AN ABSTRACT OF THE THESIS OF

Lise Marie K. Gander for the degree of Master of Science in Microbiology presented on January 8, 1990.

Title: Methionine Recycling in Fruit: Studies on the Metabolism and Function of Selected Intermediates.

Abstract approved: Redacted for Privacy

Dr. Adolph J. Ferro

This research has focussed upon both the *in vitro* and *in vivo* characterization of the recycling pathway leading from 5'-deoxy-5'-methylthioadenosine to methionine in fruit. In addition to contributing towards polyamine biosynthesis and other important cellular functions, in plant tissues these events also serve to generate the hormone ethylene. A broad range of physiological functions governing plant growth and development, including fruit ripening, are regulated by this compound. Whereas most of the enzymatic steps leading to the synthesis of ethylene from methionine have received considerable attention in efforts to suppress ethylene evolution and thereby control its effects, much less is known concerning the relative contribution of methionine recycling. Furthermore, the complete methionine cycle and the impact of its constituent reactions upon ethylene have yet to be elucidated.

To address these issues *in vitro,* cell-free extracts of two varieties of avocado

and four varieties of pear fruit were utilized. Several methods of tissue extraction, protein quantification, and enzyme purification were investigated and optimized with respect to enzyme activity. Two of the enzymes (5'-deoxy-5'-methylthioadenosine nucleosidase and 5-deoxy-5-methylthio-ribose kinase) involved in the recycling pathway were partially purified and biochemically characterized in each system. Of those compounds examined for their effect on the individual activities of these two enzymes, several were found to have significant influences.

For the *in vivo* work, one variety of suspension-cultured pear cells previously utilized for *in vitro* studies was employed. The cells were found to take up both radiolabelled preparations of methionine and two of its recycling intermediates and convert these compounds into radiolabelled ethylene and metabolites known to participate in methionine recycling. The effect of various compounds upon cellular growth was examined as well. The data suggest both that the recycling pathway is necessary for normal cellular growth and also that these cells serve well to study these events.

Additionally, the analyses of radiolabelled investigations (*i.e.*, enzyme assays and metabolic uptake studies) were modified to utilize a radioisotope detector coupled to a high performance liquid chromatograph (HPLC). This application was found to be rapid, sensitive, cost-effective, and in some cases, provided more information than previously documented methods.

Methionine Recycling in Fruit:
Studies on the Metabolism and Function of Selected Intermediates

by

Lise Marie K. Gander

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed January 8 , 1990
Commencement June 1990

APPROVED :

Redacted for Privacy

Professor of Microbiology in charge of major

Redacted for Privacy

Chairman of the Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented January 8, 1990

Typed by Lise Marie K. Gander

ACKNOWLEDGEMENTS

I am indebted to a broad number of people and organizations whose continued support and patience have made the completion of this work possible.

Dr. A. J. Ferro : for an introduction to SAM, the opportunity to pursue this research, and a strong example of scientific commitment

Dr. Lyle Brown : for computer assistance, moral support, advice

Dr. John Fryer : for a variety of special exceptions, particularly permission to continue research with relatively few changes in laboratory facilities and use of Microbiology Dept. office equipment (Laserprinter)

Dr. David Loomis, Dr. Linda Blythe : for moral support, advice

Dr. Paula Tower : for technical advice, moral support

Nick Hamel, Dave Carlson, Valerie Stallbaumer : for technical assistance

Dept. of Microbiology Office Personnel (especially **Bonnie Casper** and **Joy Asbury**) : for record-fast ordering, moral support, advice

NSF, ARF, NSI : for financial support

*Special thanks to **Stuart Gander** and all the trout I might have fished over - - - they have paid dearly for this degree.*

TABLE OF CONTENTS

Chapter		Page
I.	LITERATURE REVIEW	1
II.	MATERIALS AND METHODS	21
III.	CHARACTERIZATION OF 5'-DEOXY-5'-METHYLTHIOADENOSINE NUCLEOSIDASE AND 5- DEOXY - 5 - METHYLTHIORIKINASE FROM CRUDE FRUIT EXTRACTS OF PEAR (*Pyrus communis* L.) AND AVOCADO (*Persea americana* Mill)	41
IV.	UPTAKE AND ACCUMULATION OF METHIONINE, 5'-DEOXY - 5' - METHYLTHIOADENOSINE, AND 5 - DEOXY - 5 - METHYLTHIORIBOSE IN SUSPENSION CULTURES OF PASSE CRASSANE (*Pyrus communis* L.)	88
V.	REFERENCES	121
VI.	APPENDICES	
	A. PARTIAL PURIFICATION OF 5'-DEOXY-5'-METHYLTHIOADENOSINE NUCLEOSIDASE AND 5 -DEOXY-5 - METHYLTHIORIBOSE KINASE FROM FRUIT EXTRACTS OF PEAR (*Pyrus communis* L.) AND AVOCADO (*Persea americana* Mill) AND PROBLEMS ENCOUNTERED	130
	B. CHROMATOGRAPHIC R_f AND t_R VALUES	142
	C. CHEMICAL STRUCTURES OF VARIOUS PHENOLIC-BINDING RESINS	144
VII.	FINIS	145

LIST OF FIGURES

Figure		Page
I.1.	The interrelationship of methionine and polyamine biosynthesis in plants.	2
I.2.	Methionine cycle in relation to ethylene biosynthesis.	10
III.1.	Effect of incubation buffer pH upon relative MTA nucleosidase activity in crude extracts from two varieties of *Persea americana* Mill and four varieties of *Pyrus communis* L.	48
III.2.	Effect of incubation buffer pH upon relative MTR kinase activity in crude extracts from one variety of *Persea americana* Mill and four varieties of *Pyrus communis* L.	51
III.3.	Effect of incubation temperature upon relative MTA nucleosidase activity in crude extracts from two varieties of *Persea americana* Mill and four varieties of *Pyrus communis* L.	54
III.4.	Arrhenius plots of relative MTA nucleosidase activities.	56
III.5.	Effect of incubation temperature upon relative MTR kinase activity in crude extracts from two varieties of *Persea americana* Mill and one variety of *Pyrus communis* L.	58
III.6.	Arrhenius plots of relative MTR kinase activities.	60
III.7.	Effect of temperature and time upon MTR kinase activity from a crude extract of *Persea americana* Mill, cv Hass.	62
III.8.	Heat shock purification of MTA nucleosidase from crude extracts of *Persea americana* Mill, cv Hass and two varieties of *Pyrus communis* L.	64
III.9.	Heat shock purification of MTR kinase from two varieties of *Persea americana* Mill.	66
III.10.	Double-reciprocal plots of MTA nucleosidase activity vs. 5'-deoxy-5'-methylthioadenosine concentration in crude extract of *Persea americana* Mill and *Pyrus communis* L.	67

LIST OF FIGURES (cont.)

Figure		Page
III.11.	Chemical structures of 5'-deoxy-5'-methylthioadenosine (MTA) analogs listed in Table IV.3.	71
III.12.	HPLC System I: representative chromatograms of enzyme assay substrates and products.	72
III.13.	HPLC System II: representative chromatograms of enzyme assay substrates and products.	73
III.14.	HPLC System III: representative chromatograms of enzyme assay substrates and products.	74
III.15.	HPLC System IV: representative chromatograms of enzyme assay substrates and products.	75
IV.1.	Growth profiles of Passe Crassane cells cultured in three different media.	90
IV.2.	Viability profiles of Passe Crassane cells cultured in three different media.	91
IV.3.	Relationship between cell number and fresh and dry weights of Passe Crassane suspension cells cultured in growth medium.	92
IV.4.	Effects of MET, MTA, ETR, PUT, and ACC upon the growth of Passe Crassane fruit cells cultured in growth medium.	94
IV.5.	Ethylene production by suspension pear cells cultured in growth medium with and without the presence of the auxin IAA.	96
IV.6.	Ethylene production by suspension pear cells cultured in aging medium with and without the presence of varying concentrations of the auxin IAA.	97
IV.7.	Effects of putrescine, spermidine and spermine upon auxin-induced ethylene production in Passe Crassane suspension cells cultured in aging medium.	98

LIST OF FIGURES (cont.)

Figure		Page
IV.8.	Uptake of L-[3,4-^{14}C]-methionine by a log-phase suspension of Passe Crassane pear cells cultured in growth medium.	100
IV.9.	Uptake of L-[3,4-^{14}C]-methionine by a stationary phase suspension of Passe Crassane pear cells cultured in growth medium.	101
IV.10.	Uptakes of [$^{14}CH_3$]-MTA and [$^{14}CH_3$]-MTR by stationary phase suspensions of Passe Crassane pear cells cultured in growth medium.	102
IV.11.	Uptakes of L-[3,4-^{14}C]-MET, [$^{14}CH_3$]-MTA and [$^{14}CH_3$]-MTR by stationary phase suspensions of Passe Crassane pear cells cultured in aging medium.	103
IV.12.	HPLC chromatogram of L-[3,4-^{14}C]-MET metabolism by stationary phase suspensions of Passe Crassane.	112
IV.13.	HPLC chromatogram of [$^{14}CH_3$]-MTA metabolism by stationary phase suspensions of PasseCrassane.	113
IV.14.	HPLC chromatogram of [$^{14}CH_3$]-MTR metabolism by stationary phase suspensions of PasseCrassane.	114
VI.C.1.	Chemical structures of various phenolic-binding resins.	144

LIST OF TABLES

Table		Page
III.1.	Typical protein concentrations and enzyme activities of both MTA nucleosidase and MTR kinase in crude extracts of five varieties of *Pyrus communis* L.	44
III.2.	Typical protein concentrations and enzyme activities of both MTA nucleosidase and MTR kinase in crude extracts of two varieties of *Persea americana* Mill.	45
III.3.	Effect of MTA analogs upon relative MTA nucleosidase activity in crude extracts of three varieties of *Pyrus communis* L.	69
III.4.	Effect of MTA and ATP analogs upon enzyme activities of both MTA nucleosidase and MTR kinase in crude extracts of *Persea americana* Mill, *Pyrus communis* L., and *Escherichia coli.*	70
IV.1.	Incorporation of [3,4-^{14}C]-Methionine and [$^{14}CH_3$]-Methylthioadenosine by Stationary Phase Suspension Cells of *Pyrus communis* L. (cv Passe Crassane) Cultured in Aging Medium.	107
IV.2.	Products of [3,4-^{14}C]-MET, [$^{14}CH_3$]-MTA, and [$^{14}CH_3$]-MTR Metabolism in Cell Suspensions of Passe Crassane *(Pyrus communis* L.) Cultured in Aging Medium.	108
IV.3.	Putative Identity of Some Products of [3,4-^{14}C]-MET Metabolism in Cell Suspensions of Passe Crassane*(Pyrus communis* L.) Cultured in Aging Medium.	109
IV.4.	Putative Identity of Some Products of [$^{14}CH_3$]-MTA, and [$^{14}CH_3$]-MTR Metabolism in Cell Suspensions of Passe Crassane *(Pyrus communis* L.) Cultured in Aging Medium.	110
VI.A.1.	Representative partial purifications of MTA nucleosidase from extracts of *Pyrus communis* L., cv Packham Triumph and *Persea americana* Mill, cv Fuerte.	132

LIST OF TABLES (cont.)

Table		Page
VI.A.2.	Representative partial purifications of MTR kinase from extracts of *Pyrus communis* L., cv Packham Triumph and *Persea americana* Mill, cv Fuerte.	133
VI.A.3.	Representative concentration attempts of MTR kinase from *Persea americana* Mill, cv Hass.	136
VI.B.1.	Chromatographic t_R and R_f values.	142

Methionine Recycling in Fruit:
Studies on the Metabolism and Function of Selected Intermediates

CHAPTER I

LITERATURE REVIEW

That a salvage pathway for methionine (MET) might exist in plant tissues originated from studies elucidating the biosynthesis of the plant hormone ethylene (C_2H_4). This naturally occurring compound exists as a gas under normal physiological conditions and has been implicated in a broad range of functions governing plant growth and development, including inhibition of both DNA synthesis and cell division (10a). Other effects under C_2H_4 influence include seed germination, seedling growth, leaf abscission, floral induction, root growth, growth of leaves, a variety of stress phenomina, and the regulation of ripening, aging and senescence (for general reviews, see 80, 124, 148). Morphological changes associated with C_2H_4 are attributed to its effects upon cell division, cell expansion and auxin transport (10a, 10b, 12, 91), and may reflect differential gene expression (24, 36, 74, 81-83, 95, 126).

Catabolism of S-Adenosyl-L-Methionine in Plants

The interrelationship of S-adenosyl-L-methionine (SAM) catabolic pathways showing the involvement of this compound in reactions leading to methylations, C_2H_4 biosynthesis and polyamine (PA) formation is depicted in Figure I.1.

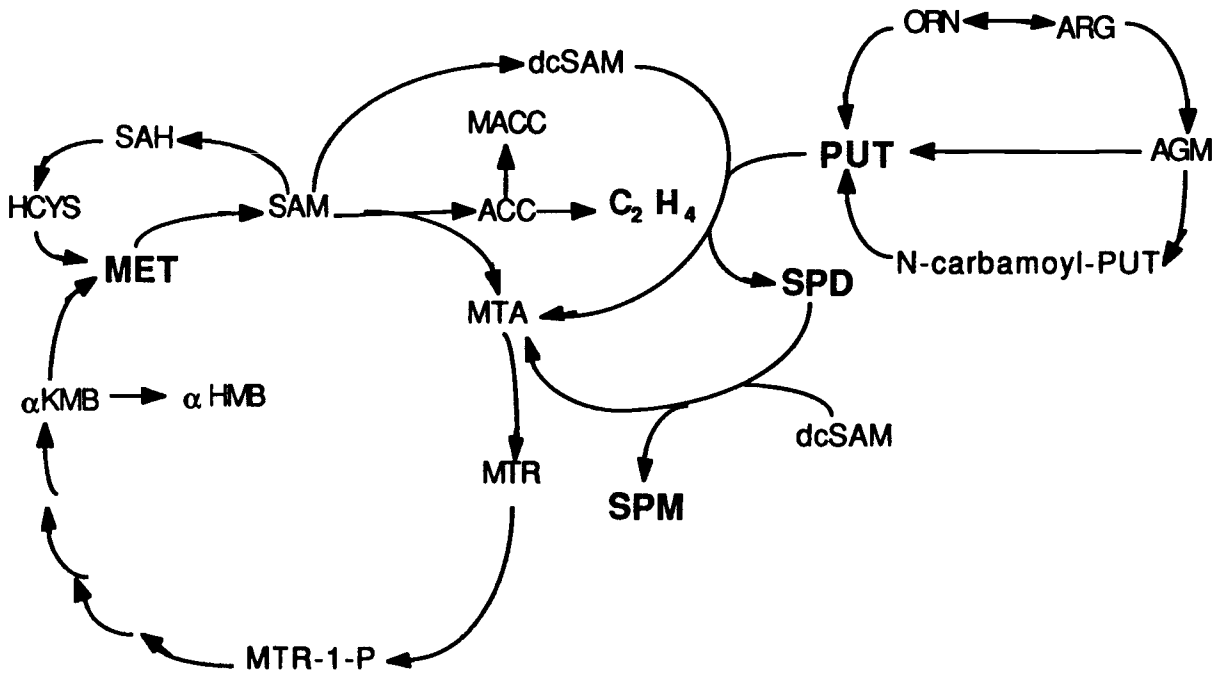
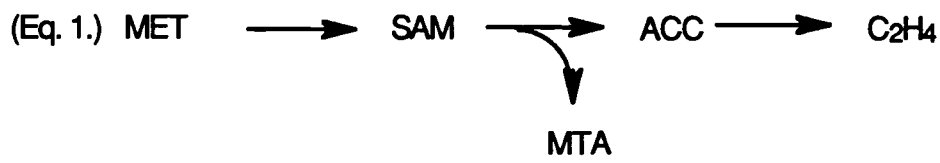


Figure I.1. The interrelationship of methionine recycling and polyamine biosynthesis in plants. Methionine, ethylene, and the predominant polyamines putrescine, spermidine and spermine appear in bold type. ACC = aminocyclopropanoic acid; ARG = arginine; AGM = agmatine; C2H4 = ethylene; dcSAM = decarboxylated SAM; HCYS = homocysteine; HMB = hydroxymethylthiobutyrate; KMB = ketomethylthiobutyrate; MACC = malonylaminocyclopropanoic acid; MET = methionine; MTA = methylthioadenosine; MTR = methylthioribose; MTR-1-P = methylthioribose-1-phosphate; ORN = ornithine; PUT = putrescine; SAH = S-adenosylhomocysteine; SAM = S-adenosylmethionine; SPD = spermidine; SPM = spermine. Reactions are discussed further in the text.

1. Ethylene Biosynthesis and Methionine Recycling

Plants produce both natural and stress-induced C_2H_4 from MET via the compounds SAM and 1-aminocyclopropane-1-carboxylic acid (ACC), as shown in Equation 1. The nucleoside 5'-deoxy-5'-methylthioadenosine (MTA) also is generated in the reaction. Although C_2H_4 is not metabolized further, its precursor ACC may be converted to malonyl-1-aminocyclopropane-1-carboxylic acid (MACC); however, this product appears to be biologically inactive and therefore does not contribute to C_2H_4 biosynthesis (148).



It has been shown that C_2H_4 is produced from essentially all parts of higher plants, including leaves, stems, buds, flowers, fruits and roots (80). In addition, the rate of C_2H_4 released, although variable with conditions and tissue type, may be both substantial and maintained for extended time periods. The fruits of Golden Delicious apples have been found to produce C_2H_4 at an hourly rate of 5.0 nmol / g during ripening (17). However, the amount of available MET in these tissues is only in the order of 60.0 nmol / g (17). For C_2H_4 biosynthesis to exceed 12 h, as it does under these conditions, a MET recycling pathway must be operative. Furthermore, studies with [^{35}S]-MET demonstrated that rather than being released as a volatile sulfoxide compound such as methanethiol or methyl disulfide as in model chemical (148) and some bacterial (62), fungal (53) and mammalian systems (132), the methylthio (CH_3S) group of higher plant MET is incorporated

into MTA (17).

Thus, Baur and Yang proposed in 1972 that the sulfur atom of MTA is recycled back into MET in order to sustain continual physiological C_2H_4 production (17). Subsequently, Murr and Yang confirmed that [$^{14}CH_3$]-MTA is recycled to [$^{14}CH_3$]-MET (93), and it was established that both the CH_3S and ribose moieties of 5-deoxy-5-methylthioribose (MTR), the hydrolyzed form of MTA, also are incorporated as units into this amino acid (3, 4, 90, 123, 152).

To date, the steps involved in MET recycling have not been elucidated fully in any system. This may be due in part to the added complexity that several of its intermediates play a role in other pathways as well. Three prominent examples are MET, the sulfonium compound SAM and its first metabolite in MET recycling, MTA. The major metabolic roles of MET are 1). utilization for protein synthesis; 2). conversion to SAM, and 3). transamination to 3-methylthiopropionate (MTP; 128).

Since its discovery in 1953 (25), SAM has been found to be involved in a wide variety of reactions, including methylations which occur in associaton with the production of S-adenosylhomocysteine (SAH); the biosynthesis of the PAs spermidine (SPD) and spermine (SPM), initiated with the formation of decarboxylated S-adenosylmethionine (dcSAM); MET recycling and C_2H_4 biosynthesis. In fact SAM is second only to ATP with reference to the number of reactions in which it participates. MTA is synthesized stoichiometrically during the formation of three different compounds: ACC, SPD, and SPM. This is of interest, given the facts that once SAM is decarboxylated it is committed to PA rather than C_2H_4 biosynthesis, and the physiological effects of these systems are antagonistic (more on this to follow).

It should be emphasized that cellular concentrations of MTA are characteristically low, indicating that the compound, regardless of its source, does not accumulate, but rather is metabolized rapidly (29, 93, 119, 123, 151). Also, since high concentrations of MTA are inhibitory to a variety of intracellular reactions including transmethylations and PA biosynthesis (119), the conversion of MTA to MET provides the cell with a means of removing MTA by conserving it as MET. However, since excessive levels of MET produce both growth reduction and tissue damage (19), accumulation of this compound is considered toxic. That MET has many metabolic fates is advantageous. Thus, the efficient removal of MTA through MET recycling may serve as a cellular regulatory mechanism in addition to contributing towards C_2H_4 biosynthesis.

2. Biosynthesis of Putrescine, Spermidine and Spermine

SPD and SPM arise from another PA, putrescine (PUT), itself generated in plants from the amino acid arginine (ARG) via either of two alternative decarboxylation reactions (46, 72). The first involves the formation of agmatine (AGM) by arginine decarboxylase (ADC) and either subsequent loss of urea to form PUT directly from this compound, or the biosynthesis of the PUT precursor N-carbamoyl-putrescine. The other is characterized by the initial release of urea to produce ornithine (ORN) which is decarboxylated to PUT by ornithine decarboxylase (ODC). In both mammalian and bacterial systems, ODC is the preferred pathway (46, 136).

However, in plants, both ODC and ADC are active, and experimental evidence suggests that each may predominate in a given species (130, 146). ARG and ORN are themselves metabolically interconvertible via the urea cycle.

3. Regulatory Roles of Polyamines and Ethylene

The regulatory role of naturally occurring PAs, such as PUT, SPD and SPM, in growth, proliferation, and replication of plant and other cells is well established (46, 136). Increased PA synthesis in rapidly dividing cells has been reported in both microorganisms (136) and plant tumors (131). Specific inhibition of PA biosynthesis has been seen to retard tomato fruit growth in a reversible manner (30). That this inhibition blocks cells in the G1 stage of the cell cycle (23, 114) implies that the processes of both nucleic acid synthesis and mitosis are dependent upon the presence of PAs. In further support of a polyamine-nucleic acid interaction, SPM has been reported to be concentrated in the nucleus and to play an important role in the configuration of DNA (83, 130, 136).

Polyamines also appear to have a stabilizing role in plants, especially under certain conditions of stress, such as low temperature, K^+ and Mg^{2+} deficiencies, and low pH (71). Presumably to counterbalance the increased anion concentration during these periods, PAs, especially PUT and SPD, have been found to accumulate (42, 71, 130, 149). These cations also have been implicated in increased membrane thermostability (9), another stabilizing property. Interestingly, stress also induces C_2H_4 formation.

Ethylene has been known to suppress growth in etiolated pea seedlings and numerous other plants (10a, 10b, 80, 124, 148). Apelbaum and Burg showed that this effect is exerted through both the inhibition of cell division in the meristems of root and shoot apices and the slowing down of the rate of cell expansion in these tissues (10a, 10b). They found that C_2H_4 interferes with cell division by blocking a stage prior to prophase, and established the existence in these tissues of a quantitative relationship between inhibition of DNA synthesis, cell division and growth caused by this hormone (10a). It is now generally believed that ethylene inhibits plant growth predominantly through inhibition of DNA synthesis (10a, 10b, 12).

Since polyamine metabolism is associated with cell growth and has been implicated as prerequisite for DNA synthesis (46, 136), it has been suggested by Apelbaum *et al.* that C_2H_4 inhibits DNA synthesis by blocking PA formation via inhibition of ADC and SAMdc activity (10a, 12). C_2H_4-promoted, reversible inhibition of both these enzymes and ODC has been documented (12, 61, 98). Thus, the C_2H_4-mediated inhibition of these processes may be responsible for, rather than serve as a consequence of, the inhibition of cell division.

Icekson *et al.* noted that in additon to inhibiting key PA enzymes, physiological concentrations of exogenously administered C_2H_4 produce an accompanying increase in both the level of cadaverine (a polyamine generally found in much lower concentrations than those of PUT, SPD, or SPM) and the activity of lysine decarboxylase (LDC), the enzyme responsible for its formation (60). They postulated that this effect is of a compensatory nature, occurring as a response to the C_2H_4-promoted inhibition of other PA biosynthetic enzymes. That PA depletion induces enhanced synthesis and accumulation of cadaverine in cultured Ehrlich ascites carcinoma cells was established earlier by Alhonen-Hongisto and Janne (6). Apparently, the requirement for PUT, SPD, and SPM in eukaryotic cells may be supplied, at least temporarily, by related amines such as cadaverine and its aminopropyl derivatives (6, 7). The effect also has been observed in LDC mutants of *E. coli* (49), suggesting two general paths for lysine: one for cadaverine synthesis (under conditions of PA starvation) and the other for newly synthesized proteins (under conditions of normal PA levels). The hormonal control of PAs by endogenous ethylene is implicated.

PAs may exert a regulatory role upon C_2H_4 evolution as well. There is some evidence that changes in endogenous SPM levels are involved in the control of both plant ovary senescence and the processes of fruit set and

development (11, 26, 37); three events generally associated with C_2H_4. Furthermore, exogenously administered PUT, SPD and SPM are known to inhibit ACC synthase (43), the enzyme catalyzing the formation of both ACC and MTA, and have been reported to markedly suppress the wound-induced enzyme (59). This is of interest because the substrate for this reaction, the compound SAM, serves as a common precursor for both ethylene and PA production. The fate of SAM is critical, as it represents a pivotal point between these two pathways. Thus, inhibition of ACC synthase activity or suppression of its induction would favor PA biosynthesis by freeing more SAM for subsequent decarboxylation. Conversely, high ACC synthase activity, such as that observed during periods of elevated C_2H_4 production, would reciprocally inhibit PA synthesis (12).

Another indication of control at this level lies in the finding by Apelbaum *et al.* that PAs at a concentration of 5 mM greatly inhibit the incorporation of [^{14}C]-leucine and [^{3}H]-uridine into protein and RNA in apple fruit discs (13). They suggested that PAs might inhibit the formation of ACC and consequently C_2H_4, by interference with *de novo* synthesis of ACC synthase. Induction of new enzymes also has been implicated in auxin-induced ethylene biosynthesis, based on two features of this process: inhibitors of protein and RNA synthesis retard the effect, and the stimulation of C_2H_4 by auxin is characterized by a substantial lag period (75). Recent studies regarding the C_2H_4-promoted accumulation of specific mRNAs have indicated that some plant defense genes (*e.g.*, chitinase, 4-coumarate, CoA ligase, and L-phenylalanine ammonia lyase) are regulated by this compound (24, 36).

Increasing evidence suggests that the pleiotropic effects of C_2H_4 upon plant growth and development reflect differential gene expression. C_2H_4-regulated gene transcription has been reported in carrot roots (95) and the fruits

of both avocado (28) and tomato (82). Rapid induction (*i.e.*, within 1-3 h of exposure to C_2H_4) of C_2H_4-regulated genes such as those involved in senescence and fruit ripening has been reported in both carnation flower petals (74) and tomato fruits (81, 88, 126). However, for climacteric fruits such as avocado, a more prolonged C_2H_4 exposure (*i.e.*, 24 h) appears to be required for this effect (140).

Enzymatic Steps of The Methionine Cycle and Ethylene Biosynthesis

The MET recycling pathway, showing chemical structures of all known principal intermediates is diagrammed in Figure I.2. At least six, possibly eight enzymes are believed to participate in the reactions involved in plant tissues. To date, the steps converting plant 5-deoxy-5-methylthioribose-1-phosphate (MTR-1-P) to the oxidized, deaminated MET precursor, α-keto-γ-methylthiobutyrate (2-keto-4-methylthiobutyrate; αKMB) have not been elucidated. However, the analogous reactions in both rat liver and the bacterium *Klebsiella pneumoniae* have been shown to involve an initial isomerization of the cis-diol MTR-1-P to its open ring, trans-diol form, 5-deoxy-5-methylthioribulous-1-phosphate (MTRu-1-P; 44, 139). Two additional intermediates linking MTRu-1-P to αKMB were found, but not identified by Trackman and Abeles in the mammalian system (139). The first of these is likely to be 2,3-diketo-5-deoxy-5-methylthiopentane-1-phosphate, recently isolated by Furfine and Abeles as an MTRu-1-P catabolite in *K. pneumoniae* (44). These researchers also showed that the immediate precursor of αKMB is a dephosphorylated compound which undergoes oxidation to form simultaneously both formate and MTP in addition to αKMB (44). The reaction in *K. pneumoniae* is noteworthy in that it represents the only reported case

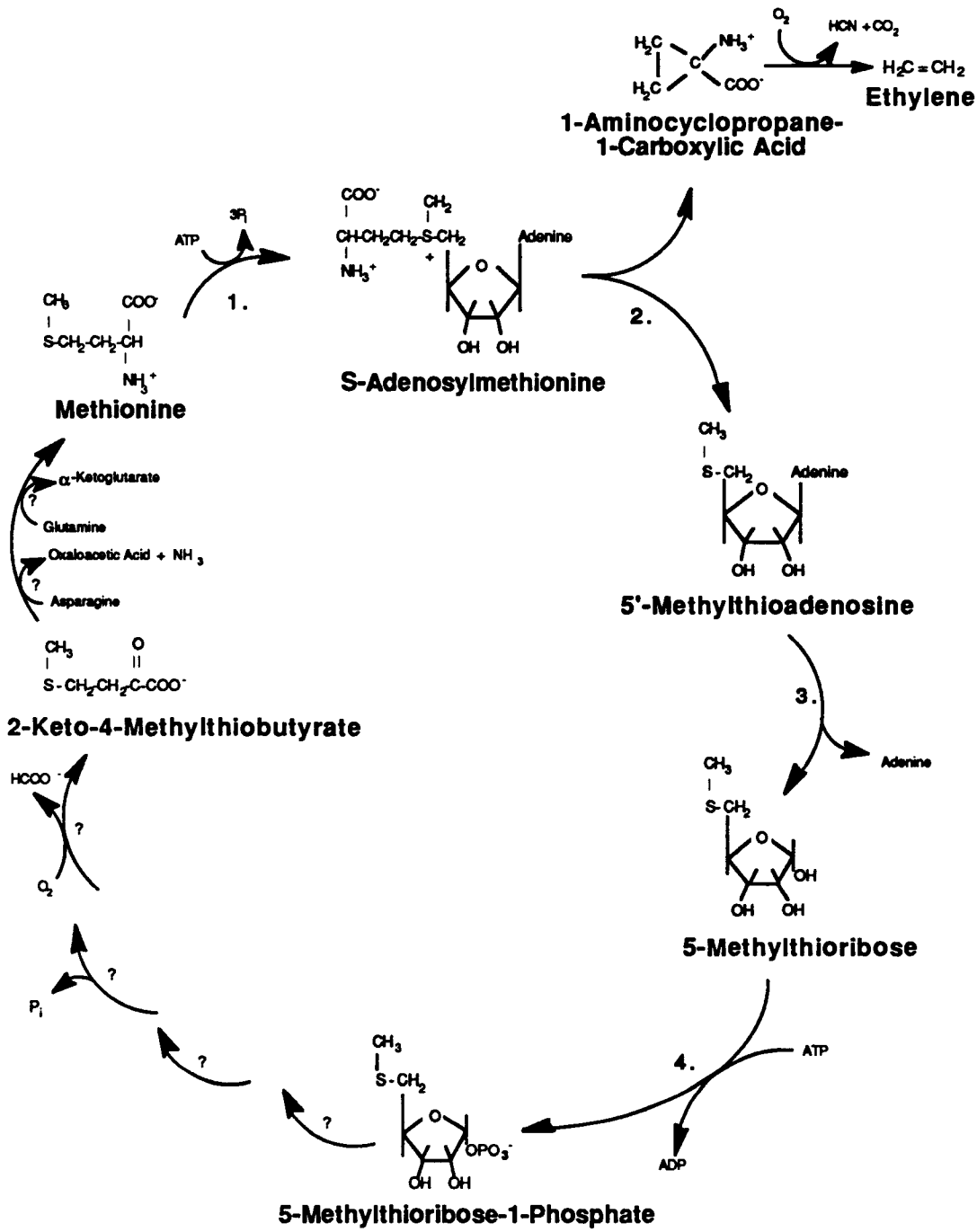


Figure I.2. Methionine cycle in relation to ethylene biosynthesis. Numbered reactions (see text) indicate those steps for which enzymes have been defined and at least partially characterized in higher plant systems. The transamination reaction converting 2-keto-4-methylthiobutyrate to methionine is believed to involve either (but not both) asparagine or glutamine.

where MTP is not formed from free αKMB. In contrast, studies utilizing both [^{14}C]-αKMB and extracts of either rat liver (132), *Xanthomonas campestris* (110), or *Saccharomyces cerevisiae* (87) suggest that MTP derives from the oxidative decarboxylation of αKMB.

Kushad *et al.* (69) demonstrated that in cell-free extracts of avocado either MTR plus ATP or MTR-1-P alone were metabolized to αKMB and its hydroxylated counterpart, α-hydroxy-γ-methylthiobutyrate (αHMB). Of these two compounds, only αKMB was found to be metabolized further to MET; a process which also released αHMB. In addition, αHMB inhibited the conversion of αKMB to MET. However, since both [U-^{14}C]-αKMB and [U-^{14}C]-MET, but not [U-^{14}C]-αHMB, were converted to C_2H_4 in tomato pericarp tissue (69), it was concluded that αHMB has no direct involvement in either MET recycling or C_2H_4 biosynthesis.

The final conversion of αKMB to MET in plant, as in mammalian tissue, is believed to be catalyzed by either glutamine or asparagine transaminase (31). The reverse reaction, *i.e.,* conversion of MET + glutamate to αKMB + glutamine, has been shown to occur in fungi (112), bacteria (113), and rat liver (38). Although considered a simple reaction, to date little research has been directed toward the biochemical characterization or purification of the enzyme(s) involved in this step.

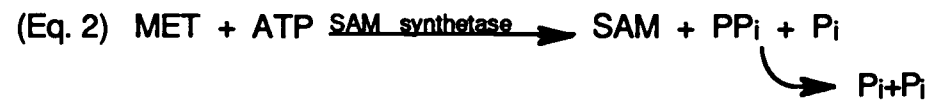
Likewise, the enzyme system responsible for the conversion of ACC to C_2H_4 has yet to be isolated *in vitro*. This is due to the fact that the enzyme is both labile and associated with an integral membrane (5). Researchers have

labelled this "ethylene forming enzyme" or EFE. Its activity *in vivo* is known to be oxygen-dependent. Based upon the chemical model of cyclopropylamine which is oxidized to C_2H_4 via a nitrenium ion intermediate Yang and Hoffman suggested that ACC is oxidized by "ACC hydroxylase" to N-hydroxy-ACC, which then fragments to C_2H_4 and cyanoformic acid (148). Since cyanoformic acid is labile, it would be expected to degrade readily to CO_2 and HCN. Using etiolated mung bean and vetch seedlings, Peiser *et al.* confirmed that CO_2 and HCN indeed evolve from ACC, and further proposed that the mechanism relies upon two sequential one-electron oxidation steps via amine cation radicals (102). Since these reactions are analogous to those involved in amine oxidase catalysis, they suggested that EFE is an oxidase, rather than an hydroxylase (102).

The four enzymatic steps known to participate in MET recycling, and therefore C_2H_4 biosynthesis, in higher plants for which enzymes have been defined and at least partially characterized are discussed below. Although most researchers consider that the degradation of MTA represents the first step in this pathway, the cycle will be presented here as going from MET back to MET with the enzyme reactions numbered accordingly.

1. S-Adenosylmethionine Synthetase

SAM is formed from MET by a single enzyme requiring the splitting of all three phosphate groups from its second substrate, ATP; the only known reaction of its kind to form pyrophosphate (PP_i) as an intermediate in the release of three inorganic phosphate (P_i) moieties. The enzyme (EC 2.5.1.6.) is known by three different names: SAM synthetase (the name by which it will be referred in this text), MET adenosyltransferase, or ATP:L-MET-S-adenosyltransferase. The reaction catalyzed by this enzyme is shown in Equation 2.



Sam synthetase has been at least partially purified from pea seedlings, *E. coli* , yeast, and rat tissue (1, 58). An unusual characteristic of the enzyme is its species- and tissue-specific existence in multiple forms: although pea seedlings, *E. coli* and extracts of rat kidney, heart and brain appear to possess but one form, *Saccharomyces cerevisiae* has two, and rat liver contains three isozymes (58). The three forms have been named I, II, and III. Only form III has been purified to homogeneity (58). Using gel filtration chromatography, the following molecular weight values were estimated for a rat liver (partially) purified preparation: form I = 208,000, form II = 120,000, and form III = 2 subunits of 47,000, yielding a native value of 94,000 daltons (58). All three demonstrated Michaelis-Menten kinetics, with K_m values for MET of 42 and 5.4 μM for forms I and II, respectively, and $S_{0.5}$ for MET = 205 μM for form III (58). Analogs of MET were found to be inhibitory to forms I and II only (58).

SAM synthetase from higher plant tissues has yet to be studied extensively. However, experimental evidence suggests that the plant enzyme shares some characteristics with those from other sources. The partially purified enzyme from pea seedlings resembles form I from rat liver in that it has a similar K_m value for MET (0.4 mM) and is inhibited by high levels of SAM (1). Konze and Kende used crude extracts from the buds of morning glory plants to show that although SAM synthetase has roughly twice the affinity for MET (K_m = 0.37 mM) than it does for selenomethionine (SeMET; K_m = 0.61 mM), the maximal rates of these reactions share the opposite relation-ship: V_{max} = 0.16 and 0.31 μmol h^{-1} mg^{-1} protein, respectively (64). This suggests that SeMET may be a better substrate for the enzyme than is MET. However,

since in model systems involving oxidation by free radicals SeMET is a less effective C_2H_4 precursor, the researchers used these data to conclude that the activation of MET by SAM synthetase and the formation of SAM (rather than its seleno analog) are more likely to be involved in C_2H_4 biosynthesis.

2. 1-Aminocylopropane-1-Carboxylic Acid Synthase

ACC synthase (S-adenosyl-L-methionine methylthioadenosine-lyase; EC 4.4.1.14) is a pyridoxal phosphate-dependent enzyme (21, 151), responsible for the formation from SAM of both ACC and MTA. The reaction is shown in Equation 3. An unusual type of SAM cleavage is involved, in which its four-carbon amino acid component is released with the concomitant formation of a cyclopropane ring. It is believed that rather than being decarboxylated to accomodate PA biosynthesis, SAM instead binds to the pyridoxal phosphate group of ACC synthase by forming a Schiff's base, which is then degraded to ACC and MTA (4, 119, 148, 151). Yu *et al.* were the first to suggest that the conversion of SAM to ACC and MTA occurs as a typical γ-elimination (1,3-elimination), facilitated by the pyridoxal enzyme and involving a carbanion intermediate (151). The positive sulfonium ion thermodynamically destabilizes SAM, thereby promoting the intramolecular nucleophilic displacement reaction by the carbanion which yields ACC and MTA. Hyodo and Tanaka have postulated that the amino group of (poly)amines may prevent SAM from reacting with the enzyme by interfering with the prosthetic group (59).

$$\text{(Eq. 3)}\quad \text{SAM} \xrightarrow{\text{ACC synthase}} \text{ACC} + \text{MTA}$$

The enzyme is key for a variety of reasons: it is induced by various stimuli; it may regulate the rate of C_2H_4 biosynthesis [ACC synthase generally is

considered to be the rate-limiting step (21, 151)], and it is located at the branch point of SAM metabolism, where SAM can be utilized for C_2H_4 production, PA formation or transmethylation. Studies of this enzyme have shed the most light on the interdependent relationship of C_2H_4 and PA biosynthesis.

As mentioned earlier, from a physiological perspective, the effects of these pathways are antagonistic: C_2H_4 induces senescence and suppresses growth, while PAs retard senescence and have a metabolic association with growth (10a, 13, 43, 46, 80, 124, 131, 148). Evidence suggests a feedback control mechanism, since each pathway, once initiated, tends to shut off the other (46). In both higher plant tissues (including leaves, fruit discs, petals and hypocotyls) and protoplasts derived from them, PAs such as PUT, SPD and SPM have been shown to inhibit C_2H_4 evolution (11, 13, 37, 43, 135), an effect seen to shunt the accumulation of 3,4-[^{14}C]-MET from [^{14}C]-ACC (and hence, [^{14}C]-C_2H_4) into [^{14}C]-SPD in orange peel discs (37).

In addition to decreased C_2H_4, the level of ACC in these tissues also was found to be reduced in the presence of PAs (135). Fuhrer *et al.* determined that in oat leaves, PAs both cause a marked inhibition of the conversion of ACC to C_2H_4, and to a lesser extent, inhibit the step of SAM to ACC (43). But Hyodo and Tanaka, working with excised mesocarp discs from winter squash treated with PUT, SPD or SPM, found insignificant inhibition of EFE (59). In fact, they even report a slight stimulation of the enzyme following a 25 h preincubation with 10 mM SPM (59). Thus, PAs appear to affect C_2H_4 biosynthesis in a selective manner, possibly dependent upon tissue type and probably involving at least two enzyme sites: the reaction(s) associated with EFE, and that involving ACC synthase. It is also possible that the effects observed reflect feedback inhibition of these enzymes by increased levels of MTA released during PA biosynthesis.

Unlike amino acids serving as precursors in PA biosynthesis, *e.g.*, ARG, ORN, and lysine, MET has been reported to be ineffective as an ACC synthase inhibitor (59). However, this is not the case for some MET recycling intermediates. Yu *et al.* reported that in tomato fruit, ACC synthase is inhibited by both MTA and SAH (151). This has been shown in winter squash as well (59), although MTA has no effect upon the enzyme from etiolated mung bean hypocotyl segments (115). The apparent contradiction may reflect differences in natural state vs. wound-induced (stress) C_2H_4 production.

Hyodo and Tanaka reported three types of inhibition of the dialyzed enzyme extracted from wounded mesocarp of winter squash fruit, depending on the inhibitors used (59). The naturally occuring PAs, PUT, SPD and SPM, and the MET recycling intermediate αKMB inhibited ACC synthase in a non-competitive manner (K_i = 4.9, 2.9, 1.6 and 9.9 mM, resp.), while both aminooxyacetic acid (AOA) and the rhizobitoxine analog aminoethoxyvinyl-glycine (AVG) acted competitively with much lower K_i values (2.1 and 32.2 μM, resp.). Although both AOA and AVG are well known inhibitors of pyridoxal phosphate-dependent enzymes, of the two, only AOA bears no structural resemblance to SAM (124, 151). AVG is also a structural analog of MET, and does not inhibit the conversion of ACC to C_2H_4 (65, 151). MTA, which serves as both a MET recycling intermediate and a byproduct released during SPD and SPM synthesis, caused an uncompetitive type of inhibition (K_i =0.39 mM) in wounded winter squash (59).

ACC synthase has been partially purified (up to a 6500-fold purification from the crude extract) from tomato tissue, where it demonstrated optimal activity (K_m = 13-20 μM, with resp. to SAM) at pH 8.5 and a relatively narrow substrate specificity (2, 20, 21, 151). Both the adenosine and sulfonium moieties of SAM are required for an active substrate (151). Its molecular weight

was estimated to be 50,000-58,000 daltons, based upon gel filtration chromatography (2) and two-dimensional electrophoresis (20).

ACC synthase in plant tissues has a short half-life: 23.5 min in etiolated mung bean hypocotyl segments at physiological concentrations of SAM (115). The cellular concentration of the enzyme is determined by a dynamic equilibrium between its synthesis and inactivation. Evidence suggests that SAM serves as an irreversible inactivator (with which AVG competes) in addition to a substrate (115). The instability of ACC synthase, characterized by its rapid turnover, has delayed and complicated purification attempts.

3. 5'-Methylthioadenosine Nucleosidase

In plants and most bacterial systems, MTR is formed enzymatically with the release of adenine from MTA by the nucleosidic action of MTA nucleosidase (EC 3.2.2.9.). The reaction is shown in Equation 4.

(Eq. 4.) MTA $\xrightarrow{\text{MTA nucleosidase}}$ MTR + adenine

In *E. coli*, this enzyme also cleaves SAH to adenine and S-ribosyl-homocysteine (which in turn is converted to ribose + homocysteine; HCYS), and is called SAH / MTA nucleosidase (33, 34). However, eukaryotes including plants utilize a different enzyme, SAH hydrolase (EC 3.3.1.1.) for the reaction leading to adenosine + HCYS. Both SAH and SAH hydrolase are involved in a separate pathway of methionine recycling. (See Figure 1.)

Plant MTA nucleosidase has been purified to homogeneity from the seeds of *Lupinus luteus* (51, 52). It is composed of two identical subunits, yielding a native molecular weight of 62,000 daltons. In this preparation, optimal MTA

nucleosidase activity with MTA as the substrate (K_m = 0.41 μM) occurred at pH 8 to 8.5. The enzyme exhibited a broad substrate specificity, accepting as substrates among others, six inosyl (deaminated) derivatives of MTA, and was competitively inhibited most potently by adenine (K_i = 11 μM) and 3-deazaadenine (K_i = 19 μM). MTR, a product of the reaction, was a poor inhibitor (K_i = 1060 μM). In apple tissue and crude tomato extract, the enzyme is so active that as soon as MTA is formed, it is hydrolyzed into MTR (3, 151). Apparently, no feedback inhibition is involved in this step.

It should be mentioned that during the first few hours of lupin seed germination, MTA nucleosidase is solely responsible for the enzymatic liberation of free adenine into the nucleotide pool (51, 119). However, on the second day of germination another adenine releasing enzyme, adenosine nucleosidase, appears, reaching peak activity on day 4-5 of seedling development (119). Adenine generated through the MTA nucleosidase reaction in duckweed, a higher plant that produces little or no C_2H_4, is salvaged efficiently into ADP and ATP (47), most probably by the actions of the enzyme adenine phosphoribosyltransferase (117) and subsequent phosphorylations.

4. 5-Methylthioribose Kinase

Although initially it was reported that MTR represents a terminal end product in prokaryotic systems (121), as mentioned earlier, Adams and Yang showed that in apple, portions of this compound are recycled back to MET (3, 4). That the first step of this process involves a phosphorylation by the enzyme MTR kinase was established by Ferro *et al.*, studying the bacterium *Enterobacter aerogenes* (40). MTR kinase activity has been found in a variety of plants as well (50, 68). The reaction catalyzed by this enzyme requires ATP, and is shown in Equation 5.

(Eq. 5.) MTR + ATP $\xrightarrow{\text{MTR kinase}}$ MTR-1-P + ADP

In mammals, fungi and the thermophilic bacterium *Caldariella acidophila* (27), the enzyme MTA phosphorylase cleaves MTA directly into adenine and MTR-1-P, thereby avoiding the two step (MTA nucleosidase + MTR kinase) reaction and the MTR intermediate utilized by other bacteria and plants. Also, the MTA phosphorylase reaction is independent of ATP, relying instead upon other phosphate sources.

The partially purified (almost 2000-fold from the crude extract) MTR kinase enzyme from lupin seeds has a native molecular weight of approximately 70,000 daltons and exhibits nearly twice as high an affinity for MTR (K_m = 0.45 μM) than it does for its other substrate, ATP (K_m = 0.83 μM) (50). The MTR analog 5-deoxy-5-ethylthioribose (ETR) may serve as both a substrate and a competitive inhibitor (K_i = 1.4 μM). In the presence of dATP, the rate of lupin MTR-1-P synthesis was seen to be 20% greater than in the presence of ATP. Active MTR kinase from this source demonstrated a strict requirement for either Mg^{2+} or Mn^{2+} and gave optimal activity at pH 10-10.5. The enzyme reaction rate was inhibited by 1 mM concentrations of αKMB (10%), adenine (25%), SAH (36%) and ADP (66%). However, the polyamines PUT, SPD, and SPM were found to have no effect upon the activities of either MTA nucleosidase or MTR kinase from *Luteus lupinus* (50, 51).

Kushad *et al.* measured both MTA nucleosidase and MTR kinase activities and related these values to the levels of ACC and C_2H_4 in crude extracts of developing tomato (70). They found that whereas MTR kinase activty peaks with ACC and C_2H_4 levels at the mature to breaker stage of these fruits, MTA nucleosidase exhibits its highest activity prior to the mature green stage.

This suggests that MTA may accumulate during the latter stages of tomato ripening, possibly as an effect of declining MTA nucleosidase activity.

Whether fluctuations in the activities of these enzymes reflect changes in their *de novo* synthesis or regulation at the level of enzyme activity is unknown. However, that the role(s) of both MTA nucleosidase and MTR kinase may be significant in regulating the synthesis of MET, ACC and C_2H_4 was implied in studies with inhibitors of their activities which were seen to inhibit C_2H_4 biosynthesis as well (70).

Concluding Remarks

The physiological events associated with plant growth and development are regulated by biosynthetic processes leading to the formation of polyamines and ethylene. Although antagonistic with respect to the effects they produce, these processes are related through the catalysis of SAM, itself a product of MET metabolism, and appear to share feedback control mechanisms. Each pathway, once initiated, tends to inhibit the other.

Whereas most of the enzymatic steps leading to the synthesis of C_2H_4 from MET historically have received considerable attention in efforts to suppress C_2H_4 evolution and thereby control its effects, much less is known concerning the relative contribution of MET recycling. The complete MET cycle and the impact of its constituent reactions upon C_2H_4 remain to be elucidated.

CHAPTER II

MATERIALS AND METHODS

Chemicals. All compounds were of the highest grade available commercially, unless stated otherwise. Prior to their use, chromatographic resins were washed free of acid- and alcohol-soluble materials as described by Cooper (32) and Loomis *et al.* (85, 86).

Radioactive Compounds: [3,4-^{14}C]-methionine ([$^{14}CH_3$]-MET; 57 mCi / mmol) and [$^{14}CH_3$]-S-adenosyl-L-methionine ([$^{14}CH_3$]-SAM; 48 mCi / mmol) were purchased from Research Products International. [$^{14}CH_3$]-5'-Deoxy-5'-methylthioadenosine ([$^{14}CH_3$]-MTA) and [$^{14}CH_3$]-5-deoxy-5-methylthioribose ([$^{14}CH_3$]-MTR) were prepared from [$^{14}CH_3$]-SAM by the acid hydrolysis method of Schlenk (119), and purified by liquid chromatography (below) prior to use. Radiolabelled MTR solutions were stored at either -20 or -70°C in dithiothreitol (DTT) to prevent oxidation of the compound to its corresponding sulfoxide.

Non-Radioactive Compounds : MTA analogs acyclic-MTA, carbocyclic-MTA, carbocyclic-3-deaza-MTA, 5'-chloro-3-deazaaristeromycin, and 3-deaza-MTA were gifts from Dr. Marvin Pankaskie (University of Nebraska, Omaha, NE). 5'-Deoxy-5'-methylthioinosine (MTI) was a gift from Dr. Michael Riscoe (Portland V. A. Hospital, Portland, OR), who synthesized the compound from MTA by nitrous acid deamination according to the method of Savarese *et al.* (118). MTR and its analogs 5-deoxy-5-ethylthioribose (ETR) and 5-deoxy-5-isobutylthioribose (iBTR) were prepared by the same basic procedure used to hydrolyze [$^{14}CH_3$]-MTR from [$^{14}CH_3$]-MTA (above). Formycin A (8-aza-9-deazaadenosine), purchased from Sigma, was halogenated to 5'-chloroformycin by a previously described method (66). Ammonium molybdate,

platinum chloride, and potassium iodide were purchased from J. T. Baker. DTT and D-mannitol were from U. S. Biochemical Corp. Aquacide I, Macerase pectinase and myo-inositol were obtained from CalBiochem. Macerozyme RS and Cellulase Onozuka R-10 were purchased from Yakult Honsha Co., Ltd. Technical grade insoluble polyvinylpyrrolidone (insoluble PVP; PVPP) was from either GAF Corp. or Aldrich, and prepared for use as described by Loomis (85). Bio-Gel HTP (hydroxyapatite), AG 1[$HCOO^-$]-X8 and Affi-Gel 601 resins were purchased from Bio-Rad, whereas AGATP Types 3 and 4 and all gel filtration chromatography media except Bio-Gel P-10 (Bio-Rad) and Sephacryl S-200 (Sigma) came from Pharmacia, Inc. Coomassie Brilliant Blue G-250 was purchased as a solid from both Sigma (approximately 75% soluble dye) and Eastman Kodak (96% soluble dye), and as liquid concentrate from Pierce. Bicinchoninic acid (BCA) reagents were also from Pierce. Most solvents, including Triton X-100, were purchased from Malinkrodt, Inc. All other reagents were obtained from Sigma.

Plant Materials. Four varieties of pear fruit (*Pyrus communis* L., cvs Comice, Packham Triumph, Bosc, and d'Anjou) were obtained from either the Hood River Experiment Station, Hood River, Oregon, or a local market. Inoculum of a strain of continuously cultured pear cells established in 1972 by Pech *et al.* (100) from young Passe Crassane (*Pyrus communis* L.) fruit was a gift from Dr. Roger J. Romani (University of California, Davis, CA). Unripe mature avocado fruits (*Persea americana* Mill, cvs Hass and Fuerte) were purchased from a local store. All whole fruits were stored at 4°C until use.

Cell Culture. Two general types of defined media were employed: growth medium and two forms of aging media.

Growth Medium: The standard medium used for cell growth and maintenance consisted of both the mineral nutrients of Murashige and Skoog

(92) and the organic nutrients of Nitsch *et al.* (96) as first modified by Pech and Fallot (99) for apple suspension cell cultures. This medium contained (per final liter of solution): 120 mM sucrose, 1.2 mM asparagine, 284 µM ascorbate, 328 µM thiourea, 5 mL Fe / EDTA solution (containing 37.25 mg Na_2EDTA and 27.85 mg $FeSO_4 \cdot 7H_2O$), 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 1.90 g KNO_3, 1.65 g NH_4NO_3, 0.44 g $ZnSO_4 \cdot 7H_2O$, 18.95 mg $MnSO_4 \cdot H_2O$, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg $CuSO_4 \cdot 5H_2O$, 0.025 mg $CoCl_2 \cdot 6H_2O$, 0.83 mg KI, and a vitamin solution consisting of 10 mg pantothenic acid, 1.0 g myo-inositol, 0.01 mg biotin, 1.0 g nicotinic acid, 10.0 mg thiamine, and 10.0 mg pyridoxine. The prepared solution, lacking appropriate amounts of Fe / EDTA, 2,4-D, and vitamins, was pH adjusted at room temperature to a value of 5.6-5.8 prior to being autoclaved. The three remaining filter-sterilized solutions (pH 5.6-5.8) were added to complete the medium once the autoclaved solution had cooled to room temperature. Cell suspensions were grown at 27-30°C in 250 mL sterile flasks covered with both foam plugs and foil caps, and kept in the dark on a rotary shaker (110 rpm), unless stated otherwise. Cells were subcultured in a Laminar flow hood every 7-12 d, depending on their density, by first allowing the cells to settle, then decanting excess medium and pipeting 10-20 mL aliquots (approximately 15-30% inoculum) of the remaining suspension into flasks containing 50 mL of fresh medium. For growth studies, 25 mL flasks were used for a final suspension of 10 mL, and the inoculum was reduced to approximately 1 mL (10^6 viable cells as determined by the Evan's Blue method; below). Three flasks were sacrificed daily over a 10 day growth period to obtain both fresh and dry cell weights post vacuum filtration through tared nitrocellulose filters (below). Excess cells were either discarded or harvested for extractions (below).

Aging Medium A: This medium (pH 5.6-5.8), used by Puschmann and Romani (106) for both maintenance and eventual senescence of cells under

auxin deprivation and used here only for the latter, consisted of 15 mM sucrose, 385 mM D-mannitol, and 25% of the concentration of all remaining mineral and organic nutrients present in growth medium, except no 2,4-D was added. Cells were kept first in growth medium for 7-12 d, then during subculturing washed twice with an equal volume of fresh growth medium lacking 2,4-D, and grown in this auxin-deprived medium 9-12 d until the next subculturing whereupon they were allowed to settle, then washed twice with aging medium and transferred in 20 mL aliquots into 50 mL of aging medium in 250 mL flasks unless stated otherwise .

Aging Medium B: This modified form of Aging Medium A was used both to make protoplasts (below) and to wash membrane filters during studies of uptake and accumulation (below), and differed from that medium only in that it contained double the concentration of D-mannitol (700 mM) and none of the following components: sucrose, asparagine, ascorbate, thiourea, Fe / EDTA, and vitamins.

Estimation of Cell Viability. Dead cells in 1-2 mL aliquots of cell suspension were selectively stained by 0.5% (w / v) Evan's Blue added to the medium (125). 5-10 min after the stain was applied, cell viability was estimated by counting under a light microscope both unstained (colorless) and stained (blue) cells in 100 µL samples evenly distributed over both chambers of a hemocytometer. Representative samplings were made at least in duplicate and averaged each time. Viability (%) was judged as the ratio of unstained to unstained + stained cells, multiplied by a factor of 100. This method was applied to samples of both intact cells and protoplasts. In both cases, values ranged from approximately 60% to greater than 90% viability. Wherever possible, cells used for metabolic studies were at least 80% viable.

Isolation of Protoplasts. The general method used was modified from

that of Hayashi and Nakajima (56) for cell suspensions of tobacco. Of the two variations presented below, Method 1 was preferred, and used wherever possible.

Method 1: Settled suspension pear cells ready to be subcultured were instead decanted of excess medium, poured into balanced centrifuge tubes and centrifuged at 2°C for 10 min at 4000 rpm in a Beckman model J2-21 centrifuge equipped with a model JA-20 rotor. The supernatant was removed and cells transferred to 250 mL plastic centrifuge bottles containing an equal volume of either enzyme mixture A (preferred) or a solution of either 1 or 2 parts enzyme mixture A + 1 part enzyme mixture B (both to follow). Enzyme mixture A was composed of 0.5% Macerozyme RS and 1.0% Cellulase Onozuka R-10 in 700 mM D-mannitol, whereas enzyme mixture B contained 0.25% Macerase pectinase, 0.5% cellulase Type I, 700 mM D-mannitol, and 2.5 mM $CaCl_2$. The digesting mixtures were incubated with occasional gentle swirling (manually) at room temperature (under normal artificial light conditions) for a period of 1.5 -2 h, after which were added two volumes of cold (4°C) Aging Medium B. These suspensions were swirled gently, left to settle either by gravity at 4°C for 1-4 h (preferred) or by gentle centrifugation (1500 rpm, 10 min) at 2-4°C, then decanted of excess liquid and replenished with 1-2 volumes of cold medium as before. Again, the cells were allowed to settle at 4°C (1-8 h) or centrifuged at 2-4°C. This wash step was repeated as often as required (3-6 times) to render at least 80% free of cellular debris a representative sample stained with Evan's Blue and viewed by light microscopy (100x magnification). The resultant protoplasts were covered with a small volume of medium and stored at 4°C no longer than 3 d, until used to prepare cell-free extracts (below).

Method 2: Cells were treated as in Method 1, except for the following: they were refrigerated overnight prior to centrifugation at 2-4°C for 10 min at 5000 rpm, incubations formerly conducted at room temperature and swirled

manually were instead shaken (110 rpm) in a 27°C shaker and all incubations were carried out in the dark.

Preparation of Cell-Free Extracts. Several methods were investigated. In general, Methods 1 and 2 were derived from Kushad *et al.* (68-70), while Methods 3-7 were patterned after techniques in enzyme isolation recommended by Loomis *et al.* (85, 86, 127). Approaches employed to harvest suspension cultures and disrupt protoplasts were loosely based upon those utilized for bacterial cells. Assays for enzyme activity (below) and protein concentration (below) were conducted on extracts that never had been frozen (-20 or -70°C), wherever possible.

Method 1 (whole pear fruits): Each fruit was peeled, quartered and cored, then suspended in a ratio of 1 g tissue / mL cold (4°C) extraction buffer A consisting of 0.2 M potassium phosphate, 3 mM DTT and 3% (w / v) soluble polyvinylpyrrolidone (PVP; 40,000 MW), at a final pH of 7.2 (adjusted at 4°C). The suspension was homogenized in an Acme Supreme Juicerator lined with Whatman #1 filter paper, then incubated at 4°C for 20-60 min (a step that seemed to improve protein yields). Pooled preparations were passed through four layers of cheesecloth and centrifuged at 4°C for 15-30 min at 12,000 x g. The supernatant fluid served as crude extract.

Method 2 (whole avocado fruits): Small pre-weighed sections of tissue were ground with reagent sand in a pre-chilled (4°C) mortar, washed into a large flask or graduated cylinder with several small volumes of cold (4°C) extraction buffer A such that the resultant slurry was 1 g tissue / mL buffer, then incubated, filtered through cheesecloth and centrifuged as described in Method 1.

Method 3 (whole pear and avocado fruits): Pears and avocados were treated as per Methods 1 and 2, respectively, except to each mL of incubating homogenate were added the following: 0.07 g dry weight (or its

equivalent, 0.33 g hydrated weight) polyvinylpolypyrrolidone (insoluble PVP; PVPP), 0.67 g hydrated weight Amberlite XAD-4 resin, and 0.25 g Dowex 1[Cl-]-X8.

Method 4 (whole pear and avocado fruits): Fruit tissues, excluding peel and core, were cut into small pieces (approximately 10 g at a time) and placed in a pre-chilled (-70°C) mortar which was first heated to drive off moisture contained within the porcelain. Tissues were ground to a fine powder under liquid N_2, then while still frozen, transferred to 4°C extraction buffer A with PVPP, Amberlite XAD-4, and Dowex 1[Cl-]-X8 in the proportions described in Method 3. After incubating at 4°C approximately 60 min, the thawed homogenate was passed through cheesecloth and centrifuged as described earlier. This crude extract, relative to those obtained via Methods 1-3, was less pigmented, less prone to oxidation, less likely to form (floating) lipoprotein complexes during subsequent salt fractionation, and in general, more stable.

Method 5 (suspension pear cells): Cells first were converted into protoplasts and washed 3-6 times as described earlier, then passed twice through a French pressure cell (12,000 psi) equipped with a 3 / 8" piston. Cellular debris was removed by centrifugation (20,000 x g) at 4°C for 30 min, either prior to freezing at -20°C (preferred) or upon subsequent thawing of the frozen suspension.

Method 6 (suspension pear cells harvested from growth medium) : Protoplasts isolated from suspension cells were centrifuged gently (2500 x g) at 4°C for 3 min, washed twice with cold extraction buffer B (pH 6.2, adjusted at 4°C) consisting of 200 mM 2-(N-morpholino)-ethane sulfonic acid (MES), 1.0 mM DTT and 5% glycerol, then resuspended in this buffer and crushed in small volumes (approximately 3 mL at a time), using a 40 mL hand-held glass homogenizer that was kept on ice. Homogenates were pooled in centrifuge tubes containing per mL of packed protoplasts (volume estimated

after centrifuging protoplasts), 0.33 g PVPP, 0.67 g Amberlite XAD-4, and 0.25 g Dowex 1[Cl-]-X8, wetted with buffer. These solutions were vortexed, allowed to sit briefly, then centrifuged at 2-4°C for 20 min at 10,000 x g to isolate supernatant solutions as crude extracts.

Method 7 (suspension pear cells harvested from aging medium A): Protoplasts were treated as described in Method 6, except the following buffer (extraction buffer C) was used: 100 mM MES, 3 mM DTT and 10% glycerol (final pH adjusted at 4°C to a value of 6.0-6.3).

Method 8 (suspension pear cells used for metabolic studies): Cultures of Passe Crassane cells were harvested by centrifugation (10,600 x g), washed twice with an equal volume of either ddH_2O or Aging Medium B, and the cell pellets were frozen at -20°C. Pellets were extracted twice, first with at least two volumes of cold (4°C) 5% perchloric acid (PCA) plus PVPP (0.05 g dry form per mL pellet volume) for 2 h on ice with gentle stirring, then overnight (4°C) with three volumes of cold 95% ethanol (EtOH). In some cases, following the 2 h extraction, PCA suspensions were frozen (-20°C) overnight and thawed during subsequent centrifugation at room temperature (10 min at 10,000 x g). Prior to extraction by EtOH, the PCA suspensions were deproteinized by centrifugation at 4°C (unless stated otherwise). Resultant supernatants were neutralized with 8.2 N KOH and centrifuged to remove salts, while pellets were resuspended in EtOH and treated comparably. Both extracts were stored at -20°C prior to chromatographic analyses (below).

Ethylene and Gas Measurements. Non-radioactive C_2H_4 samples were removed in duplicate or triplicate through rubber septa in 1 mL tuberculin syringes from the headspace of either 25 or 125 mL flasks containing 10-25 mL of suspension culture and analyzed by gas chromatography (GC), using a Perkin Elmer model GC 3920 system equipped with both a Waters

Poropak N column (80-100 mesh, 1 m x 2.3 mm) heated to 50°C and a flame ionization detector. Nitrogen (60 mL / min) served as the carrier. Peak areas were calculated from height-width measurements, and quantified by calibration against C_2H_4 standards (Supelco). Radioactive C_2H_4 was absorbed in a freshly prepared solution of 0.25 M $Hg(ClO_4)_2$ in 2.0 M PCA (69) injected through a rubber septum cap into a small hanging plastic bucket approximately 2 h prior to the termination of each uptake experiment. The buckets of complexed Hg-C_2H_4 were removed at the appropriate time and analyzed for radioactivity by liquid scintillation spectrometry (below). Although this trapping agent is much more efficient than those involving mercuric salts of acetate, sulfate, nitrate, or chloride (149), it was found to be non-specific. To test complexed gas samples for the presence of sulfur compounds, the contents of each bucket were made basic with the addition of 8.2 N KOH and checked visually for the presence of a black precipitate (HgS).

Enzyme Assays. All assay procedures were modified slightly from those of Ferro *et al.* (39, 40). Unless stated otherwise, reaction mixtures in 1.5 mL microfuge tubes were incubated by placing these closed containers in stationary beakers of solid NaCl that were kept in an oven heated to the appropriate temperature. This method was preferred to that involving a waterbath-shaker, as no isolated liquid in the form of condensation on the caps and walls of the microfuge tubes resulted. Reaction products were separated from substrate and analyzed either by gravity flow column chromatography + liquid scintillation counting (Beckman model LS8000 or LS3801), using scintillation fluid consisting of 0.4% PPO and 0.01% POPOP in 2:1 (v / v) toluene / Triton X-100, or by high performance liquid chromatography (HPLC) using a Beckman model 334 gradient liquid chromatograph equipped with the following Beckman instruments: model 421 controller, model 210 sample injection valve, model 110A and 110B pumps,

model 164 variable wavelength detector, model 156 refractive index detector and model 171 radioisotope detector. The on-line radioisotope (HPLC) detector utilized one of three flow cells: a solid scintillator cartridge cell of either 125 µL or 300 µL, or a liquid scintillator cell of variable volume. For studies assessing the effects of analogs of MTA and MTR upon enzyme activity, care was taken both to insure that these compounds were neutralized prior to their addition to the reaction mixture, and to prepare appropriate matrix-matched controls to account for nonenzymatic substrate degradations.

MTA Nucleosidase: MTA nucleosidase activity was determined by measuring the conversion of [$^{14}CH_3$]-MTA to [$^{14}CH_3$]-MTR + adenine. The standard reaction mixture contained, unless stated otherwise, 50 mM cyclohexylaminopropane sulfonic acid (CAPS) buffer (pH 11.0, adjusted at the incubation temperature to be utilized),74 µM [$^{14}CH_3$]-MTA (10^5-10^6 cpm / µmol, pH 7), and extract (or, in the case of negative controls, either ddH_2O or buffer) in a total volume of 250 µL. The reaction mixture was incubated at either 37, 45 or 52°C for the time required to convert 5-20% of substrate into product, then generally stopped by the addition of 50 µL of cold (4°C) 1.8 M trichloroacetic acid and microfuged to remove particulate. Substrate and products in a 250 µL aliquot of supernatant were separated chromatographically either by charge (cation exchange) or polarity (reverse phase HPLC System II; below). Two cation exchange methods were employed: either Dowex 50W [H+]-X4 (100-200 mesh) column (0.7 cm x 5.5 cm) chromatography as described by Ferro *et al.* (39), or HPLC System III (below). Reactions to be analyzed by HPLC were terminated with the addition of 3 volumes of EtOH.

MTR Kinase: MTR kinase activity was determined by measuring the conversion of [$^{14}CH_3$]-MTR + ATP to [$^{14}CH_3$]-methylthioribose-1-phosphate ([$^{14}CH_3$]-MTR-1-P) + ADP, according to the assay of Ferro *et al.* (40), but

modified with respect to buffer (pH), reaction volume, incubation time and temperature, and in some cases, method of analysis. The standard reaction mixture contained, unless stated otherwise, 50 mM CAPS buffer (pH 9.5, adjusted at the incubation temperature to be utilized), 1 mM ATP (either prepared just prior to use, or frozen once), 5 mM $MgSO_4$, 10 mM DTT, 0.1 mM [$^{14}CH_3$]-MTR (10^6-10^8 cpm / µmol, pH 7), and extract, in a total volume of 350 µL. Negative controls were prepared comparably, but used ddH_2O instead of ATP unless extract supply was limiting, in which case ATP was included and either ddH_2O or buffer was substituted for extract. Reactions were limited to 5-20% conversion at either 30, 45, or 52°C, terminated with 3 volumes of EtOH and microfuged. A 1000-1200 µL aliquot of supernatant was applied to an anion exchange column [2.2 mL bed volume of either AG 1[$HCOO^-$]-X8 (100-200 mesh), or Dowex 1[Cl^-]-X8 (100-200 mesh) converted to the formate form as described by Cooper (32)]. Following a 15 mL wash of 0.10 N NaCOOH (pH 5.0) to remove unreacted substrate, the product [$^{14}CH_3$]-MTR-1-P was eluted with 15 mL of 0.75 N NaCOOH (pH 5.0), 1 / 5 of which was analyzed by liquid scintillation counting. Alternatively, a 250 µL aliquot of supernatant was injected into the HPLC, using either System II or System IV (below).

Determination of Enzyme pH, Temperature Optima and K_m. Assay methods were conducted as described above, except the following reaction buffers (50 mM with respect to total reaction volume) were employed for the pH ranges indicated: Hepes-KOH (pH 7.2-8.0), Imidazole-HCl (pH 7.3-8.0),Tris-HCl (pH 8.0-9.0), CAPS-KOH (pH 9.25-11.25), and Ascorbic Acid-KOH (pH 11.5-12.5). The latter was prepared generally within one week of use, as it was found to be unstable. All buffers were pH adjusted at the incubation temperature to be utilized, and stored at 4°C. Effect of incubation temperature (at optimal pH) upon enzyme activity was

tested over a range of 4-60°C. Since pH and temperature are known to exhibit mutually dependent effects upon enzyme activities, the following 4-step strategy for optimization of these parameters was pursued: 1). determine optimal pH (pH_{opt}) at constant incubation temperatures of 37°C for MTA nucleosidase and 30°C for MTR kinase, 2). determine optimal incubation temperature (T_{opt}) at the pH_{opt} established in step 1., 3). determine pH_{opt} at the T_{opt} established in step 2., and 4). repeat temperature optimization if pH_{opt} determined in step 3. grossly differs from that established in step 1. Michaelis constants (K_m values) were determined at optimal pH and temperature.

Determination of Aldopentose Concentration. Generally, the concentration of non-radioactive standards of MTA, MTR, and their analogs was determined spectrophotometrically by the method of Ashwell (14), using ribose as the standard and 4.6% ethanolic phloroglucinol as the chromophore. Glass or quartz cuvettes were required, since the strong acidity of the reagent mixture was found to damage plastic cells. Alternatively, these compounds were quantified by HPLC, using one of the systems described below.

Protein Determinations. Two general spectrophotometric methods (below) were employed, with some variations to permit analysis of dilute solutions. One such variation involved the addition of up to 1 mL of sample (instead of the usual 0.1 mL) to increase available protein. When this was done, the volume of each standard also was increased by an appropriate amount of ddH_2O or buffer. Where Coomassie Blue dye was used, care was taken to insure that the same source (Sigma, Eastman Kodak, or Pierce) of reagent was employed within each set of experiments. Likewise, whenever treatment (*i.e.*, partial purification) of one sample caused its protein content to change from relatively high to relatively low (or vice versa) concentration,

only one dye type (Coomassie Blue or Bicinchoninic acid) was used. In all cases, bovine serum albumin (BSA) served as a standard and absorbance measurements were performed in plastic cuvettes using a Beckman model 25 spectrophotometer.

Coomassie Blue (Bradford) Method: The standard method of Bradford (22) was used to analyze samples containing protein in the range of 10-70 µg; while samples below that were quantified for protein by the variation of this method described by both Read and Northcote (107) and Peterson (103). The sensitivity of this modified method has been reported to be so great that as little as 0.1 µg of protein (2 µg / mL in 50 µL) can be determined accurately (107).

Bicinchoninic Acid (BCA) Method: This method, both in standard (10-100 µg protein) and enhanced (0.5-25 µg protein) form was used as described by Smith *et al.* (129).

Purification Schemes. The approaches utilized to partially purify MTA nucleosidase and MTR kinase from both avocado and pear extracts were patterned with little modification after those employed by Guranowski *et al.* (50-52) for the same enzymes from extracts of *Lupinus luteus* seeds. Unless stated otherwise, salt fractionation steps were performed as back-extractions (32) involving the dropwise addition of $(NH_4)_2SO_4$ previously dissolved in buffer and delivered at 4°C under gentle stirring over the course of 1-2 h. Concentrated preparations were desalted by one of three ways: dialysis against several changes of buffer, application over a gel filtration column, or repeated centrifugation at 3000 x g using Centriprep-30 concentrators (Amicon). Alternatively, Bio-Gel P-10 added in dry form, aqueous Aquacide I or Centriprep-30 units were employed to concentrate samples, thereby avoiding changes in ionic strength and reducing or eliminating subsequent dialysis time. All operations, except heat shock purifications (below), were conducted at 4°C.

MTA nucleosidase: The protocol for the partial purification of MTA nucleosidase involved the following treatments: $(NH_4)_2SO_4$ precipitation, DEAE-Cellulose chromatography, either Sephadex G-200 or Sephacryl S-200 chromatography, and hydroxyapatite chromatography. The initial back-extraction was conducted as follows: the crude extract was fractionated at 0-20% $(NH_4)_2SO_4$, and centrifuged (10,000 x g) to isolate the supernatant which then was fractionated at 20-47% and centrifuged to isolate the precipitate which in turn was resuspended in a 27% $(NH_4)_2SO_4$ buffer solution and centrifuged. The final pellet resulting from these manipulations was resuspended in nucleosidase purification buffer B (below) and desalted. Buffer solutions (nucleosidase purification buffer A, nucleosidase purification buffer B, and nucleosidase purification buffer C) were as described by Guranowski *et al.* (51, 52), except buffer C (pH 8.3, adjusted at 4°C) contained 50 mM Tris / KOH instead of 50 mM Bicine, and all buffers were prepared to include 1% PVP, 3 mM DTT, and 10 rather than 5% glycerol.

MTR kinase: The partial purification of MTR kinase employed $(NH_4)_2SO_4$ precipitation, aminohexyl (AH)-Sepharose chromatography, either Sephadex G-200 or Sephacryl S-200 chromatography, and hydroxyapatite chromatography. The initial back-extraction, conducted as described above, involved the following salt solutions: 0-30%, 30-50% and 35% $(NH_4)_2SO_4$, and the final pellet was resuspended in kinase purification buffer A (below) and desalted. Buffer solutions (kinase purification buffer A and kinase purification buffer B) were as described by Guranowski (50), except 3 mM DTT replaced 2 mM ß-mercaptoethanol, 10 rather than 5% glycerol was used, and 1% PVP was included in both solutions.

MTA nucleosidase and MTR kinase: Attempts to co-purify MTA nucleosidase and MTR kinase were conducted without back-extraction, by precipitating with 27-50% $(NH_4)_2SO_4$, resuspending the pellet in nucleosidase purification buffer A, then applying the desalted solution over an AH-Sepharose column. Fractions containing MTA nucleosidase and MTR kinase

activity were pooled separately, concentrated and individually applied to a column of either Sephadex G-200 or Sephacryl S-200, then treated as described above.

Heat Shock Purifications: In efforts both to inactivate (denature) proteases and other compounds potentially responsible for the instability of MTA nucleosidase and MTR kinase and to assess the thermostability of these enzymes, representative aliquots of crude extracts were preincubated at 45-60°C for periods ranging from 0-60 min, then microfuged, analyzed for protein content and assayed under optimal reaction conditions. Reaction blanks treated comparably were run to correct for non-enzymatic substrate conversions. Supplemental DTT was added to all solutions requiring (freezer) storage prior to analysis, to replenish that inactivated by heat. However, wherever possible, these solutions were analyzed immediately following pre-treatment.

HPLC Systems. Four isocratic systems, System I, System II, System III, and System IV (below), were employed to separate (purify) and identify compounds. (See Appendix B for retention times.) All HPLC analyses were conducted with a Beckman model 334 gradient liquid chromatograph equipped as described above. In this set-up, both counting efficiency and recovery of radioactive compounds were found to be in excess of 90%, except in two cases: 1). systems utilizing both a solid flow cell and a mobile phase of formate had counting effiencies in the range of 80-85%, and 2). when the 300 µL flow cell was used, these values were lowered by 10%. Solutions to be analyzed by HPLC first were pH adjusted to a value within the tolerance of the column to be used, then were filtered through 0.45 µm Durapore / Millex HV_4 filter units (Millipore) and injected by Hamilton syringe into the instrument. Mobile phases also were filtered prior to use, through either 0.45 µm Durapore / Millex HV membranes (Millipore) or 0.2 µm Metricel GA-8 membranes (Gelman Sciences, Inc.). Each column first was

conditioned by running KOH-neutralized samples of 5% PCA extracts of suspension pear cells (Method 8; above)until the retention times of authentic standards prepared in neutralized 5% PCA became stabilized. Where required, standards also were prepared as aqueous and ethanolic solutions, then chromatographed to verify retention times. To oxidize sulfur-containing compounds to their corresponding sulfoxides, authentic standards were incubated in 1% H_2O_2 at room temperature and the oxidation was monitored over a 1-6 h period by chromatographing aliquots of each reaction mixture.

HPLC System I: System I utilized a mobile phase of ddH_2O (0.6 mL / min flow rate) at either 85°C (preferred) or room temperature delivered through a Bio-Rad Carbo-C guard column (4.6 mm x 40 mm) also at room temperature and a Beckman μSpherogel Carbohydrate column (7.7 mm x 300 mm) heated to 85°C via a circulating waterbath and column jacket (Bio-Rad). Care was taken to insure that this system never was operated at a back pressure exceeding 1200 psi.

HPLC System II: System II was operated at room temperature, and involved the following components: a Whatman Solvecon precolumn (4.6 mm x 250 mm), a Whatman CO:PELL ODS guard column (2.1 mm x 70 mm) and a Waters μBondapak C_{18} reverse phase column (3.9 mm x 300 mm). The mobile phase was methanol (MeOH) / ddH2O (36:64, v / v), delivered at 0.7 mL / min (153).

HPLC System III: System III utilized a mobile phase of 0.5 M NH_4COOH, pH 4.0 (adjusted with HCOOH) pumped at 3 mL / min first through the Solvecon precolumn described in System II, then through a Whatman Pellicular Cation Exchanger guard column (2.1 mm x 70 mm) and a Whatman Partisil 10 SCX analytical column (4.6 mm x 250 mm; 155). All operations were conducted at room temperature.

HPLC System IV: System IV involved the same precolumn as Systems II and III in addition to a Whatman Pellicular Anion Exchanger guard column (2.1 mm x 70 mm) and a Whatman Partisil 10 SAX analytical column (4.6

mm x 250 mm). A mobile phase comprised of 0.25 M NH_4COOH, pH 4.0 (adjusted with HCOOH) was used at 1.0 mL / min (153). To effect quantitative recovery of negatively charged radioactive species, this system required the use of a liquid (rather than solid) scintillator flow cell. In these applications, the pre-mixed scintillation fluid Ready Flow III (Beckman) was employed.

Paper and Thin Layer Chromatography Systems. Both paper chromatography (PC) and thin layer chromatography (TLC) were used to verify the identification (and purity) of compounds and reaction products. Authentic standards were prepared as described above for HPLC applications. All PC systems employed sheets of Whatman paper 35-45 cm in length and TLC systems used plates backed with plastic (preferred), aluminum or glass. The systems involved are described below. Retention times (R_f values) of various compounds are either cited below or listed in Appendix B.

PC System I: $(NH_4)_2SO_4$ / Na phosphate buffer / 2-propanol, prepared by adding first 600 g $(NH_4)_2SO_4$, then 20 mL 2-propanol to 1.0 L of 0.1 M Na phosphate buffer (pH 6.8). This ascending PC system developed by Schlenk (119), generally conducted for 18 h on Whatman #1 paper, was used primarily to separate MTA (R_f = 0.08) from MTR (R_f = 0.92).

PC System II: n-butanol (ButOH) / acetic acid (HAc) / ddH_2O (60:15:25, by volume), ascending on Whatman #1 paper, approximately 12 h (123).

PC System III: ButOH / acetone / HAc / ddH_2O (70:70:20:40, by volume), descending on Whatman #3 paper, approximately 8 h (87, 108).

PC System IV: EtOH / ddH_2O / HAc (65:34:1, by volume), descending on Whatman #1 paper, approximately 9 h (108).

TLC System I: as per PC System III, but chromatographed on celluose F_{254} coated plates (EM Labs) for 3-4 h.

TLC System II: MeOH / $CHCl_3$ (85:15, v / v), silica gel 60 F_{254} (EM Labs), 1.5-2 h (87, 108).

Chromatographic Detection and Visualization of Compounds. Several methods of detection and visualization were utililzed (below).

HPLC: Three Beckman detectors were employed: model 164 variable wavelength detector (set at 254 nm), model 156 refractive index detector and model 171 radioisotope detector equipped with one of the following flow cells: variable length liquid scintillator cell, 125 µL solid scintillator cell or 300 µL solid scintillator cell. When the liquid cell was used, the pre-mixed scintillation fluid was either Ready Flow II or Ready Flow III (Beckman), delivered by a Beckman model 110B pump. Peak integrations were performed by both the model 171 detector (for radioactivity only) and a Waters model 740 data module.

PC and TLC: Dried PC and TLC chromatograms were examined for UV absorbing compounds with a short wave UV light source (254 nm). Radiolabelled substances were detected with a model 7201 Packard radiochromatogram scanner. Alternatively, chromatographed spots were cut (PC) or scraped (TLC) and counted in scintillation cocktail. Reactions utilizing spray reagents for chemical visualization were conducted directly on paper and TLC plates. Ninhydrin, 0.25% (w / v) in acetone, was used as a spray reagent for primary and secondary amino groups which appeared blue to purple in color after heating. Also, chromatographed spots of the compounds SAM and homoserine were stained pink to light orange by this reagent. Oxidizable sulfur compounds, and inexplicably adenine, were detected with platinic iodide spray prepared in acetone (94%) to contain 99 µM $H_2PtCl_6 \cdot 6H_2O$, 3 mM KI, and 9.9 mM HCl (137).

Accumulation and Uptake Studies of Radioactive Compounds. All tests were performed under aseptic conditions. In 10 mL aliquots (approximately 10^7 cells), suspensions of Passe Crassane cells were delivered into 125 mL Erlenmeyer flasks which then were capped with either rubber septa or foam plugs. For experiments involving the trapping of radioactive gases, a small

plastic bucket was inserted into each septum cap prior to its placement on the flask. The suspensions were incubated in a waterbath-shaker with slow shaking at 27°C. Radioactive substrates were added either volumetrically or gravimetrically by tuberculin syringe at time zero and allowed to mix. To monitor accumulation, representative aliquots of 100-200 µL were removed at specific times and applied onto pre-moistened 3 µm nitrocellulose filters (Millipore SSWP 025 00) under vacuum filtration. After washing with 6-10 mL cold (4°C) Growth Medium deficient in both 2,4-D and all vitamins, the filters were dried under a heat lamp and the radioactivity quantified by liquid scintillation counting. To serve as a blank in each study, one unused filter was washed, dried and analyzed for radioactivity as described. Accumulated radioactivity was expressed on the basis of the parent compound (nmol accumulated per 10^7 cells) as a function of time. In some cases, tared filters were utilized and both fresh and dry weights of washed, filtered cells were determined. Following the removal of the last desired aliquot, the remaining cells + medium were harvested and extracted by Method 8 (cell free extraction of suspension pear cells used for metabolic studies) as described above, and analyzed chromatographically using at least three of the following: PC, TLC, HPLC, cation exchange (Dowex 50W [H+]-X4; 0.7 cm x 5.5 cm) and boronate affinity (Affi-Gel 601; 0.7 cm x 5.0 cm) chromatography. The affinity columns separated cis-diol (*i.e.*, ribose) containing compounds from those lacking cis-diols by the method of Pfadenhauer and Tong (104), and were found to be at least 90% efficient. By individually passing the effluents from the Affi-Gel columns over Dowex 50W [H+] resin according to the method described by Kuttan and Radhakrishnan to isolate homospermidine from related substances (72), compounds both containing and lacking cis-diols were characterized further with respect to charge. In efforts to identify these partially characterized compounds, they were analyzed by PC or TLC, and HPLC, wherever possible both as neutralized PCA or EtOH extracts and as common components with respect to presence or absence of

cis-diols and charge.

Studies with Indoleacetic Acid (IAA). Cell suspension studies relating concentration of added IAA with C_2H_4 production, enzyme activities and metabolic uptake of non-radioactive substrates were conducted by removing at designated times 1 mL samples of gas from the headspace of septum-capped flasks, as described above. No cell suspension was removed until cultures were ready to be harvested, at which time PCA extracts (Method 8; above) were made. Control samples were prepared by adding instead of IAA an equal volume of the solvent in which this compound was dissolved (approximately 30% EtOH). Care was taken to insure that these two solutions were of the same pH value.

CHAPTER III

CHARACTERIZATION OF 5'-DEOXY-5'-METHYLTHIOADENOSINE NUCLEOSIDASE AND 5-DEOXY-5-METHYLTHIORIBOSE KINASE FROM CRUDE FRUIT EXTRACTS OF PEAR (*Pyrus communis* L.) AND AVOCADO (*Persea americana* Mill)

In order to both optimize enzyme assay conditions and understand better the species- and cultivar (cv)-related differences exhibited by the methionine (MET) recycling enzymes 5'-deoxy-5'-methylthioadenosine (MTA) nucleosidase and 5-deoxy-5-methylthioribose (MTR) kinase, these enzymes were extracted from five varieties of pear (*Pyrus communis* L., cvs Comice, Packham Triumph, Bosc, d'Anjou, and Passe Crassane) and two varieties of avocado (*Persea americana* Mill, cvs Hass and Fuerte) and biochemically characterized with respect to pH, incubation temperature, Arrhenius energy of activation (Ea), heat stability, effect of substrate analogs, and in some cases Michaelis constant (K_m). Due to the unexpected limited stability of partially purified preparations of both enzymes (see Appendix A), crude extracts were employed for these studies. Several measures undertaken to minimize loss of enzyme activity are described in Appendix A. Because even in crude extracts, both MTA nucleosidase and MTR kinase enzyme activities were found to vary somewhat from preparation to preparation and to degrade (sometimes nonuniformly) both over time and with repeated freeze / thaw steps, in this text enzyme activities generally are presented in terms of relative (%) rather than specific activity.

The initial rationale in examining so many different pear fruit cultivars was to enable a comparison to be drawn between the *in vitro* characteristics of MTA nucleosidase and MTR kinase extracted from both whole fruit (Comice,

Packham Triumph, Bosc, d'Anjou) and suspension cultures (Passe Crassane). An additional goal was to relate back the *in vivo* behavior demonstrated by suspension cultured cells to that of whole fruit (see Chapter IV). Such work would establish clearly the suitability of suspension cultured pear cells to serve as a (limited) model for whole pear fruit metabolic processes.

Early data suggested that problems of enzyme instability and low specific activity might be related to the low protein content characteristic of water-laden fruit, such as the pear. Hence, avocado fruit which when homogenized into a crude cell-free extract contains in the order of both 10 to 35 times more protein than comparable extracts of whole pear fruit and 3 to 6 times more protein than extracts of suspension pear cells, was incorporated into the study. An additional benefit of extracts of avocado was that relative to pear extracts, they consistently exhibited higher MTR kinase activities, and therefore were utilized to "work out the bugs" in the MTR kinase enzyme assay. Methods employed to prepare cell-free extracts, determine their protein concentrations, and assay the specific activities of both MTA nucleosidase and MTR kinase are described in Chapter II ("Materials and Methods").

RESULTS

Protein Concentrations and Enzyme Activities of MTA Nucleosidase and MTR Kinase in Crude Extracts of *Pyrus communis* L. and *Persea americana* Mill.

Typical protein concentrations and enzyme activities of both MTA nucleosidase and MTR kinase in crude extracts of *Pyrus communis* L. and *Persea americana* Mill are presented in Tables III.1. and III.2., respectively. As described in Chapter II, several (*i.e.*, three for whole fruits of both pear and avocado, and two for suspension pear cells in two different media) methods of preparing cell-free extracts were investigated and compared with respect to their influence upon enzyme activity and stability. Inasmuch as separate preparations of different individual fruits (of the same species) may be considered to be relatively uniform, the inclusion of the phenolic--binding resins PVPP, XAD-4 and Dowex 1[Cl^-]-X8 (extraction Methods 3, 4, 6, and 7; see Appendix C for structures) produced extracts of considerably greater stability, but not necessarily higher specific activity for either of the enzymes examined, except in cases where both these resins and liquid N_2 were utilized in the extraction procedure.

In the case of those pear extracts for which the effect of the addition of resins alone was studied (extraction Methods 1 and 5 vs. 3 and 6, respectively), a significant change in specific activity was observed only for Passe Crassane suspension cells (see Table III.1). Here, MTA nucleosidase activity was increased 2.9-fold, whereas MTR kinase activity was increased by a factor of 4.8. Why these resins would have relatively no effect upon the specific activities of MTA nucleosidase and MTR kinase in crude extracts prepared from whole pear fruits is unknown, but might relate to the fact that relative to

TABLE III.1. Typical Protein Concentrations and Enzyme Activities of Both MTA Nucleosidase and MTR Kinase in Crude Extracts of Five Varieties of *Pyrus communis* L.

EXTRACT (method)[a]	PROTEIN (mg / mL)	SPECIFIC ACTIVITIES[b] MTA nucleosidase (nmol $min^{-1}mg^{-1}$)	MTR kinase (pmol $min^{-1}mg^{-1}$)
1. Comice (1)	0.82	5.51	33.8
Comice (3)	0.97	5.04	41.1
Comice (4)	0.39	7.14	72.3
2. Packham Triumph (1)	1.00	6.97	46.4
Packham Triumph (3)	0.85	7.42	42.1
Packham Triumph (4)	0.38	8.20	83.7
3. Bosc (1)	0.67	9.28	61.7
Bosc (3)	0.59	9.04	59.1
Bosc (4)	0.28	14.2	89.7
4. d'Anjou (4)	0.31	3.56	35.1
5. Passe Crassane (5)[c]	2.68	0.194	28.3
Passe Crassane (6)[c]	3.40	0.556	136
Passe Crassane (7)[d]	1.80	0.364	66.7

[a]Extraction Methods 1-7 are described in Chapter II.

[b]Measured under conditions of optimal pH and temperature for the time required to convert 5-20% of substrate into product.

[c]Suspension pear cells harvested from growth medium.

[d]Suspension pear cells harvested from aging medium A.

TABLE III.2. Typical Protein Concentrations and Enzyme Activities of Both MTA Nucleosidase and MTR Kinase in Crude Extracts of Two Varieties of *Persea americana* Mill

EXTRACT (method)[a]	PROTEIN (mg / mL)	SPECIFIC ACTIVITIES[b] MTA nucleosidase (nmol $min^{-1}mg^{-1}$)	MTR kinase (pmol $min^{-1}mg^{-1}$)
1. Hass (2)	10.9	1.81	78.2
Hass (3)	8.67	4.63	240
Hass (4)	6.55	7.14	493
2. Fuerte (2)	7.96	2.17	112
Fuerte (3)	8.11	ND[c]	195
Fuerte (4)	6.04	8.21	367

[a]Extraction Methods 1-7 are described in Chapter II.

[b]Measured under conditions of optimal pH and temperature for the time required to convert 5-20% of substrate into product.

[c]Not determined.

other enzyme sources examined, whole pears contain the least amount of protein (0.3-1.0 mg / mL). These values represent approximately one tenth of the protein content of suspension pear cells which in turn ranged from 1.8-3.4 mg / mL.

In contrast, whole avocados extracted with PVPP, XAD-4 and Dowex 1[Cl-]-X8 included in the buffer (extraction Method 3 vs. 2) exhibited relatively uniform increases in specific activity (see Table III.2.); ranging from 2.6-fold (Hass MTA nucleosidase) to 1.7-3.1-fold (Fuerte and Hass MTR kinase, respectively). It should be noted that some of these differences might reflect variations in efficiency of extraction (protein content), age of fruit, and stability of enzyme preparation. To minimize the latter, all enzyme activities included in Tables III.1. and III.2. were measured prior to subsequent freezing, and on the same day the fruit was homogenized.

Although a visible reduction in the presence of floating lipoprotein complexes was observed (especially in avocado homogenizations), inclusion of the three resins in the extraction procedure appeared to have no strong effect upon resultant protein concentrations. However, utilization of liquid N_2 in a mortar and pestle (extraction Method 4) markedly reduced these levels, probably reflecting reduced extraction efficiency.

Lower protein concentrations of liquid N_2-extracted whole fruit crude preparations might account in part for resultant increases in enzyme specific activities (Tables III.1 and III.2.). For the nucleosidase, these increases relative to identical extractions conducted without liquid N_2 (*i.e.,* extraction Method 3 vs. 4), were typically 42%, 11%, and 57% greater for Comice, Packham Triumph, and Bosc pear, respectively, and 54% greater for Hass avocado. The effect was even more pronounced for the kinase, producing elevated relative specific activities averaging above 80% (76%, 99%, and

52% for Comice, Packham Triumph, and Bosc pear, respectively; 105% and 88% for Hass and Fuerte avocado, respectively).

Although dependent upon method of extraction, in general MTA nucleosidase activities in all whole fruit extracts examined were roughly comparable (ranging from 1.81-14.2 nmol min^{-1} mg^{-1}, but averaging about 7.2 nmol min^{-1} mg^{-1}),whereas MTR kinase activities in avocado extracts were considerably higher (78.2-493 pmol min^{-1} mg^{-1}) than those observed for whole pears (33.8-89.7 pmol min^{-1} mg^{-1}). The suspension cells behaved differently, exhibiting much lower nucleosidase activities (0.194-0.556 nmol min^{-1} mg^{-1}) than any other extracts examined, but kinase activities similar to those observed in whole pears (*i.e.*, 28.3-136 pmol min^{-1} mg^{-1}). When homogenized similarly (*i.e.*, via Methods 6 and 7), extracts of cells grown in growth medium characteristically displayed values of protein concentrations and both MTA nucleosidase and MTR kinase activities double in magnitude relative to those grown in aging medium (see Table III.1.).

Effect of pH Upon MTA Nucleosidase and MTR Kinase Activities.

Initial velocities of MTA cleavage to MTR + adenine were tested in 50 mM incubation buffers covering the pH range of 7.2-12.5. As seen in Figure III.1., except for Hass avocado, all fruit extracts examined exhibited optimal MTA nucleosidase activity in the pH range of 10.75-11.25; hence the buffer CAPS-KOH, pH 11.0 was used in subsequent nucleosidase assays for all pear extracts (Comice, Packham Triumph, Bosc and Passe Crassane) and those of Fuerte avocado. The pH optimum (pH_{opt}) for Hass avocado crude extract, on the other hand, was 9.5 (CAPS-KOH). This profile exhibited an additional anomaly in that nucleosidase activity at pH 7.3 (checked with both Hepes-

Figure III.1. Effect of incubation buffer pH upon relative MTA nucleosidase activity in crude extracts from two varieties of *Persea americana* Mill and four varieties of *Pyrus communis* L. Cell-free extraction methods and enzyme activity measurements under conditions of previously determined optimal temperature (see Figure III.3.) were performed as described in Chapter II. Each data point reflects the net average of at least two independent assays utilizing both the same extract and buffer pH. Corrections for non-enzymatic substrate degradation were conducted by subtracting out background activities of negative controls run simultaneously with test samples at each pH value. Extracts examined include Hass avocado homogenized by Method 3 (A), Fuerte avocado homogenized by Method 4 (B), Comice pear homogenized by Method 4 (C), Packham Triumph pear homogenized by Method 3 (D), Bosc pear homogenized by Method 4 (E), and Passe Crassane pear harvested from growth medium and homogenized by Method 6 (F).

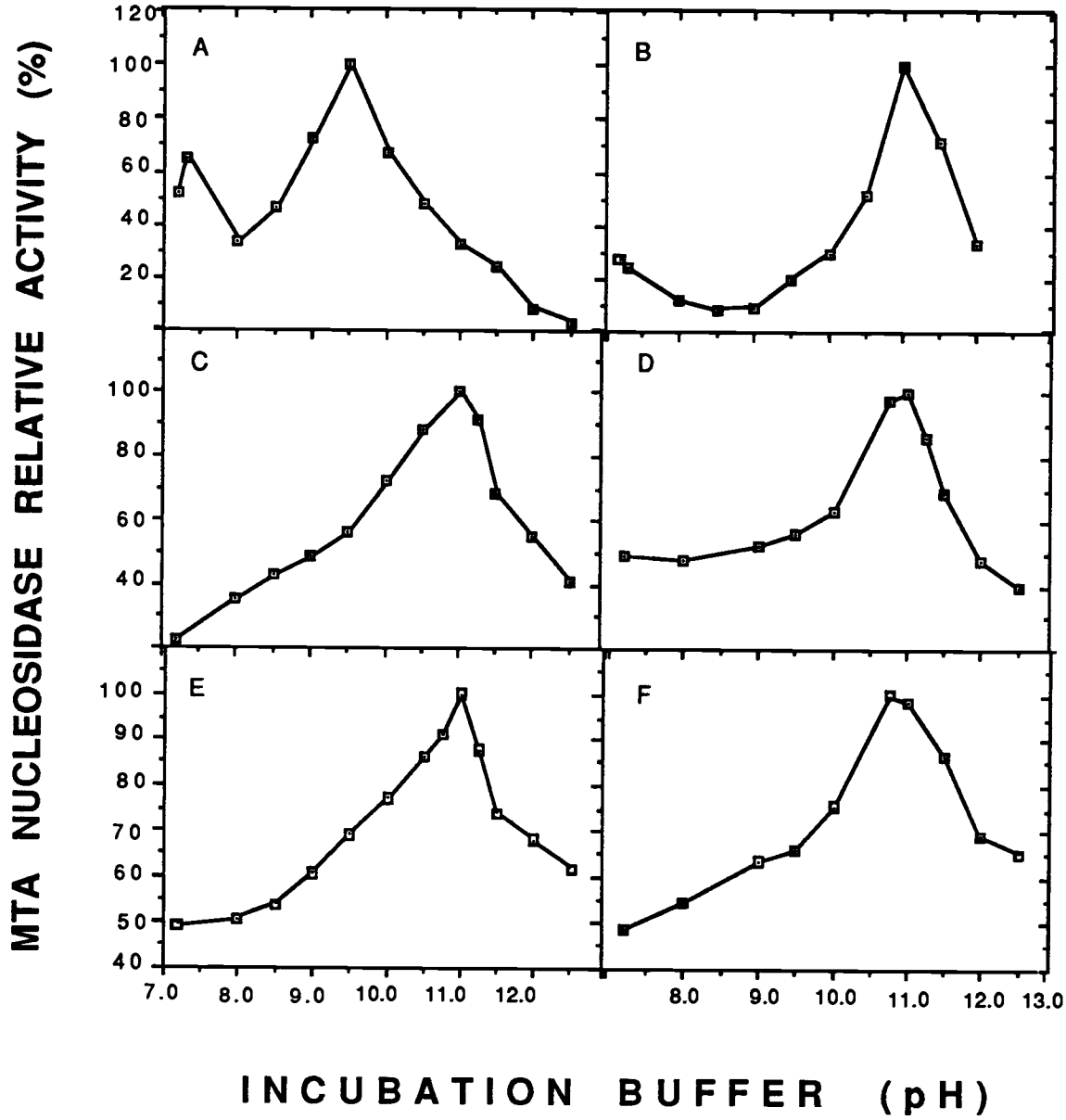

MTA NUCLEOSIDASE RELATIVE ACTIVITY (%)
A
B
C
D
E
F
INCUBATION BUFFER (pH)

Figure III.1.

KOH and Imidazole-HCl buffer) was observed to be greater than that at pH values of 7.2 (Hepes-KOH), 8.0 (Hepes-KOH, Imidazole-HCl, and Tris-HCl), 8.5 (Tris-HCl), or exceeding 10.0 (CAPS-KOH and Ascorbic Acid-KOH). Where more than one buffer type at the same pH value was used, no appreciable differences in enzyme activity were noted except in cases involving Tris-HCl, where nucleosidase activity was inhibited in the order of 20% (relative to assays employing either Hepes-KOH or Imidazole-HCl), and Ascorbic Acid which had degraded with time. (See note in Chapter II concerning the latter.)

Initial velocities of ATP-dependent phosphorylations catalyzed by MTR kinase extracted from several sources were examined in the same buffer pH range employed to test MTA nucleosidase (above). The results are shown in Figure IV.2. Extracts of Fuerte avocado were not tested. Optimal incubation buffer pH for the kinase from all crude extracts of whole pear fruit was 11.0 (CAPS-KOH), the same value representing pH_{opt} for MTA nucleosidase in these extracts. Likewise pH_{opt} for MTR kinase in Hass avocado was the same as that for the nucleosidase, *i.e.,* pH 9.5 (CAPS-KOH). However, suspension pear cell extracts behaved differently, exhibiting a pH optimum of 8.5 (Tris-HCl) for the kinase, as compared to 11.0 for the nucleosidase.

For the sake of comparison, nucleosidase assays were conducted upon cell-free extracts of both *Candida albicans* and *Escherichia coli* over a range in pH of 7.2-12.0. Typical maximal initial velocities obtained were 40.6 pmol min^{-1} mg^{-1} at pH 7.2 (Hepes-KOH) and 15.5 nmol min^{-1} mg^{-1} at pH 7.3 (imidazole-HCl), respectively (data not shown). That *Candida albicans* possesses the enzyme MTA nucleosidase rather than MTA phosphorylase has not been established; however, except for the phosphate requirement of the latter, the two assays are identical and hence, the pH study above is valid. It is noteworthy that a cell-free extract of *Candida* was able to degrade

Figure III.2. Effect of incubation buffer pH upon relative MTR kinase activity in crude extracts from one variety of *Persea americana* Mill and four varieties of *Pyrus communis* L. Cell-free extraction methods and enzyme activity measurements under conditions of previously determined optimal temperature (see Figure III.5.) were performed as described in Chapter II. Each data point reflects the net average of at least two independent assays utilizing both the same extract and buffer pH. Corrections for nonenzymatic substrate degradation were conducted by subtracting out background activities of negative controls run simultaneously with test samples at each pH value. Extracts examined include Hass avocado homogenized by Method 3 (A), Comice pear homogenized by Method 4 (B), Packham Triumph pear homogenized by Method 3 (C), Bosc pear homogenized by Method 4 (D), and Passe Crassane pear harvested from growth medium and homogenized by Method 6 (E).

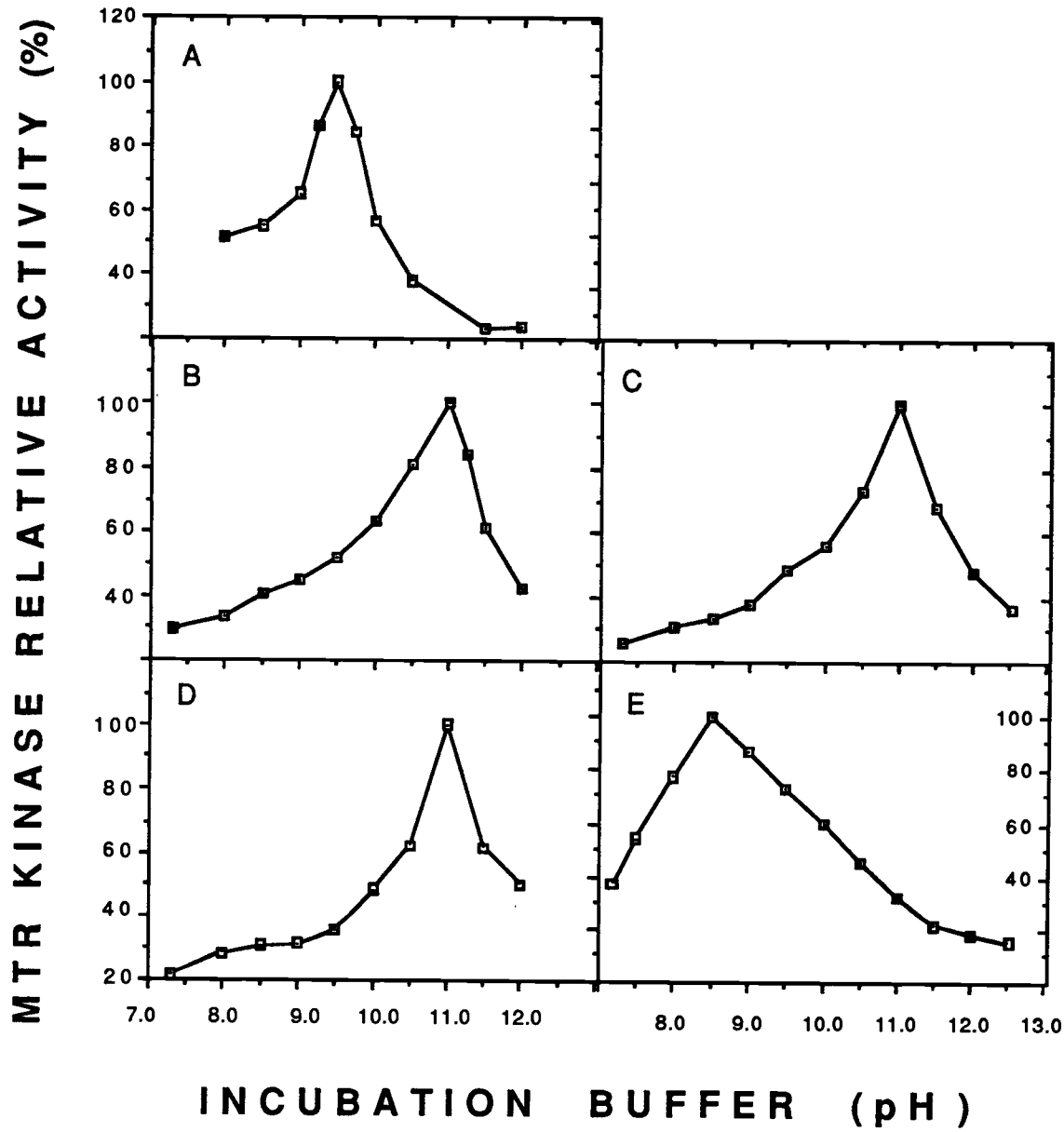


Figure III.2.

MTA without the addition of exogenous phosphate (however, sufficient levels of phosphate to drive the reaction may have been present in the extract itself).

Effect of Temperature Upon MTA Nucleosidase and MTR Kinase Activities.

Temperature influences upon enzyme activities of MTA nucleosidase and MTR kinase in crude extracts of both *Pyrus communis* L. and *Persea americana* Mill were examined in at least two ways. First, optimal temperature (T_{opt}) and energy of activation (E_a) for each reaction were determined by incubating the standard reaction mixture under conditions of optimal pH for the temperature range 23-25 to 60°C, and in some cases also 4 and 67°C. Second, enzyme thermostability was assessed by pre-incubating representative aliquots of crude extract at 45-60°C for 0-60 min prior to assaying enzyme activity at optimal temperature and pH. (These methods are described in Chapter II.) The "heat shock purifications" also served as an attempt to inactivate proteases and other compounds potentially responsible for enzyme instabilities (see Appendix A). In one case (MTR kinase from Hass avocado; Figure III.7.), the linearity of the reaction with both incubation time and temperature also was studied. Results are shown in Figures III.3.-III.9.

The data reveal that, except in one case, enzymatic catalysis of both MTA and MTR is increased significantly by elevated temperatures. For those crude extracts examined, all but Fuerte avocado and the kinase from Bosc pear showed T_{opt} for both MTA nucleosidase and MTR kinase activity at 52°C. As shown in Figure III.7., in Hass avocado extract the MTR kinase reaction is linear at this temperature for approximately 15 min; longer than the period of linearity observed for any other incubation temperature

Figure III.3. Effect of incubation temperature upon relative MTA nucleosidase activity in crude extracts from two varieties of *Persea americana* Mill and four varieties of *Pyrus communis* L. Cell-free extraction methods and enzyme activity measurements under conditions of previously determined optimal pH (see Figure III.1.) were performed as described in Chapter II. Each value reflects the net average of two independent assays utilizing the same extract. Corrections for nonenzymatic substrate degradation were conducted by subtracting out background activities of negative controls run simultaneously with test samples at each incubation temperature. Extracts examined include Hass avocado homogenized by Method 3 (A), Fuerte avocado homogenized by Method 4 (B), Comice pear homogenized by Method 4 (C), Packham Triumph pear homogenized by Method 3 (D), Bosc pear homogenized by Method 4 (E), and Passe Crassane pear harvested from growth medium and homogenized by Method 6 (F).

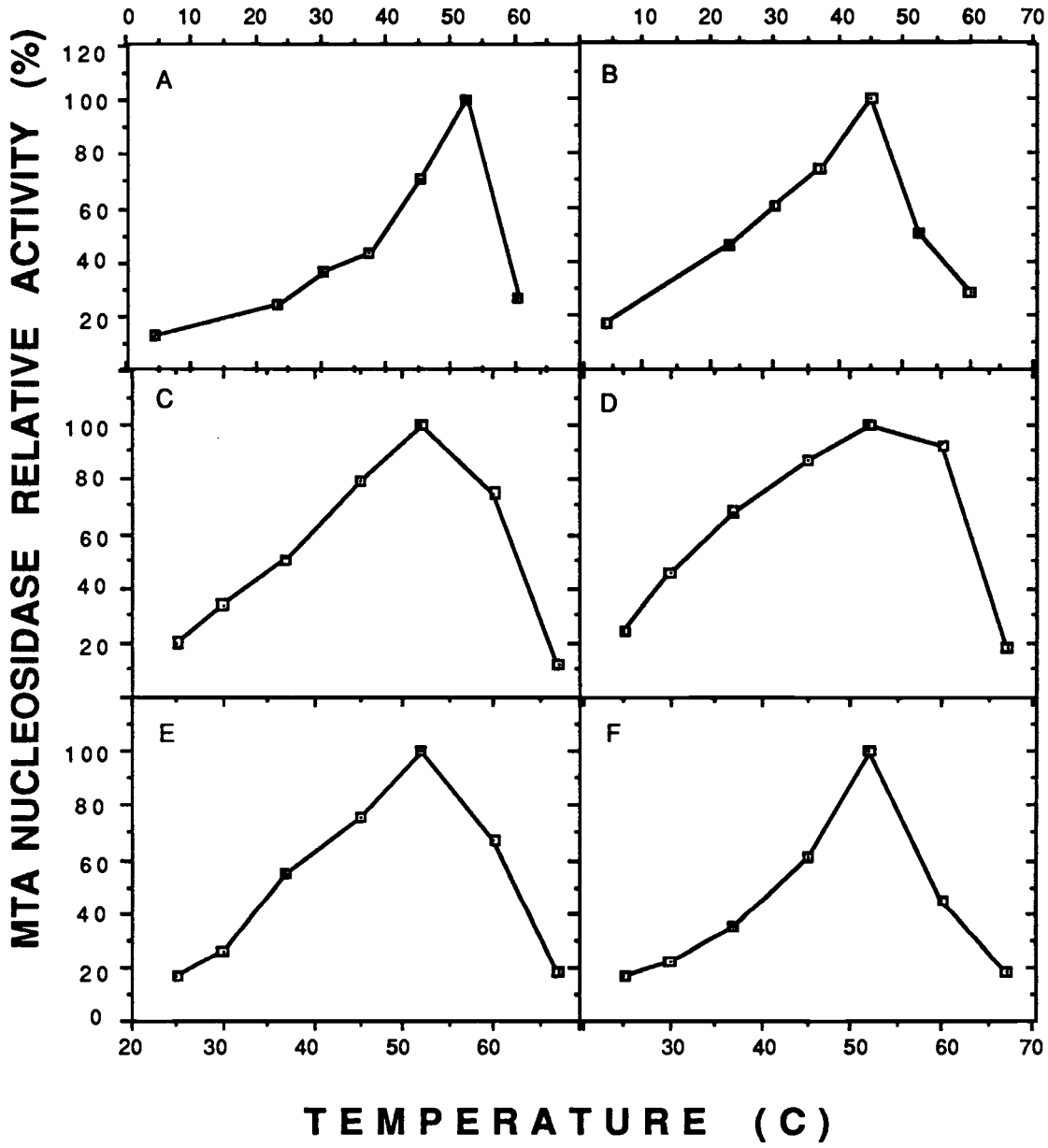


Figure III.3.

Figure III.4. Arrhenius plots of relative MTA nucleosidase activities. Initial velocity of each reaction was measured at 23-25, 30, 37, 45, and in some cases, also 4 and 52°C. Conditions and extracts examined are as described in Figure III.3.

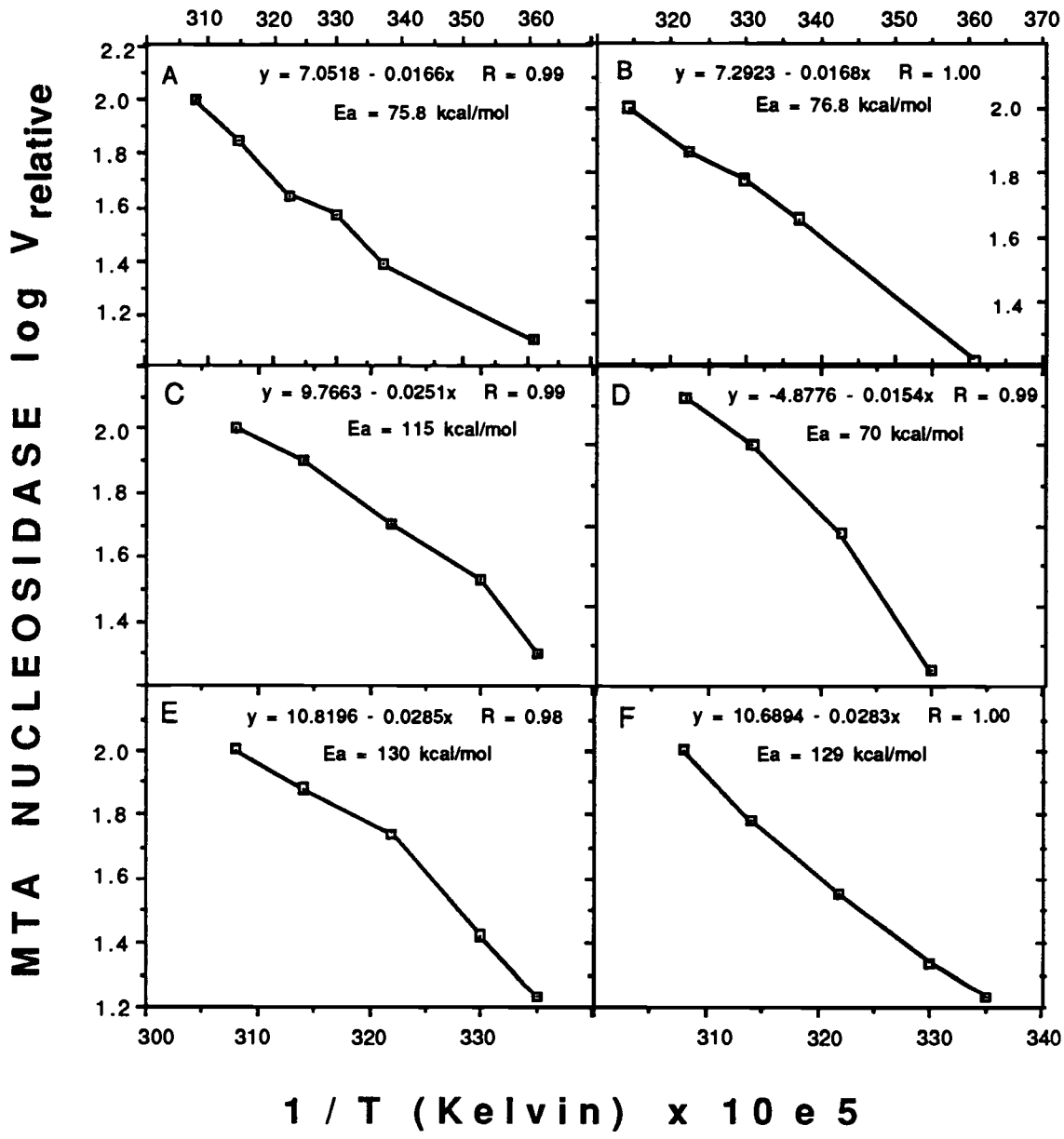

MTA NUCLEOSIDASE log V relative
A
y = 7.0518 - 0.0166x R = 0.99
Ea = 75.8 kcal/mol
B
y = 7.2923 - 0.0168x R = 1.00
Ea = 76.8 kcal/mol
C
y = 9.7663 - 0.0251x R = 0.99
Ea = 115 kcal/mol
D
y = -4.8776 - 0.0154x R = 0.99
Ea = 70 kcal/mol
E
y = 10.8196 - 0.0285x R = 0.98
Ea = 130 kcal/mol
F
y = 10.6894 - 0.0283x R = 1.00
Ea = 129 kcal/mol
1 / T (Kelvin) x 10 e 5

Figure III.4.

Figure III.5. Effect of incubation temperature upon relative MTR kinase activity in crude extracts from two varieties of *Persea americana* Mill and one variety of *Pyrus communis* L. Cell-free extraction methods and enzyme activity measurements under conditions of previously determined optimal pH (see Figure III.2.) were performed as described in Chapter II. Each value reflects the net average of two independent assays utilizing the same extract. Corrections for nonenzymatic substrate degradation were conducted by subtracting out background activities of negative controls run simultaneously with test samples at each incubation temperature. Extracts examined include Hass avocado homogenized by Method 4 (A), Fuerte avocado homogenized by Method 3 and assayed at pH_{opt} for Hass avocado (B), and Bosc pear homogenized by Method 4 (C).

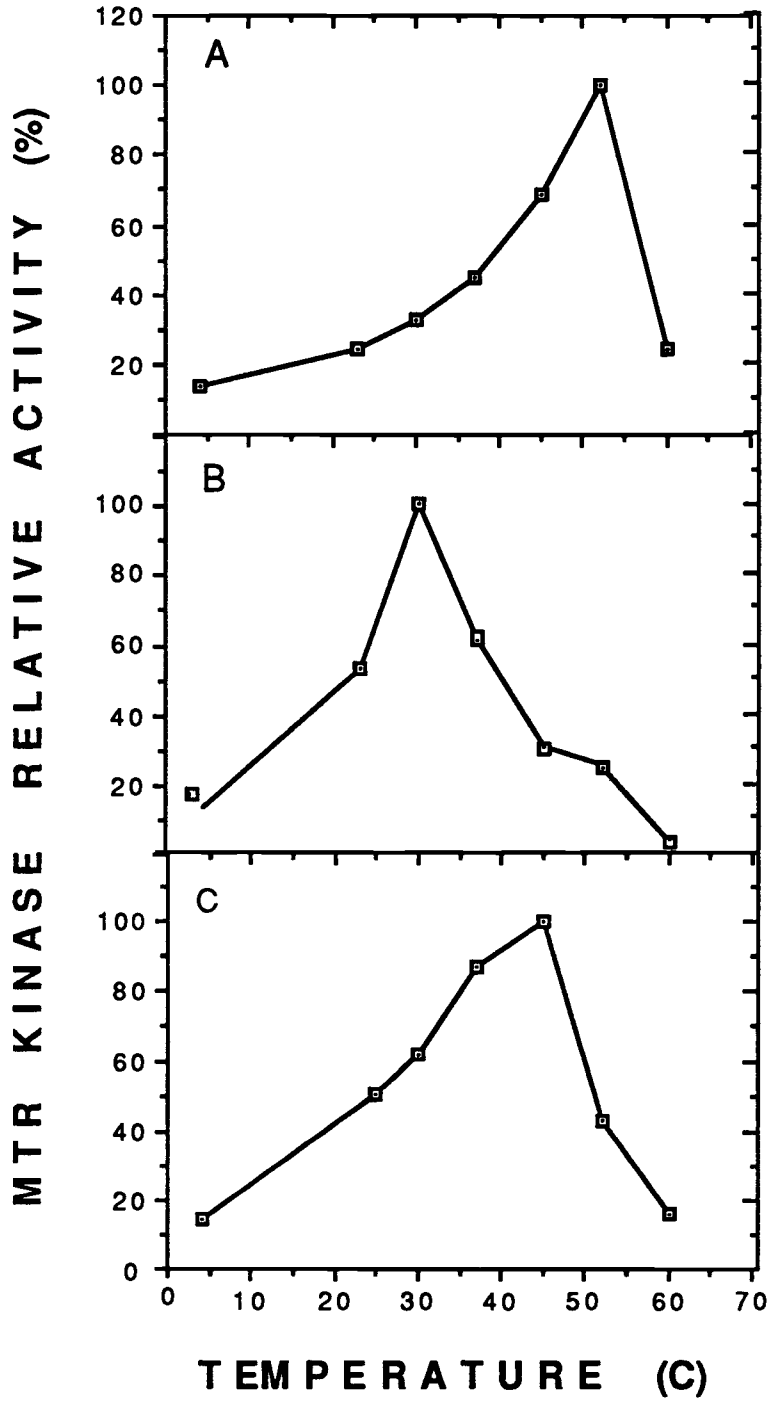

MTR KINASE RELATIVE ACTIVITY (%)
A
B
C
120
100
80
60
40
20
0
10
20
30
40
50
60
70
TEMPERATURE (C)

Figure III.5.

Figure III.6. Arrhenius plots of relative MTR kinase activities. Initial velocity of each reaction was measured at 4, 23-25, 30, and in some cases, also 37, 45 and 52°C. Conditions and extracts examined are as described in Figure III.5.

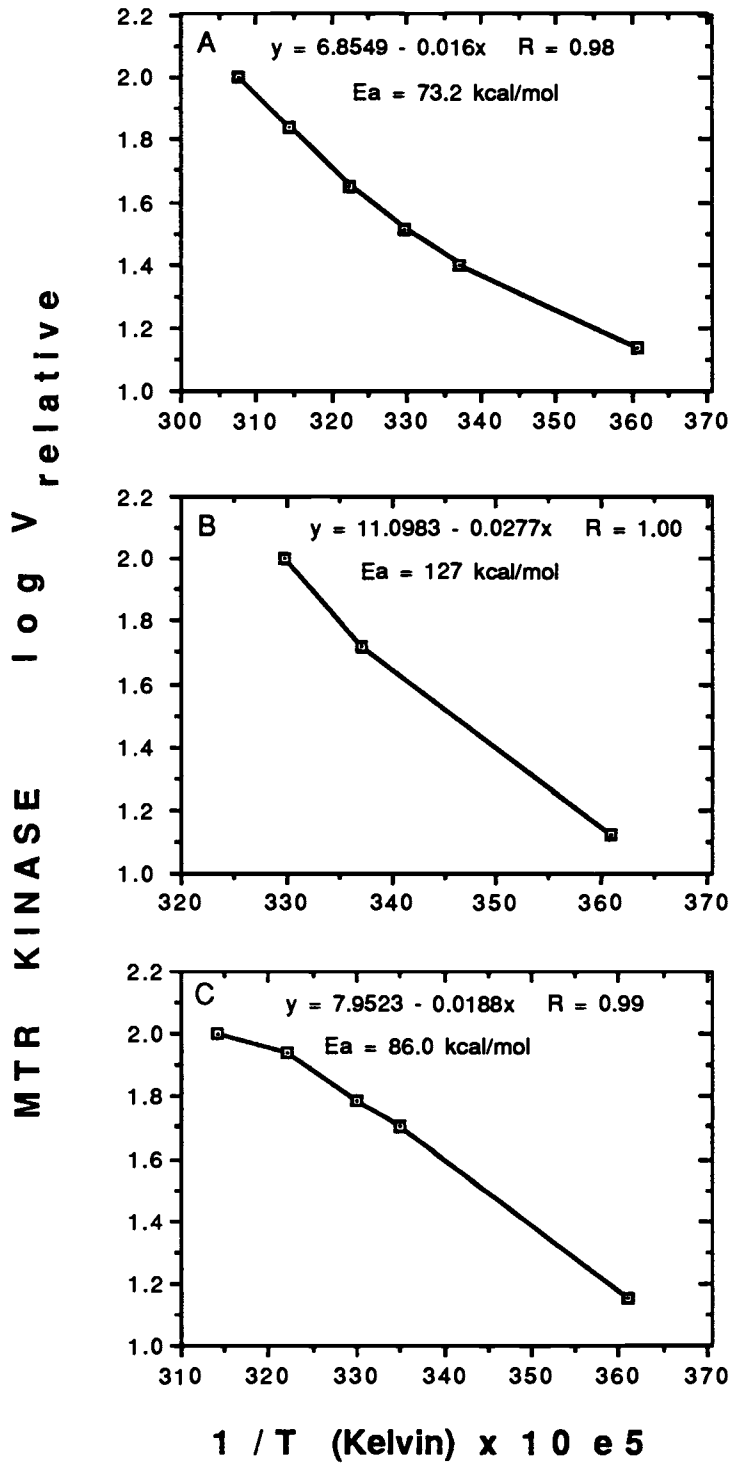

A
y = 6.8549 - 0.016x R = 0.98
Ea = 73.2 kcal/mol
B
y = 11.0983 - 0.0277x R = 1.00
Ea = 127 kcal/mol
C
y = 7.9523 - 0.0188x R = 0.99
Ea = 86.0 kcal/mol
MTR KINASE log V relative
1 / T (Kelvin) x 10 e 5

Figure III.6.

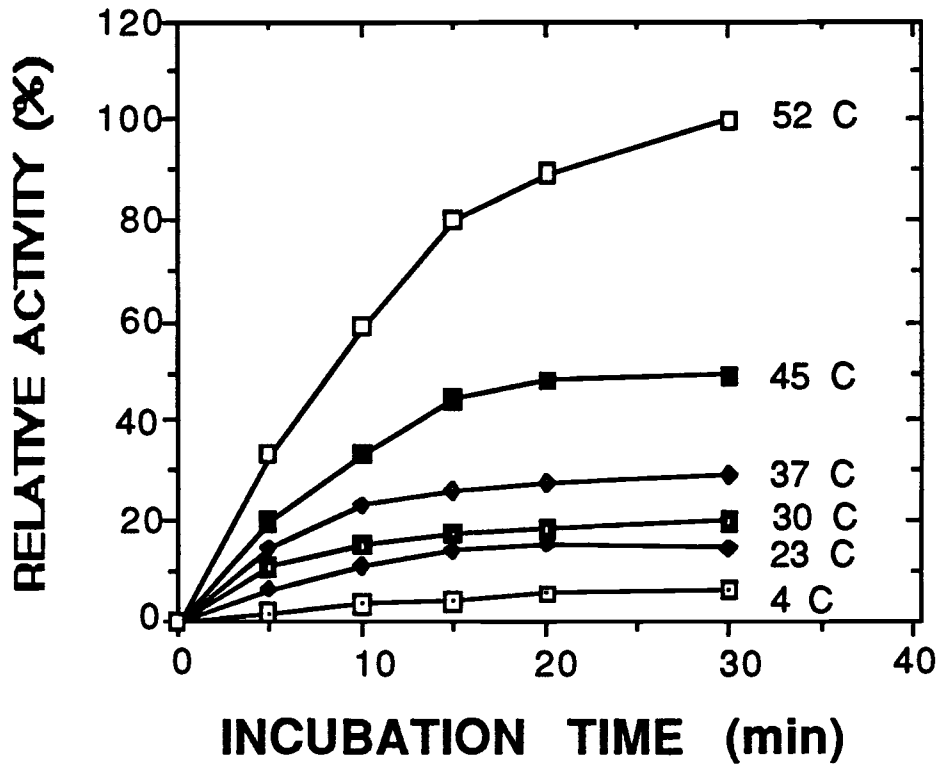


Figure III.7. Effect of temperature and time upon MTR kinase activity from a crude extract of *Persea americana* Mill, cv Hass. Enzyme activities at pH 9.5 were determined after incubating an extract homogenized by Method 3 for time intervals of 0, 5, 10, 15, 20, and 30 min as described in Chapter II at the following temperatures: 4°C (⊡), 23°C (◆), 30°C (■), 37°C (◇), 45°C (■), and 52°C (□).

examined. Crude extracts of Fuerte avocado demonstrated maximal activity at lower temperatures: 45°C for MTA nucleosidase and 30°C for MTR kinase. Similarly, MTR kinase activity in a crude extract of Bosc pear (the only variety of *Pyrus communis* L. for which the effect of temperature upon this enzyme was studied) had T_{opt} = 40-45°C.

In all cases a linear dependency was found when logarithms of specific rates of reaction at any temperature below and including T_{opt} were plotted against the reciprocal of the absolute temperature. Values of E_a for each reaction computed from Arrhenius plots (Figures III.4. and III.6.) ranged from 70-130 kcal / mol for both enzymes. No strong differences between avocado and pear fruit were observed in the latter studies.

The relatively high incubation temperatures associated with maximal enzyme activities prompted investigations of enzyme thermostability via heat shocking. In the case of MTA nucleosidase, elevated activities in the order of 18% (Hass avocado and Comice pear) to 35% (Bosc pear) above the non-preincubated control were observed under optimal assay conditions following 10 minutes of preincubation at 45°C; whereas both longer pre-incubations and higher temperatures generally resulted in either unchanged or significantly reduced activity (Figure III.8.). Both Hass and Fuerte avocado extracts showed even greater relative increases in MTR kinase activity (51 and 41%, respectively; see Figure III.9.) when assayed comparably following 30 min preincubations at 45°C. However, no increase in MTR kinase activity was observed when crude extracts of either Comice or Bosc pear were preincubated at 45, 52-55, or 60°C (data not shown).

Effect of Substrate Concentration and Other Analogs

Figure III.8 Heat shock purification of MTA nucleosidase from crude extracts of *Persea americana* Mill, cv Hass and two varieties of *Pyrus communis* L. Crude extracts homogenized by Method 4 were preincubated for various time periods (0-60 min) at three different temperatures: 45°C (⊡), 52°C (◆), and 60°C (■) prior to enzyme activity determinations under optimal reaction conditions, as described in Chapter II. In all cases, corrections were made for nonenzymatic hydrolysis. Extracts examined include: Hass avocado (A), Comice pear (B), and Bosc pear (C).

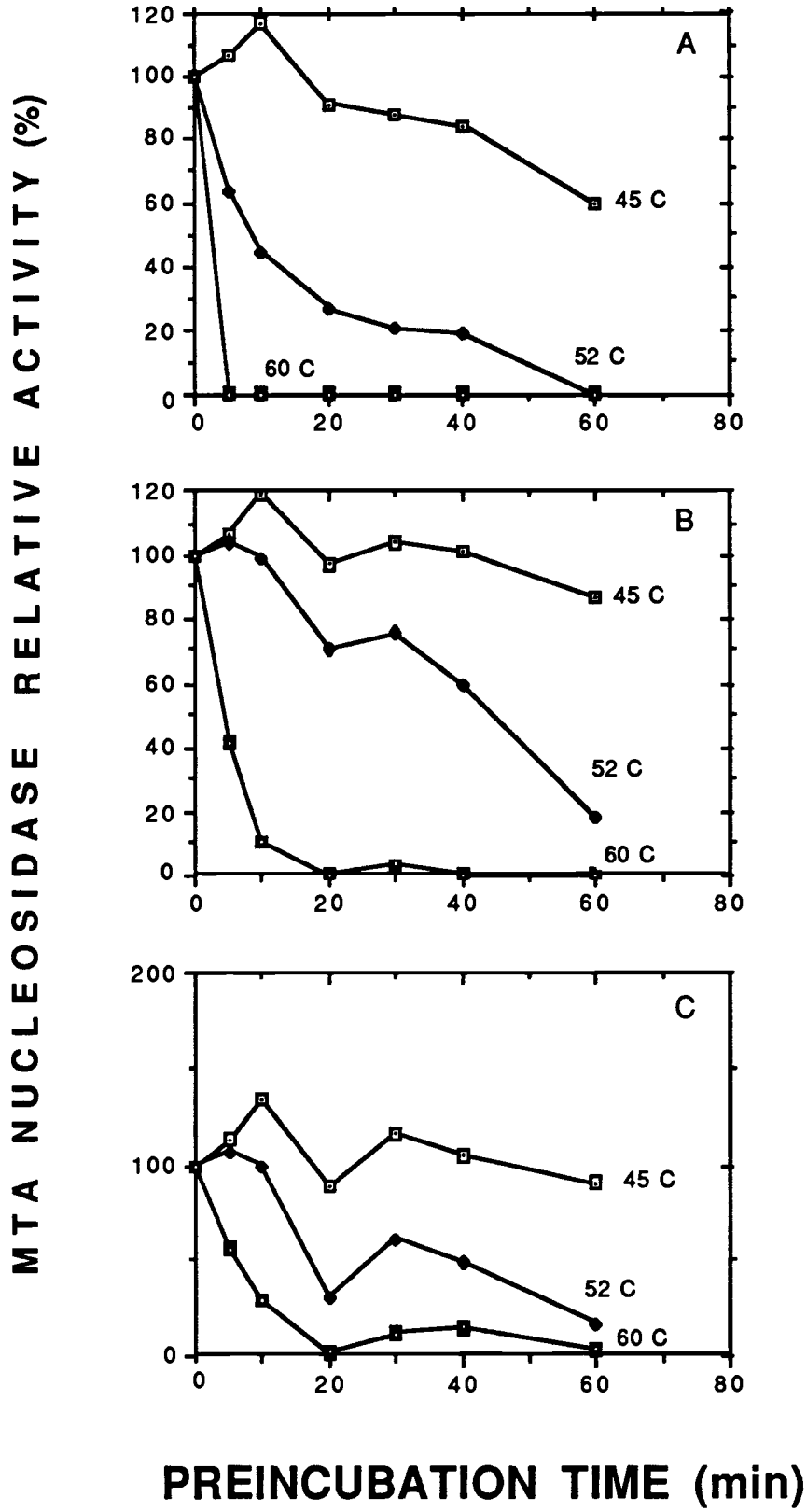

MTA NUCLEOSIDASE RELATIVE ACTIVITY (%)
A
B
C
45 C
52 C
60 C
PREINCUBATION TIME (min)

Figure III.8.

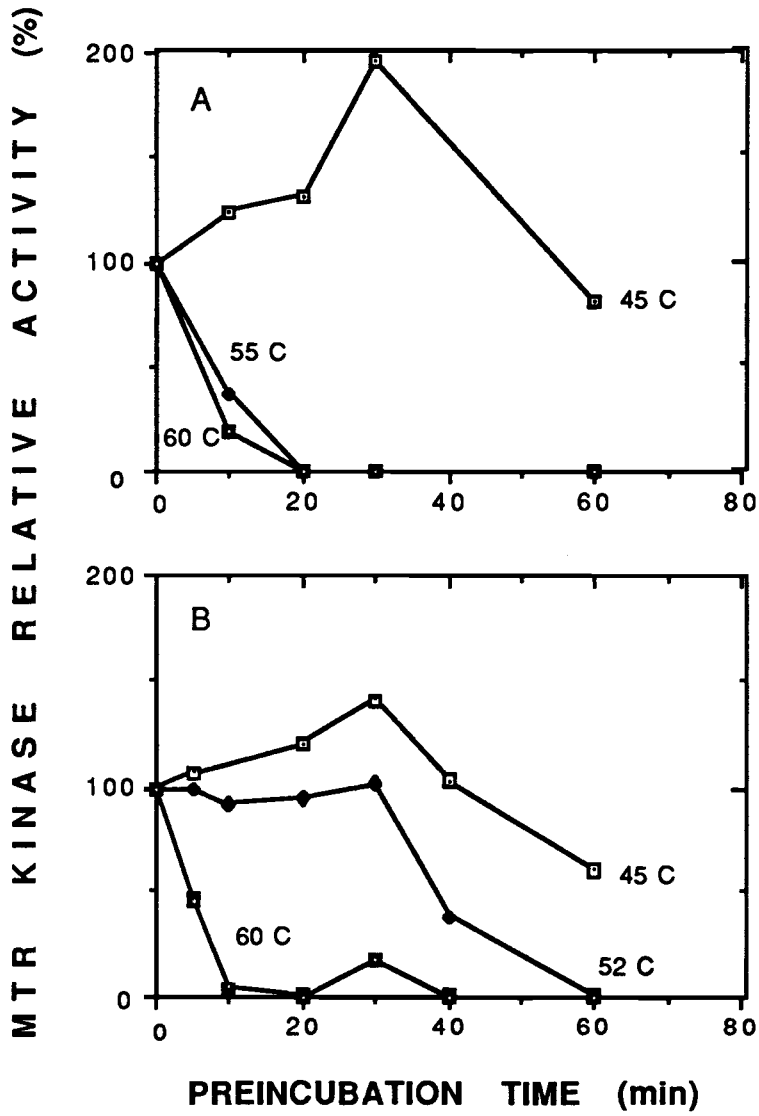


Figure III.9. Heat shock purification of MTR kinase from two varieties of *Persea americana* Mill. Crude extracts homogenized by Method 4 were preincubated for various time periods (0-60 min) at three different temperatures prior to enzyme activity determinations under optimal reaction conditions, as described in Chapter II. In all cases, corrections were made for nonenzymatic substrate degradation. Extracts examined include: Hass (A) and Fuerte avocado (B).

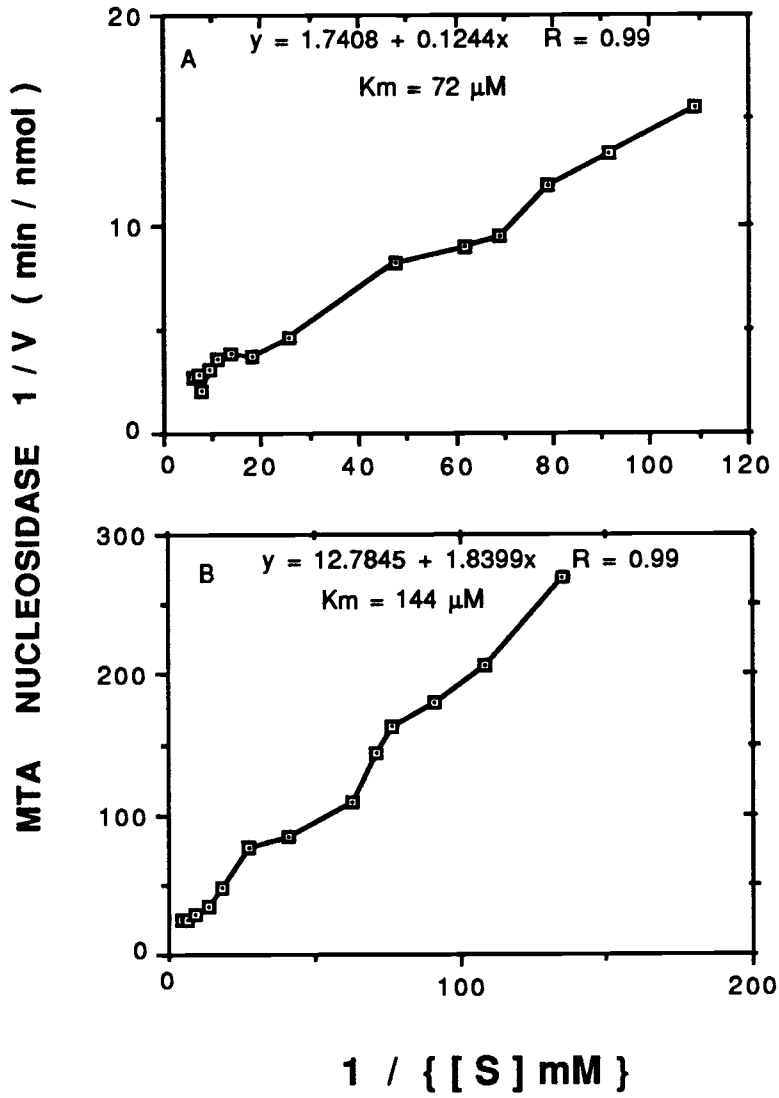


Figure III.10. Double-reciprocal plots of MTA nucleosidase activity vs. 5'-deoxy-5'-methylthioadenosine concentration in crude extracts of *Persea americana* Mill and *Pyrus communis* L. Each value represents the average of two independent assays at optimal reaction conditions, with corrections for nonenzymatic substrate degradation taken into account (see Chapter II for methods). Extracts examined include Hass avocado (A) and Bosc pear (B).

The MTA nucleosidase enzyme from crude extracts of Hass avocado and Bosc pear exhibited typical Michaelis-Menten kinetics with apparent K_m values of 72 and 144 μM, respectively, and V_{max} values of 575 pmol MTR hydrolyzed per min for the avocado and 78.2 pmol min^{-1} in Bosc extract (see double-reciprocal plots, Figure III.10.). Kinetic constants for MTR kinase were not determined.

To test as substrates several analogs of both MTA and MTR + ATP, the natural substrates of MTA nucleosidase and MTR kinase, respectively, the reaction products of each assay were separated and analyzed by either column chromatography followed by TLC or PC, or preferably one of the HPLC systems (I-IV) and detection methods described in Chapter II. The latter approach affords many advantages, as both substrate integrity (*e.g.*, purity and lack of oxidative degradation) and the ability of compounds to serve as substrate, inhibitor, or stimulator may be tested simultaneously (see representative chromatograms, Figures III.12.-III.15.). The effects of other compounds not analogous in structure but important in reactions relating to MTA, such as polyamines and S-adenosylhomocysteine (SAH) were investigated also. Some of the results are shown in Tables III.3. and III.4.

Of the seven compounds listed in Table III.3., only the first six are true analogs of MTA (see Figure III.11.) and hence only these were tested for ability to serve as substrates for MTA nucleosidase. Except possibly for carbocyclic-MTA (the cyclopentyl analog of MTA), none were found to be active in this capacity. Unlike other results in this particular study, the chromatograms generated to resolve whether or not carbocyclic MTA can act as a substrate for MTA nucleosidase in crude extracts of *Pyrus communis* L. contained enough artifacts to preclude congener peak recognition and for that reason cannot be considered definitive (data not shown). That MTA analogs with modifications in the N-3, C-8, and N-9 positions of adenine or

TABLE III.3. Effect of MTA Analogs Upon Relative MTA Nucleosidase Activity in Crude Extracts of 3 Varieties of *Pyrus communis* L.

	EXTRACT[a]	COMPOUND	RATIO OF [MTA] : [A][b]	% MTA NUCLEOSIDASE ACTIVITY[c]
1.	Comice	acyclic-MTA	1:1	63
	Packham Triumph		1:1	74
	Bosc		1:1	57
	Comice		1:2.5	33
2.	Comice	5'-Cl-3-deazaaristeromycin	1:1	111
	Packham Triumph		1:1	96
	Bosc		1:1	83
3.	Comice	carbocyclic-MTA	2:1	104
	Packham Triumph		2:1	100
	Bosc		2:1	91
4.	Comice	3-deaza-MTA	1:1	100
	Packham Triumph		1:1	106
	Bosc		1:1	108
	Comice		1:2.5	96
5.	Comice	carbocyclic-3-deaza-MTA	1:ss[d]	88
	Packham Triumph		1:ss[d]	92
	Bosc		1:ss[d]	88
6.	Comice	5'-Cl-formycin	1:1	44
	Packham Triumph		1:1	53
	Bosc		1:1	61
7.	Comice	5'-Cl-ribose	1:1	108
	Packham Triumph		1:1	85
	Bosc		1:1	98
	Comice		1:10	129
	Packham Triumph		1:10	23
	Bosc		1:10	111
	Packham Triumph		1:25	18
	Packham Triumph		1:66	20

[a]All extracts were prepared by Method 1, as described in Chapter II.

[b]Concentration of MTA relative to concentration of analog (A) being tested.

[c]Measured at buffer pH 11.0 and incubated at 37°C for the time required to convert 5-20% of substrate into product and expressed as a percentage of the reaction rate found with MTA.

[d]This compound was sparingly soluble.

TABLE III.4. Effect of MTR and ATP Analogs Upon Enzyme Activities of Both MTA Nucleosidase and MTR Kinase in Crude Extracts of *Persea americana* Mill, *Pyrus communis* L., and *Escherichia coli.*

EXTRACT	ANALOG	RATIO OF [NS] : [A] [a]	RELATIVE ACTIVITY (V_{analog} / V_{NS}) [b] MTA NUCLEOSIDASE	MTR KINASE
1. Hass avocado	MTR	1:1	1.21	0.52
Passe crassane pear [c]		1:1	1.13	ND[d]
E. coli		1:1	1.32	ND[d]
2. Hass avocado	ETR [e]	1:1	2.43	0.11
Passe crassane pear [c]		1:1	1.26	ND[d]
E. coli		1:1	1.86	ND[d]
Hass avocado		1.4 :1	1.18	ND[d]
Hass avocado		2:1	1.03	0.28
Hass avocado		4:1	ND[d]	0.44
3. Hass avocado	iBTR [f]	1:1	2.07	0.24
Hass avocado		1.4 :1	1.43	ND[d]
Hass avocado		2:1	1.00	0.42
Hass avocado		4:1	ND[d]	0.50
4. *E. coli*	adenine	1:1	1.00	ND[d]
5. Hass avocado	2'-dATP	0:1	ND[d]	0.64
6. Hass avocado	ADP	0:1	ND[d]	NA[g]

[a]Concentration of natural substrate (NS) relative to concentration of analog (A) being tested.

[b]Measured under optimal assay conditions for the time required to convert5-20% of substrate into product and expressed as a ratioof the reaction rate found with the analog relative to that found with the natural substrate (Chapter II).

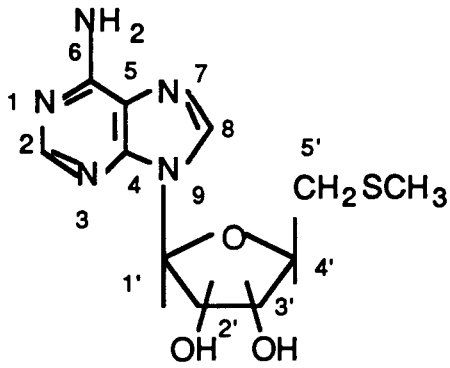
[c]Harvested from growth medium.

[d]Not determined.

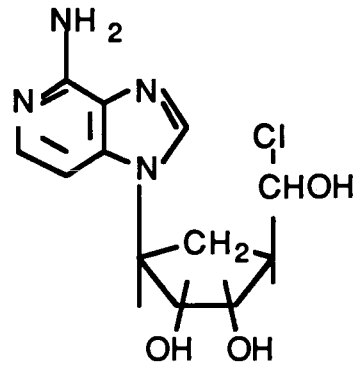
[e]The 5-ethyl analog of 5-deoxy-5-methylthioribose.

[f]The 5-isobutyl analog of 5-deoxy-5-methylthioribose.

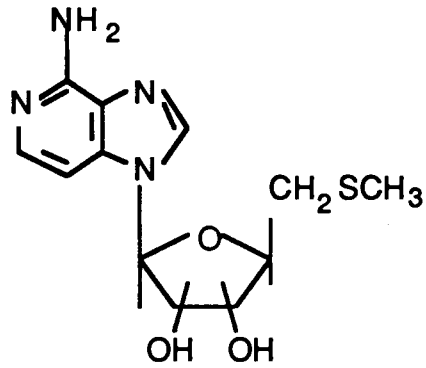
[g]No activity.



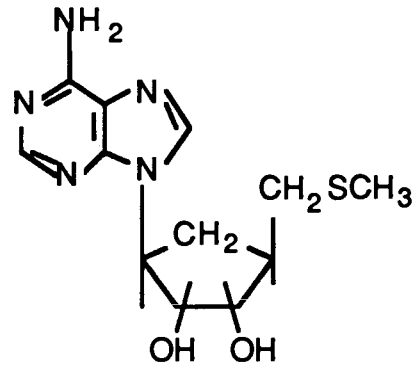
5'-DEOXY-5'-METHYLTHIOADENOSINE



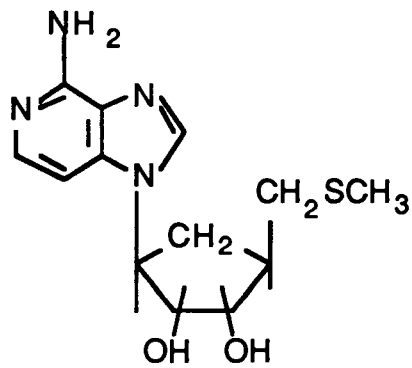
5'-CHLORO-3-DEAZAARISTEROMYCIN



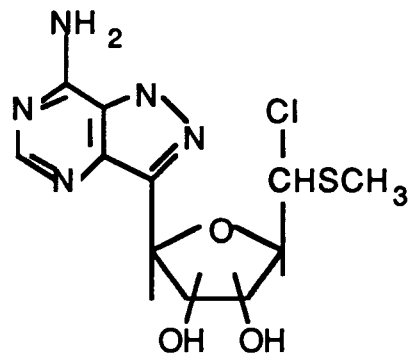
3-DEAZA-5'-DEOXY-5'-MTA



CARBOCYCLIC-5'-DEOXY-5'-MTA



CARBOCYCLIC-3-DEAZA-5'-DEOXY-5'-MTA



5'-CHLOROFORMYCIN

Figure III.11. Chemical structures of 5'-deoxy-5'-methylthioadenosine (MTA) analogs listed in Table III.3. Acyclic MTA, the only structure not listed here, has an intact adenine moiety attached to an open ring methylthioribose.

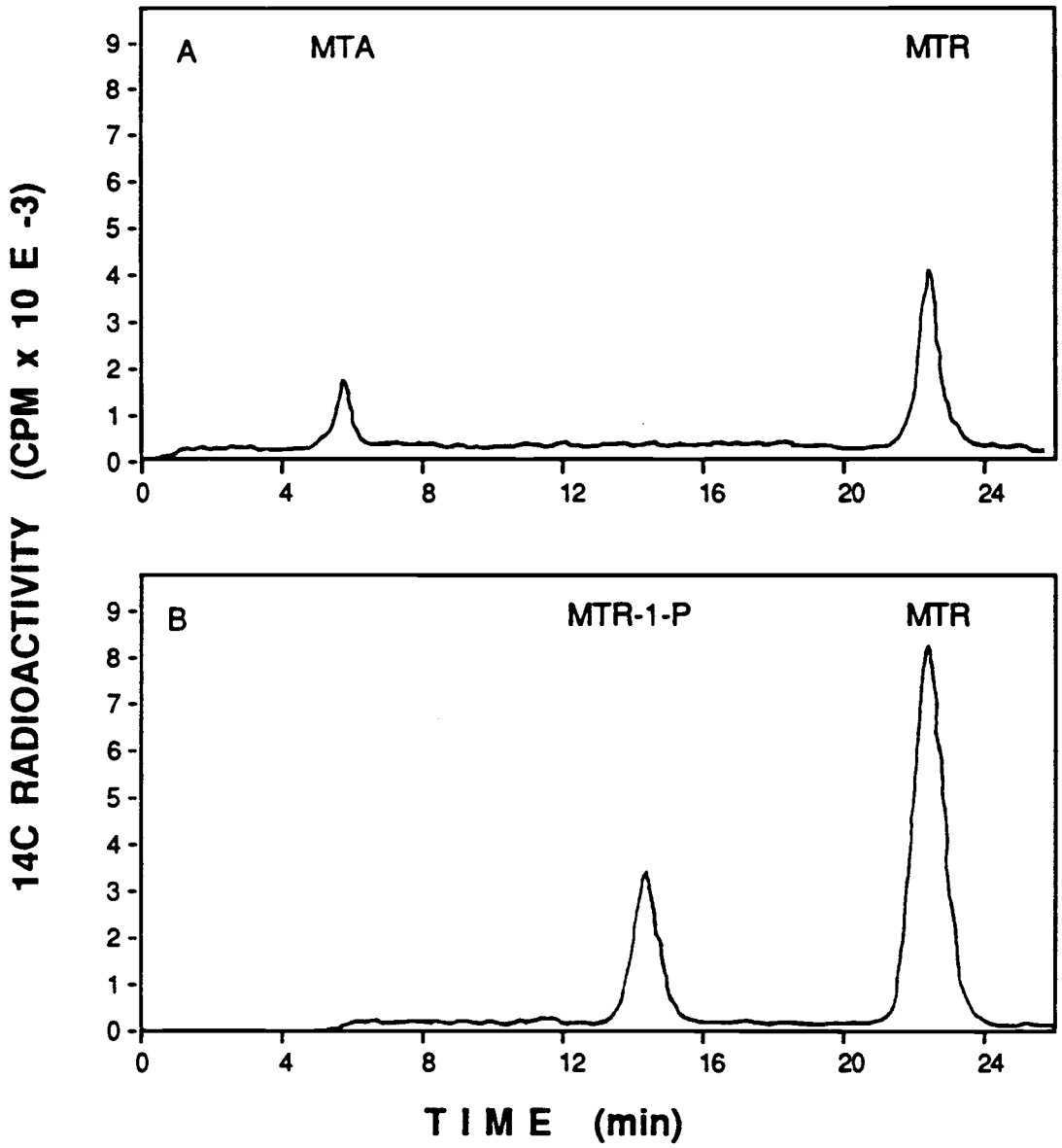


Figure III.12. HPLC System I: representative chromatograms of enzyme assay substrates and products. Panel A = MTA nucleosi- assay of a crude extract of Comice pear; panel B = MTR kinase assay of a crude extract of Hass avocado. Conditions (described in detail in both Chapter II and Appendix B) = uSpherogel column with ddH2O at 0.6 mL / min, 85 C.

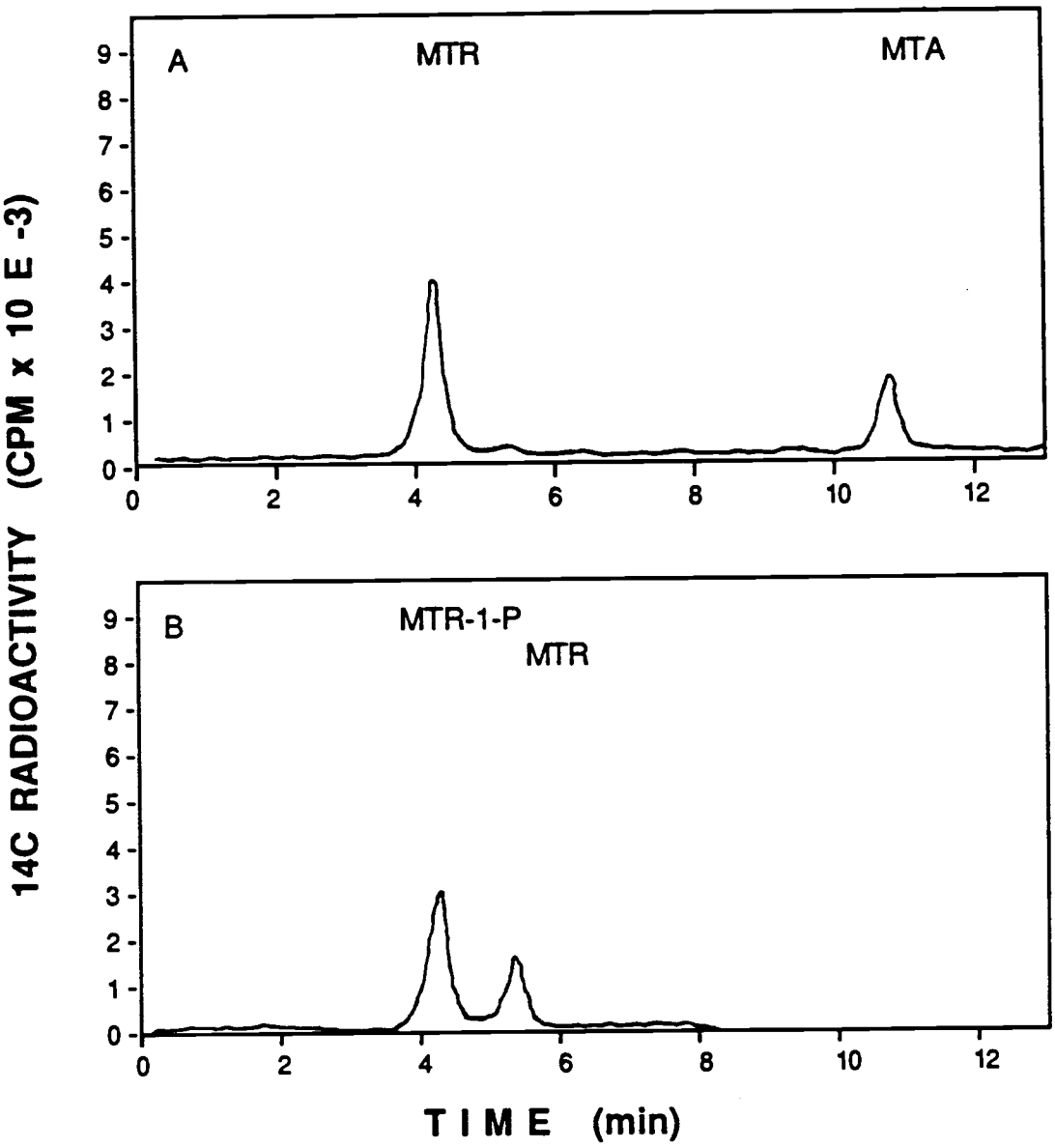


Figure III.13. HPLC System II: representative chromatograms of enzyme assay substrates and products. Panel A = MTA nucleosi- assay of a crude extract of Comice pear; panel B = MTR kinase assay of a crude extract of Hass avocado. Conditions (described in detail in both Chapter II and Appendix B) = uBondapak C18 column with methanol / ddH2O (36: 64, v / v) at 0.7 mL / min, room temperature.

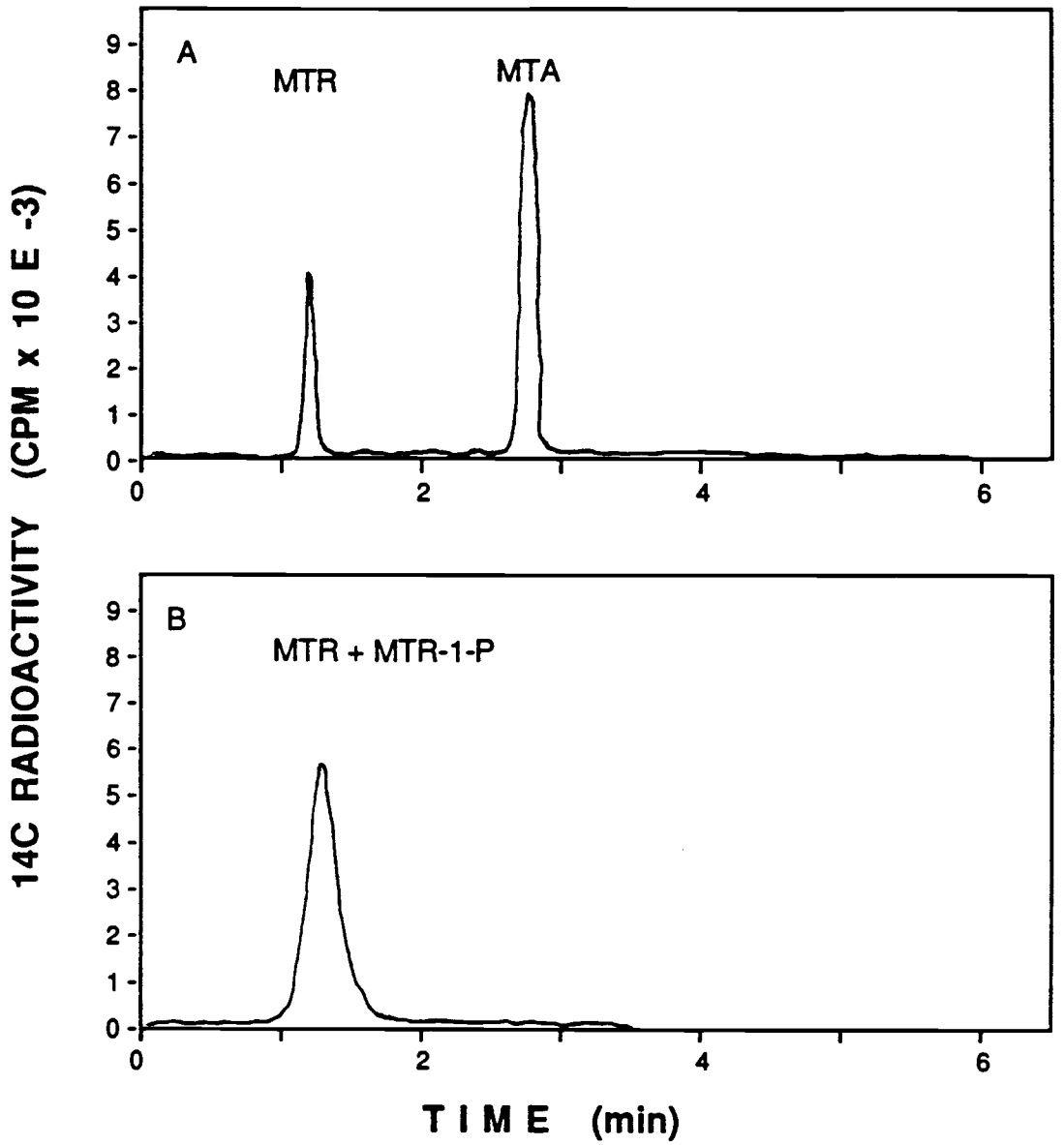


Figure III.14. HPLC System III: representative chromatograms of enzyme assay substrates and products. Panel A = MTA nucleosi- assay of a crude extract of Comice pear; panel B = MTR kinase assay of a crude extract of Hass avocado. Conditions (described in detail in both Chapter II and Appendix B) = Partisil 10 SCX column with 0.5 M NH4COOH (pH 4) 3.0 mL / min, room temperature.

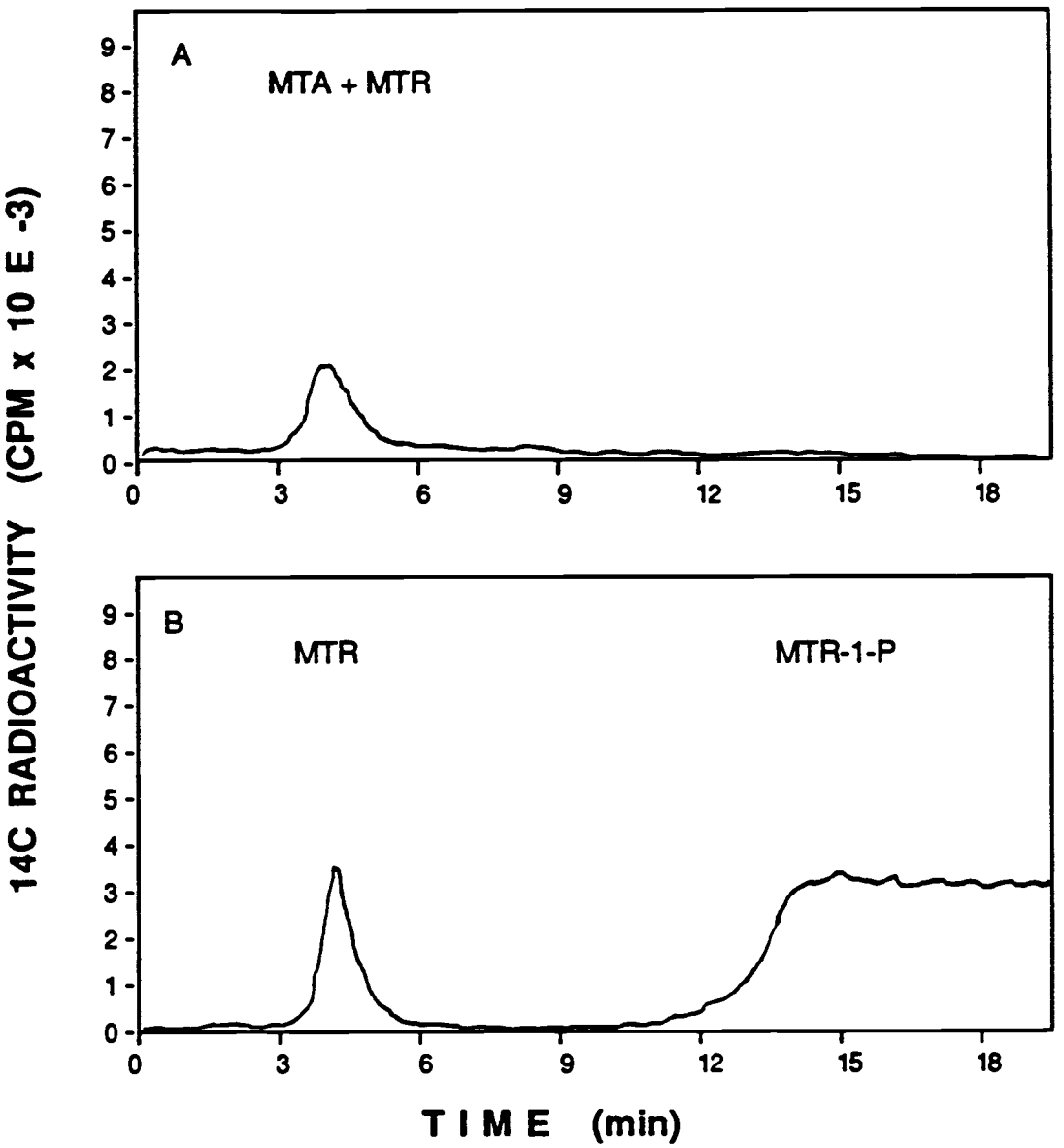


Figure III.15. HPLC System IV: representative chromatograms of enzyme assay substrates and products. Panel A = MTA nucleosi-assay of a crude extract of Comice pear; panel B = MTR kinase assay of a crude extract of Hass avocado. Conditions (described in detail in both Chapter II and Appendix B) = Partisil 10 SAX column with 0.25 M NH4COOH (pH 4) 1.0 mL / min, room temperature. Note that MTR-1-P sticks to the solid flow cell of the radioisotope detector, and thus cannot be quantitated under these conditions. (Methods to avoid this problem are discussed in Chapter II.)

lacking the cyclic structure of ribose are not substrates for MTA nucleosidase is of interest (see Discussion).

Also of note is that although the cyclopentyl analog 5'-chloro-3-deaza-aristeromycin was found to be readily soluble in 45°C water of pH 7, the other two such compounds, carbocyclic-MTA and (particularly) carbocyclic-3-deaza- MTA, exhibited limited solubility, even after being left to incubate (in the presence of the reducing agent DTT) for 12 h at 60°C. Chromatographic analyses of carbocyclic-MTA both before and after this extended incubation indicated that the compound was stable to this treatment (data not shown). No additional efforts (such as reduction of pH, for example) were made to solubilize either compound.

Nonetheless, carbocyclic-3-deaza-MTA was seen to exert mild inhibition (8-12%) upon the activity of crude pear MTA nucleosidase, even at very low concentrations (Table III.3.). Tested individually at equimolar concentrations to MTA, both acyclic-MTA and 5'-chloroformycin (a.k.a. 5'-chloro-8-aza-9-deazaadenosine) were the strongest inhibitors and demonstrated no strong cultivar-specific differences (average inhibition for all three pears examined = 66% and 53%, respectively). However, in the case of 5'-chlororibose administered comparably, only the enzyme from an extract of Packham Triumph was inhibited (15%). The effect was even more pronounced at higher concentrations (*i.e.*, 0.74 mM), where relative to standard assays run concurrently, this compound actually stimulated (11-29%) MTA nucleosidase activity measured in both Comice and Bosc pear extracts, but further inhibited that of Packham Triumph (by a factor of 77%). This degree of inhibition did not change appreciably at considerably higher concentrations (*i.e.*, 1.81 and 4.90 mM). Raising the concentration of acyclic-MTA from 74 to 185 μM resulted in an additional 30% increase in the observed inhibition of Comice MTA nucleosidase activity. When tested separately, neither the

three polyamines putrescine, spermidine, or spermine (data not shown), nor any of the other MTA analogs examined (including SAH, which was found not to be a substrate; data not shown) exerted significant influences upon this enzyme.

Although neither of the MTR analogs listed in Table III.4. were degraded by MTA nucleosidase, both these compounds as well as 2'-dATP were active substrates for MTR kinase; and when tested individually at natural substrate to analog concentration ratios of 0-1.4 : 1, all four influenced enzyme activities. Of particular note is that MTR and its 5-alkyl analogs had opposite effects upon the activities of MTA nucleosidase and MTR kinase, increasing the former in the order of 13-143% and decreasing the latter by 48-89% (Table III.4). The trend was observed in crude extracts from 1-3 different species, and was most pronounced for the compound 5-deoxy-5-ethylthioribose (ETR); the same 5-alkyl MTR analog found when tested at equimolar concentration to the natural substrate to exert the most significant inhibition (89%) of MTR kinase activity. That the former effect is independent of (residual) adenine possibly present in preparations of MTR and its analogs is suggested by the fact that at a concentration equimolar to MTA, adenine was found to have no influence upon MTA nucleosidase activity in an extract of *E. coli*.

Also of interest are the observations that in extracts of Hass avocado, whereas substitution of 2'-dATP for ATP reduced MTR kinase activity by 36%, the same treatment with ADP resulted in complete loss of enzyme activity; indicating that 2'-dATP but not ADP can serve as a phosphate donor for the reaction (see Table III.4.).

DISCUSSION

The data presented in Tables III.1. and III.2. indicate that extraction Methods 4, 6, and 7, involving both liquid N_2 and the mixture of resins including PVPP, XAD-4, and Dowex 1[Cl-]-X8 are superior with respect to corresponding MTA nucleosidase and MTR kinase enzyme activities than those lacking one or both of these features. In addition, the former preparations were found to be more stable. Similar results have been reported for other enzymes extracted both from *Pyrus communis* L. (127) and a wide variety of other plant tissues (85, 86, 143, for example).

Although this treatment produced the highest enzyme activities from crude extracts generated for the study, the values obtained were still considerably lower than those reported by Kushad *et al.* (68) for MTR kinase extracted by Method 1 or 2 from d'Anjou pear (35.1 vs. 680 pmol min^{-1} mg^{-1}) and Hass avocado (493 vs. 810 pmol min^{-1} mg^{-1}). The reason for this is unknown. Both MTA nucleosidase and MTR kinase activities observed here in whole pear fruit extracts are comparable to or greater than those reported for the same enzymes from crude extracts of *Lupinus luteus* seeds (50, 51). However, crude extracts of pear and avocado fruit utilized in this study have characteristic MTR kinase activities 1-4 orders of magnitude below those reported from crude extracts of higher plant seeds from five other species (*Glycine max, Cucumis sativus, Helianthus annuus, Zea mays,* and *Hordeum vulgare;* 50). The differences may reflect variations in protein content and even ATPase activity, the latter of which would compete for one of the substrates of the MTR kinase reaction (50; see Appendix A).

That suspension cultures of Passe Crassane pear cells both have been shown by other investigators to possess the metabolic pathway for ethylene

(C_2H_4) synthesis from methionine (MET; 78, 79, 105, 106, 137) and have been demonstrated in the present study to possess MTA nucleosidase and MTR kinase activity, suggests that they also contain the MET recycling pathway, and therefore could be used in metabolic studies examining this event (see Chapter IV). In this regard, it is of interest that extracts of Passe Crassane cells grown in aging medium, which has been reported to cause both a reduction in cell division and an increase in C_2H_4 production, thereby serving as a model for senescence (16, 101, 105, 106), have lower (approximately 50%) MTA nucleosidase and MTR kinase activities than those of cells originating from growth medium. This is contrary to what might be expected. A similar trend was observed when MTA nucleosidase activity was assayed in ripening fruits of tomato and Bosc pear (67, 70), although the effect was reversed in avocado (67, unpublished data of this author). The decline with fruit age of MTA nucleosidase (and MTR kinase) activity suggests that regulating the levels or activities of MET recycling enzymes may play an important role in the regulation of C_2H_4 biosynthesis. Thus, the impaired ability of aged fruit cells to recycle MTA could limit the synthesis of MET, and therefore C_2H_4 as well.

Except for MTA nucleosidase from Hass avocado and MTR kinase from both Hass avocado (pH_{opt} = 9.5, in both cases) and Passe Crassane pear (pH_{opt} = 8.5), the optimum pH with respect to the relative activities of both enzymes from all plant extracts examined was in the incubation buffer range of 10.75-11.25. The lower end of this range is approximately the same pH_{opt} value reported for the partially purified MTR kinase from an extract of *L. luteus* (50). However, it is 3.5 units below that for a comparable extract of *Enterobacter aerogenes* (40). Likewise, all reported values of pH associated with optimal MTA nucleosidase activity are lower: 6.8-7.3, *E. coli* and *C. albicans* (39, and this study); 8-8.5, *L. luteus* (51).

That both enzymes from the majority (*i.e.*, all but one; MTR kinase from Passe Crassane pear) of crude extracts examined would exhibit lower activities when assayed in the presence of Tris-HCl buffer is not surprising. The same effect has been reported to occur even more significantly with partially purified bacterial MTA nucleosidase assayed in the presence of either Tris-HCl or Hepes, rather than a phosphate buffer (75% and 30% inhibition of enzyme activity, respectively; 39). The explanation for this phenomenon (not given in the previous reference) most likely lies in the strong dependence of pK_a with temperature characteristic of Tris (and some other amine) buffers (122). Although temperature has a barely measurable effect upon the dissociation of phosphate, it has been shown that the pK_a of Tris changes by one pH unit between the temperatures of either 4-37°C or 20-45°C (122).

Although pH optima with respect to enzyme activities could reflect a variety of events (*e.g.*, a true, reversible effect upon reaction rate; an effect upon substrate affinity and enzyme saturation, or an effect upon enzyme stability), these data suggest that both the MTA nucleosidase and MTR kinase enzymes from fruit may be acid-sensitive. If so, the need to neutralize or remove from plant extracts various acidic (formerly vacuolar) compounds released during cell lysis in additon to phenolic and quinone substances and their oxidation products (accomplished here with the inclusion of various resins in the extracting buffer) is reinforced. As an interesting sidenote to this, it was found generally that even cell-free homogenates of fruit extracted in the presence of PVPP, XAD-4, and Dowex 1 [Cl^-]-X8 characteristically possessed solution pH values 0.5-0.8 units below that of the extraction buffer.

In those crude extracts for which the effect of temperature upon enzyme activity was examined, with the exception of MTR kinase from Fuerte

avocado (where T_{opt} = 30°C), both the nucleosidase and kinase appeared to be unusually thermostable. The phenomenon was found also to be true for MTR kinase from preparations of partially purified (approximately 30-fold) extracts of Hass avocado (data not shown), and was exploited in some cases as a purification step. These results agree in part with the only previous report from a similar study which indicates that T_{opt} for partially purified (4-fold) MTR kinase activity from an unnamed cultivar of avocado is 30°C (68). Results of the present investigation suggest that this fruit was Fuerte. Although partially purified (13-fold) human lymphocyte MTA phosphorylase is reported to catalyze at a maximal, linear rate the degradation of MTA to phosphorylated MTR (*i.e.*, MTR-1-P) at elevated temperatures (47°C; 145), this is the first report of a similar phenomenon occurring in the plant (fruit) enzymes MTA nucleosidase and MTR kinase. That the latter would exist seems reasonable, as fruit tissue development and ripening are stimulated by higher temperatures.

It is of interest that whereas significant cultivar-specific differences (with respect to profiles relating enzyme activities to the effects of pH and particularly temperature) exist in avocado, except for the lower pH requirement for maximal MTR kinase activity in Passe Crassane, crude extracts from pear behaved relatively uniformly. This reinforces the suggestion that physiologically, suspension-cultured Passe Crassane pear cells might be similar enough to other cultivars of whole pear fruit to be utilized as a model system for the MET recycling metabolic events of the latter.

That enzyme activity would increase in the majority of extracts exposed to "heat shock" treatments prior to being assayed under normal reaction conditions suggests the presence in crude extracts of heat-sensitive enzyme inhibitors (or proteases). Additional evidence for this lies in the interesting

trend in this study whereby an initial drop of at least 20% in MTA nucleosidase activity measured after a 20 min preincubation at 45 and 52°C was observed; generally at the data point immediately following the measurement of maximal activity in each profile, and often followed by a temporary rise in activity prior to a steady decline (see Figure III.8.). This might reflect the initial heat activation (at the 20 min mark) of an enzyme inhibitor of limited thermostability and its subsequent time-dependent heat inactivation. If so, the same inhibitor appears to have no influence upon MTR kinase activity (Figure III.9.).

Effects of thermostability have been reported for both arginine decarboxylase (a key enzyme in polyamine metabolism) extracted from Fuerte avocado (146) and MTA phosphorylase from the thermoacidophilic archaebacterium *Caldariella acidophila* (45). Unfortunately, in the present study, heat-purified extracts did not exhibit increased stability with respect to enzyme activity (in fact, they were found to be highly unstable with time), an event which may relate to either increased protease activity or perhaps the limited half-life at elevated temperatures of the reducing agent DTT (see Appendix A).

Previous kinetic studies of the natural substrates for MTA nucleosidase (*i.e.*, MTA) and MTR kinase (*i.e.*, MTR and ATP) from partially purified extracts of other plants have revealed the following apparent K_m values measured at 37°C, for MTA: 15 μM (*Lycopersicon esculentum;* 151), 10.3 μM (*Vinca rosea;* 18), 0.41 μM (*Lupinus luteus;* 51); for MTR: 4.3 μM (*L. luteus;* 50), and for ATP: 8.3 μM (*L. luteus;* 50). Relative to plants, bacterial sources have been reported to have higher affinities for MTA (K_m = 0.3-0.4 μM in *E. coli;* 33, 50), but lower binding capacities for both MTR and ATP (K_m = 8.1 μM and 74 μM, respectively, in *E. aerogenes;* 40). The kinetic constants generated in this study, reflecting the MTA-binding capacity of MTA nucleo-

sidase from both *Persea americana* and *Pyrus communis,* although similar to bacterial $K_{m\ (ATP)}$ for MTR kinase, are considerably higher (*i.e.,* lower substrate affinities) than other values listed above. These differences likely reflect the facts that K_m can change with temperature (122) and extracts utilized in this report were crude, rather than (partially) purified.

Based upon the behavior of those analogs examined in this study, both MTA nucleosidase and MTR kinase from fruit extracts appear to have a high degree of substrate specificity. The nucleosidase failed to accept as substrates compounds modified in either the N-3, C-8, or N-9 positions of adenine, or the cyclic ether structure of ribose. Similar results have been reported in studies of SAH / MTA nucleosidase (which, unlike its nucleosidic counterpart in plant tissues, utilizes both SAH and MTA as substrates) from *E. coli,* where four possible substrate recognition sites in two moieties were postulated: NH_2 and N-3 of adenine, and the two OH groups of ribose (33). As in the present investigation, an intact ribose ring appears to be required for the binding process of the bacterial enzyme (33).

This is in partial contrast to the finding that human placental MTA phosphorylase exhibits a low substrate affinity for various 3-deaza derivatives of MTA (154). That MTA analogs modified in the N-1 position of adenine are inactive as substrates for this enzyme suggests the presence of another site of recognition or interaction (154).

Both groups of researchers (33, 154) also stress the importance with respect to catalytic mechanism rather than substrate recognition of the N-7 position of adenine and its ability to become protonated. The protonation of this species elicits an electron withdrawal which in turn promotes a nucleophilic attack by phosphate on C-1' of the ribose moiety. The latter is accomplished via a distortion of all ribosyl bonds, resulting in an inversion at C-1'. Zappia

et al. suggest that recognition of the two hydroxyl groups at C-2' and C-3' may distort the ring so as to enable the formation of a substrate-enzyme transition state, and have found the chirality at these positions to be relevant to substrate specificity (154).

Modifications at this site were not investigated in the present study -- all MTA analogs examined possessed N-7, but only two such compounds (acyclic-MTA and 5'-chloroformycin) at the concentrations tested significantly inhibited fruit MTA nucleosidase activity. One of these, 5'-chloroformycin, when administered in an equimolar concentration to MTA, did not exert as strong an influence upon the activity of this enzyme as that previously reported in crude extracts of tomato fruit (in the order of 50% vs. 89% inhibition; 70). This conflict may reflect differences in compound purity or oxidation state (*i.e.*, oxidative degradation to the corresponding sulfoxide or sulfone).

MTR kinase from extracts of avocado fruit was found to accept as both substrates and strong inhibitors MTR analogs modified in the aliphatic chain at C-5', corroborating similar reports concerning both this enzyme, and in the case of substrate specificity only, also the nucleosidase, from other sources (33, 39, 50, 70). Of those analogs tested, the ethyl analog ETR was the most potent inhibitor, reducing MTR kinase activity from a crude extract of avocado fruit by 89%, whereas the isobutyl analog iBTR inhibited this activity 76%. Both compounds when test individually at equimolar concentrations to MTR have been shown to be inhibitors of MTR kinase activity in crude extracts of tomato fruit (70); but in the latter report, the magnitudes of inhibition are considerably different (*i.e.*, 19% and 41%, respectively). This may be a result of either the quality of the analogs (*i.e.*, purity and integrity with respect to oxidation state), or the non-optimized enzyme reaction conditions utilized in this study (*i.e.*, a 2 h incubation at 30°C in an incubation buffer of pH 7.3).

Although tomato extracts were never examined in the present investigation, if instead of assaying enzyme activity under source-specific (optimal) reaction conditions, the analyses mentioned earlier had been conducted under those conditions cited above, it is unlikely that the measurable MTR kinase activity from crude extracts of avocado would be as strongly inhibited by these two compounds.

As mentioned earlier, it was anticipated that both ETR and iBTR would inhibit MTR kinase activity. However, that this group of compounds as well as MTR itself (but not adenine) would have the opposite effect upon MTA nucleosidase activity is most unexpected and puzzling. That the phenomenon occurs (to varying extents) in crude extracts from three different species suggests that the effect is not unique. Two theories, both involving an additional (unknown) enzyme, to account for the antagonistic effects of these compounds upon the activities of MTA nucleosidase and MTR kinase are presented below. Although there is little evidence (historically or in the results of this paper) to substantiate either theory, the second seems more probable and interesting with respect to its implications.

1) In addition to inhibiting MTR kinase activity, 5'-alkyl MTR analogs (and elevated levels of MTR itself) inhibit the reaction of another enzyme ("enzyme X") also present in crude extracts. Enzyme X can utilize MTA as a substrate, and thereby competes with MTA nucleosidase. The removal via inhibition of this competitor would make available more MTA for the nucleosidase; an event that might produce an apparent increase in the activity of the latter.

2) Enzyme X does not use MTA as a substrate, and its activity is entirely unrelated to MET recycling. However, the naturally-occurring reaction product(s) of enzyme X inhibit the activity of MTA nucleosidase. Again, the inhibition of enzyme X activity (via 5'-alkyl MTR analogs) would in turn cause a stimulation of the activity of MTA nucleosidase.

In this study, MTR kinase activity also was affected by analogs of ATP. The substitution for ATP of an equimolar amount of either 2'-dATP or ADP resulted in partial (36%) to complete inhibition of enzyme activity, suggesting the presence of a third phosphate group at the 5' position is more critical to enzyme activity than is an hydroxyl group at the 2' position. Furthermore, ADP cleavage does not produce enough energy to drive this reaction. Some of these results have been reported in other systems. The first of these accompanied the first report of the existence of the enzyme, purified 38-fold from *E. aerogenes,* by Ferro and coworkers (40). These researchers describe an inhibitory effect of ADP which reduces MTR kinase activity by 52% (40). In an examination of partially purified (nearly 2000-fold) lupin MTR kinase with respect to specificity for a phosphate donor, Guranowski reported that whereas ADP is inactive in this capacity, "dATP" actually increases the rate of MTR phosphorylation by 20% (50). Whether this refers to 2'-dATP, 3'-dATP (a.k.a. cordycepin 5'-triphosphate) or the dideoxy compound 2',3'-dATP is unclear. Furthermore, it is possible that the stimulatory effect also may be a characteristic influence of dATPs upon the activity of partially purified MTR kinase from fruit extracts, but in the present investigation was checked only in crude extracts, and only with 2'-dATP.

In this report, MTA nucleosidase and MTR kinase, two enzymes involved in MET recycling, have been characterized from several sources of crude extracts. The data indicate that even at this level of impurity, some (*albeit* often minor) measurable species- and cultivar-related differences exist between them; and also with appropriate analogs, their activities appear to be inhibited (and in some cases, stimulated). Nonetheless, caution should be maintained with respect to the interpretation of these results, as artifacts relating to the impure state of the enzymes examined are likely to affect their characteristic properties. At best, enzymatic studies of crude extracts can only imply trends. The work should be repeated with preparations of greater

purity in order to verify that the effects reported here are actually specific to each enzyme, rather just than to each crude extract of a given source.

CHAPTER IV

UPTAKE AND ACCUMULATION OF METHIONINE, 5'-DEOXY-5'-METHYLTHIOADENOSINE, AND 5-DEOXY-5-METHYLTHIORIBOSE IN SUSPENSION CULTURES OF PASSE CRASSANE (*Pyrus communis* L.)

Suspension cultures of Passe Crassane (*Pyrus communis* L.) possess both the ability to synthesize ethylene (C_2H_4; 16, 105, 106) and inhibitable activities of at least two of the enzymes involved in methionine (MET) recycling (see Chapter III); facts suggesting that they might serve well in metabolic studies regarding this extended pathway. Furthermore, since crude extracts prepared within this species were found to exhibit relatively minor cultivar-specific differences with respect to the biochemical characteristics of the enzymes 5'-deoxy-5'-methylthioadenosine (MTA) nucleosidase and 5-deoxy-5-methylthioribose (MTR) kinase (Chapter III), it would seem that undifferentiated cell suspensions of Passe Crassane might be utilized as a (limited) model for some of the metabolic events occurring in whole fruit systems.

The present study was undertaken to examine the fate (uptake and accumulation) of MET, MTA and MTR in suspension-cultured cells of Passe Crassane. As preliminary investigations regarding this effort, suspension cell growth rate, viability, and C_2H_4 evolution under a variety of conditions were examined as well. Methods, including those required for cell culture under conditions of growth and senescence, are described in Chapter II.

RESULTS

Suspension Cell Growth and Viability.

Growth rates and viability (% live cells) of Passe Crassane pear cells in suspension culture were found to vary with several factors, predominately presence / absence of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D; *i.e.*, growth vs. aging medium), size of inoculum, and constancy of incubation temperature. In order to characterize cellular behavior with respect to optimal suitability for metabolic studies, the latter two factors were held as constant as possible, while the effects of the former upon growth rate (as represented by cell dry weight) and viability were examined with time.

Measurements were conducted over the course of 1 to 11-14 days in three different media-related growth stages: "growth" (standard medium, including 2,4-D), "transition" (standard medium, lacking 2,4-D), and "aging" (depleted medium lacking 2,4-D, but including mannitol). So as to initiate each of these stages, the cells were successively subcultured from growth to transition to aging media by removing aliquots (as described in Chapter II) from identical vessels which otherwise were not utilized for the study. Once measurements of growth rate and viability were initiated within a set of medium-related growth conditions, no further subculturing was conducted. The results are presented in Figures IV.1. and IV.2.

Previously it was established that 80 mg cell dry weight corresponds to approximately 10^6 cells (see Figure IV.3.). Figure IV.3. also shows the relationship between fresh and dry weights of suspension-cultured pear cells grown in standard (growth) medium. Although somewhat variable, pear cell dry weight was found to represent roughly 80% of fresh weight values.

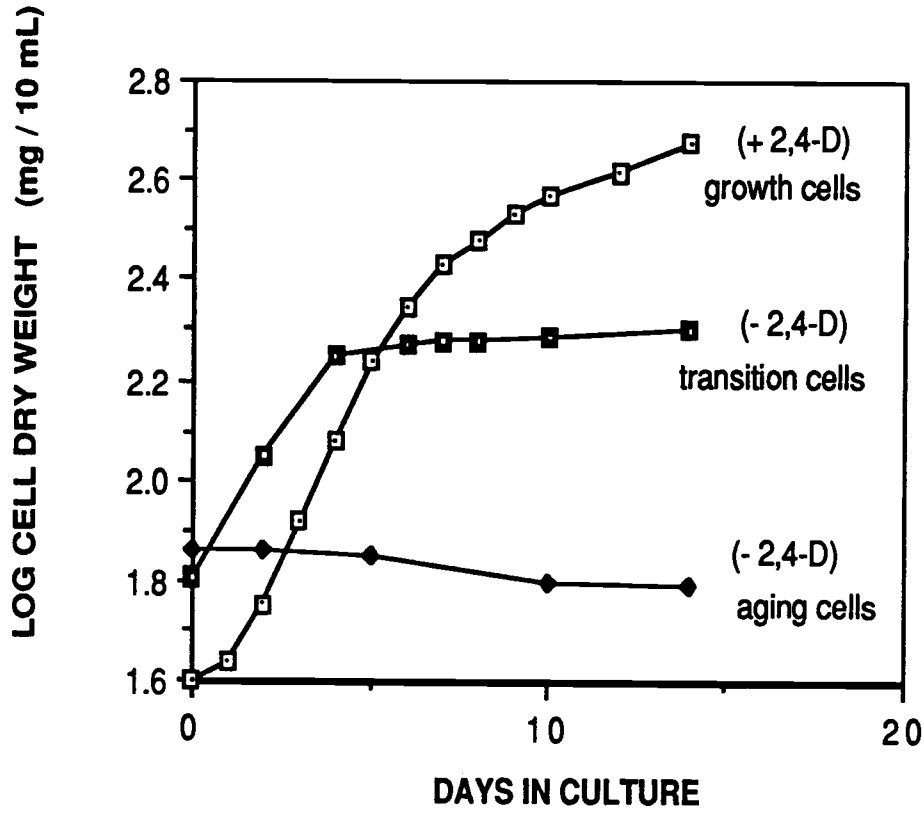


Figure IV.1. Growth profiles of Passe Crassane cells cultured in three different media. Values represent the average of three flasks, each containing a suspension of 10 mL (initial inoculum of 10^6 cells), sacrificed daily over a 14 day period. Culture methods and media (with and without the auxin 2,4-D) are described in Chapter II.

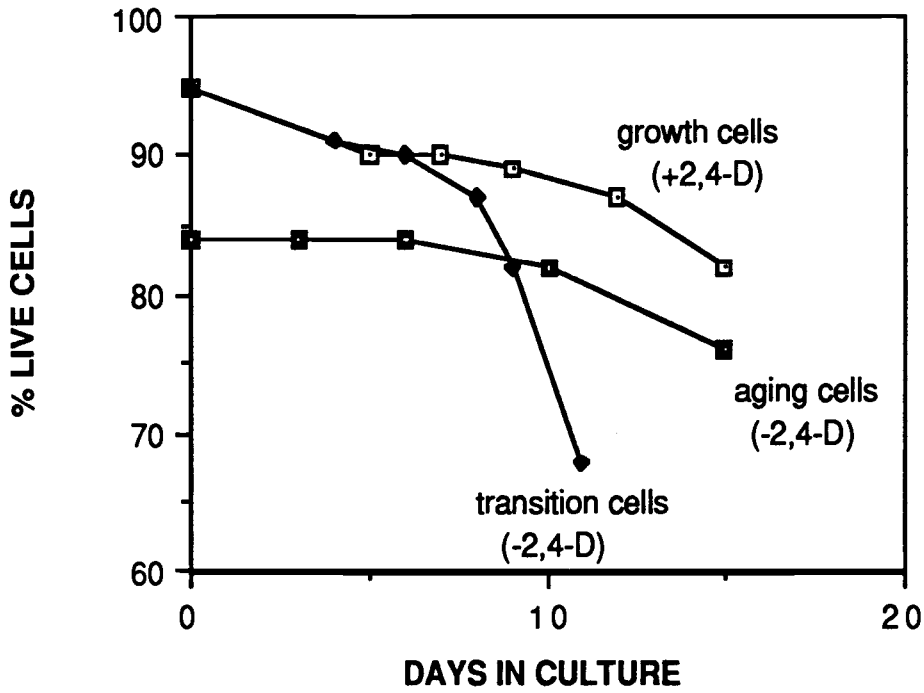


Figure IV.2. Viability profiles of Passe Crassane cells cultured in three different media. Values represent the average of three flasks, each containing a suspension of 10 mL (initial inoculum of 10^6 cells), sacrificed daily over an 11-14 day period. Culture methods and media (with and without the auxin 2,4-D) are described in Chapter II.

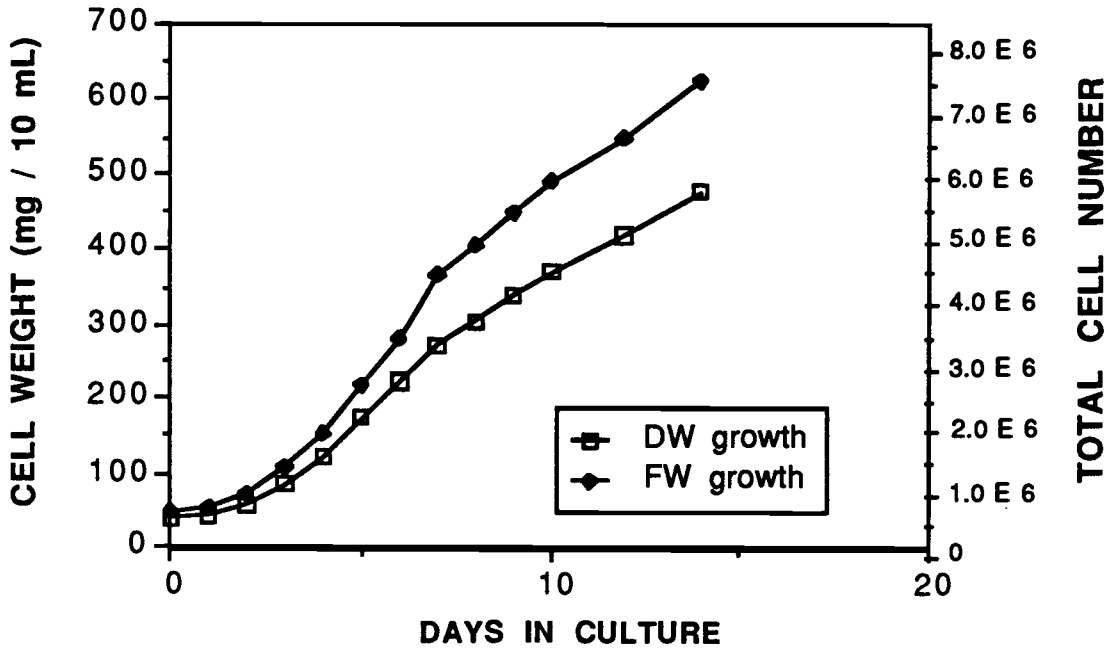


Figure IV.3. Relationship between cell number and fresh and dry weights of Passe Crassane suspension cells cultured in growth medium. Values represent the average of three flasks, each containing a suspension of 10 mL (initial inoculum of 10^6 cells), sacrificed daily over a 14 day period, as described in Chapter II.

As seen in Figure IV.I., cells cultured under conditions of normal growth (growth medium + 2,4-D) exhibited a classical growth cycle, with logarithmic growth occurring between day 2 and 5-6 (approximately 72 h doubling time), and a final density in 10 mL of about 6.0 x 10^6 cells (479 mg DW). However, cells which had been transferred to growth medium lacking 2,4-D ("transition cells") were found both to display a slower growth rate (final cell density approximately 2.4 x 10^6, or 195 mg DW per mL), and entered stationary phase more rapidly. These effects promoted an accelerated rate of cell death (Figure IV.2.). Cells transferred to aging medium appeared to undergo no cell division whatsoever (Figure IV.1.), but did not exhibit reduced viability until after approximately 9 days of culture; roughly the same behavior seen in growth cells (Figure IV.2.). Readdition of 2,4-D did not reverse these effects (data not shown). Hence, aging cells cannot be studied in log phase.

Effect of Compounds Upon Growth of Suspension Cells

Prior to initiating experiments designed to study the methionine recycling pathway in suspension-cultured pear cells, the effects of five related compounds upon cell growth were examined (Figure IV.4.). In addition to MET, these compounds included MTA, 5-deoxy-5-ethylthioribose (ETR; the ethyl analog of MTR), putrescine (PUT), and 1-aminocyclopropane-1-carboxylic acid (ACC). Each was tested individually over the course of eight days at a concentration of 2.0 mM. As shown in Figure IV.4., only MTA, ETR, and PUT were found to influence cell growth significantly. Both MTA and ETR were potent inhibitors (5.7 and 1.2%, respectively, of the control on day 8 of culture), whereas PUT was stimulatory (1.5-fold stimulation relative to the control on day 8).

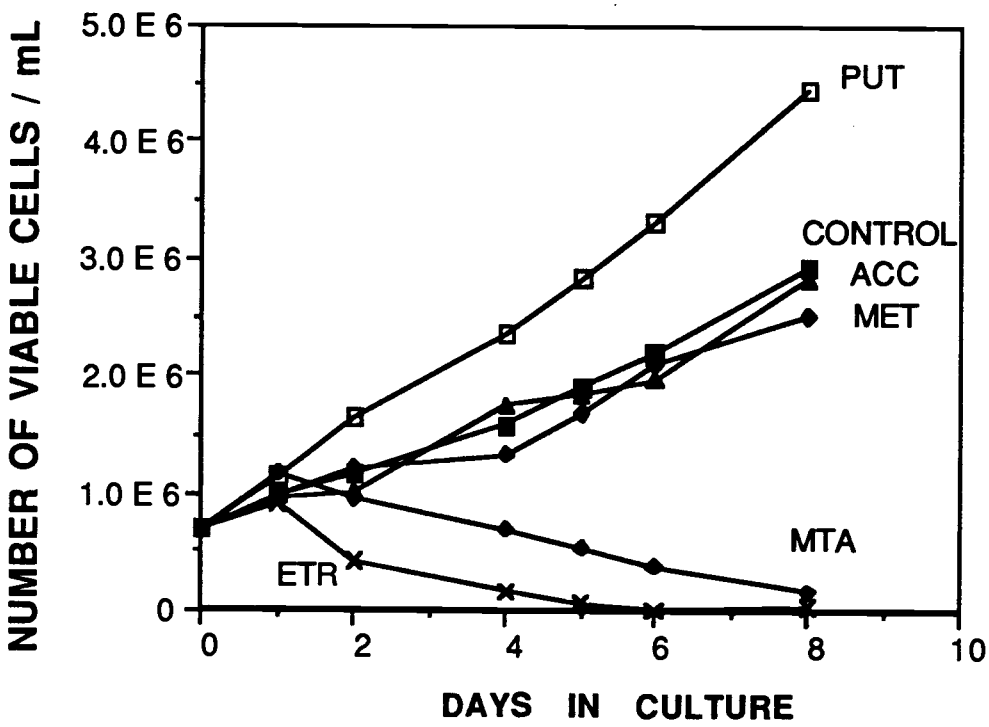


Figure IV.4. Effects of MET, MTA, ETR, PUT, and ACC upon the growth of Passe Crassane fruit cells cultured in growth medium. Log-phase cells were inoculated (7 x 10^5 cells / mL) into standard (growth) medium (20 mL suspensions) containing 2 mM concentrations of either methionine (MET), 5'-deoxy-5'-methylthioadenosine (MTA), 5-deoxy-5-ethylthioribose (ETR), putrescine (PUT), 1-aminocyclopropane-1-carboxylic acid (ACC), or no addition (control), then monitored for cell number and viability over an eight day culture period. Methods are described in Chapter II.

Ethylene Production in Suspension-Cultured Pear Cells

In a separate study regarding the individual impacts of 1.0 mM concentrations of MTA and ETR upon C_2H_4 biosynthesis in pear cell suspensions monitored over a 2.5 d period, the former appeared to be slightly stimulatory, whereas the latter had the opposite effect; however, neither was pronounced (data not shown). Since the amount of C_2H_4 produced by these cells is dependent upon a variety of factors, predominantly cell age, extent of ongoing cell division, and the presence / absence of auxin, and in fact can be induced significantly by the addition of indoleacetic acid (IAA) as well as other compounds (16, 105, 106), a study to characterize the effects of varying concentrations (0-200 μM) of IAA upon C_2H_4 evolution was initiated. The results, shown in Figures IV.5. and IV.6, indicate that maximal quantities of C_2H_4 (peak = 17 nL / 10^6 cells / hr) are produced after approximately 2.5 d by cells cultured in aging medium containing 100 μM IAA. Even in the presence of the same concentration of IAA, cells cultured in growth medium were found to produce less than half of this value (Figure IV.5.).

When the diamine PUT and the polyamines spermidine (SPD) and spermine (SPM) were added individually at 1 mM concentrations to cultures of aging pear cells previously induced by 100 μM IAA to produce maximal amounts of C_2H_4, an inhibitory effect was noted (Figure IV.7.). Of the three compounds, SPM (the largest and most positively charged) exerted the strongest effect, decreasing C_2H_4 production by over 60% after 12 h. In the same time period, the presence of SPD caused a 45% inhibition of measurable C_2H_4, whereas PUT lowered the value by only 12%. All three effects appeared to be at least partially reversible (Figure IV.7.).

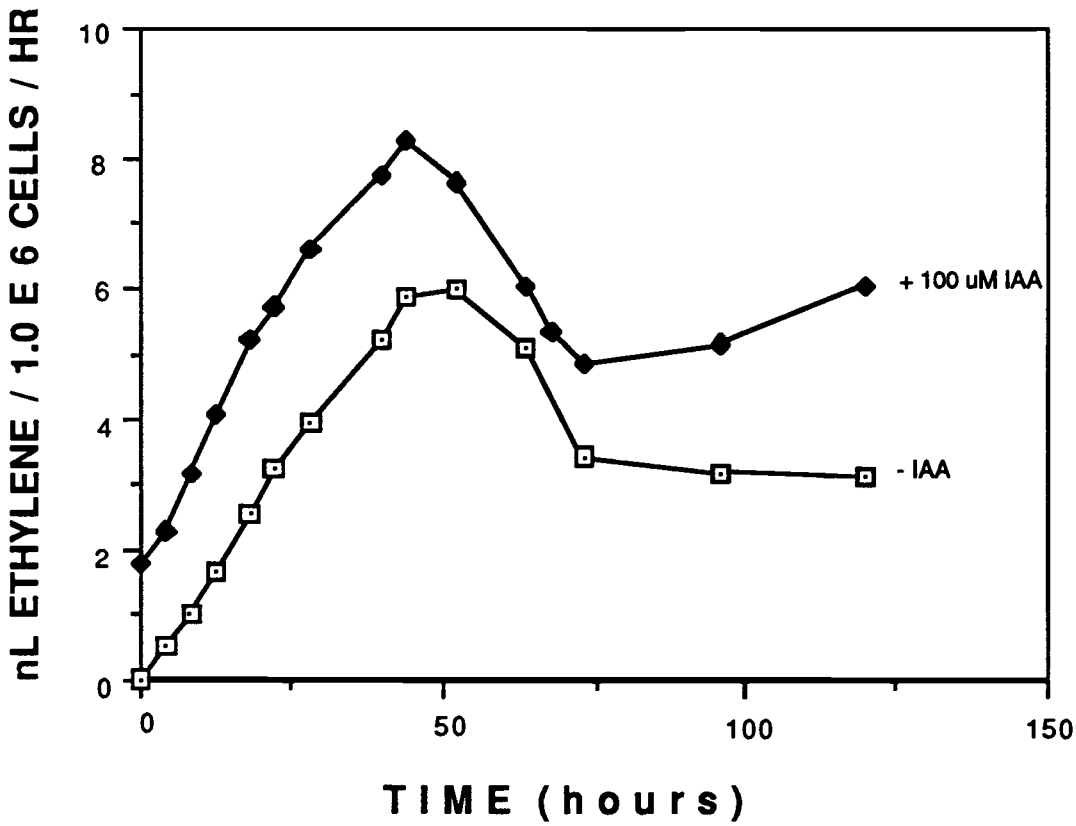


Figure IV.5. Ethylene production by suspension pear cells cultured in growth medium with and without the presence of the auxin, IAA. Log-phase cells were inoculated into standard (growth) medium (25 mL suspensions) containing either 100 uM IAA or the matrix (approximately 30% ethanol) into which this compound was dissolved. The headspaces of both 125 mL flasks were flushed with air at time 0 (prior to the initiation of ethylene sampling). Methods are described in Chapter II.

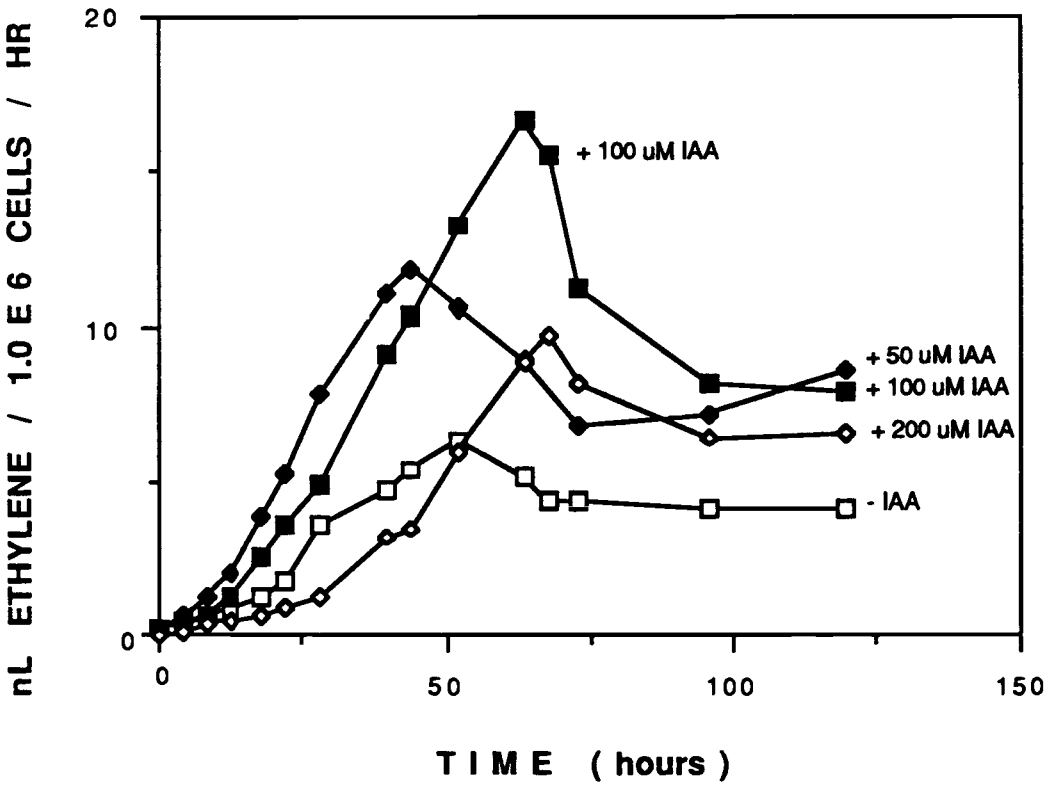


Figure IV.6. Ethylene production by suspension pear cells cultured in aging medium with and without the presence of varying concentrations of the auxin IAA. Stationary phase cells were first transferred from growth medium into transition medium (9 d) then into aging medium (25 mL suspensions) containing either 0, 50, 100, or 200 uM IAA. The headspace of each 125 mL flask was flushed with air at time 0 (prior to the initiation of ethylene sampling). Methods are described in Chapter II.

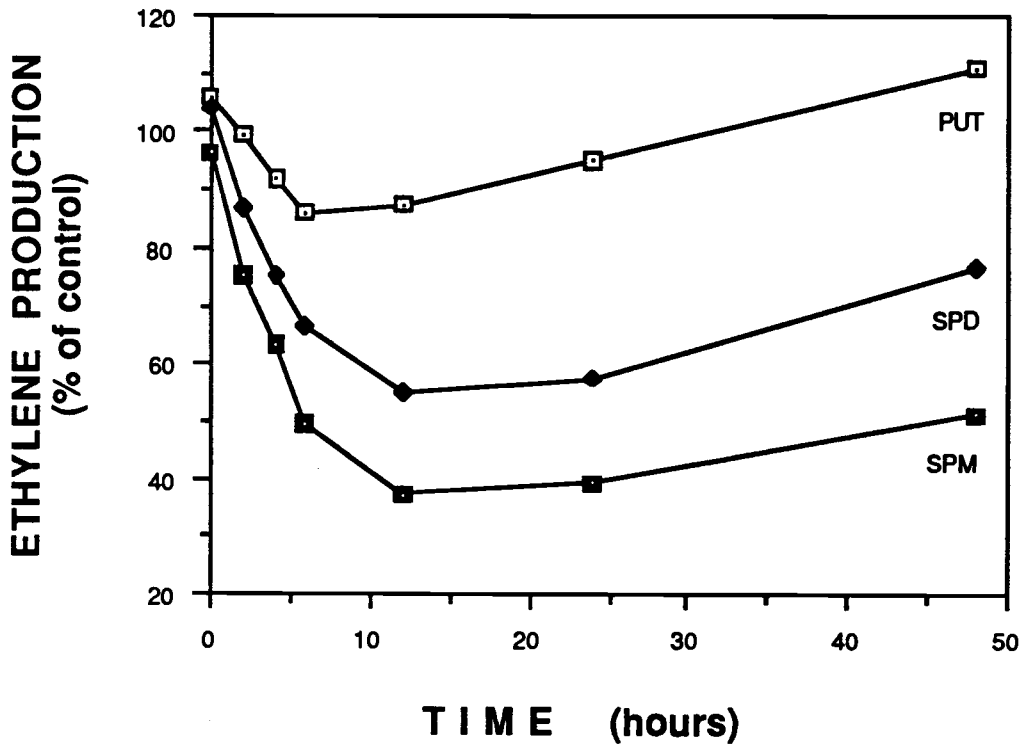


Figure IV.7. Effects of putrescine, spermidine, and spermine upon auxin-induced ethylene production in Passe Crassane suspension cells cultured in aging medium. Stationary phase cells were transferred first from growth medium into transition medium (9 d) then into aging medium containing, in a final volume of 25 mL, both 100 uM IAA and 1 mM of one of the following: putrescine (PUT), spermidine (SPD), or spermine (SPM). The control contained only 100 uM IAA, and produced ethylene at a rate comparable to that (+ 100 uM IAA) shown in Figure IV.6. The headspace of each 125 mL flask was flushed with air at time 0 (prior to the initiation of ethylene sampling). Methods are described in Chapter II.

Uptake of MET, MTA, and MTR into Suspension Pear Cells

It has been demonstrated that for suspension-cultured plant cells, growth during early log phase is due mainly to cell division (high mitosis), whereas the exponential and early stationary phases are characterized by cell expansion and vacuolization (63). Thus, although some compounds may be taken up and accumulated more readily during early log phase, the extent of their metabolism (and subsequent recovery of metabolic products) might be greater in older cells. Such behavior might be expected from compounds involved chiefly in the processes of C_2H_4 production and senescence. However, in the case of MET and some of its recycling intermediates which are also required for polyamine biosynthesis and normal cell growth (*e.g.*, both MTA and S-adenosyl-L-methionine; SAM), the opposite may be true. Based upon both these observations and the data above, it was decided that metabolic uptake and fate transport studies of MET, MTA and MTR should be conducted upon cells in both early to mid-log (2-4 d following subculture for growth cells) and early stationary phase (10-12 d or 2-3 d following subculture for growth and aging cells, respectively). All three compounds were found to be taken up by these cells. Time course uptake profiles are shown in Figures IV.8.-IV.11.

Inasmuch as experiments conducted both at different times and with different subcultures may be validly compared, in the three conditions examined the final amounts of accumulated MET were not found to differ significantly; ranging from 763-1018 nmol / 10^7 viable cells. However, great differences were seen with respect to rates of accumulation. Cells cultured in growth medium and tested during log phase were saturated with MET within 1.5 h (Figure IV.8.), whereas older cells (stationary phase) from the same medium took up only 29.2 nmol / 10^7 viable cells in that time and showed no evidence of saturation after 24 h (Figure IV.9.). Stationary cells cultured in

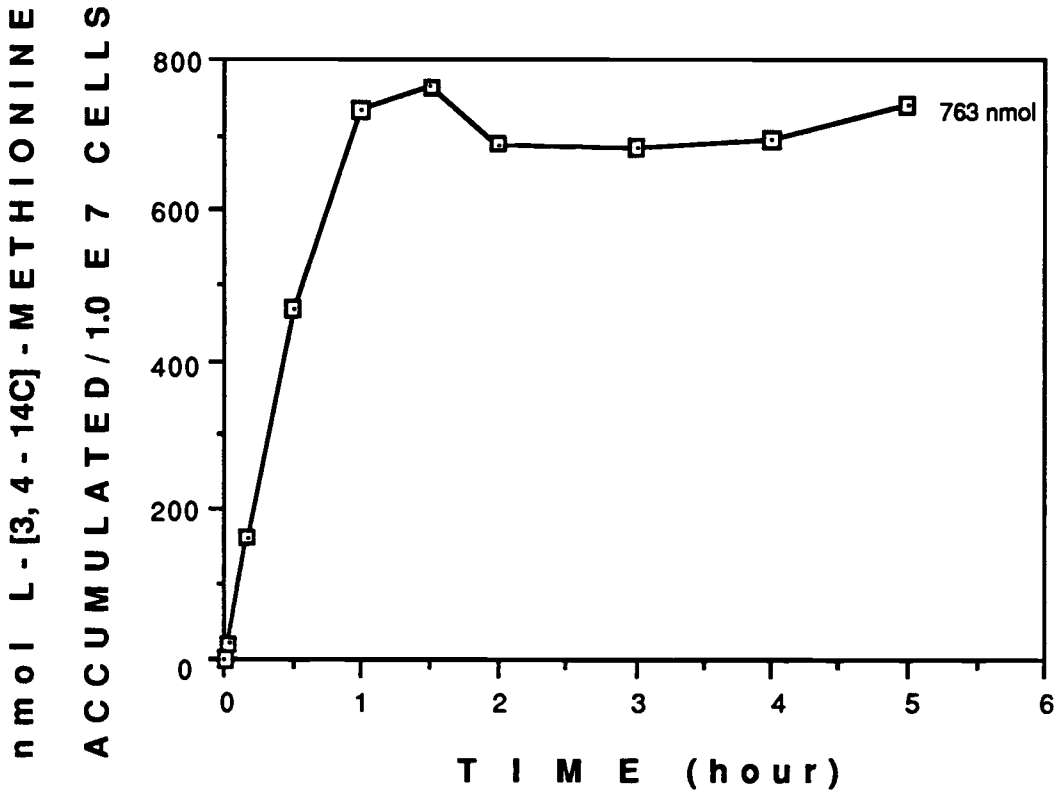


Figure IV.8. Uptake of L-[3,4-14C]-methionine by a log-phase suspension of Passe Crassane pear cells cultured in growth medium. Cells, in one flask, were grown in standard (growth) medium for 3 d following subculture prior to introduction of radiolabel (2.7 uCi; 204 uM; 2.94 E 6 cpm / umol). Cell density, as number of viable cells per flask (10 mL suspension), was measured to be 7.0 E 6. To monitor uptake, representative aliquots of 200 uL were removed at specific times then filtered, dried, and counted by liquid scintillation counting. Radioactivity is expressed on the basis of the parent compound. Methods are described in Chapter II.

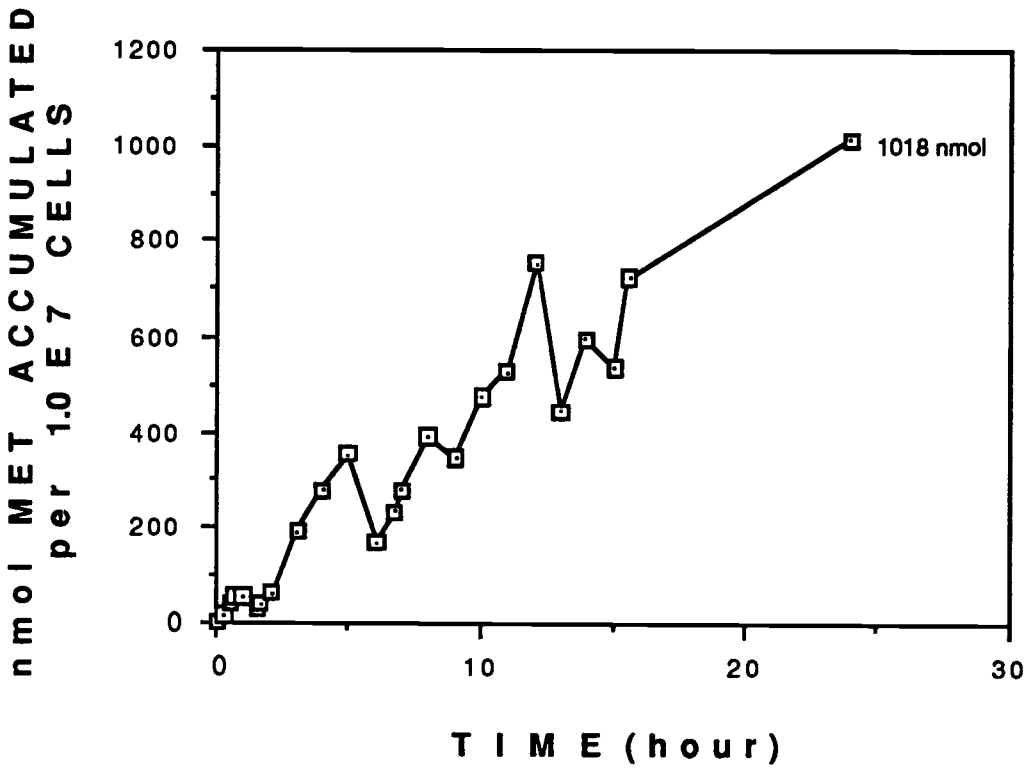


Figure IV.9. Uptake of L-[3,4-14C]-methionine by a stationary phase suspension of Passe Crassane pear cells cultured in growth medium. Cells, in one flask, were grown in standard (growth) medium for 11 d following subculture prior to introduction of radiolabel (2.5 uCi; 204 uM; 2.72 E 6 cpm per umol). Cell density, as number of viable cells per flask (10 mL suspension), was measured to be 1.5 E 7. To monitor uptake, representative aliquots of 200 uL were removed at specific times then filtered, dried, and counted by liquid scintillation counting. All radioactivity is expressed on the basis of parent compound. Methods are described in Chapter II.

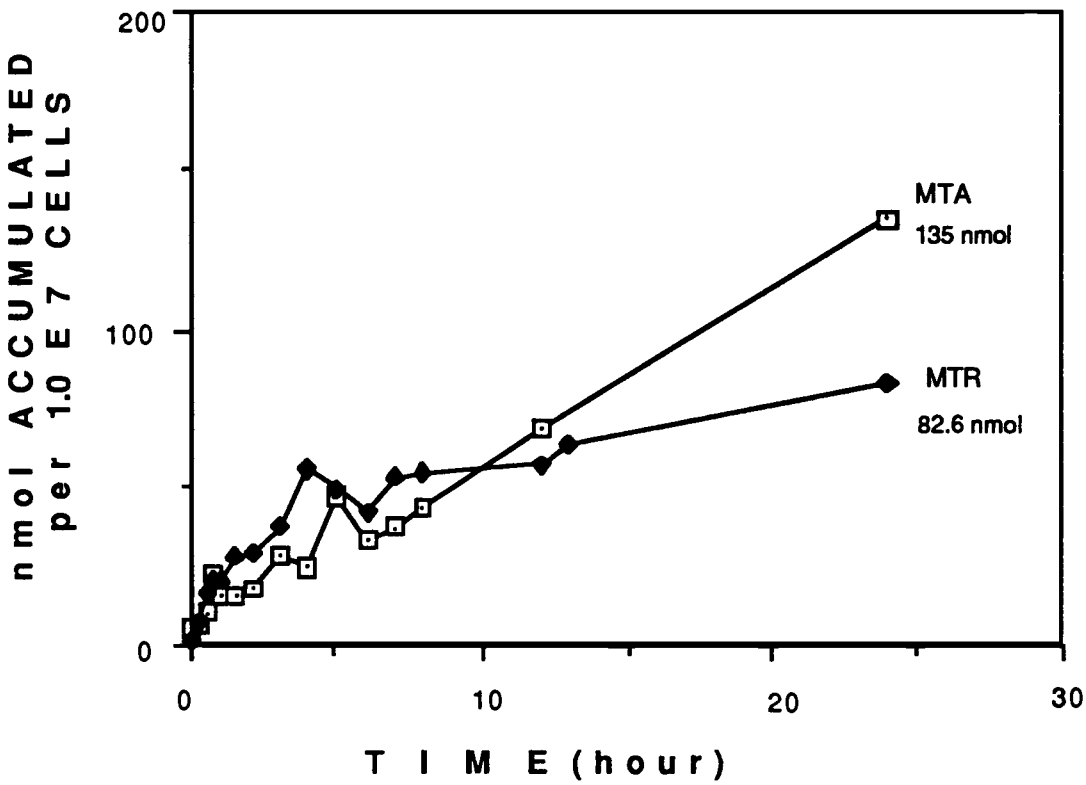


Figure IV.10. Uptakes of [14CH3]-MTA and [14CH3]-MTR by stationary phase suspensions of Passe Crassane pear cells cultured in growth medium. Cells, in two flasks, were grown in standard (growth) medium for 11 d following subculture prior to the introduction of radiolabel (either MTA at 3.0 uCi, 210 uM, 3.17 E 6 cpm per umol; or MTR at 2.3 uCi, 210 uM, 2.43 E 6 cpm / umol). Cell density, as number of viable cells per flask (10 mL suspension), was measured to be 1.6 E 7 for the MTA flask and 1.9 E 7 for the MTR flask. Radioactivity is expressed on the basis of each parent compound. Methods are described in Chapter II.

Figure IV.11. Uptakes of L- [3,4-^{14}C]-MET, [$^{14}CH_3$]-MTA, and [$^{14}CH_3$]-MTR by stationary phase suspensions of Passe Crassane pear cells cultured in aging medium. Stationary phase cells were transferred from growth medium to transition medium (9 d) then into aging medium (10 mL suspensions). Cells were allowed to equilibrate for 2 d following this last subculture prior to introduction of radiolabel (either MET at 2.6 uCi, 206 uM, 2.80 E 6 cpm / umol; MTA at 2.9 uCi, 210 uM, 3.07 E 6 cpm / umol; or MTR at 2.3 uCi, 208 uM, 2.46 E 6 cpm / umol). Cell density, as number of viable cells per flask, was measured to be 2.18 E 7 for the MET flask, 1.99 E 7 for the MTA flask, and 1.94 E 7 for the MTR flask. Radioactivity is expressed on the basis of each parent compound. Panel A shows the accumulation of all three compounds, while Panel B is an expanded scale graph of only the MTA and MTR data. Following the removal of the last desired sample, the remaining cells and media were harvested, extracted, and analyzed chromatographically. Methods are described in Chapter II.

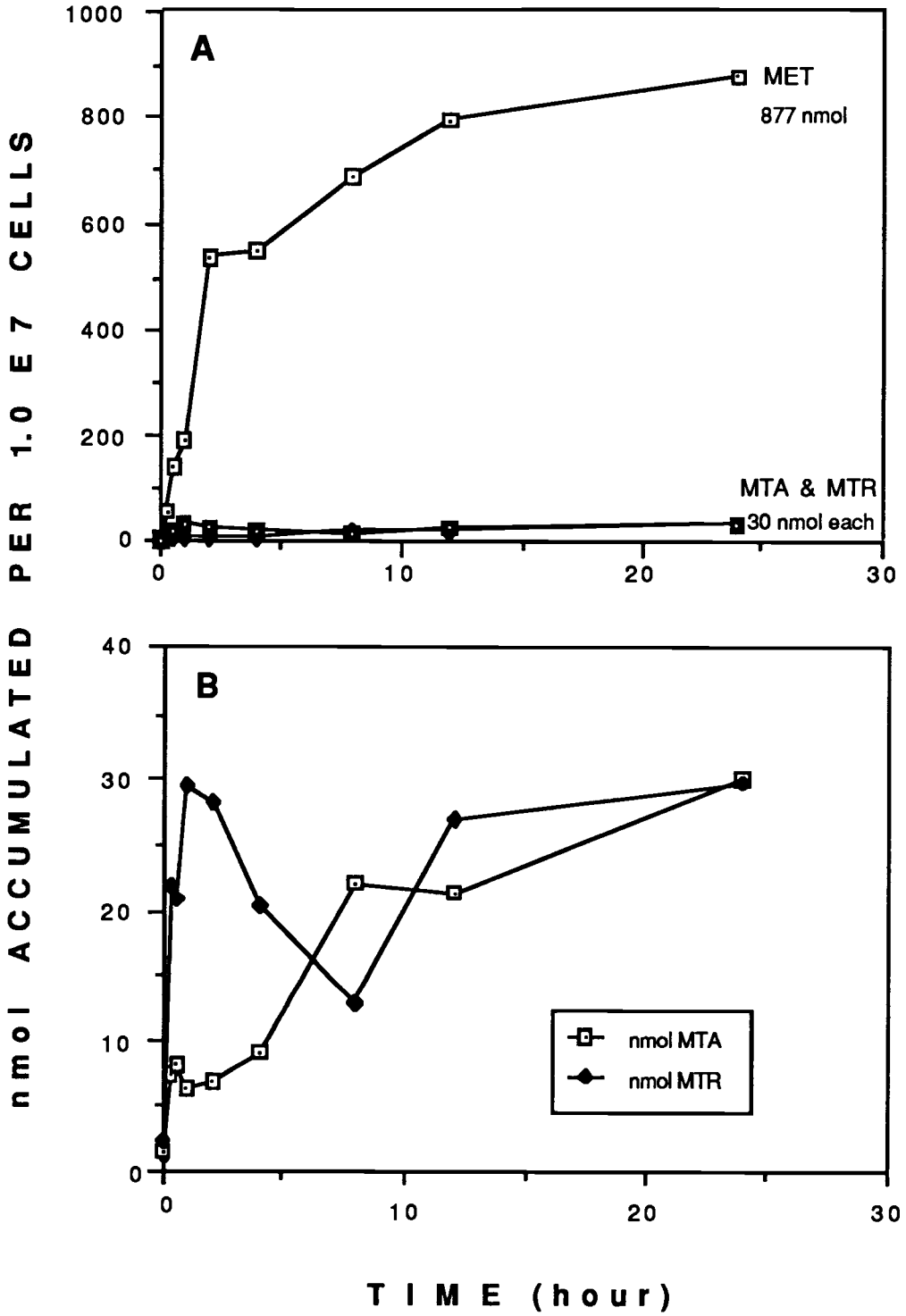

A
MET
877 nmol
MTA & MTR
30 nmol each
1000
800
600
400
200
0
0
10
20
30
B
nmol MTA
nmol MTR
40
30
20
10
0
0
10
20
30
nmol ACCUMULATED PER 1.0 E7 CELLS
TIME (hour)

Figure IV.11.

aging medium exhibited an intermediate rate of MET accumulation (*i.e.*, approximately 250 nmol / 10^7 viable cells taken up by 1.5 h), and saturation by about 12 h (Figure IV.11.).

In contrast, accumulation rates of MTA and MTR did not vary appreciably in the two test conditions examined (*i.e.*, stationary phase cells in growth and aging media). Both profiles suggest that MTA uptake is still rising after 24 h, while MTR accumulation approaches saturation within 2-5 h (Figures IV.10. and IV.11.). However, the final amounts accumulated are grossly different. Cells cultured in growth medium took up 4.5 times more MTA (135 vs. 30 nmol / 10^7 viable cells) and 2.8 times more MTR (82.6 vs. 30 nmol / 10^7 viable cells) than those in aging medium.

Since both MTA and MTR were taken up by the suspension pear cells at such low levels relative to MET uptake, two efforts were made to try to stimulate the former. Neither was found to have a significant influence (data not shown). The first was an attempt to induce enzymes, thereby promoting assimilation, and involved pre-conditioning the cells in non-radioactive MTA (approximately 200 μM). The cells were grown in this supplemented medium prior to being filtered, washed, re-suspended in normal medium, then exposed to the radiolabel. The second approach was based on work with *E. coli* by Shroeder *et al.* (121). These researchers found that conditions of sulfur deficiency promoted increases in both endogenous and secreted levels of MTR. In the case of suspension cultures of Passe Crassane, restriction of the usual sulfur supply in growth medium was found to have no pronounced effect upon MTA uptake (data not shown).

In a separate study, the addition of 100 μM IAA was found to have no influence upon the accumulation of either [3,4-^{14}C]-MET or [$^{14}CH_3$]-MTA in sta-

tionary phase cells grown in aging medium (Table IV.1.). This, and other experiments (data not shown), also indicated that the $Hg(ClO_4)_2$ solution employed to bind evolved radioactive C_2H_4 is non-specific and thus cannot be used to quantitate C_2H_4 production. Not only was Hg-complexed radioactivity recovered from flasks to which MET had been added (as would be expected since this compound is labelled in the two carbons that form C_2H_4), but it was found also in [$^{14}CH_3$]-MTA-containing flasks (Table IV.1.) which cannot contribute to $^{14}C_2H_4$ production. The addition of strong base to each of these complexed solutions (see Chapter II) sugested that sulfur-containing compounds (*e.g.*, methanethiol) were not present in any of them.

Fate of MET, MTA, and MTR in Suspension Pear Cells

Extracts of suspension cells previously utilized for uptake studies with [3,4-^{14}C]-MET, [$^{14}CH_3$]-MTA, and [$^{14}CH_3$]-MTR (see Figure IV.11.) were analyzed chromatographically in an effort to determine the fate of these compounds. As shown in Table IV.2., the majority (*i.e.*, greater than 70%) of the recovered radioactivity from [3,4-^{14}C]-MET metabolism was in the form of trapped volatiles; presumably C_2H_4. However, as indicated both earlier and by the fact that the two methyl-labelled compounds (whose radiolabelled carbons do not contribute to C_2H_4 formation) also produced varying levels (*i.e.*, 2.5-13%) of radioactive volatiles, these results should be interpreted with caution.

For each of the three compounds, the principal component of the non-volatile fraction was recovered as perchloric acid (PCA) soluble, non cis-diol metabolites (Tables IV.2.-IV.4.). For [3,4-^{14}C]-MET, the majority of these (*i.e.*, 87% of the PCA soluble fraction) were cationic; presumably polyamines,

Table IV.1. Incorporation of [3,4-^{14}C]-Methionine and [$^{14}CH_3$]-Methylthioadenosine by Stationary Phase Suspension Cells of *Pyrus communis* L. (cv Passe Crassane) Cultured in Aging Medium

ADDITION[a]	ACCUMULATION OF LABEL OVER 24 h (nmol / 10^7 viable cells)[b]	
	AMOUNT TAKEN UP BY CELLS[c]	AMOUNT TRAPPED AS VOLATILES[d]
[3,4-^{14}C]-MET	70.3	6.20
[3,4-^{14}C]-MET + IAA	78.8	27.7
[$^{14}CH_3$]-MTA	22.3	3.66
[$^{14}CH_3$]-MTA + IAA	24.7	64.1

[a]50 µM of either 2.91 x 10^6 cpm / µmol MET or 5.84 x 10^6 cpm / µmol MTA with or without 100 µM IAA delivered into 15 mL suspensions of 2 d old stationary phase cells (2.0 x 10^5 viable cells / mL) cultured in aging medium.

[b]All radioactivity is expressed on the basis of parent compound.

[c]As determined by a filtered 200 µL aliquot removed at t=24 h (see Chapter II).

[d]As complexed by 250 µL of 0.25 M $Hg(ClO_4)_2$ (see Chapter II).

Table IV.2. Products of [3,4-^{14}C]-MET, [$^{14}CH_3$]-MTA, and [$^{14}CH_3$]-MTR Metabolism in Cell Suspensions of Passe Crassane (*Pyrus communis* L.) Cultured in Aging Medium.

COMPONENT	206 µM [3,4-^{14}C]-MET	210 µM [$^{14}CH_3$]-MTA	208 µM [$^{14}CH_3$]-MTR
	% of label recovered over 24 hours [a]		
PCA soluble fraction	22.3	76.1	81.2
cis-diols	0.3	2.5	2.5
neutral + anionic	(0.006)	1.8	1.2
cationic	0.3	0.7	1.3
non cis-diols	22.0	73.6	78.7
neutral + anionic	2.7	60.8	70.6
cationic	19.3	12.8	8.1
PCA insoluble fraction	7.6	10.6	16.3
EtOH soluble fraction	4.8	8.2	12.8
cis-diols	(0.03)	0.1	0.2
non cis-diols	4.8	8.1	12.6
$Hg(ClO_4)_2$ trapped volatiles	70.1	13.3	2.5

[a]Conditions are described in Figure IV.11.; Methods are in Chapter II.

Table IV.3. Putative Identity of Some Products of [3,4-^{14}C]-MET Metabolism in Cell Suspensions of Passe Crassane (*Pyrus communis* L.) Cultured in Aging Medium.

COMPONENT[a]	% OF PCA SOLUBLE FRACTION	PUTATIVE IDENTITY[b]
PCA soluble fraction		
cis-diols		
neutral + anionic	(0.03)	unknown
cationic	1.4	SAM
non cis-diols		
neutral + anionic	12.1	MET, ACC, MACC
cationic	86.6	PUT, Polyamines

[a]Conditions are described in Figure IV.11.; Methods are in Chapter II.

[b]Abbreviations are as follows: SAM = S-adenosylmethionine; MET = methionine; ACC = 1-aminocyclopropane-1-carboxylic acid; MACC = malonyl-1-aminocyclopropane-1-carboxylic acid; PUT = putrescine.

Table IV.4. Putative Identity of Some Products of [$^{14}CH_3$]-MTA and [$^{14}CH_3$]-MTR Metabolism in Cell Suspensions of Passe Crassane (*Pyrus communis* L.) Cultured in Aging Medium.

	[$^{14}CH_3$]-MTA	[$^{14}CH_3$]-MTR	
COMPONENT[a]	% OF PCA SOLUBLE FRACTION	% OF PCA SOLUBLE FRACTION	PUTATIVE IDENTITY[b]
PCA soluble fraction			
cis-diols			
neutral + anionic	2.4	1.5	MTR, MTR-1-P, MTI
cationic	0.9	1.6	MTA, SAM, dSAM
non cis-diols			
neutral + anionic	79.9	86.9	MET, αKMB, αHMB
cationic	16.8	10.0	unknown

[a]Conditions are described in Figure IV.11.; Methods are in Chapter II.

[b]Abbreviations are as follows: MTR = 5-deoxy-5-methylthioribose; MTR-1-P = 5-deoxy-5-methylthioribose-1-phosphate; MTI = 5-deoxy-5-methylthioinosine; MTA = 5-deoxy-5-methyl-thioadenosine; SAM = S-adenosylmethionine; dSAM = decarboxylated S-adenosylmethionine; MET = methionine; αKMB = α–keto-γ-methylthiobutyric acid; αHMB = α–hydroxy-γ-methylthiobutyric acid

including the diamine putrescine (Table IV.3.). The remaining 12% were found to be neutral + anionic, and could include MET, ACC, and / or its malonylated counterpart, MACC. In the cases of both [$^{14}CH_3$]-MTA and [$^{14}CH_3$]-MTR, most of the PCA soluble radioactivity (*i.e.*, 80-87%) was recovered as non-cationic compounds; presumably MET, α–keto-γ-methylthiobutyric acid (αKMB), and / or α–hydroxy-γ-methylthiobutyric acid (αHMB; Table IV.4.).

When each of these PCA soluble, non-cationic, non cis-diol fractions was analyzed by HPLC (Systems I and II, as described in both Chapter II and Appendix B), the major component in all cases except that involving [3,4-^{14}C]-MET treatment, was identified as MET or its oxidation product, based upon co-migration of this metabolite with authentic MET (Figures IV.12.-IV.14.). In fact, in the case of the [$^{14}CH_3$]-MTR extract, no other significant peaks were detected by either UV or radioactivity. However, chromatograms of both of the other extracts (deriving from treatments with either [3,4-^{14}C]-MET or [$^{14}CH_3$]-MTA) showed considerable diversity; *i.e.*, in the order of 30-40% of the recovered, chromatographed radioactivity divided among 6-9 peaks (Figures IV.13.-IV.14.). PCA extracts composed of the non-cationic metabolites of [3,4-^{14}C]-MET incorporation were found by HPLC analysis to contain primarily ACC (Figure IV.12.).

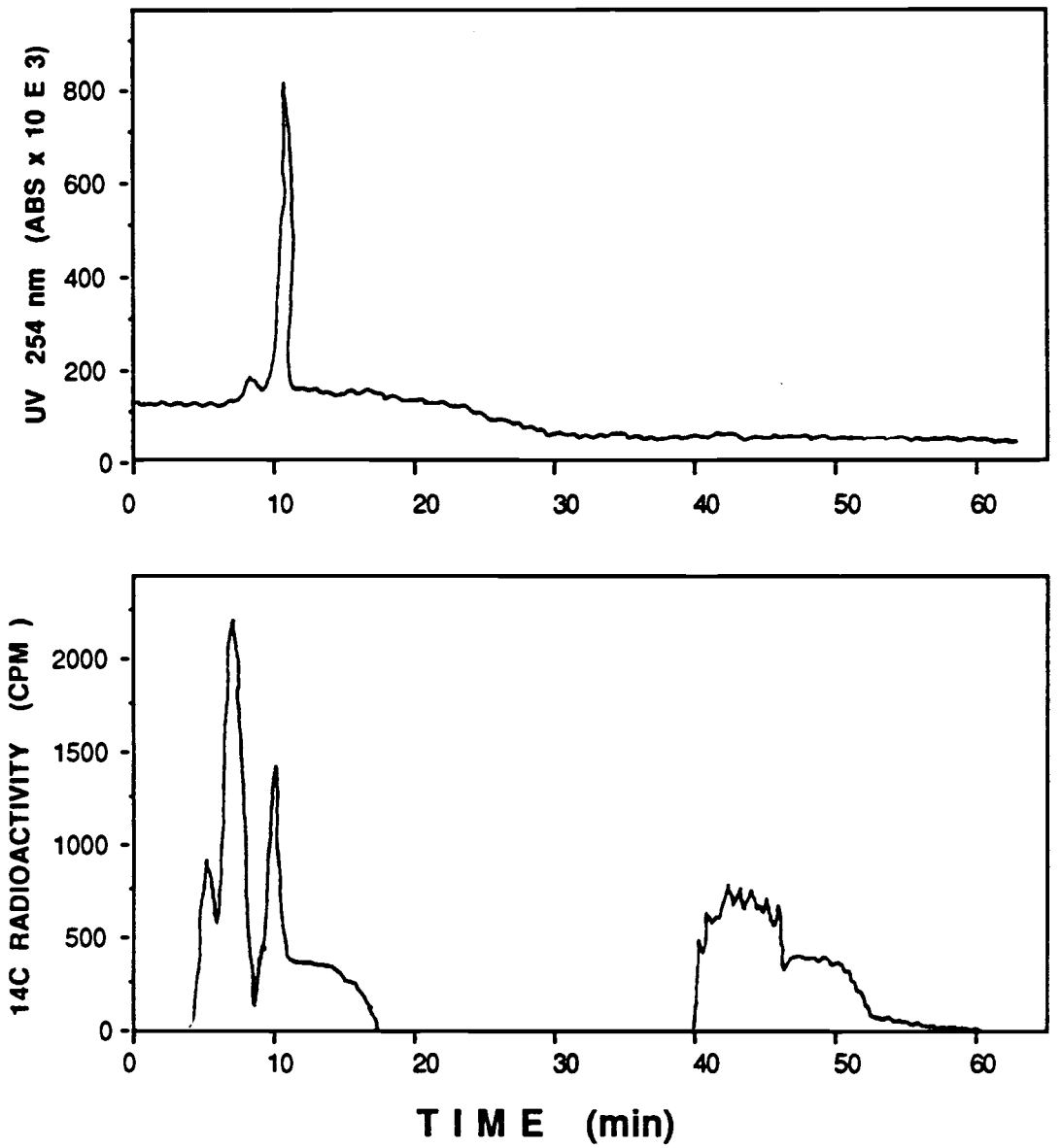


Figure IV.12. HPLC chromatogram of L-[3,4-14C]-MET metabolism by stationary phase suspensions of Passe Crassane. Conditions (described in detail in both Chapter II and Appendix B) = uBondapak C18 column with methanol / ddH2O 36:64, v / v) at 0.7 mL / min, room temperature (HPLC System II). The predominant radioactive peak has the same retention time as that of authentic ACC (7.3 min).

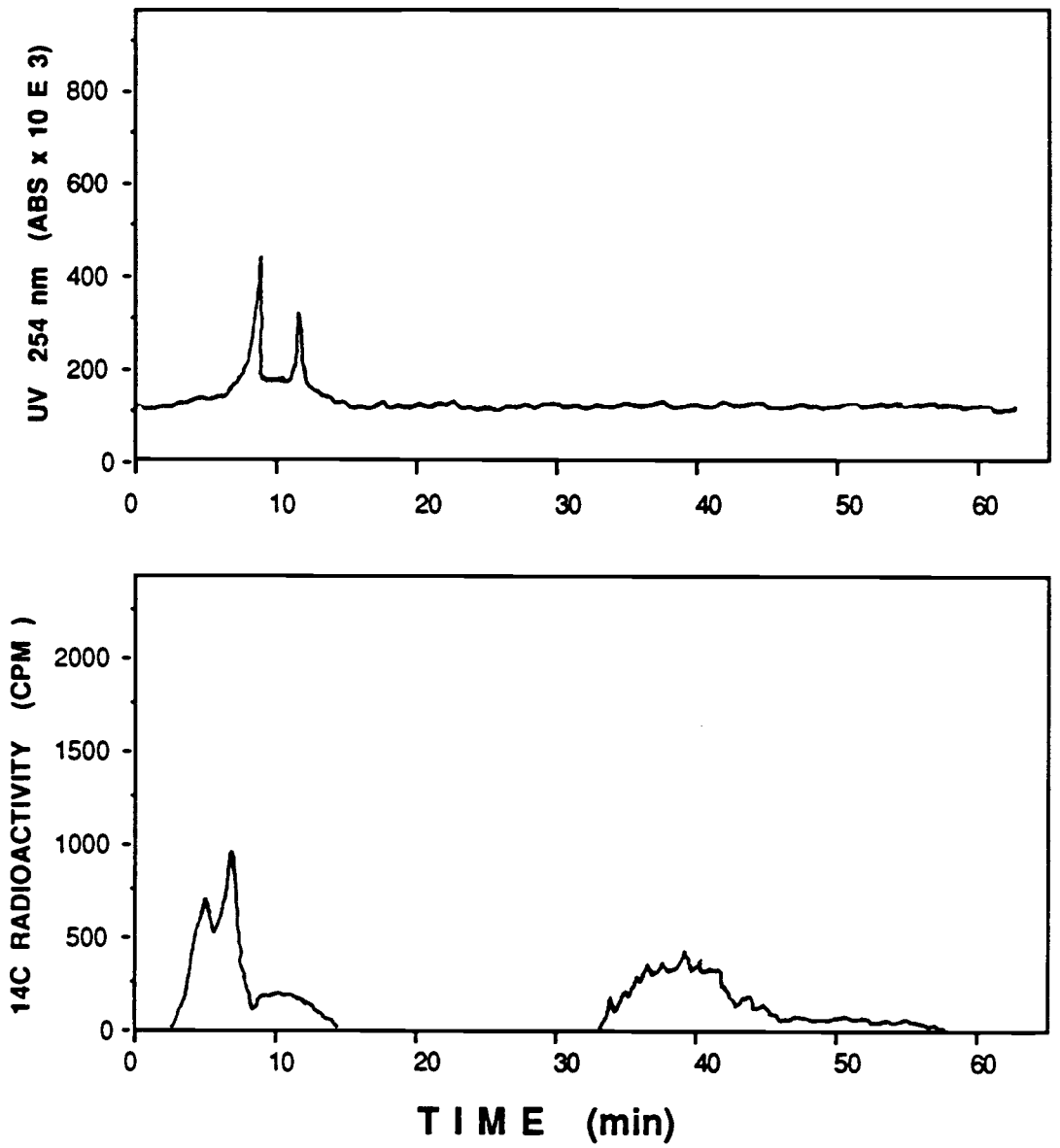


Figure IV.13. HPLC chromatogram of [14C]-MTA metabolism by stationary phase suspensions of Passe Crassane. Conditions (described in detail in both Chapter II and Appendix B) = uBondapak C18 column with methanol / ddH2O 36:64, v / v) at 0.7 mL / min, room temperature (HPLC System II). The predominant radioactive peaks have the same retention times as authentic MET (5.3 min) and MET sulfoxide (4.9 min).

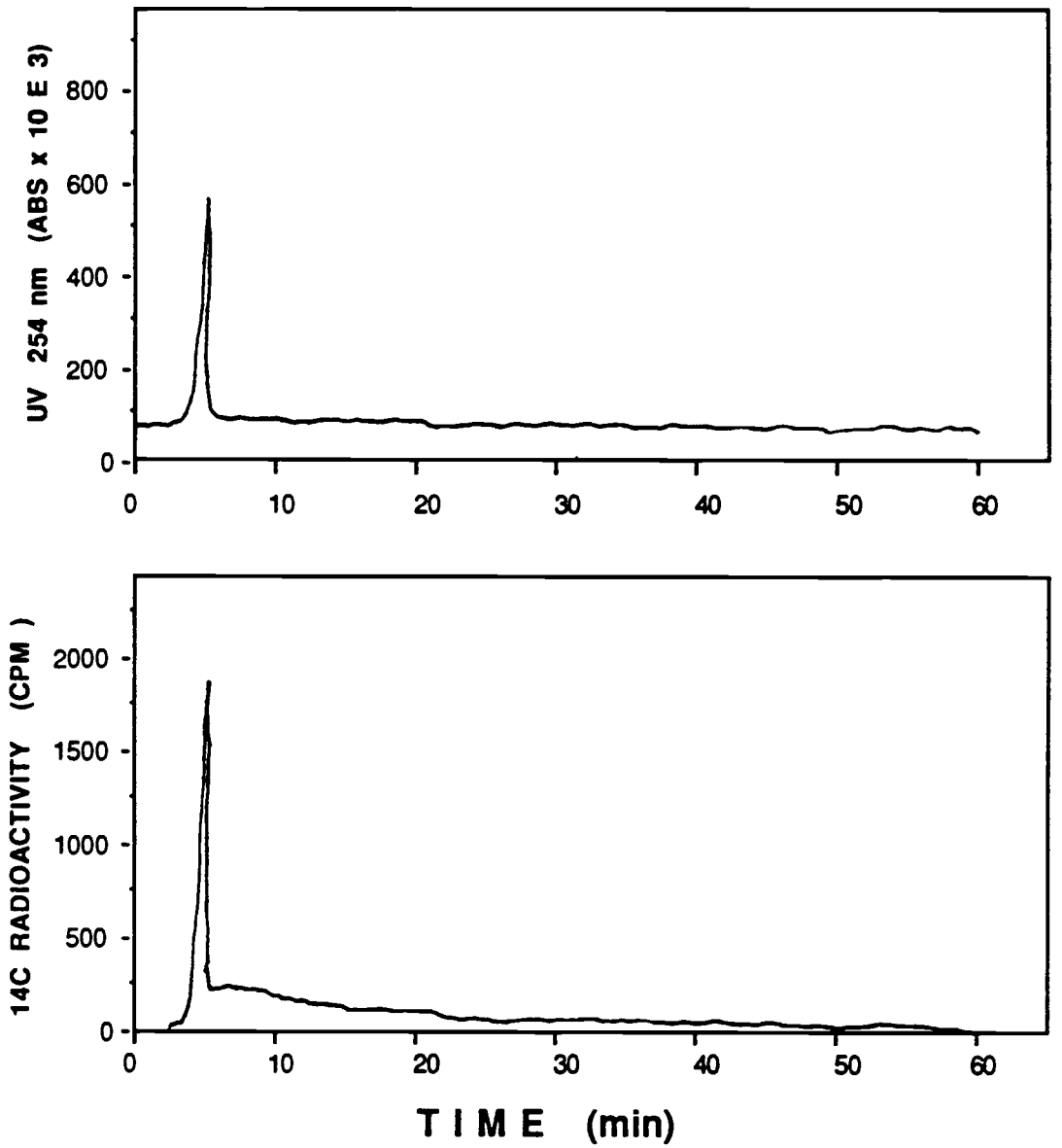


Figure IV.14. HPLC chromatogram of [14C]-MTR metabolism by stationary phase suspensions of Passe Crassane. Conditions (described in detail in both Chapter II and Appendix B) = uBondapak C18 column with methanol / ddH2O 36:64, v / v) at 0.7 mL / min, room temperature (HPLC System II). The predominant radioactive peak has the same retention time as authentic MET (5.3 min) and/or its sulfoxide (4.9 min).

DISCUSSION

Previous investigations regarding suspension fruit cells of *Pyrus communis* L., cv Passe Crassane have shown that although these cells both undergo some variability with progressive subculturing and do not always respond identically to the same growth conditions (16, 101, 111), experiments conducted with cells from the same stock yield similar results (16). In this framework, the results reported here concerning cell growth and viability (Figures IV.1.-IV.3.) are within the range of those appearing in the literature (16, 78, 105, 111). However, C_2H_4 profiles (Figures IV.5.-IV.6.) differ both with respect to maximal amounts released and rate of production.

Senescent pear cells cultured in aging medium in the presence of IAA and analyzed as described in this investigation have been reported to produce as much as 30-60 nL C_2H_4 / 10^6 cells-hr within 18-24 hr (105, 106). In the present study, peak C_2H_4 production was found to be about half of the lower end of this range (*i.e.*, 17 nL C_2H_4 / 10^6 cells-hr), and occurred in roughly triple the time (*i.e.*, >60 hr). Even under other conditions examined, maximal C_2H_4 evolution was not seen prior to approximately 45 hr. The reason for these differences is unknown, but could reflect age-related changes in cell behavior. Another possible explanation might relate to the ability of C_2H_4 to initiate cellular expansion and prolong its duration (10a, 10b,12, 91). If these effects, relative to the measurable release of C_2H_4, were to occur more quickly, a lag would be observed.

In the literature, there is one report of a similar delay in IAA-induced C_2H_4 production from senescent Passe Crassane suspensions (peak at 72 hr), in which a relatively high amount of C_2H_4 (*i.e.*, 40 nL / 10^6 cells-hr) is released

nonetheless (105). However, in this case a 1.8 L suspension was analyzed for C_2H_4 by removing 50 mL aliquots; a maneuver which effectively doubled the lag time preceeding observed C_2H_4 relative to that measured concurrently in smaller (*i.e.*, 25-50 mL), non-manipulated suspensions (105). This effect might have resulted from the "diluting out" of collected C_2H_4, since only freshly produced C_2H_4 would have been measured.

Another point of interest is that unlike suspension cultures of sunflower and other plant cells (116), these pear fruit cells are capable of producing peak amounts of C_2H_4 not just (in growth medium) during the exponential growth phase (Figure IV.5.), but also (in aging medium) during stationary phase (Figure IV.6.). This suggests that rapid cell division and C_2H_4 biosynthesis may be antagonistic. Work with pea seedlings also supports this theory (10a, 10b). Furthermore, auxin deprivation (*e.g.*, culture in either transition or aging medium) has been linked to both the cessation of cell division and a rise of inducible C_2H_4 production (105). It should be emphasized that because of its high mannitol content (*i.e.*, strong osmoticum), in addition to preventing rapid cell death, aging medium also suppresses the extent of cellular expansion (54). Thus, depending upon the extent of C_2H_4-promoted expansion, cells in this medium might exhibit neither pronounced cellular division nor expansion. Also, the C_2H_4 effect of preventing or slowing mitotic activity might reflect the ability of this compound to inhibit DNA synthesis.

Both MTA and MTR were seen to inhibit suspension pear cell growth (Figure IV.4.), presumably by different mechanisms. MTA has long been recognized as a growth regulatory compound with diverse modes of action (119). The nucleoside has been shown to cause potent inhibition of the activities of a number of enzymes, including S-adenosylhomocysteine (SAH) hydrolase (41), both SPD and SPM synthetase (57, 97), and cAMP phosphodiesterase (109). The primary site of action of MTA in murine lymphoma cells

has been attributed to the latter, *i.e.*, the perturbation of cAMP metabolism (109). A similar event may be occurring in these pear cells.

Although the MTR analog ETR, also a structural analog of MTA, might serve as a cAMP phosphodiesterase inhibitor as well, its mode of action is more likely the interference of MET recycling. ETR inhibits pear MTR kinase activity and is accepted as an alternate substrate for this enzyme (see Chapter II). The product of the reaction, ETR-1-phosphate (ETR-1-P), may represent either a dead-end metabolite, or could be converted intracellularly to ethionine, a known cytotoxic agent (8). The latter has been demonstrated in *Klebsiella pneumoniae* (94). Either fate would result in a reduction of cellular growth.

That neither MET nor ACC at the concentrations tested affected the viable cell density of log phase pear cells (Figure IV.4.) is of interest. MET has several metabolic fates; predominantly, utilization for protein synthesis, trans-sulfurization via conversion to SAM, or transamination. If converted to SAM, in addition to transmethylations, MET may contribute to three separate events: polyamine biosynthesis (normal to increased cell division), C_2H_4 production (decreased cell division and senescence), or MET recycling (normal to decreased cell division). Furthermore, high concentrations of MET are toxic (19), and in plants the compound appears to regulate its own *de novo* synthesis (48). Giovanelli *et al.* found that MET supplementation to *Lemna* colonies greatly reduced its incorporation and accumulation into both cystathionine and its products (*i.e.*, homocysteine, MET, S-methylmethionine-sulfonium salt, SAM, and SAH) without appreciably affecting that into protein cysteine (48). A combination of multiple fates and feedback regulation may be acting to dilute out the impact of exogenous MET upon pear cell growth.

Likewise C_2H_4 is capable of feedback regulating its own production via regu-

lation of ACC malonyltransferase (84), the enzyme responsible for converting ACC into the dead-end metabolite MACC. Thus, although exposure to increased ACC should promote C_2H_4 biosynthesis and therefore contribute to reduced cellular growth rates, MACC could serve as a sink for ACC instead. Also, judging from the minor impact of IAA upon C_2H_4 production from log phase pear cells cultured in growth medium relative to that from stationary phase cells in aging medium (Figure IV.5. vs. Figure IV.6.), it may be that the influence of ACC upon the former is negligible.

Exogenously administered polyamines have been shown in several plant tissues to both inhibit C_2H_4 biosynthesis (11, 37, 43, 59) and shunt the label from [3,4-^{14}C]-MET into SPD instead of ACC (37). In the present investigation, the diamine PUT was seen to reversibly inhibit IAA-induced C_2H_4 production (Figure IV.7.) and increase the rate of suspension pear cell growth (Figure IV.4.). The most abundant polyamines in developing pea ovaries are reported to be SPD and SPM (26). When the individual effects of these two compounds upon IAA-induced C_2H_4 evolution in pear fruit cell suspensions were examined, the inhibition was even more pronounced than that seen with PUT (Figure IV.7.). These results might be generally expected, given the antagonistic relationship with respect to the competitive fate of SAM in polyamine vs. C_2H_4 biosynthesis, and may reflect the inhibition of ACC synthase. However, that the extent of C_2H_4 inhibition was seen to increase with increasing cationic charge (*i.e.*, PUT < SPD < SPM) also implies that plant membrane charge neutralization may be responsible. These cations could influence transport by binding to some membrane component, thereby causing a conformational change which might favor an increased proton-motive force. In support of this theory, other studies have shown that high Ca^{2+} concentrations prevent or partially reverse the SPM-induced C_2H_4 inhibition effect (11).

The rate at which MET is transported into suspension-cultured pear cells appears to be a function, among other things, of the growth curve. Rapidly dividing cells (log phase, growth medium) were the most quickly saturated (within 1.5 h; Figure IV.8.). Stationary phase cells exhibited substantially slower uptake rates (Figures IV.9. and IV.11.), presumably due to the predominance of cellular expansion and vacuolization over mitotic activity. However, the fact that these cells were able to take up in 12-24 h at least as much MET as their log phase counterparts, in one case (Figure IV.9.) without even approaching saturation, suggests that MET is incorporated better under non-dividing conditions. The kinetic differences of cells cultured in growth vs. aging medium observed both in the latter and in the cases of MTA and MTR uptake could reflect natural variations incurred during subculturing, or changes in osmolarity introduced by the media. Aging medium has a much higher osmoticum; this could have a strong effect upon various kinetic components of both amino acid and metabolite uptake.

That MTA was taken up better in stationary phase growth cells than was MTR might reflect kinetic differences in the enzymes (MTA nucleosidase and MTR kinase, resp.) that degrade these two compounds. Evidence suggests that concentrations of >40 μM MTR feedback inhibit MTR kinase in cell-free extracts of *Klebsiella pneumoniae* (Dr. Paula Tower, Portland V. A. Hospital, Portland, OR; personal communication). Furthermore, since MTA is produced as a natural byproduct of polyamine biosynthesis, and high concentrations of this nucleoside are detrimental to the cell, it would stand to reason that the mechanisms involved in its removal and / or incorporation would be well developed.

In all cases, MET was incorporated into Passe Crassane suspensions far more effectively than was either MTA or MTR. Prolonged culture periods of the latter did little to reduce the significance of this difference. The event may

reflect a lower rate (or relative lack) of penetration of MTA and MTR into the cells. Similar results have been reported in uptake studies with the yeast *Candida albicans* (120); although in these studies unlike both the present investigation and others involving plants including duckweed (47), apple tissue (3, 152), or mungbean hypocotyls (90), little metabolism of these two compounds (especially MTR) was observed.

The three compounds, MET, MTA, and MTR not only were found to be taken up by suspension cells of Passe Crassane, but also were metabolized to known intermediates of either MET recycling (*e.g.*, predominantly MET itself; recovered in both [$^{14}CH_3$]-MTA and [$^{14}CH_3$]-MTR uptake studies) or C_2H_4 biosynthesis (*e.g.*, ACC and presumably C_2H_4 itself; recovered in [3,4-^{14}C]-MET uptake studies). That these metabolites would predominate is in agreement with several other studies utilizing a variety of plant species (3, 47, 69, 90, 152), and further supports the theory that undifferentiated suspensions of Passe Crassane *(Pyrus communis* L.) might be utilized as a (limited) model for some of the metabolic events (such as MET recycling and C_2H_4 evolution) occurring in whole fruit systems.

REFERENCES

1. Aarnes, H. 1977. *Plant Sci. Lett.* 10: 381-390.

2. Acaster, M. A. and H. Kende. 1982. *Plant Physiol.* 72: 139-145.

3. Adams, D. O. and S. F. Yang. 1977. *Plant Physiol.* 60: 892-896.

4. Adams, D. O. and S. F. Yang. 1979. *Proc. Natl. Acad. Sci. USA* 76: 170-174.

5. Adams, D. O. And S. F. Yang. 1981. *Trends Biochem. Sci.* 6: 161-164.

6. Alhonen-Hongisto, L. and J. Janne. 1980. *Biochem. Biophys. Res. Comm.* 93: 1005-1013.

7. Alhonen-Hongisto, L., Seppanen, P., Holtta, E. and J. Janne. 1982. *Biochem. Biophys. Res. Comm.* 106: 291-297.

8. Alix, J.-H. 1982. *Microbiol. Rev.* 46: 281-295.

9. Altman, A., Kaur-Sawhney, R. and A. W. Galston. 1977. *Plant Physiol.* 60: 570-574.

10a. Apelbaum, A. and S. P. Burg. 1972. *Plant Physiol.* 50: 117-124.

10b. Apelbaum, A. and S. P. Burg. 1972. *Plant Physiol.* 50: 125-131.

11. Apelbaum, A., Burgoon, A. C., Anderson, J. D., Lieberman, M., Ben-Arie, R. and A. K. Mattoo. 1981. *Plant Physiol.* 68: 453-456.

12. Apelbaum, A., Goldlust, A. and I. Icekson. 1985. *Plant Physiol.* 79: 635-640.

13. Apelbaum, A., Icekson, I., Burgoon, A. C. and M. Lieberman. 1982. *Plant Physiol.* 70: 1221-1223.

14. Ashwell, G. 1972. *Meth. in Enzymol.* 8: 85-95.

15. Backlund, P. S. and R. A. Smith. 1981. *J. Biol. Chem.* 256: 1533-1535.

16. Balague, C., Latche, A., Fallot, J. and J.-C. Pech. 1982. *Plant Physiol.* 69: 1339-1343.

17. Bauer, A. H. and S. F. Yang. 1972. *Phytochem.* 11: 3207-3214.

18. Baxter, C. and C. J. Coscia. 1973. *Biochem. Biophys. Res. Comm.* 54: 147-154.

19. Benevenga, N. J. 1974. *Agricultural and Food Chem.* 22: 2-9.

20. Bleecker, A. B., Kenyon, W. H., Somerville, S. C. and H. Kende. 1986. *Proc. Natl. Acad. Sci. USA.* 83: 7755-7759.

21. Boller, T., Herner, R. C., and H. Kende. 1979. *Planta.* 145: 293-303.

22. Bradford, M. M. 1976. *Anal. Biochem.* 72: 248-254.

23. Brawley, J. V. and A. J. Ferro. 1979. J. Bacteriol. 140: 649-654.

24. Broglie, K. E., Gaynor, J. J., and R. M. Broglie. 1986. *Proc. Natl. Acad. Sci. USA.* 83: 6820-6824.

25. Cantoni, G. L. 1953. *J. Biol. Chem.* 204: 403-416.

26. Carbonell, J. and J. L. Navarro. 1989. *Planta.* 178: 482-487.

27. Carteni-Farina, M., Oliva, A., Romeo, G., Napolitano, G., De Rosa, M., Gambacorta, A. and V. Zappia. 1979. Eur. J. Biochem. 101: 317-324.

28. Christoffersen, R. E., Tucker, M. L., and G. G. Laties. 1984. *Plant Mol. Biol.* 3: 385-391.

29. Chu, T. M., Mallette, M. F. and R. O. Mumma. 1968. *Biochem.* 77: 1399-1406.

30. Cohen, E., Arad, M., Heimer, Y. M. and Y. Mizrahi. *Plant Physiol.* 70: 540- 543.

31. Cooper, A. J. L. and A. Meister. 1977. *CRC Crit. Rev. Biochem.* 4: 281-303.

32. Cooper, T. G. The Tools of Biochemistry. John Wiley & Sons, Inc. New York: 1977.

33. Della Ragione, F., Porcelli, M., Carteni-Farina, M. and V. Zappia. 1985. *Biochem. J.* 232: 335-341.

34. Duerre, J. A. 1962. *J. Biol. Chem.* 237: 3737-3741.

35. Dunlap, J. R. and P. W. Morgan. 1977. *Plant Physiol.* 60: 222-224.

36. Ecker, J. R., and R. W. Davis. 1987. *Proc. Natl. Acad. Sci. USA.* 84: 5202-5206.

37. Even-Chen, Z., Mattoo, A. K. and R. Goren. 1982. *Plant Physiol.* 69: 385-388.

38. Everett, G. B., Mitchell, A. D. and N. J. Benevenga. 1979. *J. Nutrition.* 109: 597-605.

39. Ferro, A. J., Barrett, A. and S. K. Shapiro. 1976. *Biochim. Biophys. Acta.* 438: 487-494.

40. Ferro, A. J., Barrett, A. and S. K. Shapiro. 1978. *J. Biol. Chem.* 253: 6021-6025.

41. Ferro, A. J., Vandenbark, A. A., and M. R. MacDonald. 1981. *BBRC.* 100: 523-531.

42. Flores, H. E. and A. W. Galston. 1982. *Science.* 217: 1259-1261.

43. Fuhrer, J., Kaur-Sawhney, R., Shih, L. M., and A. W. Galston. 1982. *Plant Physiol.* 70: 1597-1600.

44. Furfine, E. S. and R. H. Abeles. 1988. *J. Biol. Chem.* 263: 9598-9606.

45. Galletti, P., DeRosa, M., Gambacorta, A., Manna, C., Festinese, R. and V. Zappia. 1981. *FEBS Lett.* 124: 62-66.

46. Galston, A. W. 1983. *BioScience.* 33: 382-388.

47. Giovanelli, J., Datko, A. H., Mudd, S. H. and G. A. Thompson. 1983. *Plant Physiol.* 71: 319-326.

48. Giovanelli, J., Mudd, S. H. and A. H. Datko. 1985. *Plant Physiol.* 77: 450-455.

49. Goldemberg, S. H. 1980. *J. Bacteriol.* 114: 1428-1431.

50. Guranowski, A. 1983. *Plant Physiol.* 71: 932-935.

51. Guranowski, A. B., Chiang, P. K. and G. L. Cantoni. 1981. *Eur. J. Biochem.* 114: 293-299.

52. Guranowski, A. B., Chiang, P. K. and G. L. Cantoni. 1983. *Meth. in Enzymol.* 994: 365-369.

53. Guranowski, A. and A. Paszewski. 1982. *Biochim. Biophys. Acta* 717: 289-294.

54. Haber, A.H. and H.J. Luippold. 1960. *Plant Physiol.* 35: 168-173.

55. Haegele, K. D., Splinter, T. A. W., Romiju, J. C., Schechter, P. J. and A. Sjoerdsma. 1987. *Cancer Res.* 47: 890-895.

56. Hayashi, M. and T. Nakajima. 1984. *Japan. J. Breed.* 34: 100-134.

57. Hibasami, H., Borchardt, R.T., Chen, S.Y., Coward, J.K., and A.E. Pegg. 1980. *Biochem. J.* 187: 419-428.

58. Hoffman, J. L. and D. M. Sullivan. in Biochemistry of S-Adenosylmethionine and Related Compounds, E. Usdin, R. T. Borchardt and C. R. Creveling, eds. MacMillan Press, Ltd. London: 1982 (pp 683-686).

59. Hyodo, H. and K. Tanaka. 1986. *Plant Cell Physiol.* 27: 391-398.

60. Icekson, I. Bakhanashvili, M. and A. Apelbaum. 1986. *Plant Physiol.* 82: 607-609.

61. Icekson, I., Goldlust, A. and A. Apelbaum. 1985. *J. Plant Physiol.* 119: 335-345.

62. Ince, J. E. and C. J. Knowles. 1986. *Arch. Microbiol.* 146: 151-158.

63. Kanabus, J., Bressan, R. A., and N. C. Carpita. 1986. *Plant Physiol.* 82: 363-368.

64. Konze, J. R. and H. Kende. 1979. *Plant Physiol.* 63: 507-510.

65. Konze, J. R. and G. M. K. Kwiatkowski. 1981. *Planta.* 151: 327-330.

66. Kukagawa, K. and M. Ichino. 1971. *Tetrahedron Lett.* 2: 87-90.

67. Kushad, M. M. 1984. Doctoral Dissertation, Oregon State University, Dept. of Horticulture.

68. Kushad, M. M., Richardson, D. G., and A. J. Ferro. 1982. *Biochem. Biophys. Res.Comm.* 108: 167-173.

69. Kushad, M. M., Richardson, D. G., and A. J. Ferro. 1983. *Plant Physiol.* 73: 257-261.

70. Kushad, M. M., Richardson, D. G., and A. J. Ferro. 1985. *Plant Physiol.* 79: 525-529.

71. Kushad, M. M. and G. Yelenosky. 1987. *Plant Physiol.* 84: 692-695.

72. Kuttan, R. and A. N. Radhakrishnan. 1972. *Biochem. J.* 127: 61-67.

73. Lanka, E., Edelbluth, C., Schlicht, M., and H. Schuster. 1978. *J. Biol. Chem.* 253: 5847-5851.

74. Lawton, K. A., Huang, B., Goldsbrough, P. B., and W. R. Woodson. 1989. *Plant Physiol.* 90: 690-696.

75. Lau, O. L. and S. F. Yang. 1973. *Plant Physiol.* 51: 1011-1014.

76. Lee, C.-Y., Charles, D., and D. Bronson. 1979. *J. Biol. Chem.* 254: 6375-6381.

77. Lee, C.-Y., Lazarus, L. H., Kabakoff, D. S., Russell, Jr., P. J., Laver, M., and N. O. Kaplan. 1977. *Arch. Biochem. Biophys.* 178: 8-18.

78. Lelievre, J. M., Balague, C., Pech, J. C. and Y. Meyer. 1987. *Plant Physiol.* 85: 400-406.

79. Leslie, C. A. and R. J. Romani. 1986. *Plant Cell Reports.* 5: 144-146.

80. Lieberman, M. 1979. *Ann. Rev. Plant Physiol.* 30: 533-591.

81. Lincoln, J. E., Cordes, S., Read, E., and R. L. Fischer. 1987. *Proc. Natl. Acad. Sci. USA.* 84: 2793-2797.

82. Lincoln, J. E. and R. L. Fischer. 1988. *Mol. Gen. Genet.* 212: 71-75.

83. Liquori, A. M., Constantine, L., Crescenzi, V., Elia, V., Giglio, E., Politi, R., DeSantis Savina, M. and V. Vitagliano. 1967. *J. Mol. Biol.* 24: 113-122.

84. Liu, Y., Su, L.-Y., and S. F. Yang. 1985. *Plant Physiol.* 77: 891-895.

85. Loomis, W. D. 1974. *Meth. in Enzymol.* 31: 528-544.

86. Loomis, W. D., Lile, J. D., Sandstrom, R. P. and A. J. Burbott. 1979. *Phytochemistry.* 18: 1049-1054.

87. Marchitto, K. S. and A. J. Ferro. 1985. *J. Gen. Microbiol.* 131: 2153-2164.

88. Maunders, M. J., Holdsworth, M. J., Slater, A., Knapp, J. E., Bird, C. R., Schuch, W., and D. Grierson. 1987. *Plant, Cell and Env.* 10: 177-184.

89. Miller, R. L., Adamczyk, D. L., and W. H. Miller. 1979. *J. Biol. Chem.* 254: 2339-2345.

90. Miyazaki, J. H. and S. F. Yang. 1987. *Plant Physiol.* 84: 277-281.

91. Mohan Ram, H. Y. and V. S. Jaiswal. 1970. *Experientia.* 26: 214-216.

92. Murashige, T. and F. Skoog. 1982. *Physiol. Plant.* 15: 473-497.

93. Murr, D. P. and S. F. Yang. 1875. *Phytochem.* 14: 1291-1292.

94. Myers, R. W. and R. H. Abeles. 1989. *J. Biol. Chem.* 264: 10547-10551.

95. Nichols, S. E. and G. G. Laties. 1984. *Plant Mol. Biol.* 3: 393-401.

96. Nitsch, J. P., Asahira, T., Rossini, L. M. E. and C. Nitsch. 1970. *Bull. Soc. Fr.* 117: 479-492.

97. Pajula, R. L. and A. Raina. 1979. *FEBS Lett.* 99: 343-345.

98. Palavan, N. R., Goren, R. and A. W. Galston. 1984. *Plant Cell Physiol.* 25: 541-546.

99. Pech, J.-C. and J. Fallot. 1974. *Fruits.* 29: 771-776.

100. Pech, J.-C., Latche, A., Austrey, M. and J. Fallot. 1975. *Bull. Soc. Fr.* 122: 183-194.

101. Pech, J.-C. and R. J. Romani. 1979. *Plant Physiol.* 63: 814-817.

102. Peiser, G. D., Wang, T., Hoffman, N. E., Yang, S. F., Liu, H. and C. T. Walsh. 1984. *Proc. Natl. Acad. Sci. USA.* 81: 3059-3063.

103. Peterson, G. L. 1983. *Meth. in Enzymol.* 91: 95-119.

104. Pfadenhauer, E. H. and S. Tong. 1979. *J. of Chromatogr.* 162: 585-590.

105. Puschmann, R., Ke, D. and R. Romani. 1985. *Plant Physiol.* 79: 973-976.

106. Puschmann, R. and R. Romani. 1983. *Plant Physiol.* 73: 1013-1019.

107. Read, S. M. and D. H. Northcote. 1981. *Anal. Biochem.* 116: 53-64.

108. Riscoe, M. K. and A. J. Ferro. 1984. *J. Biol. Chem.* 259: 5465-5471.

109. Riscoe, M. K., Tower, P. A., and A. J. Ferro. 1984. *Biochem. Pharm.* 33: 3639-3643.

110. Robeson, D. J. and D. R. Cook. 1985. *Plant Physiol.* 73: 1013-1019.

111. Romani, R. J., Bos, T. J., and J.-C. Pech. 1981. *Plant Physiol.* 68: 823-826.

112. Ruiz-Herrera, J. and R. L. Starkey. 1969. *J. Bacteriol.* 99: 544-551.

113. Ruiz-Herrera, J. and R. L. Starkey. 1970. *J. Bacteriol.* 104: 1286-1293.

114. Rupniak, H. T. and D. Paul. 1978. *Cell Physiol.* 94: 161-170.

115. Satoh, S. and Y. Esashi. 1986. *Plant Cell Physiol.* 26: 219-230.

116. Sauerbrey, E., Grossmann, K., and J. Jung. 1977. *J. Plant. Physiol.* 127: 471-479.

117. Savarese, T. M., Dexter, D. L., Parks, R. E. and J. A. Montgomery. 1983. *Biochem. Pharmacol.* 32: 1907-1916.

118. Savarese, T. M., Ghoda, L. Y., Dexter, D. L., and R. E. Parks. 1983. *Cancer Res.* 43: 4699-4702.

119. Schlenk, F. 1983. *Adv. Enzymol.* 54: 195-265.

120. Schlenk. F., Zydek-Cwick, C. R., and J. L. Dainko. 1973. *Biochim. Biophys. Acta.* 320: 357-362.

121. Schroeder, H. R., Barnes, C. J., Bohinski, R. C. and M. F. Mallette. 1973. *Can. J. Microbiol.* 19: 1347-1354.

122. Scopes, R. K. Protein Purification: Principles and Practice, 2nd ed. Springer-Verlag New York, Inc. New York, New York: 1987.

123. Shapiro, S. K. and A. Barrett. 1981. *Biochem. Biophys. Res. Comm.* 102: 302-307.

124. Sisler, E. C. and S. F. Yang. 1983. *BioScience.* 34: 234-238.

125. Smith, B. A., Reider, M. L. and J. S. Fletcher. 1982. *Plant Physiol.* 70: 1288-1230.

126. Smith, C.J.S., Slater, A., and D. Grierson. 1986. *Planta.* 168: 94-100.

127. Smith, D. M. and M. W. Montgomery. 1985. *Phytochem.* 24: 901-904.

128. Smith, E. L., Hill, R. L., Lehman, I. R., Lefkowitz, R. J., Handler, P. and A. White. Principles of Biochemistry: General Aspects. 7th ed. McGraw-Hill, Inc. San Francisco:1983 (pp 655-656).

129. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and D. C. Klenk. 1985. *Anal. Biochem.* 150: 76-85.

130. Smith, T. A. 1975. *Phytochem.* 14: 865-890.

131. Speranza, A. and N. Bagni. 1977. *Z. Pflanzen Physiol.* 81: 226-233.

132. Steele, R. D. and N. J. Benevenga. 1979. *J. Biol. Chem.* 254: 8885-8890.

133. Stevens, R., Stevens, L., and N. C. Price. 1983. *Biochem. Education.* 11: 70.

134. Suelter, C. H. A Practical Guide to Enzymology. John Wiley & Sons, Inc. New York: 1985.

135. Suttle, J. C. 1981. *Phytochem.* 20: 1477-1480.

136. Tabor, C. W. and H. Tabor. 1984. *Ann. Rev. Biochem.* 53: 749-790.

137. Toennies, G. A. and J. J. Kob. 1951. *Anal. Chem.* 23: 823-826.

138. Tong, C. B., Labavitch, J. M., and S. F. Yang. 1986. *Plant Physiol.* 81: 929-930.

139. Trackman, P. C. and R. H. Abeles. 1983. *J. Biol. Chem.* 258: 6717-6720.

140. Tucker, M. L. and G. G. Laties. 1984. *Plant Physiol.* 74: 307-315.

141. Wagner, J., Danzin, C. and P. Mamont. 1982. *J. Chromatogr.* 227: 349-368.

142. Wagner, J., Hirth, Y., Claverie, N. and C. Danzin. 1986. *Anal. Biochem.* 154: 604-607.

143. Walker, J. R. L. 1980. *What's New in Plant Physiology.* 11: 33-36.

144. Wang, S. Y., Adams, D. O., and M. Lieberman. 1982. *Plant Physiol.* 70: 117-121.

145. White, M. W., Vandenbark, A. A., Barney, C. L. and A. J. Ferro. 1982. *Biochem. Pharmacol.* 31: 503-507.

146. Winer, L., Vinkler, C. and A. Apelbaum. 1984. *Plant Physiol.* 76: 233-237.

147. Woodson, W. R. and K. A. Lawton. 1988. *Plant Physiol.* 87: 478-503.

148. Yang, S. F. and N. E. Hoffman. 1984. *Ann. Rev. Plant Physiol.* 35: 155-189.

149. Young, N. D. and A. W. Galston. 1983. *Plant Physiol.* 71: 767-771.

150. Young, R. E., Pratt, H. K. and J. B. Biale. 1952. *Anal. Chem.* 24: 551-555.

151. Yu, Y. B., Adams, D. O. and S. F. Yang. 1979. *Arch. Biochem. Biophys.* 198: 280-286.

152. Yung, K. H., Yang, S. F. and F. Schlenk. 1982. *Biochem. Biophys. Res. Comm.* 104: 771-777.

153. Zappia, V., Della Ragione, F., Cacciapuoti, G., Porcelli, M., Galletti, P. and M. Carteni-Farina. in Biochemistry of S-Adenosylmethionine and Related Compounds, E. Usdin, R. T. Borchardt and C. R. Creveling, eds. MacMillan Press, Ltd. London: 1982 (pp 701-707).

154. Zappia, V., Della Ragione, F., Pontoni, G., Gragnaniello, V. and M. Carteni-Farina. in Progress in Polyamine Research, V. Zappia and A. E. Pegg, eds. Plenum Press, New York: 1988 (pp 165-177).

155. Zappia, V., Galletti, P., Porcelli, M., Manna, C. and F. Della Ragione. 1980. *J. Chromatogr.* 189: 399-405.

APPENDICES

APPENDIX A

PARTIAL PURIFICATION OF 5'-DEOXY-5'-METHYLTHIOADENOSINE NUCLEOSIDASE AND 5-DEOXY-5-METHYLTHIORIBOSE KINASE FROM FRUIT EXTRACTS OF PEAR (*Pyrus communis* L.) AND AVOCADO (*Persea americana* Mill) AND PROBLEMS ENCOUNTERED

In order to biochemically characterize an enzyme without the interfering effects of various compounds including other proteins which may be present in a crude homogenate, the enzyme first must be isolated (purified); preferably in a homogeneous form. Attempts to realize this for the enzymes 5'-deoxy-5'-methylthioadenosine (MTA) nucleosidase and 5-deoxy-5-methylthioribose (MTR) kinase extracted from both pear and avocado fruits proved to be frustrating and unsuccessful, due to unexpected enzyme instability. Although MTA nucleosidase was purified as much as 1302-fold (from Comice pear, *Pyrus communis* L.) and MTR kinase as much as 1822-fold (from Packham Triumph pear, *Pyrus communis* L.), the preparations were far from homogeneous (as judged by gel electrophoresis; data not shown), and efforts required to achieve these levels of purification were both arduous and not conducive toward generating preparations that remained stable long enough for significant biochemical analyses. For this reason, only preliminary characterizations (using crude extracts; see Chapter IV) were obtainable. Several problems encountered and efforts to minimize their impacts upon the partial purification of both enzymes are presented. General methods employed to extract (homogenize) and partially purify the enzymes, and measure both protein content and enzyme activity are described in Chapter II.

RESULTS AND DISCUSSION

Representative partial purification schemes and their effects upon the protein concentrations and enzyme activities of extracts of *Pyrus communis* L. and *Persea americana* Mill are presented in Tables VI.A.1. (MTA nucleosidase) and VI.A.2. (MTR kinase). In order to maintain enzyme activity, none of the preparations listed were frozen at any time during the purification process, and the process itself was conducted both without interruption and as quickly as possible. In other cases, (repetitive) freezing at -20°C (or even -70°C, but to a lesser extent) followed by gentle thawing (in ice water) of partially purified (but not crude) preparations was found to promote loss of enzyme activity, except occassionally for preparations containing significant amounts of $(NH_4)_2SO_4$ (data not shown). In studying SAH / MTA nucleosidase from *E. coli,* Ferro *et al.* found similar results, although these researchers were able to stabilize the enzyme by other means (39). However, probably due to the extended time and manipulations (*e.g.*, centrifugations and resolubilizations) involved, the salting-out and subsequent dialysis steps generally were responsible for the greatest losses of total protein. Hence, alternatives to the latter were sought (Chapter II, and below).

Although some crude extracts were found to remain stable at -20° C for almost a full year, the same was not true for those which had undergone some degree of purification. Loss of enzyme activity in the latter was so rapid that in one week's time of storage at -70°C, the specific activities of 1 mL aliquots of MTA nucleosidase and MTR kinase co-purified (67-fold and 41-fold, respectively, relative to the crude extract) from Comice pear were seen to degrade to less than those characteristic of the original preparation (data not shown). The effect also was observed, *albeit* less significantly, in extracts of avocado whose crude homogenates characteristically possessed

TABLE VI.A.1. Representative Partial Purifications of MTA Nucleosidase From Extracts of *Pyrus communis* L., cv Comice and *Persea americana* Mill, cv Fuerte

PURIFICATION STEP	VOL. (mL)	PROT.[a] (mg/mL)	TOT. PROT. (mg)	S. A.[b] (units/mg)	T. A.[c] (units)	PURIF. (fold)	YIELD (%)
A. COMICE:							
1. crude extract[d]	200	1.28	256	1.38	353	1.0	100
2. dialyzed 27% $(NH_4)_2SO_4$ ppt[e]	14	4.64	65	4.29	279	3.0	79
3. DEAE Cellulose pooled frxns	69	0.123	8.5	26.1	222	19	63
4. undialyzed 60% $(NH_4)_2SO_4$ ppt	9.0	0.643	6.2	21.6	134	16	38
5. Sephadex G200 pooled frxns	18	0.0324	0.58	115	67	83	19
6. dialyzed 60% $(NH_4)_2SO_4$ ppt	3.0	0.113	0.34	73.5	25	53	7.1
7. Hydroxyapatite pooled frxns	8.0	0.0011	0.0088	1250	11	906	3.1
8. Aquacide I concentrate	2.1	0.0015	0.0032	1797	5.8	1302	1.6
B. FUERTE:							
1. crude extract[d]	191	7.96	1520	2.17	3298	1.0	100
2. dialyzed 27% $(NH_4)_2SO_4$ ppt[e]	4.8	81.9	393	5.86	2303	2.7	70
3. DEAE Cellulose pooled frxns	35	0.501	17.7	111	1964	51	60
4. undialyzed 60% $(NH_4)_2SO_4$ ppt	4.3	2.99	6.89	289	1991	133	49

[a] Protein concentrations; determined by Coomassie Blue (Bradford) Method (with Read and Northcote modification, where applicable), as described in Chapter II.

[b] Specific Activity: one unit cleaves 1 nmol per min of MTA to MTR at 37°C and pH 11.0; see Chapter II for assay method.

[c] Total Activity.

[d] Cell-free extract was prepared by Method 3 (Chapter II).

[e] Deriving from back-extractions at 0-20% and 20-47% $(NH_4)_2SO_4$, followed by resuspension first at 27% $(NH_4)_2SO_4$ then in nucleosidase purification buffer B, as described in Chapter II.

TABLE VI.A.2. Representative Partial Purifications of MTR Kinase From Extracts of *Pyrus communis* L., cv Packham Triumph and *Persea americana* Mill, cv Fuerte

PURIFICATION STEP	VOL. (mL)	PROT.[a] (mg/mL)	TOT. PROT. (mg)	S.A.[b] (units/mg)	T.A.[c] (units)	PURIF. (fold)	YIELD (%)
A. PACKHAM TRIUMPH:							
1. crude extract[d]	1200	0.236	283	0.0381	10.8	1.0	100
2. dialyzed 35% $(NH_4)_2SO_4$ ppt[e]	24	0.929	22	0.0793	1.77	2.1	16
3. AH Sepharose pooled frxns + 35% $(NH_4)_2SO_4$ precipitation	34	0.0240	0.82	0.619	0.506	16	4.7
4. Sephadex G200 pooled frxns	11	0.0183	0.201	1.52	0.306	40	2.8
5. Hydroxyapatite pooled frxns	1.0	0.0061	0.0061	69.4	0.423	1822	3.9
B. FUERTE:							
1. crude extract[f]	310	7.96	2468	0.112	276	1.0	100
2. dialyzed 35% $(NH_4)_2SO_4$ ppt[e]	7.8	66.3	517	0.233	121	2.1	44
3. AH Sepharose pooled frxns	28	0.786	21.7	4.52	98.2	19	40
4. undialyzed 60% $(NH_4)_2SO_4$ ppt	3.2	3.09	9.90	9.81	97.1	88	35

[a] Protein concentrations; determined by Coomassie Blue (Bradford) Method (with Read and Northcote modification, where applicable), as described in Chapter II.

[b] Specific Activity: one unit converts 1 nmol per min of MTR to MTR-1-P at 30°C and pH 9.5; see Chapter II for assay method.

[c] Total Activity.

[d] Cell-free extract was prepared by Method 4 (Chapter II).

[e] Deriving from back-extractions at 0-30% and 30-50% $(NH_4)_2SO_4$, followed by resuspension first at 35% $(NH_4)_2SO_4$ then in kinase purification buffer A, as described in Chapter II.

[f] Cell-free extract was prepared by Method 2 (Chapter II).

in the order of 8 to 35 times more protein than those of pear (see Chapter III). This made any attempts at co-purification impractical, for it would be impossible to run enough reactions to biochemically characterize either enzyme prior to its complete loss of activity.

Assuming that the observed enzyme instabilities of MTA nucleosidase and MTR kinase derive from four types of threat, *i.e.*, denaturation, inactivation of catalytic site, proteolysis, or sorption to glass, plastic, etc., a detailed series of measures to counteract each of these effects was embarked upon. These steps and their respective impacts are described below.

Efforts to Minimize Enzyme Denaturation

The most common effectors of enzyme denaturation include extremes of pH, temperature, and time; the presence of denaturants such as organic solvents, heavy metals, ionic detergents, and nonionic compounds such as urea; and deviations from the protein's native environment (at a characteristic range of concentration) in bound or loosely associated water, a condition sometimes referred to as "[low] water activity" (122). Of these, the latter posed the most significant threat to purification efforts attempted here, and is discussed in greater detail below. To minimize the impacts of other pertinent conditions listed (*i.e.*, temperature and time), initial practices of repetitively storing preparations at -20°C during the extended course of a purification attempt were modified such that these partially purified extracts were maintained at 4°C for short term efforts and frozen at -70°C in all other cases, and a strong effort was made to expediate the purification process. Also, wherever possible, samples to be frozen were stored in $(NH_4)_2SO_4$.

In order to minimize the time required for a purification attempt, various

modifications of the schemes presented in Tables VI.A.1. and VI.A.2. were enacted. The first of these involved an alternative to steps involving salting-out and subsequent dialysis. As mentioned earlier, these were the points generally responsible for the greatest losses of total protein. Prior to replacing completely these steps, several modifications were studied for comparison. These included solid vs. dropwise liquid (in a properly buffered solution) delivery of $(NH_4)_2SO_4$, direct vs. back-extractions (see Chapter II), and inert (N_2) vs. ambient conditions. In all but the last case where no appreciable differences were observed, the latter of each series was found to be more effective (data not shown) and thereby was incorporated into future work involving $(NH_4)_2SO_4$. However, other means of concentration and desalting were examined also.

Primary among these were molecular exclusion resins such as Sepharose (or the less fragile Sephacryl) G-200, DEAE-Sephadex, and Bio-Gel P-10 added in dry form, and Centriprep-30 concentrators. As seen in Table VI.A.3. where a comparison of the last two means of concentration (used singularly and in conjunction) is presented, these approaches not only compromised total protein recoveries, but did little to effect purification. Furthermore, in the case of the Centriprep units, the time required to complete the process approached that for salt fractionation + dialysis, so nothing was gained by the process.

More drastic measures undertaken to reduce the time requirement of the purification schemes outlined in Tables VI.A.1. and VI.B.2. involved using less steps and trying both Chromatofocusing (Pharmacia) and various affinity resins, such as S-adenosylhomocysteine (SAH; for MTA nucleosidase; 51) and ATP-agarose (AGATP; for MTR kinase). In the case of the latter, two types based upon the point of attachment were employed: Type 3, in which ATP is attached via an aminohexyl group spacer to the adenine C-8 position, and

TABLE VI.A.3. Representative Concentration Attempts of MTR Kinase From *Persea americana* Mill, cv Hass

STEP		VOL. (mL)	PROT.[b] (mg/mL)	TOT. PROT. (mg)	S. A.[c] (units/mg)	T. A.[d] (units)	PURIF. (fold)	YIELD (%)
1.	crude extract[a,e]	30	6.55	197	0.708	139	1.0	100
2.a.	Bio-Gel P-10	6.6	16.6	110	0.970	106	1.4	76.5
2.b.	Centriprep 30	~3	18.2	~55	0.817	~45	1.2	~32
2.c.	Bio-Gel P-10+ Centriprep 30	~3	21.6	~65	0.986	~64	1.4	~46

[a] One extract of >90 mL was divided into three equal volumes of 30 mL and treated individually as shown in step 2.a., 2.b., or 2.c.

[b] Protein concentrations; determined by BCA Method, as described in Chapter II.

[c] Specific Activity: one unit converts 1 nmol per min of MTR to MTR-1-P at 30°C and pH 9.5; see Chapter II for assay method.

[d] Total Activity.

[e] Cell-free extract was prepared by Method 4 (Chapter II).

Type 4, where the same spacer is bound through the hydroxyl groups of ribose. Although both ATP columns have been used successfully to purify a variety of kinases (73, 76, 77, 89) and can serve as cation exchange resins as well (76), in these applications MTR kinase was found not to stick to either resin despite a variety of chromatographic conditions tested (*e.g.*, variations in ionic strength, pH, etc.; data not shown). Hence, neither purified the enzyme to a worthwhile extent. Likewise, neither Chromatofocusing nor SAH affinity chromatography were found to be effective in purifying MTA nucleosidase. In all cases, problems of enzyme instability persisted.

To mimic "low water activity" conditions analogous to the proteins' native environment, various (elevated) concentrations of either glycerol, other sugars (glucose, sucrose), polyethylene glycol (PEG-20,000), or PVP were tested. Although each was seen to exert minor stabilizing influences, none solved the problem and some (*i.e.*, solutions of >2% PEG and ≥20% glycerol) drastically altered the partitioning properties of the enzymes during processes of salting-out and molecular exclusion chromatography. Efforts made to approach the native concentrations of both proteins by supplementing extracts with other proteins, such as BSA (and thereby minimizing losses of native protein due to sorption onto glassware), are discussed below.

Efforts to Minimize Enzyme Catalytic Site Inactivation

The inactivation of an enzyme catalytic site generally is attributed to either oxidation (*i.e.*, the absence of adequate reducing agents), lack of sufficient amounts of substrates and / or cofactors (in the case of the latter, sometimes evoked by the presence of either chelators, or other divalent cations), or the presence of some specific inactivator. With respect to these purification applications, non-enzymatic and enzymatic oxidations alike were controlled

by several means; listed below.

1). the use of the sulfhydryl (SH) reducing agent DTT, a more effective antioxidant than β-mercaptoethanol by virtue of both the inability of any (cyclic) disulfide formed in the former to exchange with a still active sulfhydryl group from the protein, and the increased stability of this intramolecular disulfide (122);

2). the addition of various resins (PVP, PVPP, XAD-4, and Dowex 1 [Cl^-]; see Chapters III, IV, and Appendix C) to bind quickly phenolic and quinone compounds present in plant tissues prior to their oxidative and binding interactions with proteins;

3). the avoidance of conditions known to enhance the non-enzymatic oxidation of plant tissues, such as high pH and the presence of metal ions (85); the latter enacted by the use of both (ultra-)pure reagents, particularly $(NH_4)_2SO_4$, which in lower grades has been shown to be contaminated by both Mg^{2+} and other metal ions (122), and, in the case of MTA nucleosidase (which, unlike MTR kinase, does not require Mg^{2+}), the inclusion of 2 mM EDTA;

4). the addition of substrates (MTA for MTA nucleosidase and both MTR and Mg-ATP for MTR kinase) to the buffer medium.

Of the antioxidation measures described above, only the inclusion of EDTA and substrates were not conducted routinely. This was because neither was found to affect significantly the stability of either enzyme. Ferro *et al.* reported similar results with SAH / MTA nucleosidase from *E. coli* (39).

One limitation in the use of sulfhydryl reducing agents which may have played a strong role in the observed instabilities of partially purified MTA nucleosidase and MTR kinase in this study concerns their limited half-lives. Stevens *et al.* have noted that a solution of DTT (of undefined concentration) dissolved in 0.1 M potassium phosphate buffer (pH 8.5) has a half-life

of 1.4 h at 20°C, and 11 h at 0°C (133). It is likely that failure to replenish DTT in both buffer and enzyme solutions either maintained for prolonged periods at 4°C, or in the case of the latter, subjected to repetitive freezing and thawing, would promote loss of enzyme activity by oxidation; particularly in preparations of MTR kinase, whose activity in lupin seeds has been shown (via complete irreversible inactivation by *p*-hydroxymercuribenzoate) to require intact sulfhydryl groups (50). The same is not true for MTA nucleosidase from the same source (51). Also of interest is the fact that not only were the two partially purified (1979- and 8943-fold, respectively) lupin enzymes stable enough to permit complete biochemical characterization, but also the authors report no observable losses in activity following repeated freezing (at -20°C) and thawing in the course of at least three months (50, 51). The reason for this behavior difference is unknown, but could reflect variations of both protease and protein levels in seed vs. whole fruit (see below).

Another effect regarding sulfhydryl reagents which was not exploited in this study relates to the beneficial impact of EDTA upon these compounds. The inclusion of EDTA prolongs the half-life of all sulfhydryl reagents (which also serve as minor chelators of some divalent cations; 134) by complexing trace metals which otherwise would either bind to enzyme sulfhydryl groups, or catalyze their oxidation by molecular oxygen (134). However, in the case of MTR kinase, both non-chelated Mg^{2+} (to form the Mg-ATP complex) and reduced SH groups are required for activity, so the event is of limited value unless a considerable (unquantified) excess of Mg^{2+} (in the form of $MgSO_4$ or $MgCl_2$) is added to the reaction mixture.

Efforts to Minimize Enzyme Proteolysis

The four commonly acknowledged groups of proteases include metalloproteases, serine proteases, acid (carboxyl) proteases, and thiol (sulfhydryl) proteases (134). Also of import to the MTR kinase reaction are other proteolytic enzymes referred to as nucleases or "ATPases". Guranowski reports significant ATPase activity (*i.e.*, as much as 5.5% of the measured MTR kinase activity) in a variety of extracts from higher plant seeds (50).

In this study, no efforts were made to inhibit the activities of either acid proteases or ATPases; although the former would be expected to exhibit reduced activity at the buffer pH range utilized (*i.e.*, pH $\geq$ 7; 134). However, the effects of 2 mM EDTA (to bind metalloproteases) and 1.0 mM phenylmethylsulfonyl fluoride (PMSF), the latter of which inactivates serine proteases as well as some thiol proteases and some carboxypeptidases (but not acid proteases; 122), were tested. EDTA was used only in MTA nucleosidase preparations, for reasons cited above. Although some increased stability was observed, neither additive provoked as significant an effect as that anticipated; suggesting either that concentrations utilized were too low, or acid proteases might be at work. A third possibility is that the PMSF, which was added via dissolution into extraction buffer, hydrolyzed prior to exerting its (complete) effect. Researchers have suggested that because PMSF undergoes rapid hydrolysis in aqueous solutions (its half-life in water at pH 7, 25°C is 110 min; 134), the compound should be taken up first (and later stored) in acetone or isopropanol, then added directly to enzyme extracts rather than to homogenization solutions (122, 134).

Efforts to Minimize Enzyme Sorption to Glassware

Loss or denaturation of proteins by sorption onto glass, plastic, or dialysis membranes should have been minimized by inclusion in the purification

buffers of glycerol, PVP, and (in some cases) Triton X-100. However, because proteins in solution can displace compounds coating a container surface, and for solutions of very low protein concentration (such as whole pear extracts), even a small percentage of exchange would constitute a significant loss, additional measures of this sort of protection were explored.

It was hoped that because of its (initial) higher protein concentration, the substitution as a fruit enzyme source of avocado for pear would avoid the problem. That this was not the case suggests other factors contributing to loss of enzyme activity (above) may have predominated.

To reduce further the sensitivity to dilution exhibited by both MTA nucleosidase and MTR kinase in all extracts for which purification was attempted, the individual effects of BSA and PEG-20,000 were tested in several ways; described below. Initially these compounds, as solutions of 0.1 mg / mL and 1% respectively, were added directly to buffer solutions; later they were precoated onto test tubes which were oven-heated to dryness prior to being utilized to fraction collect column eluates. In one case, 1.5 mg / mL BSA was added to an extract of very low protein content (*i.e.*, a crude extract of Comice pear diluted to contain 2 ng / mL protein) prior to freezer storage. Although the first two approaches were found to enhance enzyme stabilities, only in the latter was the effect especially pronounced. The dilute crude extract so tested was found to retain complete MTA nucleosidase activity (that of MTR kinase was not assayed) after three months of storage at -70°C. Unfortunately, this information was obtained after attempts to purify prior to biochemically characterizing either enzyme had been abandoned. Another drawback to the inclusion of BSA (MW = 68,000) in partially purified extracts of either MTA nucleosidase (MW $\simeq$ 62,000; 51) or MTR kinase (MW $\simeq$ 70,000; 50) relates to its preclusion of molecular weight estimations based upon gel electrophoresis under non-denaturing conditions.

APPENDIX B

Table VI.B.1. Chromatographic t_R and R_f Values. High performance liquid chromatography (HPLC), paper chromatography (PC), and thin layer chromatography (TLC) systems and detetection methods are described in detail in Chapter II. Briefly, HPLC System I = μSpherogel Carbohydrate column with ddH_2O at 0.6 mL / min, all heated to 85°C; HPLC System II = μBondapak C_{18} reverse phase with methanol / ddH_2O (36:64, v /v) at 0.7 mL / min, room temperature (RT); HPLC System III = Partisil 10 SCX with 0.5 M NH_4COOH (pH 4.0) at 3.0 mL / min, RT; HPLC System IV = Partisil 10 SAX with 0.25 M NH_4COOH (pH 4) at 1.0 mL / min, RT; PC System I = $(NH_4)_2SO_4$ / Na phosphate buffer / 2-propanol, ascending; PC System II = n-butanol / acetic acid / ddH_2O (12:3:5) (12:3:5, by vol.), ascending; PC System III = n-butanol / acetone / acetic acid / ddH_2O (7:7:2:4, by vol.), descending; PC System IV = ethanol / ddH_2O / acetic acid (65:34:1, by vol.), descending; TLC System I = PC System III, above; TLC System II = methanol / chloroform (17:3, v / v). "Sulf." designates "sulfoxide"; all other abbreviations are explained in the text.

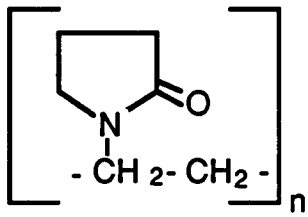
Table VI.B.1. Chromatographic t_R and R_f Values.

	t_R VALUES (min) FOR HPLC SYSTEMS				R_f VALUES FOR PC SYSTEMS				R_f VALUES FOR TLC SYSTEMS	
COMPOUND	I	II	III[1]	IV[1]	I	II	III	IV	I	II
adenine	6.2	4.6	2.4	6.2	0.13	0.54	—	—	0.57	0.63
adenosine	6.0	—	—	—	—	—	—	—	0.45	—
ACC	10.8	7.3	—	—	—	—	—	—	—	—
ETR	31.7	—	—	—	0.62	—	—	—	—	—
HSER	18.5	4.6	—	—	0.90	0.28	—	—	—	—
HSER sulf.	10.8	3.9	—	—	—	—	—	—	—	—
αHMB	9.0	4.5	—	—	—	0.85	0.87	0.84	—	0.65
αHMB sulf.	7.5	3.9	—	—	—	—	—	—	—	—
αKMB	12.0	4.3	—	—	—	0.72	0.76	0.56	0.73	0.79
αKMB sulf.	10.5	3.9	—	—	—	—	—	—	—	—
MET	28.0	5.3	—	—	0.78	0.51	0.49	0.40	0.48	0.39
MET sulf.	10.9	4.9	—	—	0.87	—	—	—	—	—
MTA	5.6	11.3	2.8	3.8	0.08	0.66	0.67	0.67	0.71	0.67
MTA sulf.	11.2	10.5	—	—	0.08	0.34	—	—	0.34	—
MTI	10.5	7.4	1.7	—	0.25	0.56	—	—	0.61	—
MTI sulf.	—	5.2	—	—	—	—	—	—	—	—
MTR	23.0	5.5	1.2	3.8	0.92	0.67	0.76	0.73	0.75	0.78
MTR sulf.	11.0	4.8	2.1	—	—	0.34	—	—	0.60	—
MTR-1-P	14.0	4.2	1.4	14.9[2]	—	0.22	0.87	0.20	0.26	0.00
SAH	>49	5.5	1.5	—	0.13	0.15	—	—	0.21	—
SAM	5.6	4.1	3.8	—	0.37	0.05	—	—	0.11	0.00
SAM sulf.	11.2	3.7	—	—	—	—	—	—	—	—

[1]HPLC systems involving both a formate mobile phase and solid flow cell on the radioisotope detector were found to exhibit reduced (by 15-20%) but constant counting efficiencies for radioactive materials. The problem can be circumvented by using a liquid flow cell, or other detection methods (see Chapter II).

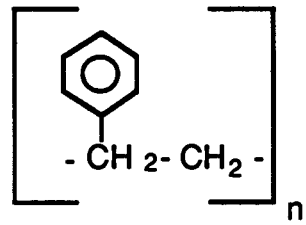
[2]This compound was found to stick to the solid flow cell of the radioisotope detector, producing a broad peak. The problem can be avoided as described in note 1.

APPENDIX C

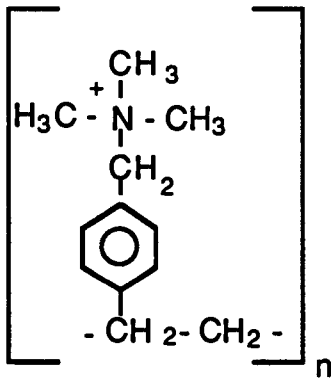


POLYVINYLPYRROLIDONE
POLYMERS

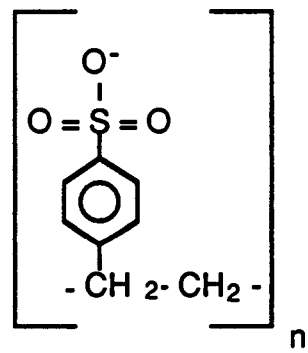
(PVP = soluble form; PVPP = insoluble form)



AMBERLITE XAD-4
POLYSTYRENE



DOWEX 1
ANION EXCHANGE
POLYSTYRENE



DOWEX 50
CATION EXCHANGE
POLYSTYRENE

Figure VI.C.1. Chemical structures of various phenolic-binding resins.

FINIS

"Luck's a chance, but trouble's sure."
A. E. Housman

"There ain't nothing more to write about, and I am rotten glad of it, because if I'd a knowed what a trouble it was to make a book, I wouldn't a tackled it and ain't a-going to no more."
Mark Twain in *Adventures of Huckleberry Finn*