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Methionine Metabolism in Apple Tissue

IMPLICATION OF S-ADENOSYLMETHIONINE AS AN INTERMEDIATE IN THE CONVERSION OF METHIONINE TO ETHYLENE[1]

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ABSTRACT

If S-adenosylmethionine (SAM) is the direct precursor of ethylene as previously proposed, it is expected that 5'-S-methyl-5'-thioadenosine (MTA) would be the fragment nucleoside. When [Me-^{14}C] or [^{35}S]methionine was fed to climacteric apple (*Malus sylvestris* Mill) tissue, radioactive 5-S-methyl-5-thioribose (MTR) was identified as the predominant product and MTA as a minor one. When the conversion of methionine into ethylene was inhibited by L-2-amino-4-(2'-aminoethoxy)-*trans*-3-butenoic acid, the conversion of [^{35}S] or [Me-^{14}C]methionine into MTR was similarly inhibited. Furthermore, the formation of MTA and MTR from [^{35}S]methionine was observed only in climacteric tissue which produced ethylene and actively converted methionine to ethylene but not in preclimacteric tissue which did not produce ethylene or convert methionine to ethylene. These observations suggest that the conversion of methionine into MTA and MTR is closely related to ethylene biosynthesis and provide indirect evidence that SAM may be an intermediate in the conversion of methionine to ethylene.

When [^{35}S]MTA was fed to climacteric or preclimacteric apple tissue, radioactivity was efficiently incorporated into MTR and methionine. However, when [^{35}S]MTR was administered, radioactivity was efficiently incorporated into methionine but not MTA. This suggests that the sulfur of MTA is incorporated into methionine via MTR. A dual label experiment with [^{35}S, Me-^{3}H]MTA indicates that the CH_3S group of MTA was transferred as a unit to form methionine.

A scheme is presented for the production of ethylene from methionine, the first step being the activation of methionine by ATP to give SAM. SAM is fragmented to give ethylene, MTA, and other products. MTA is then hydrolyzed to MTR which donates its methylthio group to a four-carbon acceptor to reform methionine.

Methionine has been shown to be a precursor of ethylene in a number of plant tissues and the characteristics of this conversion of methionine to ethylene *in vivo* have been the subject of a number of investigations (20). It has been shown that carbons 3 and 4 of the methionine molecule are incorporated into ethylene (5, 8). In apple tissue, Burg and Clagett (5) have shown that ethylene production is the main metabolic pathway of methionine and that the sulfur atom of methionine is not volatilized during ethylene production, but is retained in the tissue and metabolized.

Although apple is known to maintain a substantial rate of ethylene production for extended periods, its methionine level is quite low (2). This led Baur and Yang (2) to argue that in order for the apple to continue to produce ethylene, the sulfur of methionine must be recycled so as to provide an adequate supply of methionine for continued ethylene production.

2,4-Dinitrophenol, an uncoupler of oxidative phosphorylation, has been shown to inhibit the conversion of methionine to ethylene in apple tissue suggesting a requirement for ATP (4, 11). Murr and Yang (11) have since postulated that in the conversion of methionine to ethylene, SAM[2] is an intermediate which is degraded into CO_2, formic acid, ammonia, ethylene, and MTA. In order to test Murr and Yang's proposal, this investigation was carried out to determine: (*a*) whether MTA is the fragment nucleoside from methionine; (*b*) whether the formation of MTA is closely linked to ethylene production from methionine; and (*c*) whether MTA is readily recycled back to form methionine.

MATERIALS AND METHODS

Plant Material. Apples, *Malus sylvestris* Mill, var. Golden Delicious, were obtained from two sources. Preclimacteric fruits were obtained from a local grower. Fruits were harvested at the beginning of the commercial harvest and used as preclimacteric before they began to produce ethylene. Some of these fruits were stored at 25 C and subsequently used as climacteric fruit when they produced ethylene. Climacteric fruits were also purchased from a local market and stored at 20 C until used.

Chemicals. L-[^{35}S]Methionine, L-[methyl-^{14}C]methionine, and [methyl-^{3}H]SAM were purchased from Amersham/Searle. [^{35}S]SAM was prepared from [^{35}S]methionine using baker's yeast as described by Schlenk *et al.* (16). Unlabeled MTA was prepared from SAM by the method of Schlenk and Ehninger (15) and recrystalized from water. [Methyl-^{3}H]MTA and [^{35}S]MTA were similarly prepared from [methyl-^{3}H]SAM and [^{35}S]SAM, respectively. Labeled MTA was further purified before use by TLC on silica gel as described by Chu *et al.* (6) using chloroform-methanol-water (65:25:4, v/v) as a solvent system. [^{35}S]MTR was prepared by hydrolysis of [^{35}S]MTA in 0.01 N HCl and purified by ion exchange with Dowex 50-H$^+$ to remove adenine. AAB was a gift from J. P. Scannell of Hoffmann-La Roche Inc., Nutley, N.J.

Feeding Experiments and Characterization of Metabolites. Apple plugs 1 cm in diameter and 2 cm long were cut from whole apples with a cork bore and a scalpel. Plugs were quickly rinsed in 2% KCl (w/v) and blotted dry with a paper towel. The desired substrates in 2% KCl were introduced by a vacuum infiltration technique as previously described (1). After incubation, plugs were homogenized in 80% ethanol, centrifuged, and the pellet reextracted with 80% ethanol. The combined supernatants were concentrated *in vacuo* at 40 C and applied to

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[2] Abbreviations: SAM: S-adenosylmethionine; MTA: 5'-S-methyl-5'-thioadenosine or 5'-methylthioadenosine; MTR: 5-S-methyl-5-thioribose or 5-methylthioribose; AAB: 2-amino-4-(2'-aminoethoxy)-*trans*-3-butenoic acid; TMS: trimethylsilyl.

Whatman 3MM paper and chromatographed in 1-butanol-acetic acid-water (4:1:5, v/v). The chromatograms were scanned for radioactivity with a Packard radiochromatogram scanner. Radioactive spots were cut from the chromatograms and the material eluted from the paper with 50% ethanol and concentrated *in vacuo* at 40 C.

Sulfides were oxidized to the corresponding sulfoxides by treatment with 2% H_2O_2 at room temperature for 3 hr or by treating with 0.05% dimethylsulfoxide in 3 N HCl at 100 C for 5 min (10). Sulfoxides were reduced to the sulfide by treatment with 1% mercaptoethanol (v/v) at 100 C for 1 hr.

Radioactive metabolities were characterized by paper co-chromatography and by paper co-electrophoresis with authentic compounds in various buffer systems. For pH 2.2 and 10.8, 10% acetic acid and 0.02 M Na_2CO_3 were used, respectively. MTA and MTR were also subjected to paper electrophoresis in 0.05 M sodium borate buffer at pH 11. All electrophoresis was carried out on a Savant high voltage electrophoresis apparatus.

MTA on paper electrophoretograms of chromatograms was visualized by quenching under UV light (254 nm). Methionine and methionine sulfoxide were visualized with ninhydrin. MTR and its sulfoxide were visualized by spraying the paper with an aniline-phosphoric acid solution (7) and heating briefly at 105 C.

MTR obtained from the L-[methyl-^{14}C]methionine-feeding experiment was further characterized by gas co-chromatography with authentic MTR after silylation with bis-trimethylsilylacetamide. A 2% SE-30 column was used on a Loenco 160 series gas chromatograph equipped with a thermal conductivity detector. Column temperature was 160 C. Material emerging from the column was collected in several fractions by passing the effluent through Pasteur pipettes in which a piece of toluene-soaked cotton had been placed. The cotton was subsequently washed with scintillation fluid and counted in a Beckmann model 100 liquid scintillation counter.

Dual Label Experiment. For the dual label experiment [methyl-^{3}H]MTA and [^{35}S]MTA were mixed to give a ^{3}H/^{35}S ratio (cpm) of 1.5. This material was mixed with 0.1 μmol of unlabeled methionine to give a final volume of 100 μl in 2% KCl and was infiltrated into apple plugs. After incubation the plugs were homogenized and extracted as described above. After chromatography, MTA, MTR, and methionine spots were cut from the paper and further purified by electrophoresis in 10% acetic acid. Radioactivity in regions corresponding to methionine, MTA, and MTR were cut from the paper, eluted with 50% ethanol, concentrated, and the ^{3}H/^{35}S ratio determined by liquid scintillation counting.

RESULTS

Identification of MTA and MTR as Products of Methionine in Climacteric Fruit. Figure 1 illustrates the formation of MTA and MTR when climacteric apple plugs were fed with L-[methyl-^{14}C]methionine and incubated for 6 hr. Figure 1A shows plugs fed with labeled methionine alone and Figure 1B shows plugs fed with labeled methionine plus 0.25 μmol of MTA. Figure 1C shows plugs fed with labeled methionine in the presence of 0.25 μmol of MTA and 1.2 nmol of AAB. In this paper chromatography system, both MTA and MTR have the same mobility (R_F = 0.64) and are not separated from each other. Paper electrophoresis at pH 2.2 afforded complete resolution of MTA and MTR and revealed that the radioactive material with R_F of 0.64 that markedly increased when labeled methionine was administered with unlabeled MTA (Fig. 1B) was mostly MTR (about 90%), with only a small amount of radioactivity as MTA. Chemical identification of the radioactive material as MTR was carried out according to the following criteria: (*a*) the radioactive material co-chromatographed with authentic MTR in the butanol-acetic acid-water system; (*b*) upon oxidation with dimethylsulfoxide with authentic unlabeled

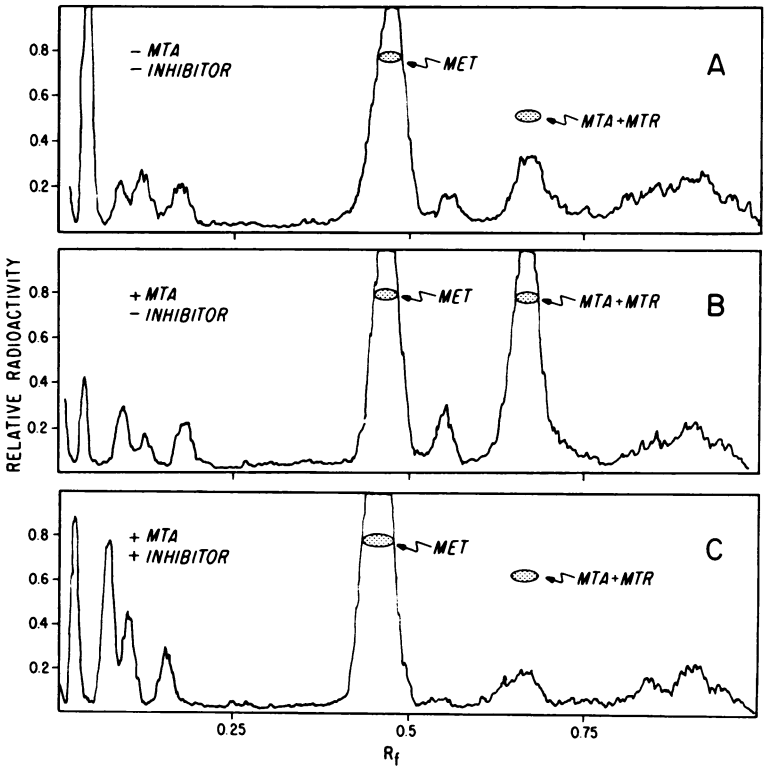

FIG. 1. Radiochromatogram scans of ethanol extracts prepared from climacteric apple plugs infiltrated with (A) 0.5 μCi of [methyl-^{14}C]methionine; (B) 0.5 μCi of [methyl-^{14}C]methionine plus 0.25 μmol of MTA; or (C) 0.5 μCi of [methyl-^{14}C]methionine plus 0.25 μmol of MTA plus 1.2 nmol of AAB. All plugs were incubated for 6 hr and the specific radioactivity of methionine was 49 μCi/μmol.

MTR, the radioactive material co-chromatographed on paper with the sulfoxide of MTR with an R_F of 0.34; (*c*) reduction of the sulfoxide with mercaptoethanol yielded a compound which was indistinguishable from MTR on paper chromatography indicating that the radioactive compound is a thioether; (*d*) the radioactive material did not move on paper electrophoresis at pH 2.2 and 10.8, however, when subjected to electrophoresis at pH 11 in borate buffer the compound co-migrated with authentic MTR toward the anode suggesting that it possessed a sugar moiety; (*e*) for further characterization a TMS derivative was prepared and subjected to gas chromatography along with an authentic TMS derivative of MTR; most of the radioactivity (75%) emerged from the column in the same fraction as the authentic MTR derivative. Similar results were obtained when the plugs of climacteric apple were infiltrated with L-[^{35}S]methionine as shown in Fig. 2. The radioactive material with R_F of 0.64 on paper chromatography was again characterized as MTR by electrophoresis at pH 2.2 and 10.8 and in borate buffer at pH 11. The material was likewise oxidizable to the sulfoxide as described above and again gave the original sulfide when reduced with mercaptoethanol. An increase in MTR is again seen when unlabeled MTA is infiltrated with labeled methionine and this accumulation is inhibited by AAB. AAB is a rhizobitoxine analog and an irreversible inhibitor of pyridoxal phosphate-dependent enzymes (14). It has been shown to inhibit ethylene evolution from apple tissue effectively and likewise inhibit the conversion of labeled methionine to ethylene (9). When labeled methionine was fed along with AAB and unlabeled MTA (Figs. 1C and 2C), ethylene evolution was greatly (70%) inhibited as compared to those plugs without AAB (Figs. 1B and 2B) and so was the formation of MTA and MTR. These data indicate that the formation of MTA and MTR from methionine is closely associated with the formation of ethylene from methionine.

Metabolism of L-[^{35}S]Methionine in Preclimacteric Apple. Figure 3 shows the scans of paper chromatograms of extracts prepared from preclimacteric apple plugs which were fed L-[^{35}S]methionine (Fig. 3A) or L-[^{35}S]methionine plus unlabeled MTA (Fig. 3B) and incubated for 6 hr. There was little conversion of methionine into MTR and MTA and the conversion was not enhanced by feeding unlabeled MTA. Thus, the ability of apple to convert methionine to MTA and MTR is parallel to its ability to convert methionine to ethylene.

Metabolism of [^{35}S]MTA in Climacteric Apple Plugs. Figure 4 shows scans of paper chromatograms of extracts prepared from climacteric apple plugs after feeding [^{35}S]MTA. The material at R_F 0.64 was shown to be mostly (78%) MTR by electrophoresis in pH 2.2 acetic acid buffer and in pH 11 borate buffer. The radioactive material at R_F 0.45 was identified as methionine by the following criteria: (*a*) the radioactivity co-chromatographed with authentic methionine on paper chromatography and co-migrated with authentic methionine on paper electrophoresis at pH 2.2 and 10.8; (*b*) after oxidation with dimethylsulfoxide in acid it co-chromatographed with methionine sulfoxide; and (*c*) upon reduction of the sulfoxide with mercaptoethanol it again co-chromatographed with authentic methionine. The radioactive material at R_F 0.34 is probably the sulfoxides of MTA and MTR because after reduction they

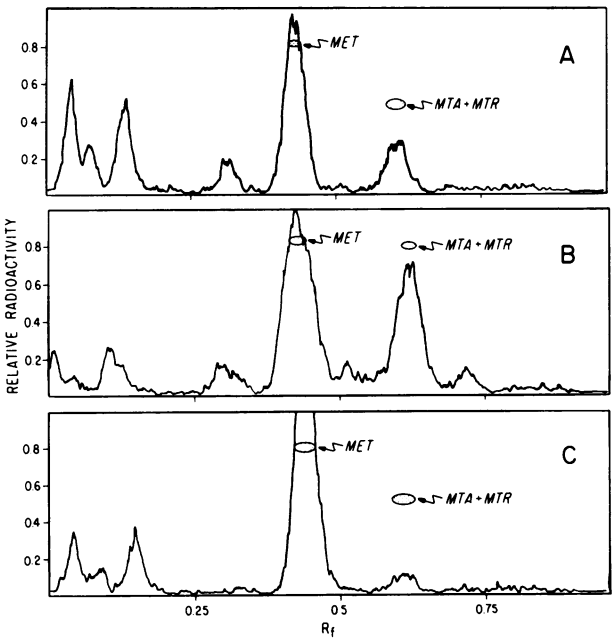

FIG. 2. Radiochromatogram scans of ethanol extracts prepared from climacteric apple plugs infiltrated with (A) 0.5 μCi of [^{35}S]methionine; (B) 0.5 μCi of [^{35}S]methionine plus 0.25 μmol of MTA; or (C) 0.5 μCi of [^{35}S]methionine plus 0.25 μmol of MTA plus 1.2 nmol of AAB. All plugs were incubated for 6 hr and the specific radioactivity of methionine was 265 μCi/μmol.

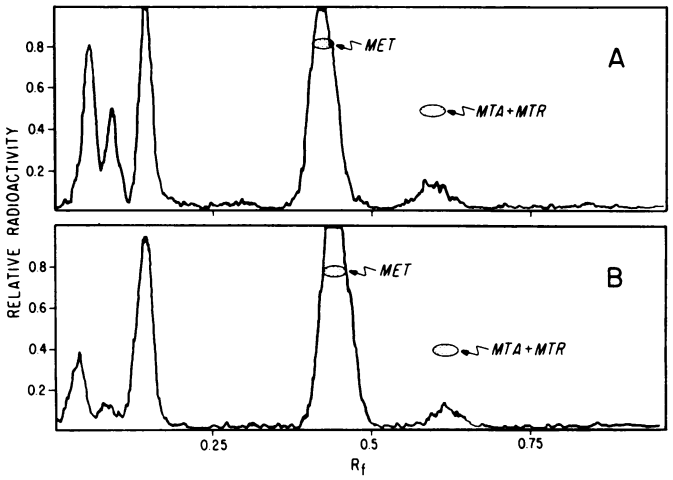

FIG. 3. Radiochromatogram scans of ethanol extracts prepared from preclimacteric apple plugs infiltrated with (A) 0.5 μCi of L-[^{35}S]methionine; (B) 0.5 μCi of L-[^{35}S]methionine plus 0.25 μmol of MTA. Plugs were incubated for 6 hr and the specific radioactivity of methionine was 212 μCi/μmol.

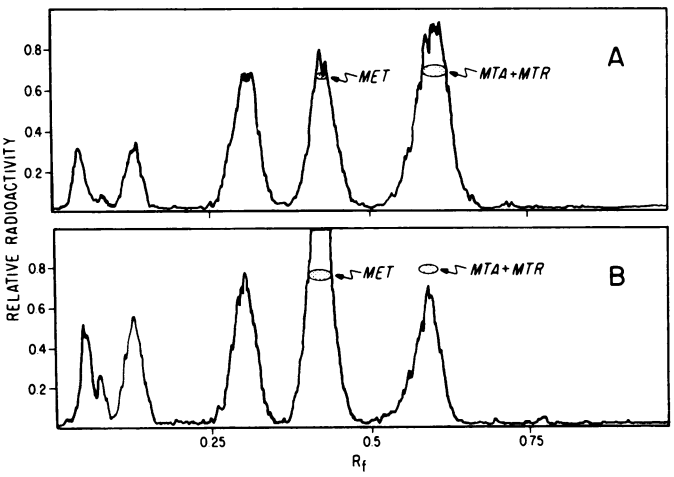

FIG. 4. Radiochromatogram scans of ethanol extracts prepared from climacteric apple plugs infiltrated with 0.5 μCi of [^{35}S]MTA and incubated for (A) 3 hr or (B) 6 hr. The specific radioactivity of MTA was 188 μCi/μmol.

yielded MTA and MTR which were characterized by paper co-electrophoresis in pH 2.2 buffer, pH 11 borate buffer, and co-chromatography with authentic material. The radioactive material at R_F 0.12 is believed to be methionine sulfoxide because it co-chromatographed with authentic methionine after reduction in 1% mercaptoethanol. The yield of methionine plus methionine sulfoxide at the end of 3- and 6-hr incubation was estimated to be 31% and 46% of the ethanol-extractable radioactivity, respectively.

Metabolism of [^{35}S]MTA in Preclimacteric Fruit. Paper chromatography of extracts prepared from preclimacteric apple plugs after feeding [^{35}S]MTA for 6 hr revealed that 27% of the MTA was converted to methionine. These data indicate that although preclimacteric tissue has little ability to convert methionine to MTA, the MTA once formed is actively converted to methionine.

Metabolism of [^{35}S]MTR in Climacteric Tissue. Figure 5 is a scan of a radiochromatogram of an extract from a climacteric apple plug infiltrated with [^{35}S]MTR and incubated for 6 hr. The peak at R_F 0.64 was found to be MTR, the starting material, and there was no MTA in this region as revealed by paper electrophoresis at pH 2.2. The radioactive compound at R_F 0.45 was shown to be methionine by co-electrophoresis with authentic methionine at pH 2.2 and by paper co-chromatography with authentic methionine.

Characteristics of Formation of Methionine from MTR. The present study established that both MTA and MTR were converted into methionine by apple plugs. When MTA was administered, both MTR and methionine were found but when MTR was administered methionine but not MTA was found. This observation suggests that MTA is converted to methionine via MTR. It has been shown that both [methyl-^{14}C]MTA and [^{35}S]MTA are converted to methionine. In order to determine whether the sulfur atom and the methyl groups are incorporated into methionine separately or as a unit, [^{35}S]MTA and [methyl-^{3}H]MTA were mixed and infiltrated into a climacteric apple plug along with unlabeled methionine. The inclusion of unlabeled methionine was intended to reduce the specific radioactivity of the methionine pool so that further metabolism of labeled methionine could be minimized. As shown in Table I, the ratio of ^{3}H/^{35}S in MTA recovered from the plug was very close to that of administered MTA. Although the ratio in MTR was slightly lower than that of MTA the ratio in methionine was essentially identical to that of MTR. This suggests that the methyl group and the sulfur atom of MTR are incorporated into methionine as a unit.

Table I. Recovery of radioactive MTA, MTR and methionine from apple plug after infiltration with [Me-^{14}C, ^{35}S]MTA

One climacteric apple plug was infiltrated with 0.1 ml of 2% KCl solution containing 0.1 µmol of methionine and 1.57 µCi of [Me-^{14}C, ^{35}S]MTA. The ratio of ^{3}H/^{35}S (in µCi) was 6.5. After incubation for 1.5 hr the plug was extracted and the metabolites were separated by paper chromatography followed by paper electrophoresis.

	MTA	MTR	Methionine
µCi	0.68	0.30	0.22
^{3}H/^{35}S	6.8	5.8	5.9

DISCUSSION

If SAM is an intermediate in the conversion of methionine to ethylene and is degraded in the manner proposed by Murr and Yang (11), it would be expected that: (*a*) MTA would be the fragment nucleoside; (*b*) the formation of MTA would be closely associated with ethylene formation; and (*c*) MTA must be cycled back to methionine to maintain ethylene evolution. In this investigation our experiments were designed to test these predictions.

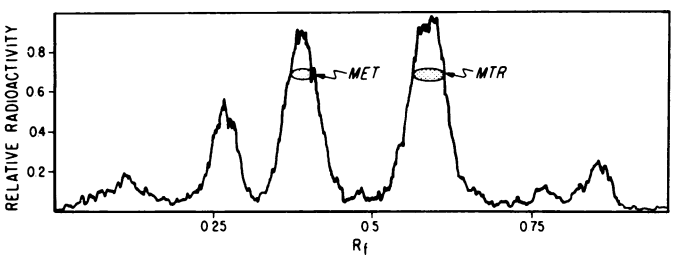

FIG. 5. Radiochromatogram scan of an ethanol extract prepared from a climacteric apple plug infiltrated with 0.5 µCi of [^{35}S]MTR and incubated for 6 hr. The specific radioactivity of MTR was 97 µCi/µmol.

When [methyl-^{14}C] or [^{35}S]methionine (Figs. 1 and 2) were administered to apple plugs with unlabeled MTA, radioactive MTR was identified as the predominant product while MTA was a minor one. These results are expected if MTA is hydrolyzed to MTR as rapidly as it is formed and MTR is further metabolized. As a result, little labeled MTA or MTR accumulates in the absence of exogenously supplied MTA (Figures 1A and 2A). When unlabeled MTA is administered it is rapidly converted to MTR and furthur metabolism of labeled MTR is greatly reduced due to the lower specific radioactivity of the MTR pool. Consequently, more radioactive MTR accumulates when unlabeled MTA is supplemented (Figures 1B and 2B) than when no unlabeled MTA is given (Figures 1A and 2A). A number of workers have reported the hydrolysis of MTA to MTR by enzymes from a wide variety of organisms including higher plants (3, 13, 18). We have also observed the enzymic hydrolysis of MTA to MTR catalyzed by apple extract (data not shown).

Although we identified MTA and MTR as metabolites of methionine, these reactions could well have been reactions of methionine unrelated to ethylene biosynthesis. It was therefore important to establish that the conversions of methionine to MTA and to ethylene are closely interrelated. In order to test this we compared the ability of preclimacteric and climacteric tissue to convert methionine to MTA and MTR and compared the conversion of methionine to MTA and MTR in climacteric tissue in the presence and absence of AAB, a known inhibitor of ethylene production (9). By feeding [U-^{14}C]methionine, we verified that preclimacteric apple tissue which produced little ethylene was unable to convert labeled methionine into ethylene; climacteric tissue, however, produced large amounts of ethylene and was capable of converting methionine to ethylene (data not shown). Thus, the ability of preclimacteric and climacteric apple tissue to convert methionine to MTA and MTR (Figs. 1-3) is correlated with their ability to convert methionine to ethylene. Further evidence supporting the view that the conversion of methionine to MTR is related to the conversion of methionine to ethylene is shown in Figures 1 and 2. AAB inhibited the conversion of methionine to ethylene as well as the conversion of methionine to MTA and MTR. Since the only known pathway by which MTR is synthesized in biological systems is via MTA from S-adenosylmethionine, our data support the suggestions of Burg (4) and Murr and Yang (11) that SAM is an intermediate in the conversion of methionine to ethylene.

Baur and Yang (2) have argued that the sulfur of methionine must be recycled for the continuous production of ethylene by apple tissue. If this is the case, then the sulfur atom of MTA must be reincorporated into methionine. Murr and Yang (12) showed that the methyl group of MTA was incorporated into methionine, but presented no evidence that the sulfur was likewise transferred. Figure 4 shows that the sulfur atom of MTA is also incorporated into methionine. Since MTA was

rapidly converted into MTR which was in turn readily incorporated into methionine, it is suggested that MTA is hydrolyzed to MTR prior to incorporation of the methyl group and sulfur atom into methionine. This does not, however, answer the question posed previously (12) as to whether the methylthio group of MTA is transferred as a unit to form methionine or whether the methyl group is transferred to methionine independent of the sulfur atom. The results of the present dual label experiment suggest that the methylthio group is transferred to methionine as a unit since no change was found in the $^3H/^{35}S$ ratio when MTR was converted to methionine by apple plugs (Table I). It is pertinent to note the work of Schwartz and Shapiro (17), who found that in *Aerobacter aerogenes* the sulfur and methyl groups of MTA are incorporated into protein methionine. From tracer studies they concluded that methionine was synthesized by methylthiolation involving MTA as a methylthio donor. Recently Sugimoto *et al.* (19) have shown that the metabolic fate of the methylthio group of MTA cannot be distinguished from that of L-methionine in a protozoan, *Ochromonas malhamensis*. They further suggested that MTA is converted to methionine via MTR.

Based on the above results a pathway of methionine metabolism in relation to ethylene biosynthesis in apple tissue is summarized in Figure 6. Methionine is first activated at the sulfur atom to give SAM which is fragmented subsequently to CO_2 (C-1), formic acid (from C-2), ammonia (from the amino group), ethylene (from C-3,4), and MTA. This is in keeping with the earlier proposal of Murr and Yang (12). The methylthio group of MTR is transferred as a unit to some four-carbon acceptor to form methionine. Baur and Yang (22) have shown that homoserine is readily incorporated into ethylene. Thus, homoserine or its derivative appears to be a logical acceptor for the methylthio group of MTR. The scheme indicates that the methylthio group of methionine is retained and recycled during the continuous production of ethylene and accounts for the observation of Burg and Clagett (5) that no volatile sulfur is lost from apple tissue during ethylene production.

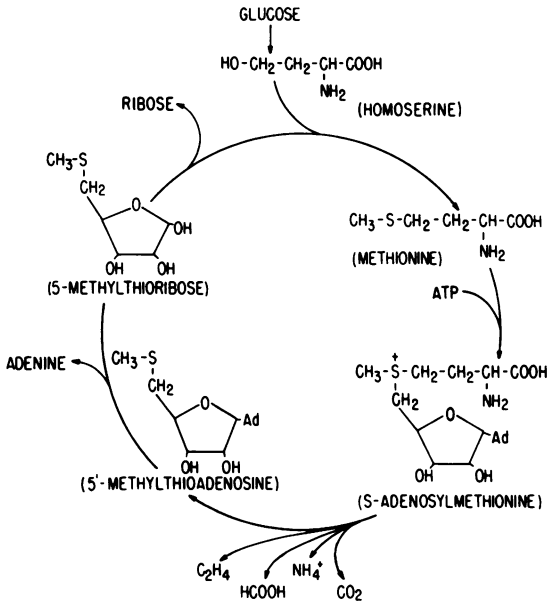

FIG. 6. A proposed scheme of methionine metabolism in relation to ethylene biosynthesis in apple.

It should be noted that the scheme depicted in Figure 6 is at variation with that presented previously by Baur and Yang (2). Their methionine-sulfur cycle was based on the assumption that the fragment produced from the methylthio group of methionine is methanethiol. Since methanethiol has never been identified as a product of methionine during ethylene biosynthesis (2) their scheme lacks experimental support.

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