AN ABSTRACT OF THE THESIS OF

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Title: Ethylene Biosynthesis: New Intermediates in the Regeneration of Methionine in Fruits

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The recycling of 5-methylthioribose (MTR) to methionine in cell free extracts from fruit tissues was examined. Addition of [$^{14}CH_3$]MTR alone to the tissue extracts was not metabolized while [$^{14}CH_3$]MTR plus ATP or [$^{14}CH_3$]5-methylthioribose-1-phosphate (MTR-1-P) were metabolized to two new products by these extracts. The data indicated that MTR was converted to MTR-1-P by MTR kinase (a previously unidentified plant enzyme) before further metabolism could 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. [^{35}S]α-KMB was found to be further metabolized to methionine and to α-HMB by these extracts, whereas α-HMB was not metabolized. However, α-HMB inhibited the conversion of α-KMB to methionine and ethylene. Both [U-^{14}C]α-KMB and [U-^{14}C]methionine, but not [U-^{14}C]α-HMB, were converted to ethylene in tomato pericarp tissue plugs. Aminoethoxyvinylglycine inhibited the conversion of α-KMB to ethylene. Thus the recycling pathway

leading to ethylene is MTR → MTR-1-P → α-KMB → methionine → S-adenosylmethionine → 1-aminocyclopropane-1-carboxylic acid → ethylene.

5'-Methylthioadenosine (MTA) nucleosidase and 5-methylthioribose (MTR) kinase activities were measured in crude extracts of developing tomato fruits (Lycopersicon esculentum Mill cv Rutgers). The highest activity of MTA nucleosidase (1,250 pmol/mg protein/min) was observed in small green fruits, then the activity decreased during ripening and was only 6.5% in the over-ripe stage. MTR kinase activity, conversely, was low at the small green stage and increased thereafter until it reached peak activity (700 pmol/mg protein/min) at the breaker stage then sharply declined at latter stages. 1-Aminocyclopropane-1-carboxylic acid (ACC) levels peaked at the red stage and ethylene reached its highest level at the light-red stage. Several analogs of MTA and MTR were tested as both enzyme and ethylene inhibitors. 5'-chloroformycin showed the highest inhibition (88%) of MTA nucleosidase and iBRT showed the highest inhibition (59%) of MTR kinase activity.

Ethylene Biosynthesis: New Intermediates in the Regeneration of Methionine in Fruits

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ETHYLENE BIOSYNTHESIS: NEW INTERMEDIATES IN THE REGENERATION OF METHIONINE IN FRUITS

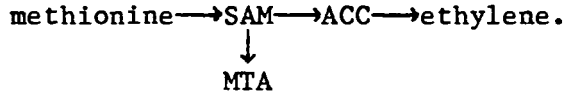
INTRODUCTION

Ethylene is a natural plant hormone recognized to regulate many facets of plant growth, development, and senescence. Methionine, the natural precursor of ethylene in all plant tissues (89,91,150), has been reported to exist in small quantity even in tissues that are known to produce significant amounts of ethylene (15). This observation prompted plant physiologists to examine the fate of methionine during ethylene biosynthesis.

Burg et al. (30) and Murr et al. (110,111) observed that inhibitors of the oxidative phosphorylation inhibited the conversion of methionine to ethylene. They suggested that a high energy requiring step existed between methionine and ethylene and they proposed S-adenosylmethionine (SAM) as an intermediate between methionine and ethylene. In order to test this proposal Adams et al. (6) incubated climacteric apple fruit plugs with [^{35}S]methionine. Paper radiochromatogram scans of the ethanol extracts (developed in butanol:acetone:water 4:1:5 v/v) of the apple plugs showed the presence of 5'-methylthioadenosine (MTA) (R_f 0.72), 5-methylthioribose (MTR) (R_f 0.72) and several other unidentified metabolites (R_f 0.10, 0.20, 0.23, and 0.85). These results presented an indirect evidence that SAM is an intermediate between methionine and ethylene. When apple plugs were incubated with [^{35}S]MTA the radioactivity was efficiently incorporated into

MTR and methionine. However, when [^{35}S]MTR was administered to the tissue, the radioactivity was found in methionine and other unidentified products. These results suggested that the sulfur of MTA is incorporated into methionine via MTR.

Adams et al. (7) and Lürssen et al. (94,95) simultaneously showed that 1-aminocyclopropane-1-carboxylic acid (ACC) is an intermediate step in the conversion of SAM to ethylene. They proposed the following sequence of reactions for ethylene synthesis in plants:



5'-methylthioadenosine nucleosidase then catalyses the conversion of MTA to 5-methylthioribose (MTR), which is recycled to methionine through a partially known mechanism.

Previously it has been reported that the methylthiol group of MTR accepts a 4-carbon unit from homoserine to form a new methionine molecule (53,87,153,156). However, recently it has been established that the ribose moiety of MTA and MTR formed the 4-carbon (2-aminobutyrate portion of methionine) acceptor (146,163).

Ferro et al. (48) examined the fate of MTR in Enterobacter aerogenes. They purified an enzyme, 5-methylthioribose kinase, that catalyzes an ATP dependent phosphorylation of MTR to 5-methylthioribose-1-phosphate and proposed that this enzyme may be a primary enzyme involved in the recycling of MTR to methionine. The

metabolism of MTR-1-P examined in rat liver homogenate (10,141), showed that MTR-1-P oxidatively converted to α-keto-γ-methylthiobutyric acid (α-KMB) (10,141) and α-hydroxy-γ-methylthiobutyric acid (α-HMB) (141). Backlund et al. (10) showed that α-KMB is an intermediate in the conversion of MTR-1-P to methionine. Trackman et al. (141) showed that α-KMB converted to α-HMB prior to its conversion to methionine.

The objectives of this thesis are to examine possible intermediates between MTR and methionine, specifically the involvement of 5-methylthioribose-1-phosphate (MTR-1-P), α-keto-γ-methylthiobutyric acid (α-KMB), and α-hydroxy-γ-methylthiobutyric acid (α-HMB) in the ethylene biosynthetic pathway, to examine the changes of MTA nucleosidase and MTR kinase specific activities during fruit development and ripening and the possible role of the methionine recycling pathway in regulating ethylene biosynthesis.

LITERATURE REVIEW

Observations on the effects of illuminating gas on plant growth are reported in the literature dated back to 1858 (45). Neljubow (115) observed that pea seedlings germinated in laboratory air contaminated with burning fumes grew horizontally, showed more radial expansion, and less vertical growth compared to uncontaminated air. Later studies by Sievers et al. (137) showed that green lemon fruits exposed to such fumes turned yellow. Ethylene, however, was not recognized as the active component of these fumes until the work of Denny (38) who observed that ethylene duplicated the effect of burning fumes in inducing lemon fruit degreening.

The first established report that ethylene is a natural plant product was presented by Elmer (44) who showed that apple fruits produced a volatile substance that inhibited the growth of potato sprouts, similar to an inhibition induced by burning fumes. Gane (52) and Kidd and West (72,73,74) also observed that the gaseous emanation from ripe apples induced the ripening of unripe apple fruits. Chemical proof that ethylene is produced during fruit ripening was first reported by Gane (51). Despite the dramatic physiological effects of ethylene, research in this field was very slow until the early 1960's.

Studies on ethylene production in plants have established that nearly all plant parts produce ethylene including leaves, stems, buds, flowers, fruits, and roots (18,31,69,86,99,103,116,151). The

amount produced, however, varies with the types of tissue and their stage of development (26,27,121). In etiolated pea stems, for example, the highest rate of ethylene production is associated with the meristem and node regions, while the internode tissue produced lower amounts of ethylene (43).

The discovery of ethylene as a natural plant product that can induce significant physiological changes in plant growth, development, and senescence (1), and that it can interact with other plant hormones to induce these changes at very low concentration, prompted plant physiologists to conclude that ethylene functions as a natural plant hormone (28,33). Postharvest physiologists have since recognized the essential role of ethylene as a fruit ripening hormone (1,119) and a general senescence promoter (127).

Physiological Role of Ethylene in Higher Plants

Ethylene appears to regulate many aspects of plant growth, development and senescence (1). The most important effect of ethylene in the postharvest physiology of fruits is the induction of ripening (1,27). Other important aspects of ethylene include: abscission and senescence of leaves, flowers, and fruits (66,79,138), inhibition of vertical growth (134,140), promotion of lateral expansion (54,117), promotion of seed germination (42,71), breaking of bud dormancy (98,109), promotion of adventitious root primordia (80,113), acceleration of chlorophyll degradation (40,147), increased synthesis of anthocyanin pigments in fruits

(58,120), change of sex expression in some cucurbitaceous plants (more female flowers) (123,125), epinasty of leaves (37), and induction of flowering in the bromeliad family (28,124).

Role of Ethylene in Fruit Ripening

Ripening is a complex physiological process, involving many biochemical and physical changes (59,122). Kidd and West (75) first suggested that ethylene is a ripening hormone. This concept was challenged by Biale (17) who found that some fruits (i.e. mangoes) ripened without increased ethylene synthesis. Biale, therefore, suggested that ethylene is a by-product, not an initiator of ripening. However, the development of gas chromatography as a tool for measuring very small quantities of ethylene (32,63,108), helped provide evidence that all fruits including mangoes produce ethylene during their ripening, and that different fruits vary in the amount of ethylene they produce, depending on the type of fruit and its stage of development (26,96).

Support of the concept that ethylene is a ripening hormone was presented by Burg *et al.* (27,28) who showed that fruit ripening was delayed by treatments that removed ethylene from the fruits. Storing fruits under vacuum, for example, reduced ethylene synthesis and delayed ripening significantly. Mapson *et al.* (102) demonstrated that storing bananas under low oxygen (below 10%) increased fruit shelf life. Similarly, chemicals that inhibit ethylene synthesis were found to be effective in delaying fruit

ripening (90,107,129). Physiological studies of rin and nor, non-ripening tomato mutants, showed that ethylene biosynthesis did not increase in these fruits during climacteric (41,61). These results, along with the observation that exogenously applied ethylene can induce apparently normal fruit ripening without changes in fruit quality, provided evidence that ethylene triggers the changes that take place during fruit ripening.

The mechanism by which ethylene initiates fruit ripening is still not clear (59). One of the earliest conceptions as to the mode of action of ethylene was presented by Blackman et al. (19) who suggested that the effect of ethylene is to lower the "organization resistance" (protoplasmic control of metabolic rate) of the protoplasm to allow contact of the reactants. Sacher (126) reported that in banana fruit slices the amount of cell free space significantly increased 44 h before the beginning of the climacteric period, suggesting a progressive increase in the proportion of cells which become completely permeable to solutes. He concluded, therefore, that the initiation of permeability marked the onset of ripening and that ethylene triggers these changes. Lyons et al. (97) suggested that an increase in the amount of ATP movement across the mitochondrial membrane following the increase in permeability would result in an increase of available energy to drive ripening.

Burg et al. (29) suggested that caution must be taken in interpreting results based on increased membrane leakage, since an

increase in the synthesis of sugars and protein during the ripening process may account for the increased solute movements. Mehard (106) showed that, in vitro, ethylene does not act by causing overt changes in membrane permeability. Hulme et al. (64) reported that increased membrane permeability was not a result of ethylene action.

Ribonucleic acid and protein synthesis were reported to be necessary for fruit ripening to occur (50,81,85,93). Hansen (59) noted that an important early event in fruit ripening is the synthesis of new enzymes. He observed that application of ethylene stimulated the synthesis of new proteins in immature pea fruits. Jones (67) reported that low concentrations of ethylene enhanced the secretion of α-amylase by barley aleurone. The relationship between ethylene and the cell wall degrading enzymes, for example, polygalacturonase and cellulase is not clear. Sawamura et al. (131) observed that ethylene triggered the synthesis of cell-wall degrading enzymes. Polygalacturonase and cellulase are reported to increase during the ripening process in tomato (24,118), pear and avocado fruits (105). Polygalacturonase activity was low in green tomato fruits, but increased as the fruits started to ripen (142). Rin tomato fruits lack of polygalacturonase activity has been attributed to its lack of ability to synthesize ethylene (24,118). Recently, however, it was found that endopolygalacturonase protein and endopolygalacturonase activity in ripening tomato fruits increased 2 to 3 days after the ripening

process has started (55), contradicting earlier findings that the increase in enzyme activity is an early event in fruit ripening and perhaps even the primary event (139).

The action of ethylene in inducing fruit ripening has also been associated with other plant hormones (8,144,145). The mechanism involved in these interrelationships is still not clear, but there is evidence to suggest a general antagonism between ethylene and abscisic acid on one hand and auxin, gibberellins, and cytokinins on the other.

Although the effects of ethylene on fruit ripening are still not clear, the changes that take place during ripening have been associated with the synthesis of ethylene in either a direct or indirect manner.

Ethylene Biosynthesis in Higher Plants

Several compounds have been suggested to be precursors of ethylene in higher plants. Among these are: ethanol, methanol, acetate, β-alanine, linolenic acid, IAA, propionic acid, glucose, acrylic acid, methional, keto acids, glutamic acid, glycerol, and methionine (33).

Methionine as Precursor of Ethylene

Methionine and the methionine recycling pathway is now accepted as the main, if not the only source of precursors of ethylene in higher plants (1,2,33). This is based on the observations that methionine is converted to ethylene in a model

system containing copper ions and ascorbic acid (88). Using radioactive methionine in the same model system Lieberman *et al.* (88) showed that ethylene is derived from carbons 3 and 4 of methionine. Methionine labelled at the 2 carbon emitted radioactive CO_2 but no ethylene. Similarly, methyl labelled methionine did not produce labelled ethylene. These results indicate that only carbon 3 and 4 of the methionine molecule are converted into ethylene.

Abeles *et al.* (4) showed that in crude extracts of pea seedlings, ethylene could be formed non-enzymatically in the presence of flavinmononucleotide (FMN). Methionine was determined to be the active substance in the pea extract. Yang *et al.* (157,158) observed that in the FMN model system ethylene is also derived from carbons 3 and 4 of methionine.

In vivo conversion of methionine to ethylene was first shown by Lieberman *et al.* (89) who observed that Rome Beauty apple plugs incubated with methionine showed an increase in ethylene synthesis. Similar experiments conducted with radioactive methionine demonstrated that ethylene is also derived from carbons 3 and 4 in a manner similar to that in the previously described (FMN and ascorbate) model systems. Methionine has served as an ethylene substrate in apple slices (89,128), cauliflower florets (101), banana (30), morning glory flowers and pea seedlings (60) and tomato (100).

In conclusion, the extent of ethylene derivation from methionine comes from the following observations: (a) In fruit tissues the L-form of methionine is converted to ethylene, not the D-form, suggesting a stereospecific enzymatic reaction (13,14,15,65). (b) IAA stimulates ethylene synthesis from methionine only in tissues that are capable of producing significant amounts of ethylene (30,128). (c) Treatments that increase ethylene production, such as copper sulfate, ozone, wounding, stress, etc. increase methionine conversion to ethylene (3,65). (d) When the concentration of methionine added to apple plugs is increased, the specific radioactivity of ethylene recovered approaches that of the added methionine (3,65), and (e) aminoethoxyvinylglycine (AVG), a known inhibitor of ethylene biosynthesis, inhibits the conversion of methionine to ethylene (114,156).

S-Adenosylmethionine as an Intermediate in Methionine Conversion to Ethylene

Murr et al. (111) reported that 2,4 dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, inhibited the conversion of radioactive methionine to ethylene by about 90% at a concentration of 100 uM. It was suggested that the inhibition occurred at a step subsequent to methionine. Burg (25) stated that the DNP inhibition of ethylene synthesis suggested the existence of a high energy step between methionine and ethylene. He proposed S-adenosylmethionine (SAM) as a possible intermediate. Murr et al. (111) confirmed the

previous finding that DNP inhibits ethylene production through an inhibition of an ATP requiring step. Other researchers have subsequently supported the hypothesis that SAM is an intermediate in ethylene biosynthesis (6,65,77,110).

Adams and Yang (6) presented the first data demonstrating that SAM is an intermediate in ethylene biosynthesis from methionine. They showed that incubating apple plugs with [^{35}S]methionine and [^{14}C]methionine resulted in the synthesis of ^{35}S and ^{14}C 5'-methylthioadenosine (MTA) and 5-methylthioribose (MTR). They also showed that preclimacteric apple tissues which have not started to produce ethylene did not synthesize MTA or MTR upon incubation with radioactive methionine. When AVG or DNP were added to the apple plugs in the presence of radioactive methionine, no radioactive MTA or MTR were formed. Instead a build up in a radioactive compound identified as SAM occurred, suggesting that SAM is an intermediate in the conversion of methionine to ethylene.

1-Aminocyclopropane-1-carboxylic Acid (ACC)

Methionine conversion to ethylene has been shown to be an aerobic process (15,25). Baur et al. (15) found that in apple tissues O_2 is absolutely required for methionine conversion to ethylene. They suggested a two-step mechanism in which the first step is an O_2 independent reaction followed by an O_2 requiring step.

Burg et al. (25) suggested that during the anaerobic conditions, an intermediate accumulated and subsequently was

converted to ethylene upon exposure of the tissue to oxygen. Adams et al. (7) later examined the metabolism of methionine in apple tissue in air and under nitrogen atmosphere. They observed that in air L-[^{14}C-U]methionine was efficiently converted to ethylene, while under nitrogen atmosphere ethylene synthesis ceased and a new radioactive compound appeared.

When the new radioactive compound was eluted from the chromatogram and administered to apple plugs in air it was efficiently converted to ethylene. However, when [$^{14}CH_3$]methionine and [^{35}S]methionine were used in similar experiments, no radioactive ethylene or the new radioactive compound were formed. Adams and Yang (7) identified this new compound as 1-aminocyclopropane-1-carboxylic acid (ACC). About the same time, but independently, another group found that ACC is an intermediate in ethylene biosynthesis from methionine in soybean leaf discs (94,95). Both groups postulated that ACC was derived from SAM.

The conclusion that ACC is an intermediate in the conversion of methionine to ethylene is supported by the following observations: (i) Labelled ACC administered to apple plugs is efficiently converted to ethylene. (ii) The conversion of labelled methionine to ethylene is greatly reduced in the presence of excess unlabelled ACC, but the conversion of labelled ACC to ethylene is unaffected by the presence of excess methionine, and (iii) AVG, a potent inhibitor of pyridoxal phosphate mediated enzymes (153) inhibited the conversion of ACC to ethylene. The following pathway

for ethylene has therefore been suggested: methionine → SAM → ACC → ethylene.

Lürssen et al. (94) reported that in order for ACC to be converted to ethylene, intact cell structure is required. In contrast, Boller et al. (20) showed that ACC could be produced from SAM by a crude enzyme preparation. Protoplasts obtained from flower tissues of Ipomoea tricolor did not form ethylene from methionine, but ethylene was produced upon incubation of the intact tissue with ACC (76). These results indicate that an intact cell structure is required for the synthesis of ethylene from ACC, but not for the synthesis of ACC from methionine or SAM.

ACC synthase, the enzyme catalyzing the conversion of SAM to ACC, has been reported in extracts of tomato (20,21,70,159), avocado and apple fruits (159). The non-ripening tomato mutant rin, which produces very low levels of ethylene, has been shown to have lower amounts of ACC and ACC synthase activity than the normal ripening variety Rutgers (20). ACC synthase has been shown to be a pyridoxal enzyme, specifically utilizing SAM as substrate, and has a molecular weight of 55,000 (5,20,104,149,159). The conversion of ACC to ethylene is thought to involve a very labile stereospecific enzyme or enzymes (20).

Physiological Role of ACC

ACC was first isolated by Burroughs (34) from pear fruit juice in 1957, but its physiological significance was not known until the discovery that ACC is an intermediate in ethylene biosynthesis

(7). Cameron et al. (35) showed that excised tissue from roots, stems, leaves, inflorescences, and fruits showed an increase in ethylene production from 10 to 1,000 times over the control, when treated with different concentrations of ACC. Similar results were also obtained by Lürssen et al. (94).

Evaluating the changes in the internal ACC level during the ripening of avocado, banana, and tomato fruits and comparing it to the internal ethylene biosynthesis (62), it was found that pre-climacteric fruits in general contain approximately less than 0.1 nmol. ACC/g. As the fruits reach the climacteric stage, the ACC level increases sharply then decreases at the end of the climacteric period. In avocados, for example, the ACC concentration starts at about 0.1 nmol/g at the pre-climacteric stage, increases to 45.0 nmol/g during the climacteric period, after which it drops to 5.0 nmol/g. It has been suggested that ACC synthesis is the rate limiting step in ethylene biosynthesis (159,161,162), that ACC increases prior to the increase in ethylene level and that the decrease in ACC concentration is a result of the increase in ethylene synthesis (125).

Recycling of MTA to Methionine

Plant tissue contains very limited amounts of free and bound methionine compared to the rate of ethylene production. Apple fruits, for example, contain approximately 60.0 nmol/g fresh weight methionine, whereas their rate of ethylene production is 5.0 nmol/g/h (16,155). Theoretically one would expect ethylene

production to last for only 12 h, but apples are known to sustain ethylene production for months (16).

Further investigations by Baur et al. (15) compared the rate of ethylene production to the endogenous methionine levels in avocado fruits (cultivar Feurte) at the climacteric peak; they observed that the methionine concentration of these fruits was 12.5 nmol/g fresh weight, while their ethylene production was 3.7 nmol/g/h. Thus, without replacement, the endogenous methionine could sustain ethylene production for only 3 h. Avocados are known to produce ethylene at a rate as high as 100 nmol/g/h for days (15). The highest amount of ethylene production recorded in plant tissue was that of fading flowers of Vanda Orchids, 3,000 nmol/g/h (9).

In order for plant tissues to sustain high rates of ethylene production with such a limited supply of methionine and sulfur, a pathway to salvage the methylthiol group (CH_3S-) of methionine was proposed (16). Adams and Yang (6) observed that in apple tissue the CH_3S- group of methionine was released as MTA from SAM during ACC formation. The MTA released was rapidly converted to MTR and adenine, similar to reactions previously reported by Shapiro and Barrett in cell free extracts of Enterobacter aerogenes (135).

MTA nucleosidase activity has been shown in bacteria (47) and plants (57). Plant MTA nucleosidase has been purified to homogeneity from lupin (Lupinus luteus) seeds, it has a native molecular weight of 62,000, with two identical subunits. It is very specific to MTA, with a pH optimum between 8 and 8.5 (57).

The end products of MTA degradation in plants are MTR and adenine (6). Schroeder et al. (133) reported that MTR in E. coli represents a terminal product. However, Ferro et al. (48) purified MTR kinase from E. aerogenes. This enzyme catalyzes the ATP-dependent phosphorylation of MTR yielding MTR-1-P and ADP as products. The K_m values were, 7.4×10^{-5} M for ATP, and 8.1×10^{-6} M for MTR. Ferro et al. (48) have suggested the involvement of MTR kinase in the recycling of the methylthiol group of MTR to methionine.

Subsequently Shapiro and Barrett (135) demonstrated that MTR-1-P is an intermediate in the biosynthesis of methionine in E. aerogenes and that 2-keto-4-methylthiobutyric acid (α-KMB) and 2-hydroxy-4-methylthiobutyric acid (α-HMB), might be involved in this pathway. In vitro incubation of rat liver extract with MTR-1-P revealed that α-KMB is indeed an intermediate in the conversion of MTR-1-P to methionine (10). Trackman et al. (141) proposed a mechanism for the conversion of MTR-1-P to α-KMB and formate. Their results suggest that α-HMB was formed upon conversion of α-KMB to methionine. Baur et al. (15) showed that α-KMB stimulated ethylene biosynthesis in apple fruit tissues, but less efficiently than methionine. Based on these results, Lieberman et al. (86) suggested that α-KMB may have stimulated ethylene production by conversion to methionine.

Previously Adams and Yang (6) suggested that MTR donates its methylthiol group to a 4-carbon acceptor, such as homoserine, to

form methionine. Recent studies on yeast cells (136), cell-free extracts of E. aerogenes (135), and rat liver homogenate (43) indicated that the radioactivity of the uniformly labelled ribose of MTA or MTR was recovered in methionine. It was suggested that the ribose portion of MTA and MTR provided the 4-carbons that were thought to come from homoserine.

Yung et al. (163) examined the fate of the ribose portion of MTR in apple plugs. They carried out a double labelling experiment with [methyl-^{3}H]MTR and [ribose-U-^{14}C]MTR as substrates. Their results showed that the ratio of $^{3}H/^{14}C$ of the precursor MTR was 1.1 and for the product methionine the ratio was 1.35. If the pathway for methionine synthesis involves modification of the 5-carbon ribose portion of MTR into the 4-carbon, 2-aminobutyrate of methionine with the CH_3S- remaining attached, the expected ratio would be 1.37 close to the value they observed.

More recently Wang et al. (146) showed that when [U-^{14}C-adenosine]MTA was infused to tomato pericarp plugs, the radioactivity was recovered in MTR, methionine, ACC, and ethylene. However, when [U-^{14}C-ribose]MTR was administered to the tomato plugs, no radioactivity was recovered in MTA, but the radioactivity was observed in methionine, ACC, and ethylene. These results established that the ethylene carbons are derived from the ribose portion of MTA and MTR, which are originally incorporated into the ethylene pathway from the ribose portion of ATP (Fig. 1).

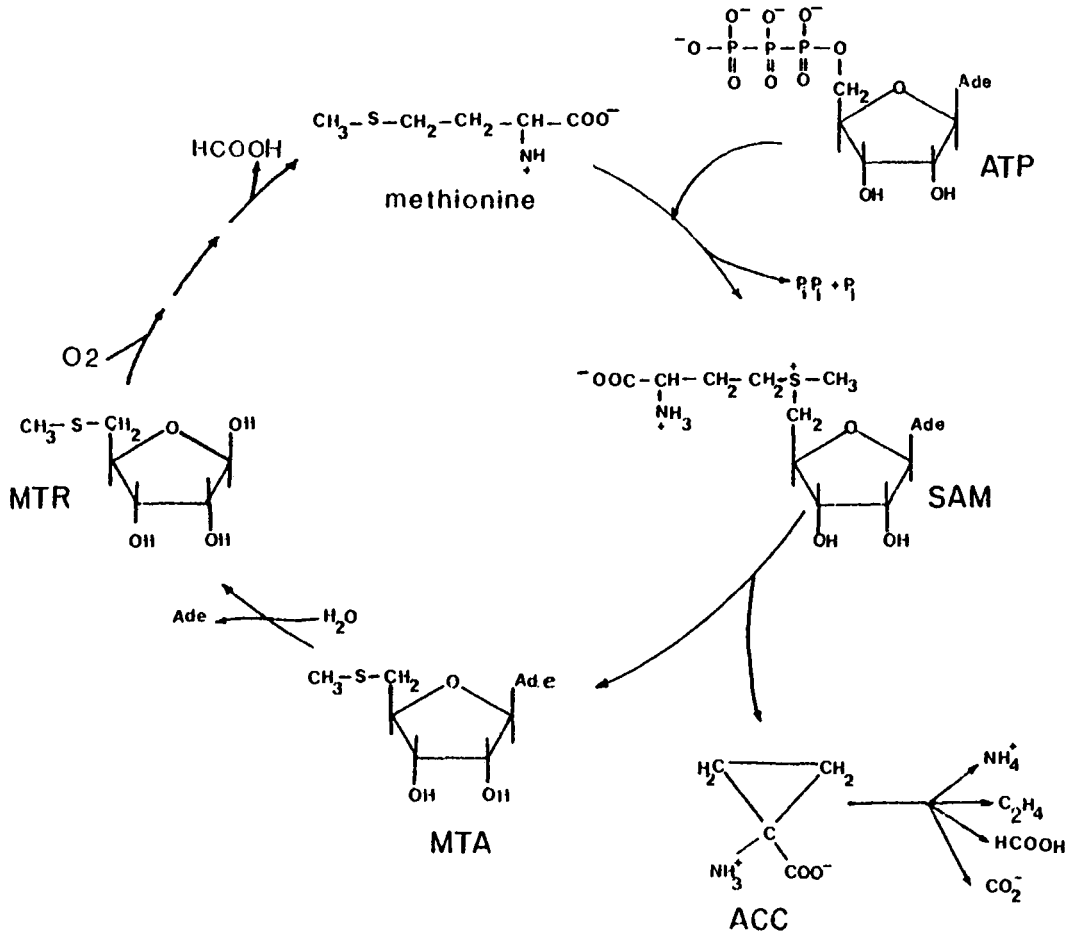


Fig. 1. Summary of the ethylene biosynthetic pathway in plants.

5-METHYLTHIORIBOSE KINASE ACTIVITY IN PLANTS

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ABSTRACT

The presence of a previously unidentified plant enzyme, 5-methylthioribose kinase, has been demonstrated in cell-free extracts from several fruit tissues. The enzyme catalyzes the ATP-dependent phosphorylation of 5-methylthioribose to 5-methyl-thioribose-1-phosphate. Enzyme activity has been found in avocado, pear, apple, strawberry and tomato tissues. The significance of the presence of the enzyme in relation to ethylene biosynthesis is discussed.

INTRODUCTION

Ethylene is a plant hormone regulating many aspects of growth, development and senescence in higher plants (1). The identification of S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) as precursors of ethylene (2,3,9) has led to the elucidation of the following metabolic sequence for the biosynthesis of ethylene:

$$\text{Methionine} \rightarrow \text{SAM} \rightarrow \text{ACC} \rightarrow \text{ethylene} + \text{MTA}$$

ACC synthase catalyzes the conversion of SAM to ACC and 5'-methylthioadenosine (MTA) (3). Various moieties of MTA have been shown to be recycled back into methionine (5,12,13). The first step in this recycling pathway is degradation of MTA. In most microorganisms and plants, MTA is degraded by a nucleosidic cleavage via MTA nucleosidase to 5-methylthioribose (MTR) and adenine, whereas in animal tissue a phosphorylytic cleavage results in the formation of 5-methylthioribose-1-phosphate (MTR-1-P) and adenine (6). Regardless of whether the direct product of MTA degradation is MTR or MTR-1-P, MTA has been shown to be recycled back into methionine. It has been proposed that in order for this recycling to occur, MTR-1-P must first by synthesized (7). In Enterobacter aerogenes (7), a new enzyme, MTR kinase, was identified and found to catalyze the ATP-dependent phosphorylation of MTR to MTR-1-P. In this communication, we report for the first time the presence of MTR kinase activity in tissue from higher plants.

MATERIALS AND METHODS

[$^{14}CH_3$]MTR was prepared from [$^{14}CH_3$]MTA which was obtained by the acid hydrolysis of [$^{14}CH_3$]SAM, (Amersham, 58 mCi/mmol) (11). [$^{14}CH_3$]MTR-1-P was prepared and purified as described by Ferro et al. (8) from the reaction mixture of an MTA phosphorylase assay utilizing a partially purified enzyme from guinea pig liver. Unripe mature avocado fruit was purchased from a local store, tomato and strawberry fruits were grown in a greenhouse, and apples and pears were harvested from the Hood River Experiment Station, Hood River, Oregon. Cell extracts were prepared by homogenization of the tissues suspended in buffer consisting of 0.2 M potassium phosphate (pH 7.2), 1% polyvinylpyrrolidone, 1% Triton X-100, and 3 mM dithiothreitol. The homogenate was passed through four layers of cheese cloth and centrifuged at 20,000 x g for 20 min. The supernatant fluid was utilized as a source of enzyme. The avocado extract was further purified by ammonium sulfate precipitation. Powdered $(NH_4)_2SO_4$ was added to 40% saturation and the precipitate obtained after centrifugation (14,000 x g) was resuspended in buffer. This step resulted in a 4-fold purification of the enzyme and this preparation was used in those experiments designed to identify reaction products.

The radiochemical assay used to measure MTR kinase activity (7) measures the conversion of MTR to MTR-1-P by chromatographic separation of the reactants and products on Dowex 1-X8 columns. For product identification, descending paper chromatography was

performed using Whatman No. 3 paper. The solvent system was 2-butanol:acetone:acetic acid:H_2O (70:70:20:40). Ultraviolet-absorbing substances were detected with a Mineralight lamp, and sulfur-containing compounds were observed by spraying the chromatograms with potassium iodoplatinate. Radioscans of paper chromatograms were performed with a Packard radiochromatogram scanner. Alkaline phosphatase treatment was performed by incubation with 0.10 unit of calf intestine alkaline phosphatase (Sigma) for 30 min at 30°C in 0.05 M Tris-HCL, pH 8.0.

RESULTS

Cell free extracts of avocado incubated with 5-[$^{14}CH_3$]methylthioribose for 6 hr at 30°C did not catalyze the formation of any products as revealed by radiochromatographic analysis (Fig. 2a). The addition of [$^{14}CH_3$]MTR and 1 mM ATP to the cell extract, however, resulted in the conversion of [$^{14}CH_3$]MTR to two radioactive compounds which migrated at R_f values of 0.17 and 0.26, compounds A and B, respectively (Fig. 2b). Thus, indogenous ATP was insufficient to drive the reaction. The formation of compounds A and B were both dependent on the presence of enzyme protein and ATP, and the disappearance of [$^{14}CH_3$]MTR (R_f = 0.72) was proportional to the amount of compounds A and B recovered. Compound B co-migrated with authentic [$^{14}CH_3$]MTR-1-P (R_f = 0.26). Exposure of authentic [$^{14}CH_3$]MTR to 0.1% H_2O_2 resulted in the conversion of [$^{14}CH_3$]MTR to [$^{14}CH_3$]MTR sulfoxide (R_f = 0.45), whereas, the oxidation of the isolated [$^{14}CH_3$]MTR-1-P (compound B) resulted in its conversion to compound A (R_f = 0.17) upon subsequent chromatography. Peroxide oxidation of the radioactive reaction mixture resulted in the disappearance of [$^{14}CH_3$]MTR, the appearance of a new peak at R_f 0.45 ([$^{14}CH_3$]MTR sulufoxide), a reduction in the [$^{14}CH_3$]MTR-1-P peak and an increase in compound A (Fig. 2c). Reduction of compound A with 6.7 mM dithiothreitol resulted in the shifting of the R_f value back to 0.26 which again co-migrated with [$^{14}CH_3$]MTR-1-P. By analogy with MTR and MTR sulfoxide, the compound (compound A) formed as a result of peroxide

treatment of MTR-1-P appears to be the sulfoxide (MTR-1-P sulfoxide). In addition, treatment of either compound A or compound B with alkaline phosphatase in the presence of 10 mM dithiothreitol resulted in the formation of MTR. On the basis of these results, it is suggested that cell-free extracts of avocado contain MTR kinase, which catalyzes the phosphorylation of MTR to MTR-1-P. The MTR-1-P formed, in turn, is partially oxidized by the crude reaction mixture to MTR-1-P sulfoxide.

The enzyme was partially purified from a cell-free extract of avocado by ammonium sulfate fractionation. MTR kinase activity, as determined by ion-exchange chromatography (7), was dependent upon the presence of ATP over a 2 hr incubation period (Fig. 3). The temperature optimum of the enzyme was 30°C (Fig. 4) and the enzyme activity was linear with protein up to about 400 µg protein per assay.

Several cell-free extracts of fruits, in addition to avocado fruit, were also assayed for MTR kinase activity (Table 1). Of the extracts assayed, avocado contained the highest enzyme activity (810 pmole/mg protein/min); this activity is very similar to the specific activity found in crude extracts from E. aerogenes (7). Following the avocado, in descending order of activity were extracts prepared from pear, strawberry, apple, and tomato.

Table 1. MTR kinase activity in several cell free fruit extracts incubated with 0.1 mM [$^{14}CH_3$]MTR (3 mCi/mmol) for 2 h at 30°C.

Fruit Type	pmol MTR-1-P formed/mg protein/min
Avocado	810
'd'Anjou' pear	680
Strawberry (red)	230
Golden delicious apple	140
Tomato (red)	110

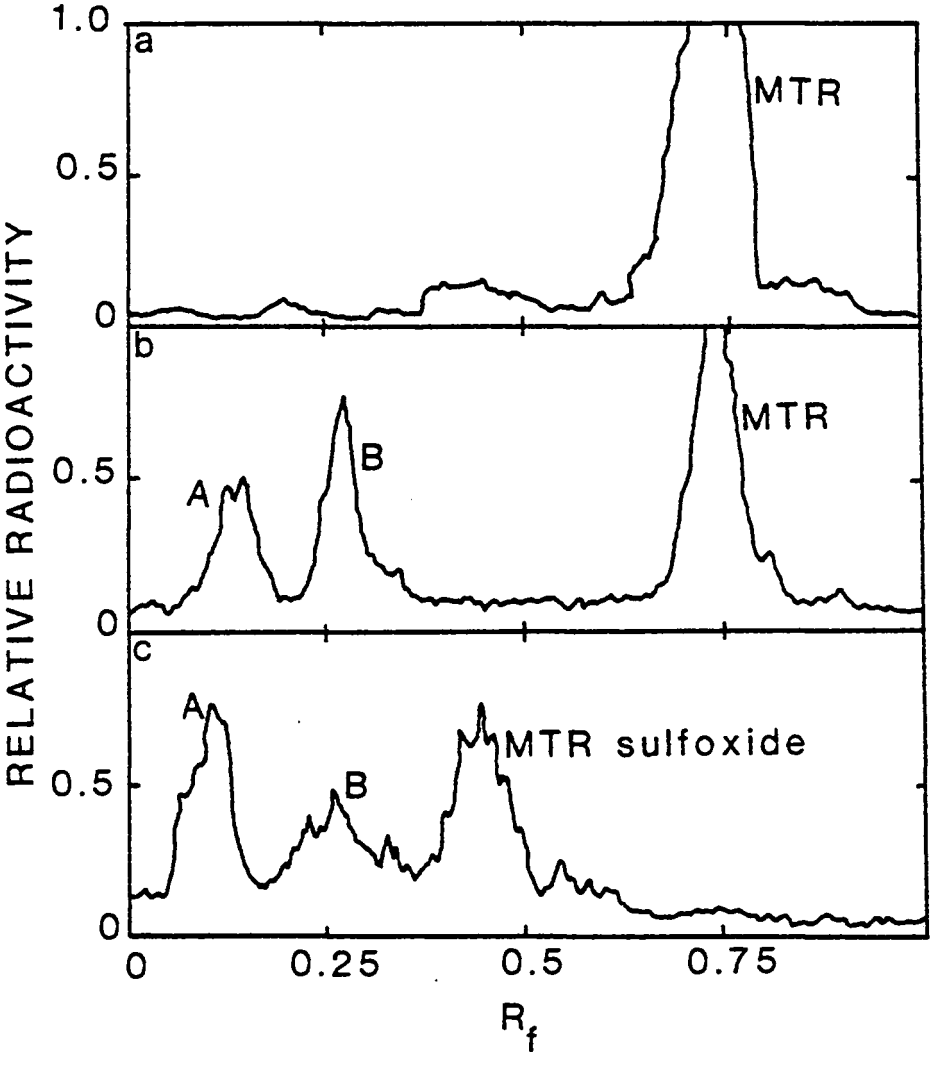

Fig. 2. Paper radiochromatogram scans of cell free extracts of avocado fruit incubated with 0.1 mM 5-[$^{14}CH_3$]MTR (3 mCi/mole) for 6 h at 30°C. (a) Crude extract incubated with 5-[$^{14}CH_3$]MTR; (b) crude extract incubated with 5-[$^{14}CH_3$]MTR and 1 mM ATP; (c) peroxide oxidation of the reaction mixture consisting of crude extract, 5-[$^{14}CH_3$]MTR and ATP.

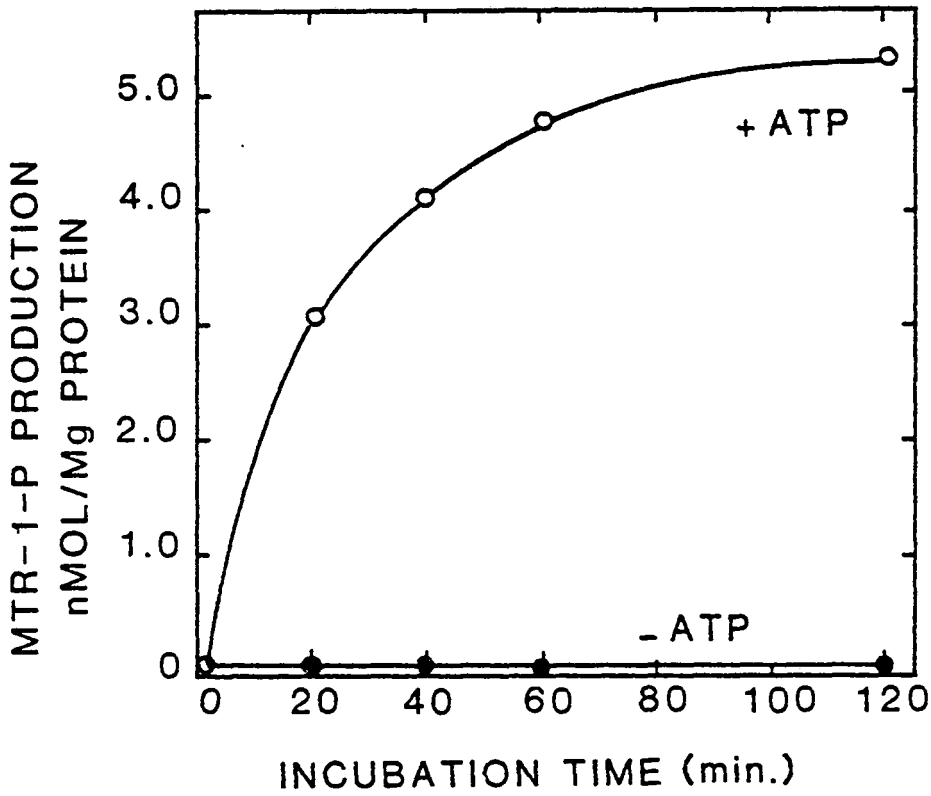

Fig. 3. MTR kinase activity of cell free extracts of avocado fruit incubated with 0.1 mM [$^{14}CH_3$]MTR (3 mCi/mmol) for 2 h at 30°C. (o - o) activity in the presence of 1 mM ATP, (● - ●) activity in the absence of ATP.

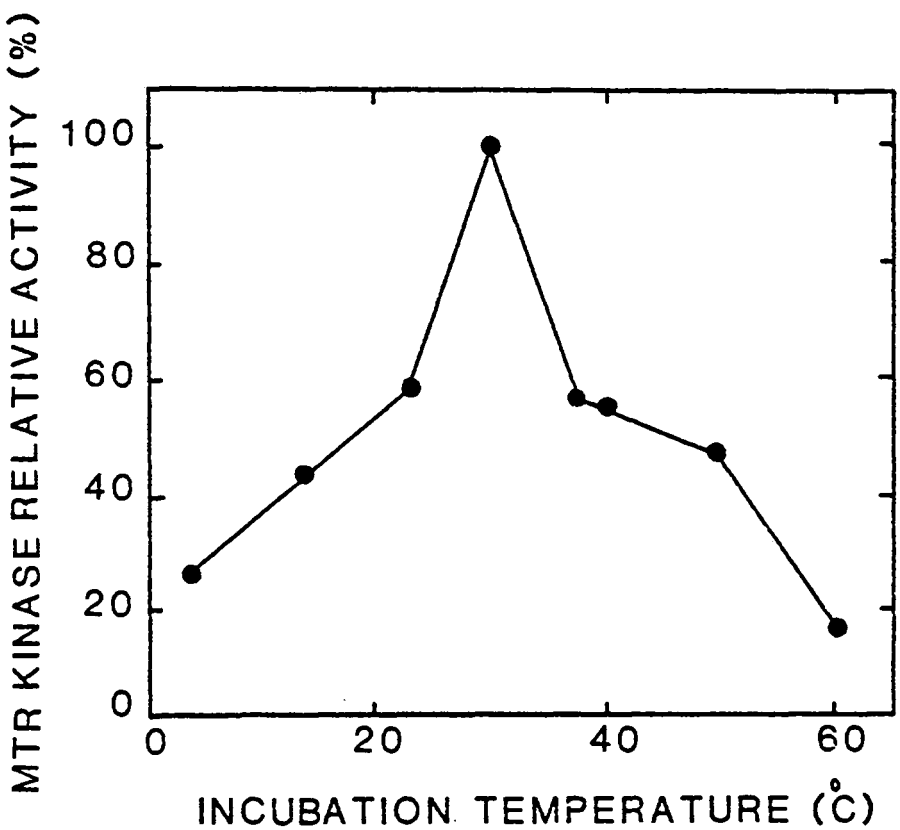

Fig. 4. Effect of incubation temperature on MTR kinase activity in cell free extracts of avocado fruit incubated with 0.1 mM [$^{14}CH_3$]MTR (3 mCi/mmol) for 2 h at 30°C. Controls were run at each temperature treatment.

DISCUSSION

In this study, MTR kinase activity has been demonstrated to exist in cell-free extracts from several fruit tissues. This is the first report of the enzymatic synthesis of MTR-1-P in plant tissues. Therefore, as in E. aerogenes, MTR-1-P is synthesized in a two step reaction from MTA:

1) MTA → MTR + adenine

2) MTR + ATP → MTR-1-P + ADP

This is in contrast to mammalian tissue where MTR-1-P is synthesized directly from MTA via MTA phosphorylase.

The primary fate of MTR in plant tissue appears to be its conversion to methionine; both the methyl group and the sulfur atom have been shown to be incorporated into methionine (2,10). Recently, Yung et al. (14) showed that in apple tissue, like yeast (13), rat liver (5) and E. aerogenes (12), the ribose portion of MTR is also incorporated into methionine. In E. aerogenes (7), it has been suggested that the conversion of MTR to MTR-1-P via MTR kinase may be the first step in this recycling pathway. More recently, MTR-1-P, but not MTR has been shown to be an intermediate in the recycling of MTA to methionine in rat liver cell extract (4). Since rat liver apparently lacks MTR kinase activity, this demonstrates that only the phosphorylated sugar is recycled in this tissue. These observations suggest that MTR-1-P, formed via MTR kinase in plant tissue, may be an essential intermediate in the biosynthesis of methionine from MTR. Studies investigating this

possibility are now in progress. The phosphorylation step may be a prerequisite to the "correct" stereochemical ring opening of ribose prior to subsequent reaction steps toward methionine.

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INTERMEDIATES IN THE RECYCLING OF 5-METHYLTHIORIBOSE TO METHIONINE IN FRUITS

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ABSTRACT

The recycling of 5-methylthioribose (MTR) to methionine in avocado (Persea americana Mill, cv. Hass) and tomato (Lycopersicum esculentum Mill, cv. unknown) was examined. [$^{14}CH_3$]MTR was not metabolized in cell free extracts from avocado fruit. Either [$^{14}CH_3$]MTR plus ATP or [$^{14}CH_3$]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. [^{35}S]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-^{14}C]α-KMB and [U-^{14}C]methionine, but not [U-^{14}C]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 → MTR-1-P → α-KMB → methionine → SAM → ACC → C_2H_4.

INTRODUCTION

Methionine serves as a precursor of ethylene (15), SAM 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 and Table 1, p. 26 this thesis) 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 (Lycopersicum esculentum Mill cv. unknown) in the breaker stage were purchased from a local store.

Chemicals. [^{35}S]methionine (1,000 Ci/mmol), [U-^{14}C]methionine (285 mci/mmol), and [$^{14}CH_3$]SAM (58 mci/mmol) were purchased from Amersham. [$^{14}CH_3$]MTA, was prepared from [$^{14}CH_3$]SAM (16), [$^{14}CH_3$]MTR was obtained by acid hydrolysis of the corresponding [$^{14}CH_3$]MTA (16), and [$^{14}CH_3$]MTR-1-P was prepared by incubating [$^{14}CH_3$]MTA with partially purified calf liver MTA phosphorylase as described by Ferro et al. (10). [^{35}S]α-KMB, and [U-^{14}C]α-KMB were obtained according to a procedure described by Dixon (8) with α-KMB extracted from the reaction with diethyl ether. [U-^{14}C]α-HMB was prepared from [U-^{14}C]α-KMB using $NaBH_4$. [U-^{14}C]α-KMB (2 μCi) was adjusted to pH 3 with 1 N acetic acid, then added to 2 mg $NaBH_4$ 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 (see chromatographic procedures below) 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 Pharmaceutical Company.

Cell-Free Extract and Enzyme Assay. Fruit extracts were prepared by homogenization of the tissue in 0.2 M potassium 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 20,000 x g. The supernatant 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 (Whatman No. 3) chromatography was carried out in butanol:acetic acid:acetone:water (70:20:70:40, v/v) (solvent System I). Thin layer chromatography 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 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

$Hg(ClO_4)_2$ injected into the bucket. After 6 h, the buckets were placed in scintillation vials containing scintillation fluid and the radioactivity determined. Non-radioactive ethylene was measured by gas chromatography.

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 2. The apparent 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 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 [$^{14}CH_3$]MTR incubated with or without avocado extract for 8 h remained unaltered (Fig. 5A). The addition of 5 mM ATP to the reaction mixture, however, resulted in the formation of a new radioactive peak (Rf = 0.85, solvent system I) and a smaller peak at Rf = 0.70, which presumably was unreacted MTR (Fig. 5B). Chromatography of this same assay mixture in solvent system II (Fig. 6), however, demonstrated that the substrate [$^{14}CH_3$]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 3) 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-HMB and α-KMB were eluted and re-chromatographed in the other three systems, they co-migrated with authentic samples of each of these compounds. Apparently the extract had such low ATP that no endogenous conversion of MTR was possible.

Metabolism of MTR-1-P in Avocado Extract. Since MTR was metabolized only when ATP was present and since MTR kinase activity has been found in these extracts, the ability of avocado extract to metabolize MTR-1-P was examined (Fig. 7). Incubation of [$^{14}CH_3$] MTR-1-P with avocado extract (in the absence of ATP) for 30 h yielded two radioactive products, α-KMB and α-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 α-KMB and α-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, α-KMB, and α-HMB as described in Materials and Methods. MTR-1-P was metabolized (Fig. 8) to both α-KMB and α-HMB at approximately equal rates during the first 6 h. Thereafter, however, the rate of α-HMB accumulation exceeded that of α-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 α-HMB. That the increase in α-HMB plus α-KMB is greater than the decrease in MTR-1-P between 18 to 30 h incubation may indicate the presence of an intermediate compound(s) synthesized from MTR-1-P during the first 18 h and converted to α-KMB and α-HMB during the latter stages of the incubation period.

Metabolism of α-KMB and α-HMB by Avocado Extract. Avocado extracts were incubated for 3 h with 1 mM asparagine and either [^{35}S]α-KMB or [^{35}S]α-HMB and analyzed for [^{35}S]methionine, [^{35}S]α-KMB and [^{35}S]α-HMB (Table 4). More than 95% of the [^{35}S] α-KMB was metabolized to methionine (63%) and α-HMB (32%), whereas [^{35}S]α-HMB incubated with extract and asparagine remained unaltered. The addition of 2.5 mM unlabelled α-HMB to the [^{35}S]α-KMB reaction mixture resulted in a 26% decrease in the conversion of α-KMB to methionine and a concomitant increase in the level of [^{35}S]α-KMB. The data 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 inhibit slightly 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 5). Of the three compounds tested, only α-KMB and methionine served as substrates for the synthesis of ethylene; α-HMB was 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 enzymatic 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 6). α-KMB at 1.0 and 2.0 mM increased ethylene production in a dose-dependent manner. The addition of 0.1 mM AVG inhibited ethylene production in these tissues in the presence of α-KMB, these data suggesting that α-KMB is metabolized to ethylene via SAM and ACC.

Table 2. MTR kinase activity in avocado fruit extract incubated with 0.1 mM [$^{14}CH_3$]MTR (3 mCi/mmol) for 2 h at 30°C.

Tissue	Enzyme activity (pmol product formed/mg protein/min)
Peel	508
Outer exocarp	1,805
Inner exocarp	1,080

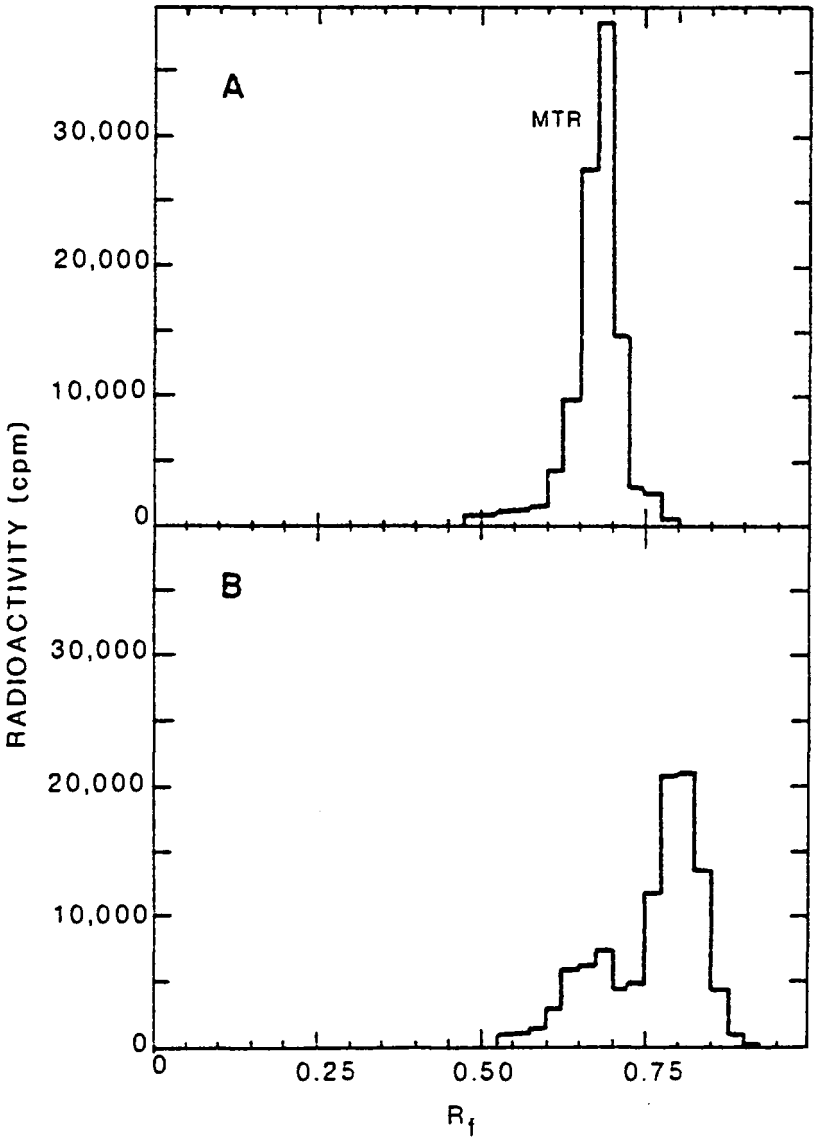

Fig. 5. Paper radiochromatogram scans in solvent system I [butanol: acetic acid: acetone: water (70:20:70:40)] of cell free extracts of avocado fruit incubated for 8 h at 30°C. (A) [$^{14}CH_3$]MTR or (B) [$^{14}CH_3$]MTR plus ATP.

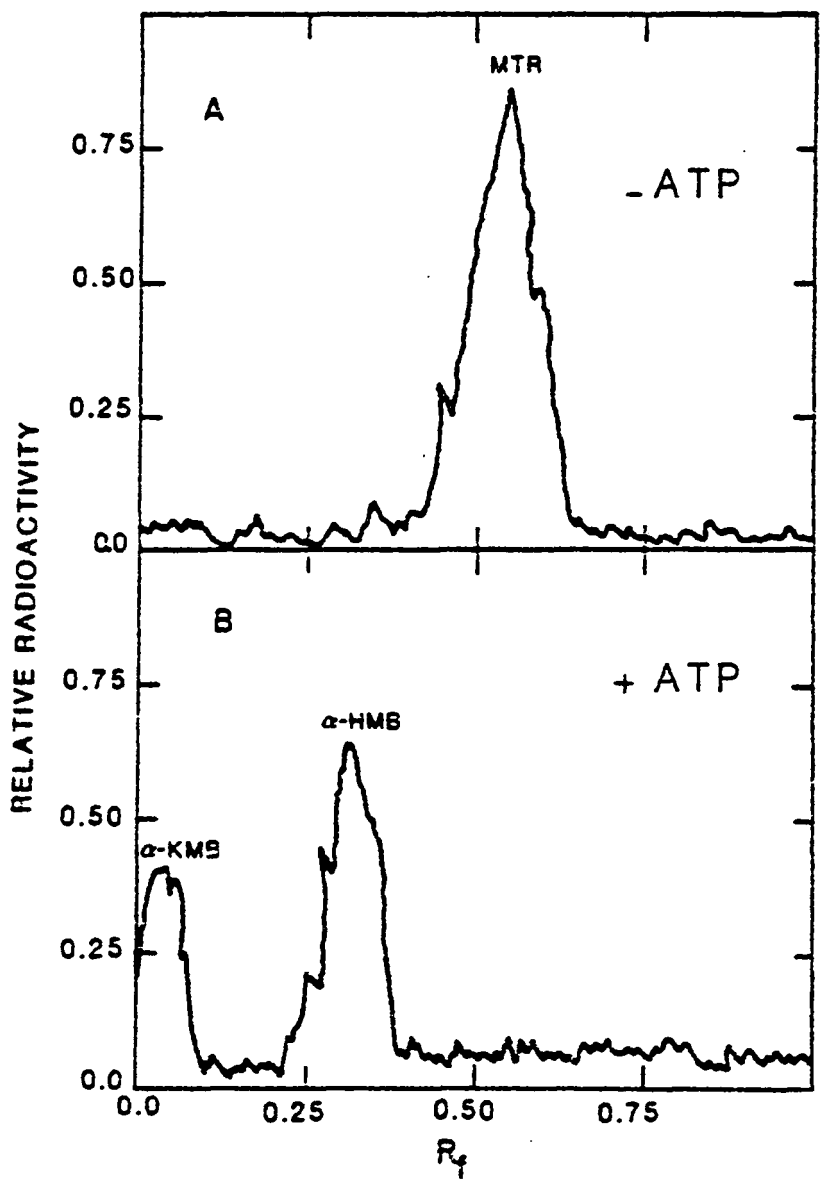

Fig. 6. Silica gel radiochromatogram scans in solvent system II [isobutanol: acetic acid: water (68:10:2)] of cell free extracts of avocado fruit incubated for 8 h at 30°C. (A) [$^{14}CH_3$]MTR or [$^{14}CH_3$]MTR plus ATP.

Table 3. R_f values of MTR, MTR-1-P, α-KMB, α-HMB and methionine in several chromatographic solvent systems. System I; descending paper chromatography [butanol: acetic acid: acetone: water (70:20:70:40)], system II; silica gel TLC [isobutanol: acetic acid: water (68:10:2)], system III; silica gel TLC [ethyl acetate: ethanol: water (9:1:2)], system IV; silica gel TLC [isobutanol: ethanol: water (68:20:10)].

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

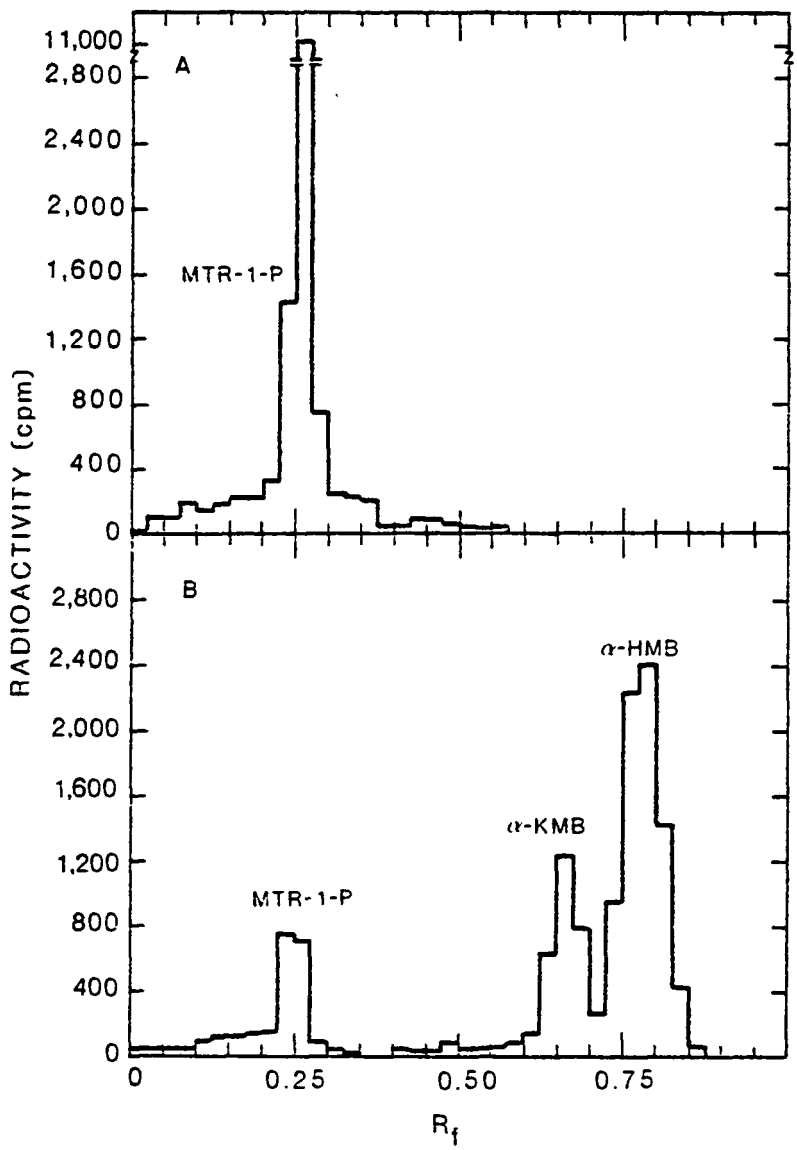

Fig. 7. Paper radiochromatogram scans in solvent system I [butanol: acetic acid: acetone: water (70:20:70:40)] of cell free extracts of avocado fruit incubated for 8 h at 30°C. (A) no extract plus [$^{14}CH_3$]MTR-1-P and (B) extract plus [$^{14}CH_3$]MTR-1-P.

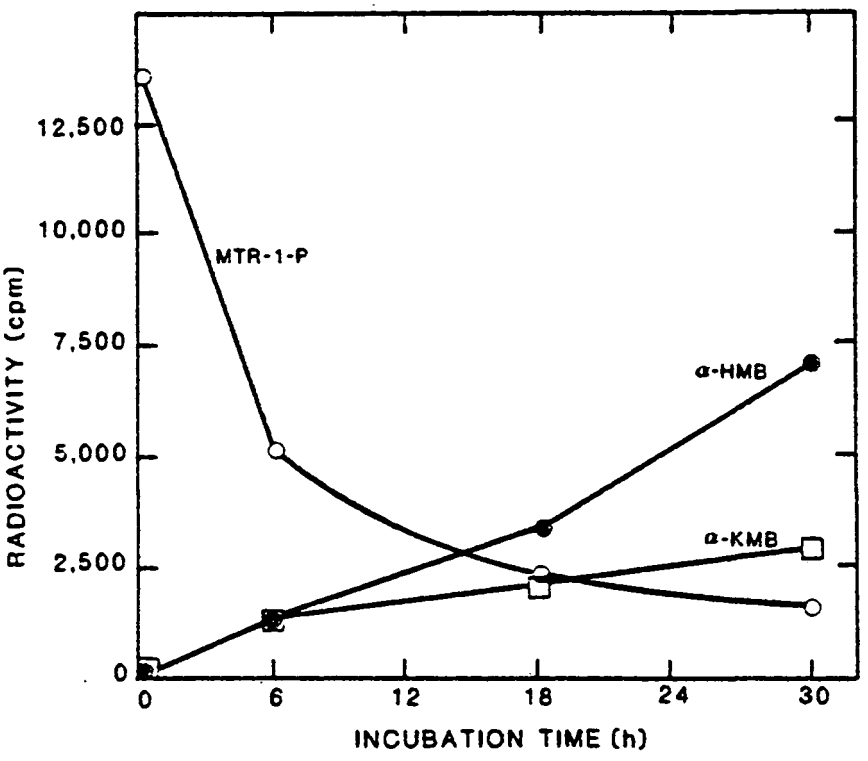

Fig. 8. Kinetics of product formation from MTR-1-P in cell free extracts of avocado fruit incubated at 30°C. (o - o) MTR-1-P; (□ - □) α-KMB; (● - ●) α-HMB.

Table 4. Methionine formation in avocado extract from α-KMB and α-HMB incubated for 5 h at 30°C.

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

Less than 0.1% of other metabolites were detected, but were not significant enough to be considered in the calculations of the percent recovery.

Table 5. Synthesis of ethylene from α-KMB, α-HMB and methionine in plugs of tomato pericarp tissue incubated for 6 h at 20°C.

Substrate[a]	C_2H_4 produced (nCi/g/h)
[U-^{14}C]α-KMB	0.294
[U^{14}C] methionine	0.671
[U-^{14}C]α-HMB	0
[U-^{14}C]α-KMB + 2.5 mM α-HMB	0.133

[a]All radioactive compounds were added at 1.29 μCi.

Table 6. Effect of AVG on ethylene production in plugs of tomato pericarp tissue incubated for 6 h at 20°C.

Addition	Ethylene Produced % of control
Control	100
1.0 mM α-KMB	125
2.0 mM α-KMB	148
1.0 mM α-KMB + 0.1 mM AVG	6
1.0 mM α-KMB + 1.0 mM α-HMB	106

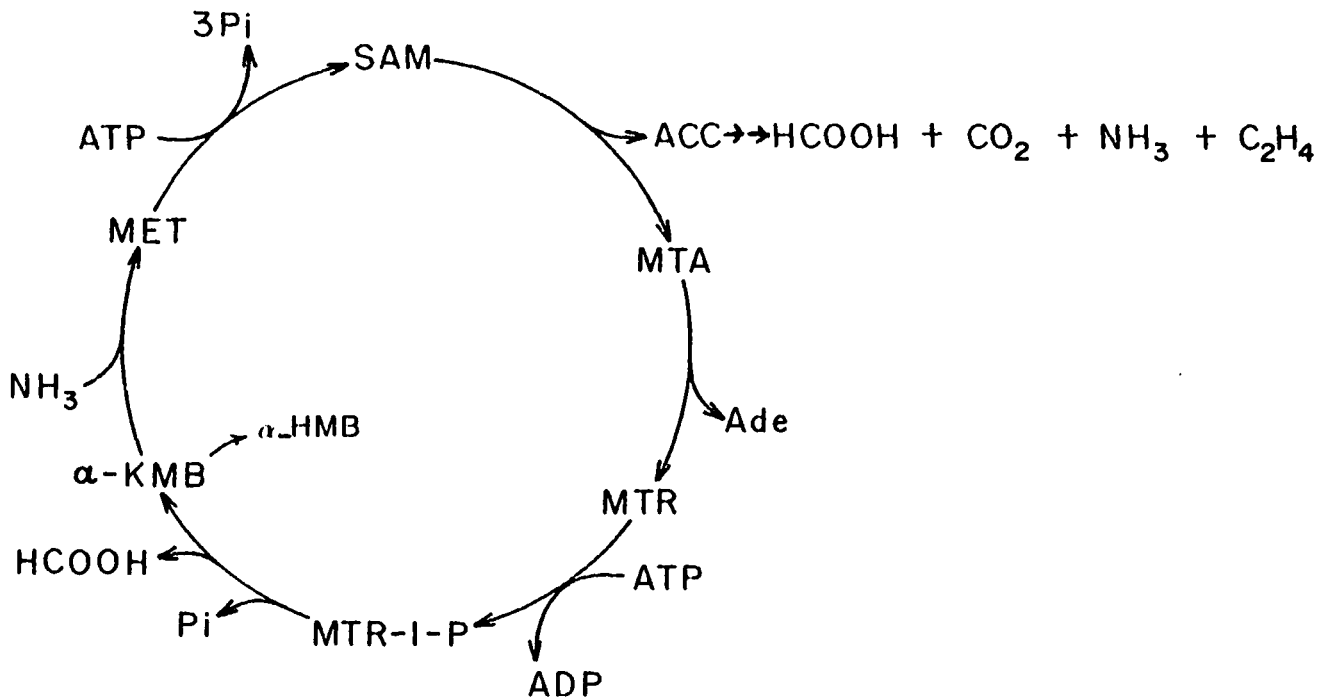

Fig. 9. Proposed recycling pathway from MTR to methionine.

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 9.

The significance of MTR kinase activity in this recycling is illustrated by the inability of MTR to be metabolized by cell extracts unless ATP is added or until it is first activated to MTR-1-P. In mammalian tissue, MTA is converted directly 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 pathway similar to plants (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 the absence of MTR kinase renders the yeast cell unable to 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 tissues (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 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:

$$2ATP \rightarrow ADP + adenine + 4Pi + 2HCOOH + CO_2 + C_2H_4$$

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 total de novo synthesis of methionine in those systems in which this recycling pathway is present.

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5'-METHYLTHIOADENOSINE NUCLEOSIDASE AND 5-METHYLTHIORIBOSE KINASE ACTIVITIES DURING TOMATO FRUIT DEVELOPMENT AND RIPENING

Mosbah M. Kushad

ABSTRACT

5'-Methylthioadenosine (MTA) nucleosidase and 5-methylthioribose (MTR) kinase activities were measured in crude extracts of tomato fruits (Lycopersicon esculentum Mill cv Rutgers) during fruit development and ripening. The highest activity of MTA nucleosidase (1,250 ρmol/mg protein/min) was observed in small green fruits. The activity decreased during ripening; at the over-ripe stage only 6.5% of the peak activity remained. MTR kinase activity was low at the small green stage and increased thereafter until it reached peak activity at the breaker stage (700 ρmol/mg protein/min) followed by a sharp decline at the later stages of fruit development. 1-Aminocyclopropane-1-carboxylic acid (ACC) levels peaked at the red stage, while ethylene reached its highest level at the light-red stage. Several analogs of MTA and MTR were tested as both enzyme and ethylene inhibitors. Of the MTA analogs examined for their ability to inhibit MTA nucleosidase, 5'-chloroformycin reduced enzyme activity 88% whereas 5'-chloroadenosine, 5'-isobutylthioadenosine, 5'-isopropylthio-adenosine, and 5'-ethylthioadenosine inhibited the reaction with MTA by about 40%. 5'-Chloroformycin and 5'-chloroadenosine inhibited ethylene production over a period of 24 h by about 58%

and 36%, respectively. Other analogs of MTA were not effective inhibitors of ethylene production, whereas aminoethoxyvinylglycine (AVG) showed a 66% inhibition over the same period of time. Of the MTR analogs tested, 5-isobutylthioribose was the most effective inhibitor of both MTR-kinase (59%) and ethylene production (65%).

INTRODUCTION

ACC synthase catalyzes the conversion of SAM to ACC and MTA in plants (2,5). Various moieties of MTA have been shown to be recycled to methionine (22). MTA is degraded in plant tissues by a nucleosidic cleavage via MTA nuleosidase to MTR and adenine (13). Adams and Yang (1) first observed the presence of MTA nucleosidase activity in crude extracts prepared from apple fruit tissue. Guranowski et al. (13) later purified this enzyme from Lupinus luteus seeds, and established that it has a molecular weight of 62,000, a Km of 0.41 μM and exhibited broad substrate specificity.

Kushad et al. (16) established that cell-free extracts from several fruit tissues contain MTR kinase actvity. This enzyme catalyzes the ATP-dependent phosphorylation of MTR to MTR-1-P. Recently, Guranowski et al. (12) partially purified MTR kinase from Lupinus luteus seeds and found that the native molecular weight of this enzyme is 70,000 and exhibits a Km value of 0.45 μM for MTR and 0.83 μM for ATP. Kushad et al. (17) subsequently showed that MTR-1-P is metabolized to α-KMB which is converted to methionine and then to ethylene. Yung et al. (25) and Wang et al (22) showed that the ribose moieties of MTA and MTR are incorporated into methionine. They therefore suggested that ethylene is formed from the ribose portion of MTA, which originated from an ATP molecule.

The objectives of the present study were to evaluate the specific activity changes of both MTA nucleosidase and MTR kinase during tomato fruit development and ripening and to relate these

changes to the rate of ACC and ethylene synthesis. In addition we have examined the effect of MTA and MTR analogs on MTA nucleosidase and MTR kinase activities and on the rate of ethylene synthesis.

MATERIALS AND METHODS

Plant Materials. Tomato fruits (Lycopersicon esculentum Mill cv Rutgers) grown at the Oregon State University, Department of Horticulture greenhouse were used for these experiments.

Chemicals. [$^{14}CH_3$] SAM (58 mCi/mmol) was purchased from Amersham, [$^{14}CH_3$]MTA was prepared from [$^{14}CH_3$]SAM (20), [$^{14}CH_3$]MTR was synthesized by acid hydrolysis of [$^{14}CH_3$]MTA (20), ACC was purchased from Sigma, AVG was a gift from Hoffman LaRoche, MTA analogs (ETA, iBTA, iPTA, MTT, 5'-chloroadenosine, 5'-chloroarabinosyladenosine, 5'-chloroformycin, and 5'-chlorotubercidin) were prepared in our laboratory according to previously described procedures (6,8,15,21). MTR analogs (ETR, iBTR, and iPTR) were prepared by the same basic procedure used to prepare MTR from MTA (20).

Preparation of Cell-Free Extracts. Fruit tissues were suspended in buffer (1 g/ml) consisting of 0.2 M K-phosphate, 3 mM DTT and 3% PVP (pH 7.2), and homogenized in an Acme Supreme Juicerator. The homogenate was passed through four layers of cheesecloth and centrifuged for 20 min at 20,000 x g. The supernatant was used as the cell free extract. Protein concentration was determined according to Bradford (7).

Enzyme Assays. MTA nucleosidase activity was determined by measuring the conversion of 5'-[$^{14}CH_3$]MTA to 5-[$^{14}CH_3$]MTR (9). The standard reaction mixture contained in a total volume of 0.25 ml, 0.1 M sodium Hepes buffer (pH 7.2), 100 µM 5'-[$^{14}CH_3$]MTA (8.0×10^6 cpm/µmol), and enough protein to allow for about 20% total substrate consumption. The reaction mixture was incubated at 30°C for 1 h and was terminated by the addition of 3 volumes of chilled 95% ethanol. The resulting precipitate was removed by centrifugation at 11,000 x g for 4 min, and the supernatant was applied to a Dowex 50 W-H^+ X 4 (100-200 mesh) column (0.5 x 3.0 cm). The columns were eluted with 3 ml distilled water directly into scintillation vials containing toluene/triton X-100 (2:1, v/v), and the vials were counted in a Beckman LS 8000 scintillation counter. When MTA analogs were tested as potential substrates for MTA nucleosidase, both the depletion of the analogs and the formation of adenine were measured by HPLC according to a previously described procedure (23). MTR kinase activity was determined according to the previously described procedure of Ferro et al. (10).

Feeding Experiments. Tomato plugs (1 cm in diameter) in the breaker stage were excised and immediately rinsed in 2% KCl and blotted dry. Chemicals were introduced in the plugs by the vacuum injection technique previously described (4). Each experiment was replicated three times. Three plugs were sealed in a 25 ml flask and at the end of each incubation period 1 ml air sample was

withdrawn from each flask and injected into a flame ionization detector GC for ethylene measurement.

Determination of ACC. ACC levels were monitored throughout the course of ripening. Three fruits from each stage of ripening were randomly selected and a total of 2.0 g of pericarp tissue was weighed and blended in 5 ml of 9% TCA in a Polytron homogenizer (Brinkman Instruments). The homogenate was centrifuged at 20,000 x g for 20 min and the supernatant was passed through a Dowex 50 $W-H^+$ column. ACC was eluted from the column with 2N NH_4OH. After concentration under reduced pressure at 50°C, the eluates were assayed for ACC according to the procedure described by Lizada and Yang (18). The conversion of ACC to ethylene was determined by including an internal standard of known concentration to determine the efficiency of conversion.

RESULTS

Enzyme Activities During Fruit Development and Ripening. Tomato fruits were classified according to the following system: 1, small green; 2, medium green; 3, mature green; 4, breaker; 5, pink; 6, red; 7, over-ripe red. Extracts were prepared from fruits in each of these stages and the specific activities of both MTA nucleosidase and MTR kinase were determined (Fig. 10-A) and compared to the levels of ACC and ethylene (Fig. 10-B). The highest MTA nucleosidase activity was observed in the small green stage (stage 1), approximately 1,200 ρmol/mg protein/min. As the fruits progressed to the mature green stage (stage 3), the activity of MTA nucleosidase declined to about one-half the original level. A slight increase in enzyme activity was observed in the breaker stage (stage 4) followed by another sharp decline in MTA nucleosidase activity. Only 84 ρmol/mg protein/min activity was observed in the over-ripe stage (stage 7). In contrast, MTR kinase activity (Fig. 10-A) was low in the early two stages of fruit development followed by a marked increase in enzyme activity during the mature green stage. The highest activity of 700 ρmol/mg protein/min was observed in the breaker stage (stage 4). A sharp decline in MTR kinase activity occurred when fruits were at the red stage. Only 200 ρmol/mg protein/min of enzyme activity was observed in the over-ripe stage (stage 7).

Changes in ACC and Ethylene Levels During Tomato Fruit Development. We also examined the ACC and ethylene levels during the developmental stages of tomato fruits (Fig. 10-B). ACC was not detected until fruits reached the breaker stage (stage 4) and peaked at the red stage (stage 6), approximately 8 nmol/g, whereas ethylene production was first observed at the mature green stage (stage 3) and reached a plateau of about 3 nmol/g/h at the pink stage (stage 5).

Effect of MTA Analogs on MTA Nucleosidase Activity. The addition of unlabeled MTA analogs at an equimolar concentration to [$^{14}CH_3$]MTA showed variable effects on MTA nucleosidase activity (Table 7). 5'-chloro-2'-deoxyadenosine, MTT, 5'-chloroadenosine and 5'-chlorotubercidin had no significant effect on the degradation of [$^{14}CH_3$]MTA to [$^{14}CH_3$]MTR. ETA, iPTA, iBTA and 5'-chloroadenosine, however, each caused a 35-40% inhibition of enzyme activity (Table 7). HPLC determination of adenine formation in crude extracts of small green tomato fruits in the presence of ETA, iPTA, iBTA, 5'-chloroadenosine, and 5'-chloroformycin have confirmed earlier findings in other systems (8,23) that ETA, iPTA, iBTA and 5'-chloroadenosine are substrates for MTA nucleosidase, while 5'-chloroformycin is not (19). However, 5'-chloroformycin, when added to tomato extract in the presence of [$^{14}CH_3$]MTA at equimolar levels, inhibited the conversion of [$^{14}CH_3$]MTA into [$^{14}CH_3$]MTR by approximately 89%. An AVG treatment was included for comparative purposes and showed no significant effect on MTA nucleosidase activity.

Effect of MTA Analogs on Ethylene Synthesis. MTA analogs which exhibited an inhibition of MTA nucleosidase activity were further tested for their ability to inhibit ethylene synthesis in breaker stage tomato plugs. The compounds (25 nmoles) were injected into tomato plugs and incubated at room temperature. Table 7 shows that ETA, iPTA and iBTA moderately inhibited (13-30%) ethylene synthesis. AVG, a pyridoxal phosphate enzyme inhibitor which inhibits the conversion of SAM to ACC (5,24), caused a 34% inhibition of ethylene synthesis. Of the analogs which were found to be substrates for MTA nucleosidase, 5'-chloroadenosine was the most effective inhibitor of ethylene synthesis (42%). 5'-chloroformycin, the potent inhibitor of MTA nucleosidase, was also the most potent inhibitor of ethylene synthesis (64%). A time course study of AVG, 5'-chloroadenosine, and 5'-chloroformycin inhibition of the rate of ethylene synthesis (Fig. 11) showed that 5'-chloroformycin and 5'-chloro-adenosine were more effective inhibitors at the early period of incubation than was AVG. 5'-chloroadenosine, however, lost some of its effectiveness over-time, while 5'-chloroformycin inhibition remained unchanged. AVG, on the other hand, was an ineffective inhibitor of ethylene synthesis up to 8 h of incubation, but as time of incubation progressed AVG showed the same rate of inhibition as that of 5'-chloroformycin.

The relationship between MTA nucleosidase activity and ethylene levels can be seen more clearly in Fig. 12. A direct correlation appears to exist between the amount of ethylene

produced and the ability to convert MTA to MTR. As the specific activity of MTA nucleosidase decreases from control values, the levels of ethylene produced decline accordingly. Extrapolation of these data suggests that in the absence of MTA nucleosidase activity, only about 25% of the control levels of ethylene would be measured.

Effect of MTR Analogs on MTR Kinase Activity. The effect of MTR analogs on MTR kinase activity was determined at the breaker stage (stage 4), the stage at which MTR kinase activity was at its peak (Fig. 10-A). Table 8 shows that of the MTR analogs tested, iBTR caused a 41% reduction in the conversion of [$^{14}CH_3$]MTR to [$^{14}CH_3$]MTR-1-P whereas ETR and iPTR caused only a slight decrease in MTR kinase activity. AVG had no effect on MTR kinase activity.

Effect of MTR Analogs on Ethylene Synthesis. iBTR, ETR and iPTR, known substrates for MTR kinase (11), also have been evaluated for their effects on ethylene synthesis in breaker tomato plugs (Table 8). Of the compounds tested, iBTR caused a 35% inhibition of ethylene synthesis followed by iPTR (22%) and ETR (no effect). MTR did not demonstrate any significant stimulation or inhibition of ethylene synthesis.

Time course studies of iBTR inhibition of ethylene synthesis showed that, compared to the control and AVG treatment (Fig. 13), iBTR markedly reduced ethylene synthesis of breaker tomato plugs (stage 4). This inhibition, however, was negated as the time of

incubation increased. In contrast, AVG showed no effect during the first 8 h of incubation, but the inhibitory effect steadily increased over time.

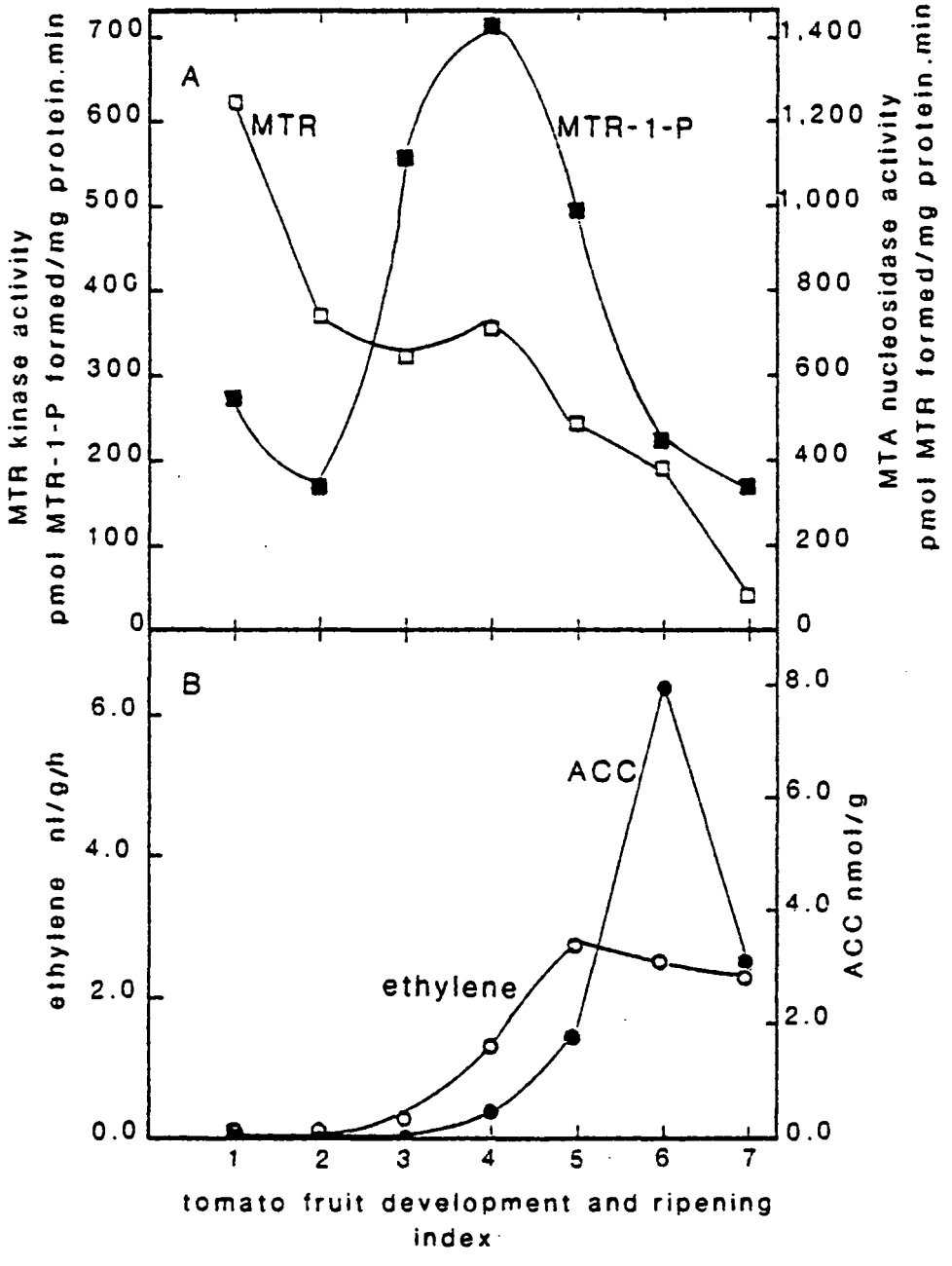

Fig. 10-A. Changes in MTA nucleosidase and MTR kinase activities during tomato fruit development and ripening incubated at 30°C for 1 and 2 h, respectively.
(□ - □) MTR formed, (■ - ■) MTR-1-P formed.

Fig. 10-B. Changes in ACC and ethylene levels during tomato fruit development and ripening. (● - ●) ACC, (o - o) ethylene.

Table 7. Effect of MTA analogs on MTA nucleosidase activity and ethylene production from breaker tomato fruits incubated for 1 h at 30°C for the nucleosidase activity and 24 h at 20°C for ethylene.

Compound[2]	Substrate	MTA nucleosidase Activity	Ethylene Production
		% of Control	% of Control
Control	+	100	100
MTA	+	50	107
5'-chloro-2'-deoxyadenosine	ND[b]	100	ND
5'-chloroarabinosyl-adenosine	ND	100	ND
MTT	-	100	ND
5'-chlorotubercidin	-	100	ND
AVG	-	98	66
ETA	+	65	87
iPTA	+	62	81
iBTA	+	61	70
5'-chloroadenosine	+	53	58
5'-chloroformycin	-	11	36

[2][$^{14}CH_3$]MTA was added at 25 nmol as were each of the analogs.

[b]Not determined.

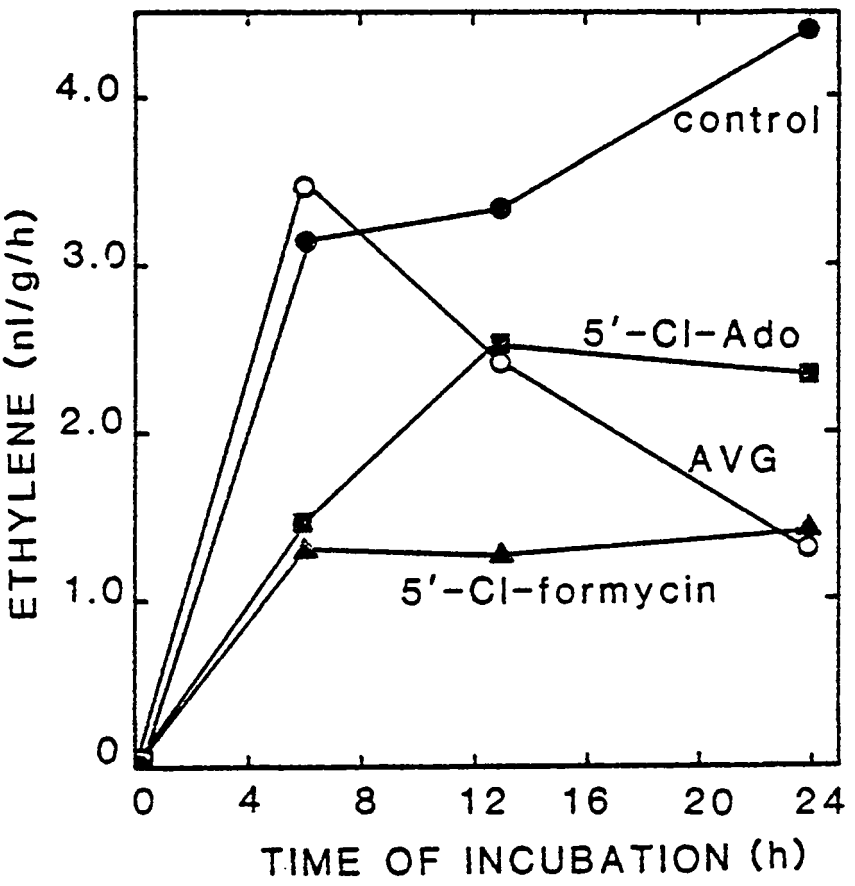

Fig. 11. Time course study of MTA analogs effect on ethylene synthesis in breaker tomato plugs incubated at 20°C. (● - ●) control, (o - o) AVG, (■ - ■) 5-Cl-adenosine, and (▲ - ▲) 5'-Cl-formycin.

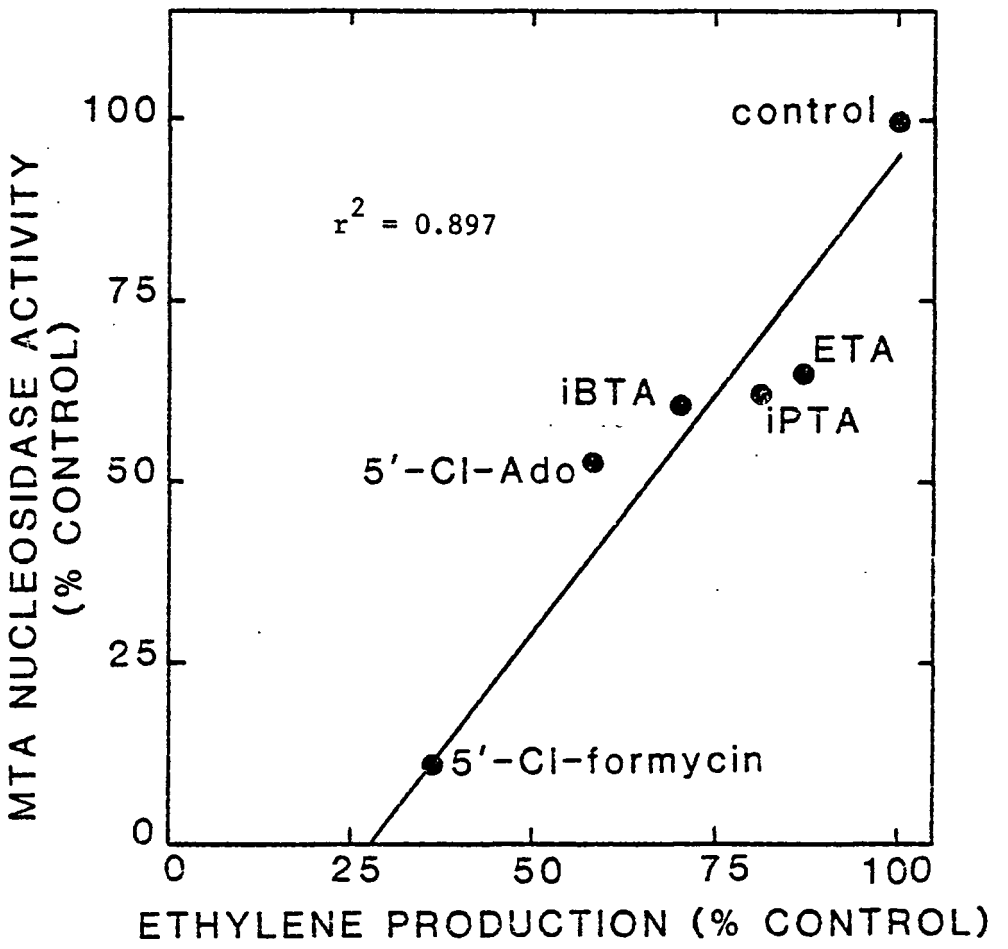

Fig. 12. Fitted linear regression between MTA nucleosidase activity and ethylene synthesis in the presence of MTA analogs.

Table 8. Effect of MTR analogs on MTR kinase activity and ethylene production from breaker stage tomato fruits incubated for 2 h at 30°C for the kinase activity and 24 h at 20°C for ethylene.

Compound[2]	MTR Kinase Activity	Ethylene Production
	% of Control	% of Control
Control	-	100
MTR	52	102
AVG	100	66
iPTR	82	78
ETR	81	100
iBTR	59	65

[$^{14}CH_3$]MTR was present at 25 nmol as were each of the analogs.

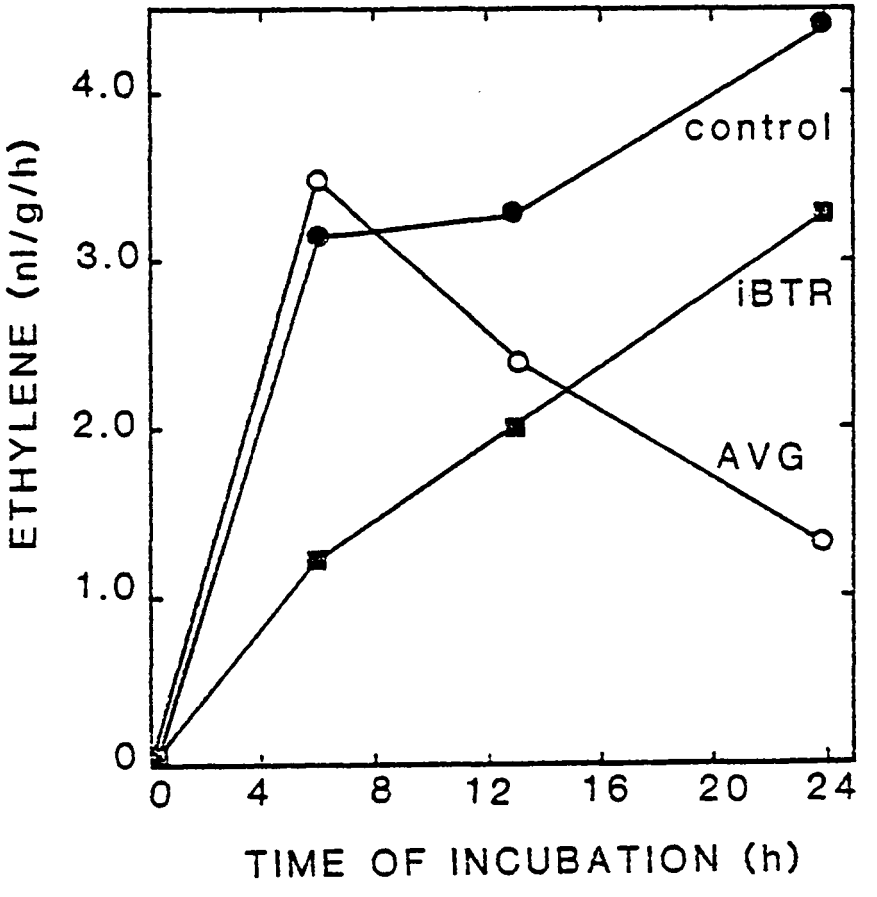

Fig. 13. Time course study of MTR analogs effect on ethylene synthesis in breaker tomato plugs incubated at 20°C. (● - ●) control, (o - o) AVG and (■ - ■) iBTR.

DISCUSSION

During the conversion of methionine to ethylene in ripening fruit, MTA is synthesized stoichiometrically with the production of ACC. The recycling of MTA to methionine has been proposed to be an essential salvage pathway necessary for the synthesis of large quantities of ethylene. We have initiated a detailed investigation to elucidate the contribution made by the methionine salvage pathway to total ethylene production.

MTA nucleosidase and MTR kinase are the first two enzymes involved in this recycling pathway. Since ethylene and ACC both begin to accumulate in mature green to breaker stage tomatoes (14, present study), we had anticipated that both MTA nucleosidase and MTR kinase activities would also be elevated at these stages in order to initiate the recycling of the MTA synthesized. As expected, MTR kinase activity followed this pattern by peaking in extracts prepared from breaker stage fruit. MTA nucleosidase activity, however, exhibited its highest specific activity prior to the mature green stage. These data suggest that MTA may accumulate during the latter stages of tomato ripening due to the declining MTA nucleosidase activity. Efforts, however, to detect elevated levels of MTA at these stages were unsuccessful (data not shown). This suggests that the activity of MTA nucleosidase present in these tissues is sufficient to accommodate the MTA which is synthesized. Whether the fluctuations in MTA nucleosidase and MTR kinase activities are due to changes in the _de novo_ synthesis of

these enzymes or to regulation at the level of enzyme activity has not been determined. Regardless, the alterations in enzyme activities observed during tomato fruit ripening suggest that the regulation of these enzymes may play an important role in regulating the synthesis of methionine, and therefore, ACC and ethylene synthesis.

To examine this possibility further, several analogs of MTA and MTR were synthesized and tested as inhibitors of MTA nucleosidase and MTR kinase activities, and also as potential inhibitors of ethylene biosynthesis. Five analogs of MTA were found to be inhibitors of both MTA nucleosidase activity and ethylene synthesis. Of the MTA analogs examined, 5'-chloroadenosine and 5'-chloroformycin were found to be the most effective inhibitors of MTA nucleosidase activity and of ethylene production. Utilizing these analogs, a direct correlation ($r^2 = 0.897$) was observed between the activity of this enzyme and the ability of fruit plugs to synthesize ethylene (Fig. 12). The data suggest that the recycling of MTA to methionine is required for maximum ethylene synthesis. Our data predicts that even in the complete absence of MTA nucleosidase activity, ethylene synthesis would still occur (25% of normal). This is probably due to the existence of ethylene precursors (methionine, SAM, ACC) whose conversion to ethylene would not be blocked by inhibitors of the nucleosidase. Obviously, before these conclusions can be confirmed, the specificity of these analogs must be established.

Baker et al. (3) observed that tomato fruits at the pink and red stage were relatively insensitive to AVG inhibition of ethylene synthesis during 6 h of tissue incubation. We have observed similar results when tomato plugs in the breaker stage were incubated with AVG (Figs. 11,13). However, we have also observed that as the time of incubation increased ethylene production declined considerably. Based on the data of Baker et al. (3) and on the higher levels of ACC observed during these developmental stages, Boller et al. (5) suggested that the ineffectiveness of AVG at these two stages may have resulted from an AVG insensitive step in the conversion of ACC to ethylene.

Of MTR analogs tested as inhibitors of MTR kinase activity and ethylene biosynthesis, only iBTR caused a significant effect. The decrease in iBTR-induced inhibition of ethylene synthesis with time is probably due to the further metabolism of this compound, since it is a substrate for MTR kinase. The continual investigation of the specific effects of MTA and MTR analogs should help understand the role of the methionine recycling pathway in ethylene biosynthesis during fruit development and ripening.

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CONCLUSIONS

1. This work has demonstrated the existence of a previously unidentified enzyme, 5-methylthioribose kinase in cell-free extracts from several fruit tissues including avocado, pear, apple, strawberry and tomato. This enzyme catalyzed the ATP-dependent phosphorylation of 5-methylthioribose to 5-methylthioribose-1-phosphate and exhibited a temperature optimum of 30°C. An examination of the specific activity of this enzyme from three morphological regions of an avocado fruit revealed that the highest enzyme activity was in the outer exocarp tissues.

2. The kinetics of product formation from MTR-1-P showed that incubation of avocado extract with MTR-1-P yielded two new products, identified as α-keto-γ-methylthiobutyric acid (α-KMB) and α-hydroxy-γ-methylthiobutyric acid (α-HMB).

3. Further metabolism of α-KMB and α-HMB were examined and the data suggest that α-KMB is a precursor of both methionine and α-HMB but that α-HMB cannot be further metabolized at least in the system that was used. It was also observed that α-HMB slightly inhibited the conversion of α-KMB to methionine.

4. The ability of tomato pericarp tissue to metabolize α-KMB and α-HMB to ethylene confirmed that only α-KMB served as substrate for the synthesis of ethylene.

5. 5'-Methylthioadenosine nucleosidase and 5-methylthioribose kinase activities have been examined during tomato fruit development and ripening. The results showed that the highest activity of MTA nucleosidase was observed when fruits were in the small green stage and dropped sharply as the fruits reached the ripening stage.

6. MTR Kinase activity, in contrast, exhibited low activities at the green stage and reached peak activity at the breaker stage. The decline in MTA nucleosidase activity during tomato fruit development and ripening suggest that this enzyme may play an important role in the regulation of ethylene synthesis.

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APPENDIX

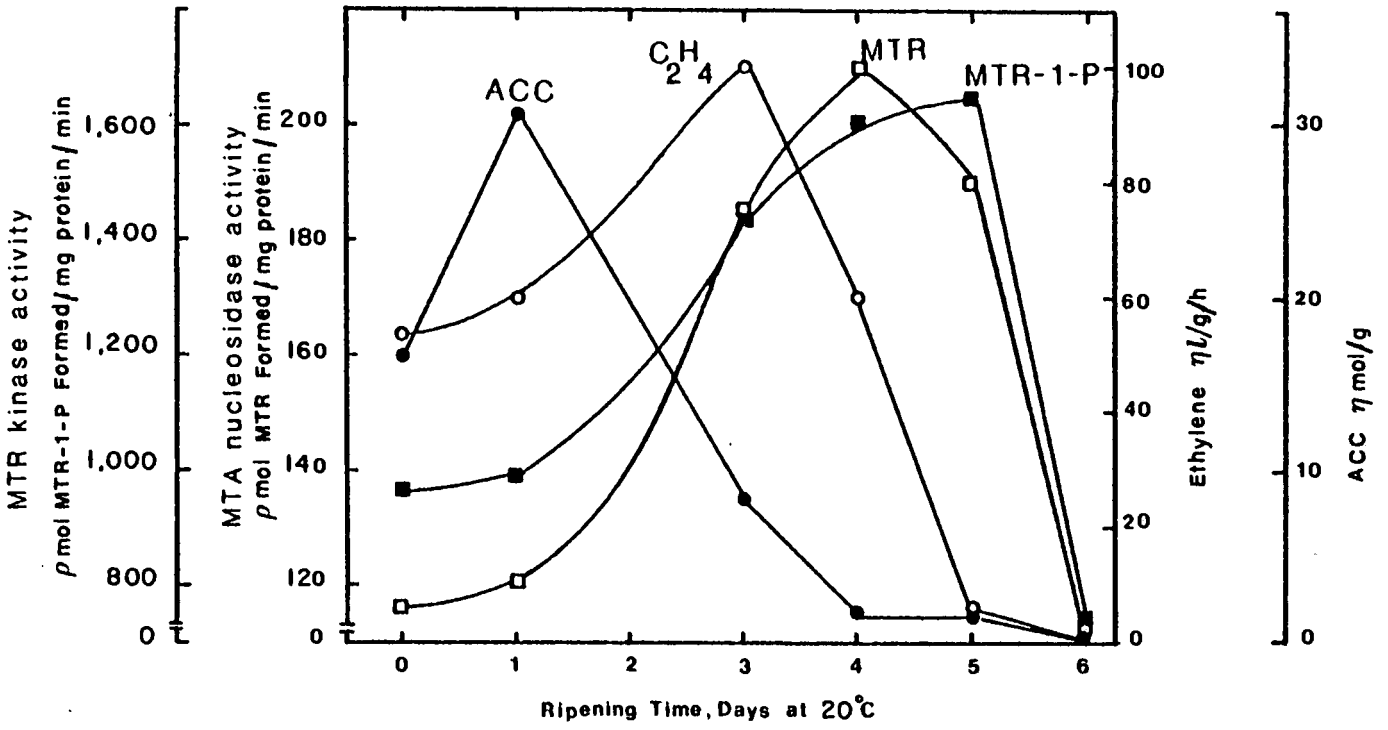

Fig. 14. Changes in MTA nucleosidase and MTR kinase apparent activities in relation to ACC and ethylene levels during ripening of avocado fruits (cv Hass). (□ -□) MTR formed, (■-■) MTR-1-P formed, (● - ●) ACC and (o - o) ethylene.

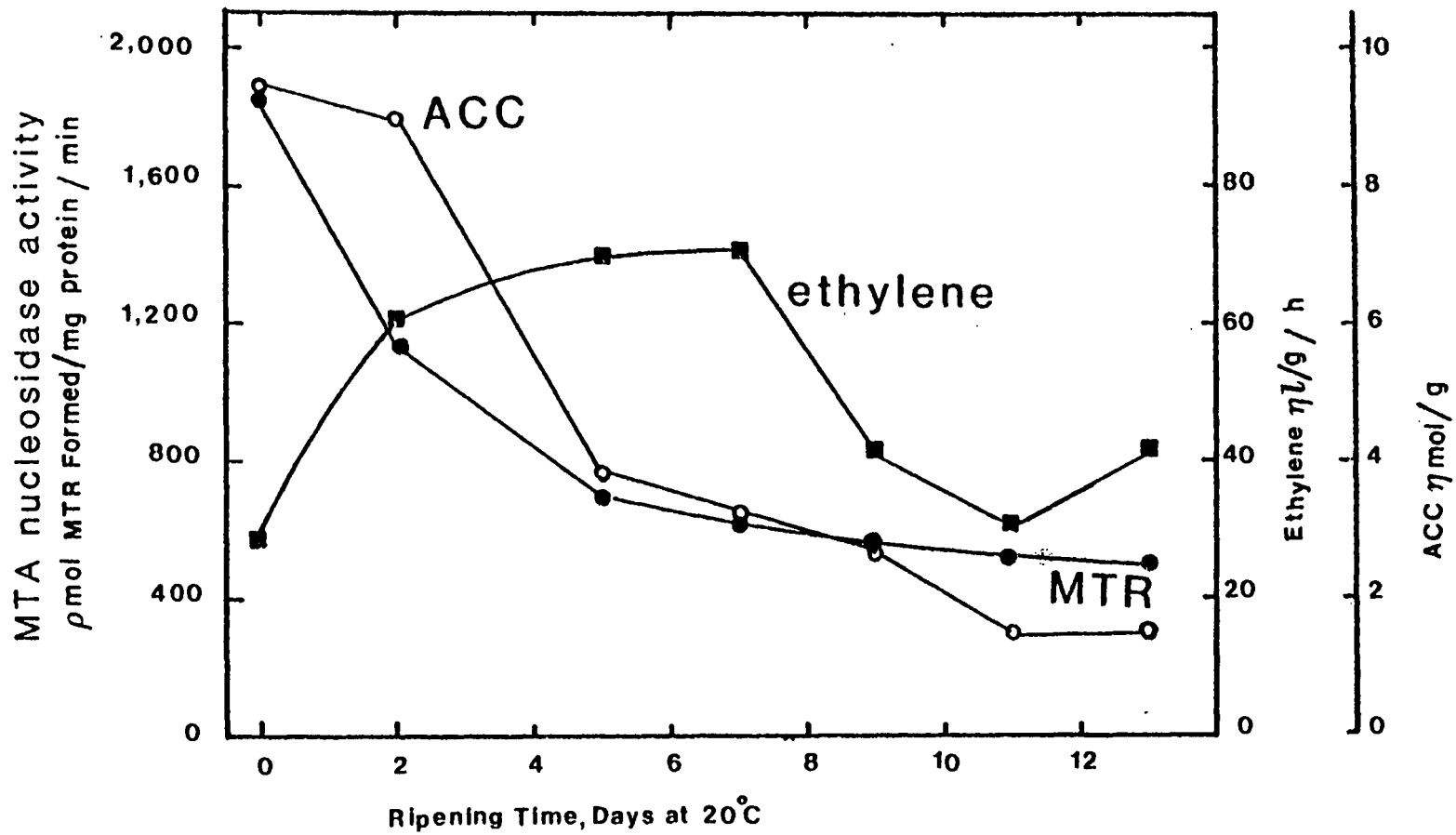

Fig. 15. Changes in MTA nucleosidase apparent activity in relation to ACC and ethylene levels during ripening of pear fruits (cv Bosc). (● - ●) MTR formed, (o - o) ACC and (■ - ■) ethylene.

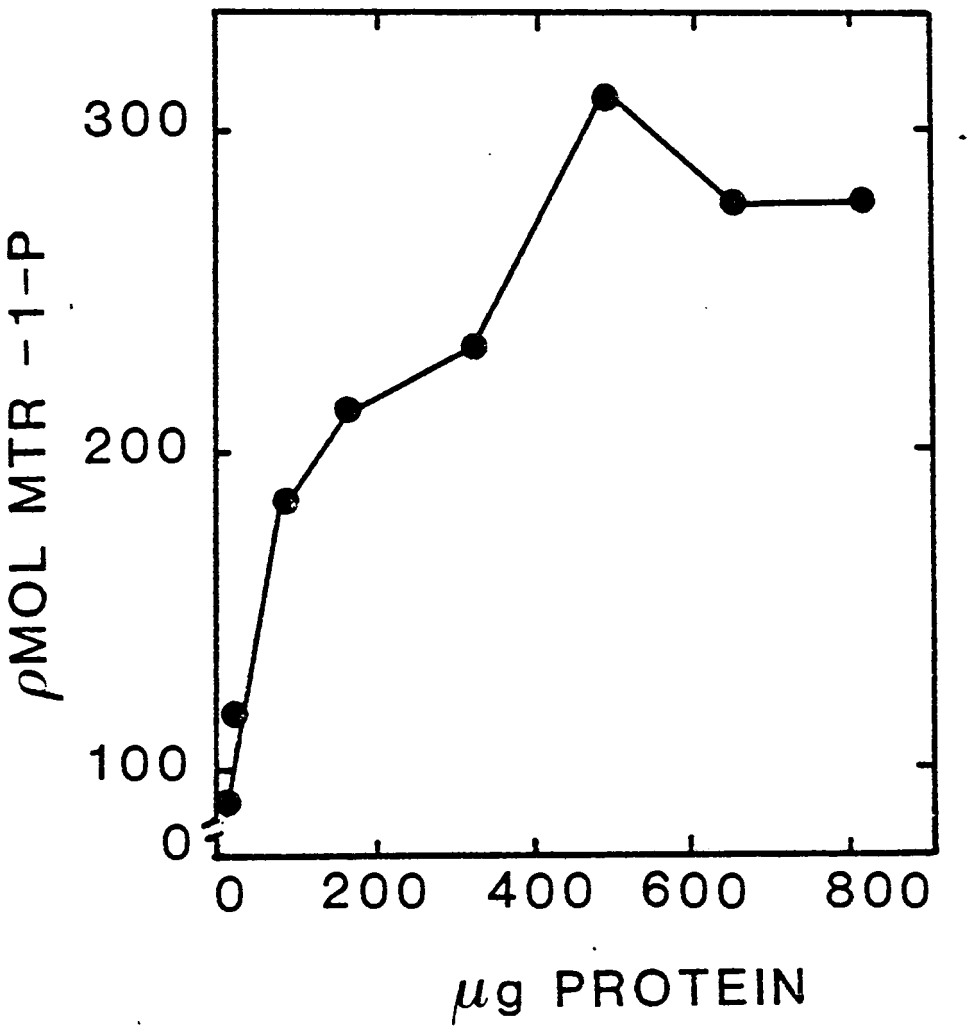

Fig. 16. Relationship between the amount of avocado cell free extract protein added to the reaction mixture and the amount of MTR-1-P formed. Reaction mixtures were incubated for 2 h at 30°C.