴CH₃]MTR was obtained by acid hydrolysis of the corresponding [¹⁴CH₃]MTA (16), and [¹⁴CH₃]MTR-1-P was prepared by incubating [¹⁴CH₃]MTA with partially purified calf liver MTA phosphorylase as described by Ferro *et al.* (10). [³⁵S] α -KMB and [U-¹⁴C] α -KMB were obtained according to a procedure described by Dixon (8) with α -KMB extracted from the reaction with diethyl ether. [U-¹⁴C] α -HMB was prepared from [U-¹⁴C] α -KMB using NaBH₄. [U-¹⁴C] α -KMB (2 μ Ci) was adjusted to pH 3 with 1 N acetic acid and was added to 2 mg NaBH₄ in 95% ethanol in a ratio of 1:1 (v/v). The mixture was incubated 10 min at room temperature and an aliquot was chromatographed in solvent system I to separate the product from the substrate. α -KMB and α -HMB were purchased from Sigma Chemical Co. AVG was a gift from Hoffman LaRoche.

Cell-Free Extract and Enzyme Assay. Fruit extracts were prepared by homogenization of the tissue in 0.2 M K-phosphate (pH 7.2), 3 mM DTT, 1% Triton-X-100 (v/v), and 3% PVP (w/v). The homogenate was passed through four layers of cheese cloth, and centrifuged for 20 min at 20,000g. The supernatant fluid was used as the cell free extract. MTR kinase activity was determined according to the procedure of Ferro *et al.* (9).

Chromatographic Procedures. Radioactive metabolites were separated and identified by four chromatographic systems. Descending paper chromatography was carried out in butanol:acetic acid:acetone:water (70:20:70:40, v/v) (solvent system I). TLC was performed on silica gels, F60 (EM Laboratories), with isobutanol:acetic acid:water (68:10:2, v/v) (solvent system II), ethyl acetate:ethanol:water (9:1:2, v/v) (solvent system III), and isobutyl alcohol:ethanol:water (68:20:10, v/v) (solvent system IV). All chromatograms were scanned with a Packard radiochromatogram scanner. For quantification of radioactivity, paper chromatograms were cut into 1-cm strips which were counted in a

¹ Supported by funds from the Oregon State University Agricultural Research Foundation. Oregon State University Agricultural Experiment Station Technical Paper 6739.

² Abbreviations: SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; MTA, 5'-methylthioadenosine; MTR, 5-methylthioribose; MTR-1-P, 5-methylthioribose-1-phosphate; α -KMB, α -keto- γ -methylthiobutyric acid; α -HMB, α -hydroxy- γ -methylthiobutyric acid; AVG, aminoethoxyvinylglycine (2-amino-4-[2'-aminoethoxy]trans-3-butenic acid).

Beckman liquid scintillation counter.

Feeding Experiments. Plugs from breaker ripening stage of tomato pericarp tissue (1 cm in diameter) were excised with a cork borer and immediately rinsed in 2% KCl and blotted dry. Radioactive substrates were introduced into the plugs by the vacuum injection technique previously described (1). Two replicates of three plugs each were used for all experiments. The plugs were sealed in a 25-ml Erlenmeyer flask with a plastic bucket hanging through a rubber serum cap. Radioactive ethylene was adsorbed in 0.2 ml of 0.25 M $\text{Hg}(\text{ClO}_4)_2$ injected into the bucket. After 6 h, the buckets were placed in scintillation vials containing scintillation fluid and the radioactivity determined. Nonradioactive ethylene was measured by GC.

RESULTS

MTR Kinase Activity in Avocado Extracts. MTR kinase activity was determined from three morphological regions of the avocado fruit: the peel, outer exocarp, and inner exocarp. The corresponding enzyme activities are presented in Table I. The specific activity of the peel was lowest, being one-third that of the outer exocarp and one-half that of the inner exocarp. In subsequent experiments, therefore, the fruit was peeled and all

Table I. MTR Kinase Activity in Avocado Extract

Tissue	Enzyme Activity
	<i>pmol product formed/mg protein · min</i>
Peel	508
Outer exocarp	1,805
Inner exocarp	1,080

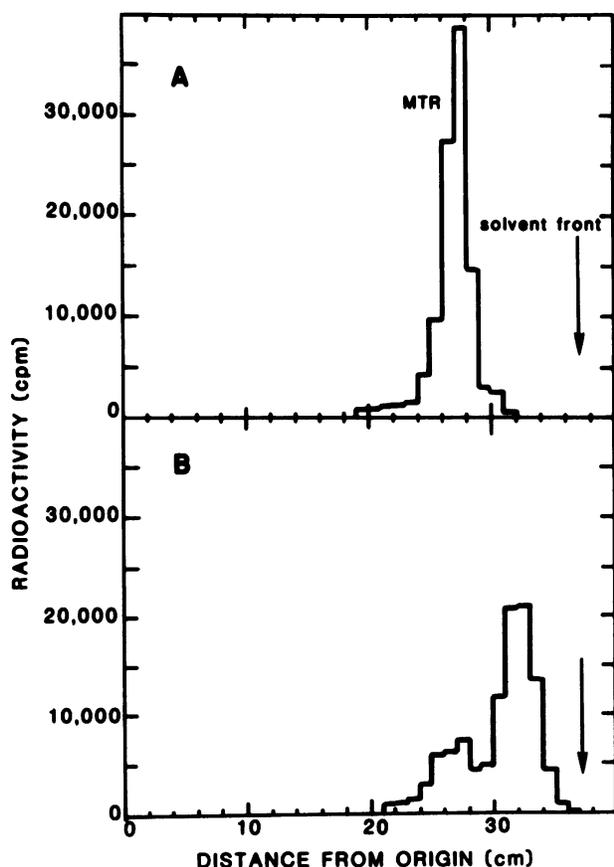


FIG. 1. Radiochromatogram scans in solvent system I of cell free extracts of avocado fruit containing (A) $[^{14}\text{CH}_3]\text{MTR}$ or (B) $[^{14}\text{CH}_3]\text{MTR}$ plus ATP.

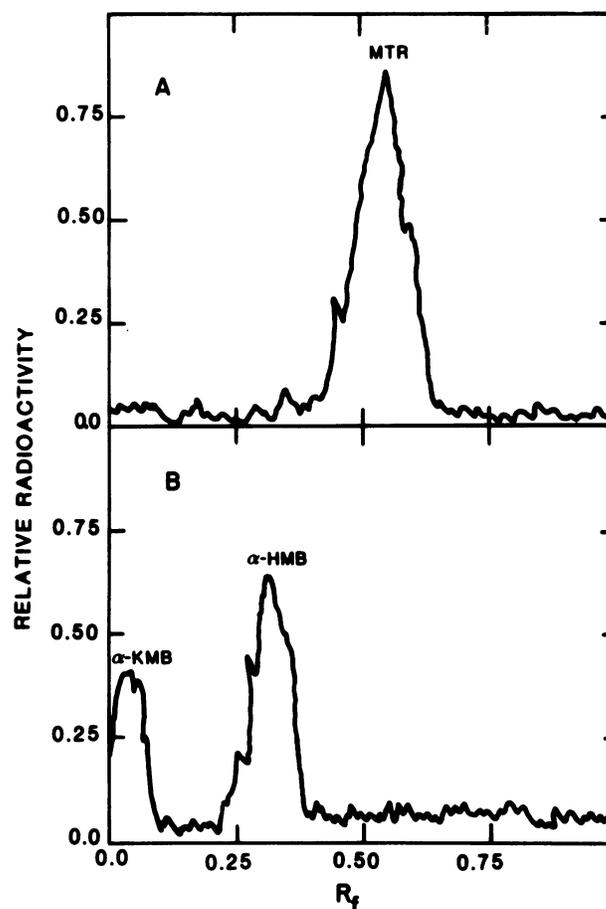


FIG. 2. Radiochromatogram scans in solvent system II of cell free extracts of avocado fruit containing (A) $[^{14}\text{CH}_3]\text{MTR}$ or (B) $[^{14}\text{CH}_3]\text{MTR}$ plus ATP.

Table II. R_f Values

Compound	Solvent System			
	I	II	III	IV
MTR	0.70	0.55	0.67	0.57
MTR-1-P	0.27			
α -KMB	0.70	0.05	0.43	0.37
α -HMB	0.85	0.30	0.43	0.56
Methionine	0.49			0.11

of the outer and some of the inner exocarp utilized.

Metabolism of MTR in Avocado Extract. Inasmuch as MTR has been shown to be metabolized when fed to tomato pericarp tissue slices (20), the ability of avocado cell-free extracts to utilize MTR as a substrate was explored. Radiochromatogram scans revealed that $[^{14}\text{CH}_3]\text{MTR}$ incubated with or without avocado extract for 8 h remained unaltered (Fig. 1A). The addition of 5 mM ATP to the reaction mixture, however, resulted in the formation of a new radioactive peak ($R_f = 0.85$, solvent system I) and a smaller peak at $R_f = 0.70$, which presumably was unreacted MTR (Fig. 1B). Chromatography of this same assay mixture in solvent system II (Fig. 2), however, demonstrated that the substrate $[^{14}\text{CH}_3]\text{MTR}$ ($R_f = 0.55$) was completely degraded and that two new peaks at R_f 0.05 and 0.30 were formed. Chromatography in two other solvent systems (Table II) confirmed that two products were formed and that these products co-chromatographed with α -KMB and α -HMB. The inability to detect both products in solvent system I was due to the overlapping migration of MTR and α -KMB in this system. When α -

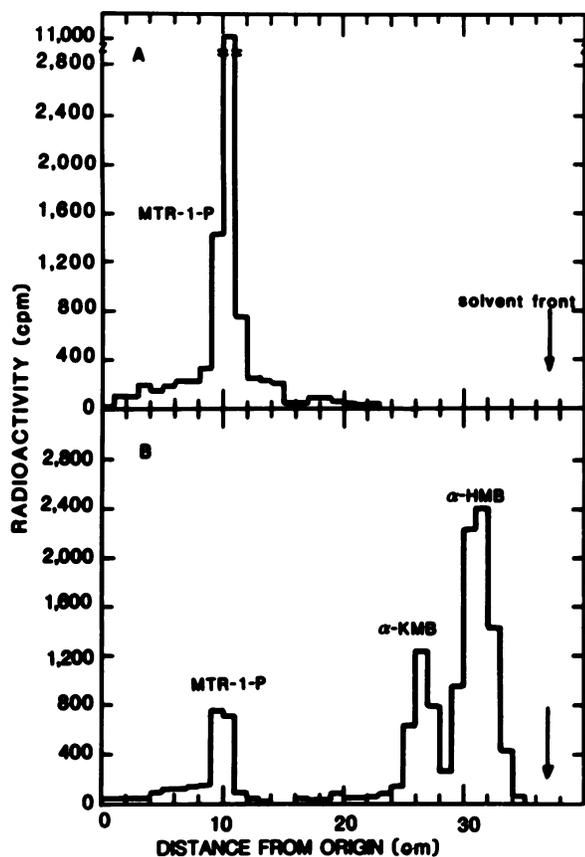


FIG. 3. Radiochromatogram scans of cell free extracts of avocado fruit containing (A) no extract plus $[^{14}\text{CH}_3]\text{MTR-1-P}$ and (B) extract plus $[^{14}\text{CH}_3]\text{MTR-1-P}$.

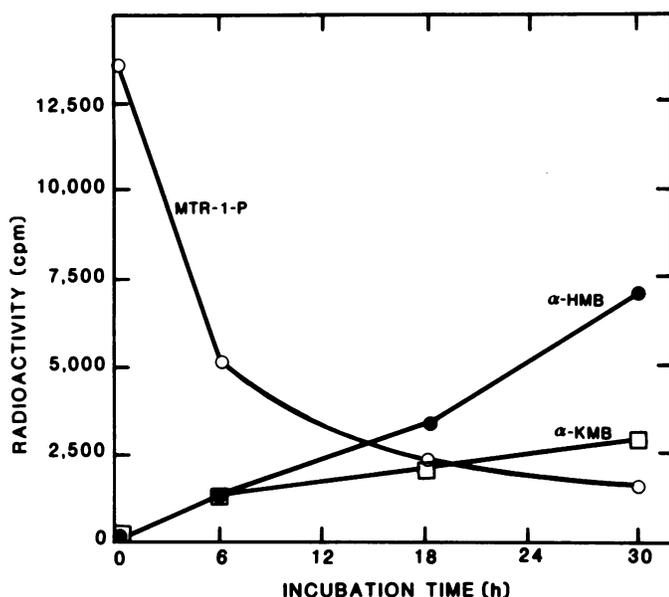


FIG. 4. Kinetics of product formation from MTR-1-P. (○), MTR-1-P; (□), $\alpha\text{-KMB}$; (●), $\alpha\text{-HMB}$.

HMB and $\alpha\text{-KMB}$ were eluted and rechromatographed in the other three systems, they co-migrated with authentic samples of each of these compounds.

Metabolism of MTR-1-P in Avocado Extract. Because MTR was metabolized only when ATP was present and since MTR kinase activity has been found in these extracts, the ability of

Table III. Methionine Formation in Avocado Extract from $\alpha\text{-KMB}$ and $\alpha\text{-HMB}$

Addition	Recovery in		
	Methionine	$\alpha\text{-KMB}$	$\alpha\text{-HMB}$
	%		
$[^{35}\text{S}]\alpha\text{-KMB}$	63.4	4.3	32.3
$[^{35}\text{S}]\alpha\text{-HMB}$	0	0	100
$[^{35}\text{S}]\alpha\text{-KMB} + 2.5 \text{ mM } \alpha\text{-HMB}$	46.9	14.7	38.4
$[^{35}\text{S}]\alpha\text{-KMB} + 2.5 \text{ mM methionine}$	63.0	4.6	32.4

Table IV. Synthesis of Ethylene from $\alpha\text{-KMB}$, $\alpha\text{-HMB}$, and Methionine in Plugs of Tomato Pericarp Tissue

Substrate ^a	C ₂ H ₄ Produced
	nCi g ⁻¹ h ⁻¹
$[\text{U-}^{14}\text{C}]\alpha\text{-KMB}$	0.294
$[\text{U-}^{14}\text{C}]\text{Methionine}$	0.671
$[\text{U-}^{14}\text{C}]\alpha\text{-HMB}$	0
$[\text{U-}^{14}\text{C}]\alpha\text{-KMB} + 2.5 \text{ mM } \alpha\text{-HMB}$	0.133

^a All radioactive compounds were added at 1.29 μCi .

Table V. Effect of AVG on Ethylene Production in Plugs of Tomato Pericarp Tissue

Addition	Ethylene Produced
	% of control
None	100
1.0 mM $\alpha\text{-KMB}$	125
2.0 mM $\alpha\text{-KMB}$	148
1.0 mM $\alpha\text{-KMB} + 0.1 \text{ mM AVG}$	6
1.0 mM $\alpha\text{-KMB} + 1.0 \text{ mM } \alpha\text{-HMB}$	106

avocado extract to metabolize MTR-1-P was examined (Fig. 3). Incubation of $[^{14}\text{CH}_3]\text{MTR-1-P}$ with avocado extract (in the absence of ATP) for 30 h yielded two radioactive products, $\alpha\text{-KMB}$ and $\alpha\text{-HMB}$, as measured by scanning chromatograms developed in solvent systems I and II. MTR was not formed under these conditions. The metabolism of MTR-1-P to $\alpha\text{-KMB}$ and $\alpha\text{-HMB}$ by this extract, therefore, does not require the presence of ATP, indicating that MTR must first be activated to MTR-1-P via MTR kinase before it can be further metabolized.

To examine the kinetics of product formation from MTR-1-P, the reaction was stopped 6, 18, and 30 h after addition of the substrate and analyzed for MTR-1-P, $\alpha\text{-KMB}$, and $\alpha\text{-HMB}$ as described in "Materials and Methods" (Fig. 4). MTR-1-P was metabolized to both $\alpha\text{-KMB}$ and $\alpha\text{-HMB}$ at approximately equal rates during the first 6 h. Thereafter, however, the rate of $\alpha\text{-HMB}$ accumulation exceeded that of $\alpha\text{-KMB}$ accumulation. By 30 h, only 11% of the MTR-1-P remained and of the two radioactive products formed, 70% was in the form of $\alpha\text{-HMB}$. That the increase in $\alpha\text{-HMB}$ plus $\alpha\text{-KMB}$ is greater than the decrease in MTR-1-P between the 18 to 30 h of incubation may indicate the presence of an intermediate compound synthesized from MTR-1-P during the first 18 h and converted to $\alpha\text{-KMB}$ and $\alpha\text{-HMB}$ during the latter stages of the incubation period.

Metabolism of $\alpha\text{-KMB}$ and $\alpha\text{-HMB}$ by Avocado Extract. Avocado extracts were incubated for 3 h with 1 mM asparagine and either $[^{35}\text{S}]\alpha\text{-KMB}$ or $[^{35}\text{S}]\alpha\text{-HMB}$ and analyzed for $[^{35}\text{S}]\text{methionine}$, $[^{35}\text{S}]\alpha\text{-KMB}$, and $[^{35}\text{S}]\alpha\text{-HMB}$ (Table III). More than 95% of the $[^{35}\text{S}]\alpha\text{-KMB}$ was metabolized to methionine (63%) and $\alpha\text{-HMB}$ (32%), whereas $[^{35}\text{S}]\alpha\text{-HMB}$ incubated with extract and asparagine remained unaltered. The addition of 2.5 mM $\alpha\text{-HMB}$ to the $[^{35}\text{S}]\alpha\text{-KMB}$ reaction mixture resulted in a 26% decrease in the conversion of $\alpha\text{-KMB}$ to methionine and a concomitant increase in the level of $[^{35}\text{S}]\alpha\text{-KMB}$. The data

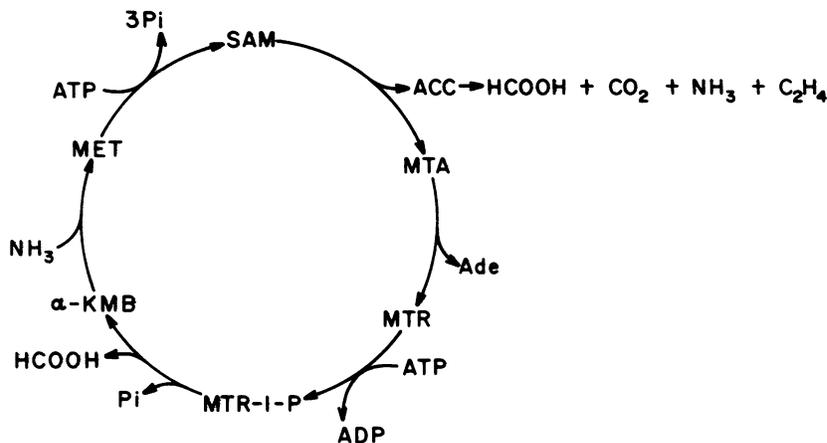


FIG. 5. Proposed recycling pathway from MTR to methionine.

suggest that α -KMB is a precursor of both methionine and α -HMB, but that α -HMB cannot be further metabolized under these conditions. In addition, α -HMB appears to slightly inhibit the conversion of α -KMB to methionine.

Ethylene Formation from α -KMB, α -HMB, and Methionine. The ability of tomato pericarp tissue to metabolize [U - 14 C] α -KMB, [U - 14 C]methionine, and [U - 14 C] α -HMB to ethylene was examined (Table IV). Of the three compounds tested, only α -KMB and methionine served as substrates for the synthesis of ethylene; α -HMB was completely inactive. α -HMB at 10 mM did, however, inhibit the conversion of α -KMB to ethylene by 55%. These data are consistent with the data obtained with cell-free extracts which indicate that α -KMB, but not α -HMB, is a precursor of methionine and that α -HMB inhibits the conversion of α -KMB to methionine which is a precursor of ethylene.

Effect of AVG on the Conversion of α -KMB to Ethylene. AVG is an inhibitor of the enzymic step leading from SAM to ACC and MTA. It has recently been shown that AVG inhibits the conversion of MTR to ethylene (20). If α -KMB is an intermediate between MTR and ACC, then AVG should also inhibit the conversion of α -KMB to ethylene. Therefore, tomato pericarp discs were infiltrated with α -KMB in the presence and absence of AVG, and the ethylene formed was measured (Table V). α -KMB at 1.0 and 2.0 mM increased ethylene production in a dose-dependent manner. The addition of 0.1 mM AVG almost completely inhibited ethylene production in these tissues in the presence of α -KMB, suggesting that α -KMB is metabolized to ethylene via SAM and ACC.

DISCUSSION

Wang *et al.* (20) have recently shown that ethylene is produced in tomato pericarp tissue from the ribose portion of MTA via MTR, methionine, and ACC. Collectively, the data presented here show that MTR-1-P and α -KMB are also intermediates in this recycling pathway. Based on these studies, this methionine salvage system and its relationship to ethylene biosynthesis is summarized in Figure 5.

The significance of MTR kinase activity in this recycling is illustrated by the inability of MTR to be further metabolized by cell extracts unless ATP is supplemented or until it is first activated to MTR-1-P. In mammalian tissue, MTA is directly converted to MTR-1-P by MTA phosphorylase (21) and, therefore, the action of MTR kinase is not required. MTR kinase activity has also been found in *Enterobacter aerogenes* (9), an organism shown to recycle MTA to methionine via a similar pathway (18). Conversely, we have not detected MTR kinase activity in extracts from *Escherichia coli*. Interestingly, *E. coli* has been shown to export MTR directly into the medium where

it accumulates (17). Thus, it would appear that the inability to form MTR-1-P via either MTA phosphorylase or the sequential action of MTA nucleosidase and MTR kinase renders the cell unable to further metabolize MTR.

α -KMB has been found to be an intermediate in the recycling pathway in animal (3, 4, 19) and bacterial (18) cells. It also has been shown to be converted to methionine in apple tissue (5). α -HMB has also been shown to be produced during the recycling of MTA in mammalian tissue (19). Our data, however, suggest that α -HMB is not a direct intermediate in this salvage pathway, but rather is synthesized from α -KMB in a side reaction not leading to ethylene synthesis. Although α -HMB does not appear to be an intermediate in the synthesis of ethylene from MTR-1-P, it does decrease ethylene synthesis, presumably by inhibiting the conversion of α -KMB to methionine.

According to the proposed recycling scheme, there is no new net synthesis of methionine. Rather, ethylene appears to be synthesized, after the first turn of the cycle, from the continual input of ATP. The balanced reaction of this scheme is:



Since ethylene has been shown to be synthesized from the ribose moiety of MTR (20) (presumably carbons 4' and 5'), which originated from the ribose moiety of ATP via SAM and MTA, the continuous generation of ATP could result in the continuous production of ethylene without the *de novo* synthesis of methionine in those systems in which this recycling pathway is operative.

LITERATURE CITED

- ADAMS DO, SF YANG 1977 Methionine metabolism in apple tissue: implication of S-adenosylmethionine as an intermediate in the conversion of methionine to ethylene. *Plant Physiol* 60: 892-896
- ADAMS DO, SF YANG 1979 Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proc Natl Acad Sci USA* 76: 170-174
- BACKLUND PS, RA SMITH 1981 Methionine synthesis from 5'-methylthioadenosine in rat liver. *J Biol Chem* 255: 1533-1535
- BACKLUND PS, CP CHANG, RA SMITH 1982 Identification of 2-keto-4-methylthiobutyrate as an intermediate compound in methionine synthesis from 5'-methylthioadenosine. *J Biol Chem* 257: 4196-4202
- BAUR AH, SF YANG, HK PRATT 1971 Ethylene biosynthesis in fruit tissues. *Plant Physiol* 47: 696-699
- BOLLER T, RC HERNER, H KENDE 1979 Assay for and enzymatic formation of an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid. *Planta* 145: 293-303
- BOLLER T, H KENDE 1980 Regulation of wound ethylene synthesis in plants. *Nature (Lond)* 286: 254-260
- DIXON JL, NJ BENEVENGA 1980 The carboxylation of α -keto- γ -methylthiobutyrate in rat liver mitochondria. *Biochem Biophys Res Commun* 97: 939-946
- FERRO AJ, A BARRETT, SK SHAPIRO 1978 5-Methylthioribose kinase, a new enzyme involved in the formation of methionine from 5-methylthioribose. *J Biol Chem* 253: 6021-6025

10. FERRO AJ, NC WROBEL, JA NICOLETTE 1979 5-Methylthioribose-1-phosphate: a product of partially purified rat liver 5'-methylthioadenosine phosphorylase activity. *Biochim Biophys Acta* 570: 65-73
11. GURANOWSKI AB, PK CHIANG, GL CANTONI 1981 5'-Methylthioadenosine nucleosidase, purification and characterization of the enzyme from *Lupinus luteus* seeds. *Eur J Biochem* 114: 293-299
12. JONES JF, H KENDE 1979 Auxin-induced ethylene biosynthesis in subapical stem sections of etiolated seedlings of *Pisum sativum* L. *Planta* 146: 649-656
13. KONZE JR, GMK KWIATKOWSKI 1981 Rapidly induced ethylene formation after wounding is controlled by the regulation of 1-aminocyclopropane-1-carboxylic acid synthesis. *Planta* 151: 327-330
14. KUSHAD MM, DG RICHARDSON, AJ FERRO 1982 5-Methylthioribose kinase activity in plants. *Biochem Biophys Res Commun* 108: 167-173
15. LIEBERMAN M 1979 Biosynthesis and action of ethylene. *Annu Rev Plant Physiol* 30: 533-591
16. SCHLENK F, DJ EHNINGER 1964 Observations on the metabolism of 5'-methylthioadenosine. *Arch Biochem Biophys* 106: 95-100
17. SCHROEDER HR, CJ BARNES, RC BOHINSKI, MF MALLETT 1973 Biological production of 5-methylthioribose. *Can J Microbiol* 19: 1347-1354
18. SHAPIRO SK, A BARRETT 1981 5-Methylthioribose as a precursor of the carbon chain of methionine. *Biochem Biophys Res Commun* 102: 302-307
19. TRACKMAN PC, RH ABELES 1981 The metabolism of 1-phospho-5-methylthioribose. *Biochem Biophys Res Commun* 103: 1238-1244
20. WANG SY, DO ADAMS, M LIEBERMAN 1982 Recycling of 5'-methylthioadenosine-ribose carbon atoms into methionine in tomato tissue in relation to ethylene production. *Plant Physiol* 70: 117-121
21. WILLIAMS-ASHMAN HG, I SEIDENFELD, P GALLETI 1982 Trends in the biochemical pharmacology of 5'-deoxy-5'-methylthioadenosine. *Biochem Pharmacol* 31: 277-288
22. YU YB, DO ADAMS, SF YANG 1979 1-Amino-cyclopropanecarboxylate synthase, a key enzyme in ethylene biosynthesis. *Arch Biochem Biophys* 198: 280-286
23. YU YB, DO ADAMS, SF YANG 1980 Inhibition of ethylene production by 2,4-dinitrophenol and high temperature. *Plant Physiol* 66: 286-290
24. YU YB, SF YANG 1980 Biosynthesis of wound ethylene. *Plant Physiol* 66: 281-285
25. YUNG KH, SF YANG, F SCHLENK 1982 Methionine synthesis from 5-methylthioribose in apple tissue. *Biochem Biophys Res Commun* 104: 771-777