PHOSPHOETHANOLAMINE *N*-METHYLTRANSFERASE FROM SPINACH

PURIFICATION AND CHARACTERIZATION OF *S*-ADENOSYL-L-METHIONINE: PHOSPHOETHANOLAMINE *N*-METHYLTRANSFERASE FROM SPINACH

by

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ABSTRACT

During conditions of osmotic stress, some plants accumulate compatible osmolytes such as glycine betaine or choline-*O*-sulphate. Choline is required as a precursor for synthesis of both osmolytes and choline is also required by all plants as a component of phospholipids. In the betaine accumulator spinach, choline synthesis requires three sequential *N*-methylations of phosphoethanolamine (PEA) to generate phosphocholine (PCho), with the first *N*-methylation being catalyzed by *S*-adenosyl-L-methionine: PEA *N*-methyltransferase (PEAMeT). Choline synthesis and, more particularly the activity of PEAMeT, are up-regulated by salinity (Summers and Weretilnyk, 1993). This thesis reports on the partial purification and preliminary characterization of PEAMeT from spinach.

A variety of column chromatography matrices including DEAE Sepharose, phenyl Sepharose, ω-aminohexyl agarose, hydroxylapatite, phenyl Superose, Mono Q and adenosine agarose, have been used to purify PEAMeT. A 5403-fold purified preparation yielded a specific activity of 189 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein. SDS-PAGE analysis of this preparation revealed a number of polypeptide bands but only one which photoaffinity cross-linked to [^{3}H]SAM. The estimated native molecular weight (MW) of PEAMeT was found to be 77 kDa by gel filtration chromatography and an estimated MW of 54 kDa was

determined by SDS-PAGE. SDS-PAGE analysis of samples photoaffinity cross-linked to [^{3}H]SAM gave a slightly higher estimated MW of 57 kDa.

Effects of various factors on PEAMeT assay conditions were evaluated using partially purified PEAMeT preparations. PEAMeT activity as a function of pH gave a unimodal curve with an apparent pH optimum at 7.8 with 100 mM HEPES-KOH buffer. *In vitro* PEAMeT activity was inhibited by phosphate, PCho, *S*-adenosyl-L-homocysteine, Ca^{+2}, Mn^{+2} and Co^{+2} but not by choline, betaine, ethanolamine, mono- and dimethylethanolamine or Mg^{+2}.

Phosphobase *N*-methyltransferase activities present in preparations enriched for PEAMeT activity can catalyse the reaction sequence PEA → PMEA → PDEA → PCho. Under optimized assay conditions using PEA as the sole substrate, PMEA, PDEA and PCho were quantified and were detected in the order: PMEA (77%) > PDEA (17%) > PCho (6%). Thus a single enzyme, PEAMeT, is capable of converting PEA to PCho in leaves of spinach. The existence of a second enzyme which converts PMEA to PCho has also been reported for leaves and roots of spinach (Weretilnyk and Summers, 1992). The presence of two enzymes with overlapping activities raises questions regarding the roles of these two enzymes in choline metabolism. For example, do these enzymes also have overlapping functions in choline synthesis, particularly under conditions of osmotic stress?

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AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BADH	Betaine aldehyde dehydrogenase
BSA	Bovine serum albumin
CAM	Crassulacean acid metabolism
CAPS	3-[Cyclohexylamino]propanesulfonic acid
cDNA	Complementary DNA
CDP	Cytidine diphosphate
CHES	2-[*N*-Cyclohexylamino]ethanesulfonic acid
cpm	counts per minute
DEA	Dimethylethanolamine
DEAE	Diethylaminoethyl
DMS	Dimethylsulphide
DMSP	3-Dimethylsulfoniopropionate
DTT	Dithiothreitol
EA	Ethanolamine
EDTA	Ethylenediaminetetraacetic acid
Hepes	*N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]
HPLC	High performance liquid chromatography
MEA	Monomethylethanolamine
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NMR	Nuclear magnetic resonance
P5CS	Δ1-Pyrroline-5-carboxylate synthetase
PAR	Photosynthetically active radiation
PCho	Phosphocholine
PDEA	Phosphodimethylethanolamine
PDEAMeT	Phosphodimethylethanolamine *N*-methyltransferase
PEA	Phosphoethanolamine
PEAMeT	Phosphoethanolamine *N*-methyltransferase
PMEA	Phosphomethylethanolamine
PMEAMeT	Phosphomethylethanolamine *N*-methyltransferase
Ptd	Phosphatidyl- (prefix)
QAC	Quaternary ammonium compound
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase
SAH	*S*-Adenosyl-L-homocysteine
SAM	*S*-Adenosyl-L-methionine
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane

LITERATURE REVIEW

Environmental challenges due to lack of water, extreme temperatures, oxygen deficiency and lack of essential nutrients are capable of causing stress responses in all organisms. In particular, water stress can occur due to high or fluctuating salinity, desiccation or freezing (Yancey *et al.*, 1982). In plants, water stress results from a change in cellular water potential.

WATER RELATIONS

Water potential (Ψ_w) is a term used to represent the chemical potential of water (Hanson and Hitz, 1982). Ψ_w is defined as the sum of the component potentials $\Psi_s + \Psi_p + \Psi_m$, where Ψ_s is the solute or osmotic potential, Ψ_p is the turgor or pressure potential and Ψ_m is the matric potential (Hanson and Hitz, 1982; Rhodes, 1987). The values for Ψ_s are negative and those for Ψ_p are positive. Osmotic potential (Ψ_s) represents the solute activity (or solute concentration) either inside or outside of the cell. Turgor pressure (Ψ_p) is maintained by the Ψ_s and is important for controlling cell growth, maintaining structural integrity, maintaining gas exchange capacity in leaves and may also regulate many cellular metabolic functions (Hanson and Hitz, 1982). Matric potential (Ψ_m) is the overall reduction in water potential mediated by soil

particles or cell walls, where surface interactions reduce the tendency of water molecules to react chemically or evaporate (Kramer, 1988). Ψ_m does not make a significant contribution to the overall Ψ_w, and therefore Ψ_w can be accurately represented by $\Psi_s + \Psi_p$ (Kramer, 1988).

DROUGHT RESISTANCE IN PLANTS

Plant adaptation to water deficient environments can be accomplished by developmental, morphological, physiological or metabolic changes in the plant.

Non-Metabolic Mechanisms

Plants have evolved many unique methods for combating water stress or salinity. Reviewed by Jones *et al.* (1981); non-metabolic mechanisms of drought resistance falls under two main categories: drought escape or avoidance and drought tolerance at high tissue water potential (low Ψ_s). Plants capable of drought escape, for example desert ephemerals, are able to complete their life cycle before a serious plant water deficit develops and therefore do not rely entirely on physiological, biochemical or morphological mechanisms to cope with water deficits.

Plants capable of drought tolerance at high tissue water potential restrict water loss by a number of methods. One method involves increasing water uptake by increasing hydraulic conductance through the roots. These plants have more roots which grow deeper and to a greater density. Some plants can

reduce the amount of water lost via transpiration by changing their evaporative surface area during dry conditions by the shedding of large, thinner winter leaves, production of small thicker leaves, leaf rolling or by rapid senescence of older leaves (Jones *et al.*, 1981; Morgan, 1984). Other plants can change the quantity of radiation absorbed by leaves via leaf movements and changes in leaf reflective characteristics. Changes in radiation absorption by leaves can be achieved by production of hairs, cuticular surface waxes or salt secretion in order to increase leaf reflectance (Jones *et al.*, 1981; Hanson and Hitz, 1982). The reduction of water loss by stomatal control is another way some plants are capable of drought tolerance at high tissue water potentials. A large loss of water from the plant can occur across the stomata. Stomatal closure only occurs after a threshold Ψ_w is reached and this closure is probably turgor driven (Hanson and Hitz, 1982). In C_4 plants, water loss across the stomata is minimized by reducing the stomatal aperture (Hatch, 1987) and in Crassulacean acid metabolism (CAM) plants, by only opening stomata at night when evaporation of water is minimized (Ting, 1985). Some plants may also actively excrete Na^+ and/or Cl^- via salt glands under saline or drought conditions (Gorham and Wyn Jones, 1983; Hanson and Gage, 1991).

Metabolic Mechanisms: Solute Accumulation

Plants capable of drought tolerance at low tissue water potentials (high Ψ_s) respond metabolically by accumulating solutes. Under conditions of water

stress, low external water potentials (or high external solute concentrations) relative to internal cell water potentials develop. Therefore there is a tendency for solutes to enter cells and water to leave. In order to adapt to these conditions, plant cells of some species gradually accumulate compatible osmotic solutes (osmolytes). These compatible solutes function to adjust the osmotic strength of the cytoplasm with that of the environment to prevent water loss and create a Ψ_w gradient which favours water uptake (Wyn Jones and Gorham, 1983). These osmolytes must not be toxic to metabolic functions (especially to the functions of membranes and enzymes) and are excluded from the protein surface and its immediate hydration sphere (Rhodes, 1987; Le Rudulier *et al.*, 1984; Wyn Jones and Gorham, 1983). High cytoplasmic concentrations of salts can affect the catalytic rate and K_m of enzymes as well as affecting transmembrane potentials (Yancey *et al.*, 1982). Compatible solutes accumulate in the cytoplasm and chloroplasts and are not readily metabolized (Rhodes, 1987). It has been hypothesized that accumulated non-compatible solutes (Na^+, Cl^- and organic acids) from outside the cell plus those already existing inside the cell are sequestered into the vacuole (Binzel *et al.*, 1988). Because the vacuole makes up about 90% of the cell volume and the cytoplasm and chloroplasts 5% each, the bulk of the osmotic adjustment is performed by the large accumulation of Na^+ and Cl^- within the vacuole (Hanson, 1992). Therefore the osmolytes, which are energetically expensive to synthesize, are only used for osmotic adjustment within the cytoplasm and

chloroplast (Hanson, 1992).

The most common osmolytes existing in plants which have been suggested to act as compatible solutes consist of amino acids such as proline (Kemble and MacPherson, 1954; Singh *et al.*, 1972), polyols such as pinitol (Paul and Cockburn, 1989) and sorbitol (Brown and Hellebust, 1978), the tertiary sulphonium compound, 3-dimethylsulfoniopropionate (DMSP) (Paquet *et al.*, 1994) and quaternary ammonium compounds (QACs) such as glycine betaine, proline betaine, hydroxyproline betaine, *β*-alanine betaine (Wyn Jones and Storey, 1981; Rhodes and Hanson, 1993) and choline-*O*-sulphate (Hanson *et al.*, 1991).

Amino Acids: Proline

In bacteria, proline was shown to act as an osmoprotectant. Using *Salmonella oranienburg* it was shown that proline has the ability to act as an osmoprotectant (Christian, 1955ab) while proline overproducing mutants of *Salmonella typhimurium* and *Serratia marcescens* showed increased osmotic stress tolerance (Csonka, 1981). However, the role for the accumulation of proline in osmotically stressed plants has been debated in the past (Delauney and Verma, 1993). Proline accumulation could provide relief from osmotic stress or could be simply an osmotic stress-induced change in amino acid metabolism that has no role in the alleviation of the effects of osmotic stress. Indeed, few plant species show a positive correlation of proline accumulation

with osmotic stress tolerance (Delauney and Verma, 1993). In these plants, proline accumulation may provide an alternative protective function.

Increased proline levels in leaves in response to water deficits result from three metabolic changes. The first change is the increased synthesis of proline from glutamate due to the loss of feedback regulation of the first step(s) of the pathway catalyzed by Δ1-pyrroline-5-carboxylate synthetase (P5CS) (Boggess *et al.*, 1976ab) and the increased synthesis of P5CS mRNA (Hu *et al.*, 1992). The remaining two changes are the decreased proline oxidation rate in mitochondria and decreased incorporation of proline into protein (Hanson and Hitz, 1982).

Polyols: Pinitol

D-Pinitol (D-3-*O*-methyl-chiro-inositol) is another cytoplasmic osmolyte which accumulates in certain plants exposed to drought conditions (Nguyen and Lamont, 1988; Ford, 1984) or NaCl exposure (Gorham *et al.*, 1981; Gorham *et al.*, 1988). In *Mesembryanthemum crystallinum* L. plants exposed to 400 mM NaCl, CAM is induced and pinitol is accumulated (Keiller *et al.*, 1987; Paul and Cockburn, 1989). Pinitol accumulation was found to occur in the chloroplast and cytoplasm where pinitol was not metabolized further (Paul and Cockburn, 1989). CAM induction was not induced nor was pinitol accumulated in *Mesembryanthemum crystallinum* suspension culture cells exposed to 400 mM NaCl, indicating that communication between specific tissues is required

(Adams *et al.*, 1992).

Pinitol is synthesized from the conversion of glucose-6-phosphate to myo-inositol 1-phosphate by glucose-6-phosphate cycloaldolase and the methylation of myo-inositol 1-phosphate to pinitol by myo-inositol-*O*-methyltransferase (Dittrich and Korak, 1984; Vernon and Bohnert, 1992a). The activities of both enzymes are up-regulated during salt stress (Paul and Cockburn, 1989; Vernon and Bohnert, 1992b).

Tertiary Sulphonium Compounds: DMSP

A tertiary sulphonium compound contains a fully methylated sulphur atom giving it a positive charge (Wyn Jones and Storey, 1981). One type of TSC which exists in plants is 3-dimethylsulfoniopropionate (DMSP). DMSP exists as a zwitterion with a zero net charge at physiological pH.

DMSP was shown to act as an osmoprotectant in studies using bacteria. DMSP supplied exogenously to *Salmonella typhimurium* cells growing in 750 mM NaCl and *Escherichia coli* cells growing in 600 mM NaCl prevented inhibition of growth caused by the salt-induced osmotic stress (Paquet *et al.*, 1994). In plants, DMSP accumulates in some salt-marsh species of *Spartina* of the family Gramineae (Dacey *et al.*, 1987), sugarcane (Paquet *et al.*, 1994) and *Wedelia biflora* of the family Compositae (Storey *et al.*, 1993). In *Spartina alterniflora* growing under nitrogen limiting conditions, DMSP accumulates to a level which is greater than another nitrogen-bearing osmolyte, glycine betaine

(Dacey *et al.*, 1987).

DMSP is synthesized from methionine and methylated to *S*-methylmethionine (Hanson *et al.*, 1994b). *S*-methylmethionine is deaminated, decarboxylated and oxidized to DMSP in an undetermined series of reactions (Hanson *et al.*, 1994b). DMSP is also the biological precursor of atmospheric dimethylsulphide (DMS), a major component of gaseous sulphur emitted into the atmosphere (Charlson *et al.*, 1987). Therefore, plants which synthesize DMSP may contribute to atmospheric DMS emissions (Dacey *et al.*, 1987).

Quaternary Ammonium Compounds

A quaternary ammonium compound (QAC) contains a fully methylated nitrogen atom with a positive charge (Wyn Jones and Storey, 1981). Many QACs exist in plants including choline-*O*-sulphate and the betaines; glycine betaine, *β*-alanine betaine, proline betaine and hydroxyproline betaine (Rhodes and Hanson, 1993). All of the above QACs exist as zwitterions with a zero net charge at physiological pH.

Choline-O-Sulphate

Choline-*O*-sulphate was shown to act as an osmoprotectant using studies in bacteria. Choline-*O*-sulphate prevented the inhibition of growth caused by salt-induced osmotic stress when supplied exogenously to *Salmonella typhimurium* cells in liquid medium containing 750 mM NaCl and *Escherichia*

coli cells in liquid medium containing 600 mM NaCl (Hanson *et al.*, 1991). Choline-*O*-sulphate is synthesized in certain species of *Limonium* in the family Plumbaginaceae and has been shown to accumulate as the plant cell osmotic potential decreases (Hanson *et al.*, 1991). In addition to choline-*O*-sulphate accumulation, members of the Plumbaginaceae also have salt glands which secrete Na^+ and Cl^- but not SO_4^{-2} (Hanson and Gage, 1991).

In *Limonium*, choline-*O*-sulphate is synthesized from choline by choline sulfotransferase using 3′-phosphoadenosine-5′-phosphosulphate as the sulphate donor (Rivoal and Hanson, 1994). Choline sulfotransferase activity is present in both roots and leaves of *Limonium* and is induced four-fold in the leaves and six-fold in the roots by salt shock with 20% artificial sea water. Osmotic shock of *Limonium perezii* cell cultures with 19% polyethylene glycol 6000 results in a two- to four-fold increase in choline sulfotransferase activity (Rivoal and Hanson, 1994). Interestingly, choline-*O*-sulphate may also sequester SO_4^{2-} ions since SO_4^{2-} salinity led to increased choline-*O*-sulphate levels (Hanson and Gage, 1991).

Betaines

It has been shown that in the presence of high NaCl, exogenously supplied glycine betaine or proline betaine to *Klebsiella pneumoniae* (LeRudulier and Bouillard, 1983), glycine betaine, *β*-alanine betaine, proline betaine or hydroxyproline betaine to *Salmonella typhimurium* (Hanson *et al.*, 1994a) and

glycine betaine, β-alanine betaine, proline betaine or hydroxyproline betaine to *E. coli* (LeRudulier *et al.*, 1984; Hanson *et al.*, 1991; Hanson *et al.*, 1994a), prevents the inhibition of growth of these bacteria due to salt-induced osmotic stress. There is extensive evidence that various plant species accumulate betaines in tissues exposed to salinity and/or drought (see lists in Wyn Jones and Storey, 1981; Rhodes and Hanson, 1993). Glycine betaine or β-alanine betaine (for *Limonium*) accumulation within plant cells help these cells maintain their osmotic potential below that of the external environment. In the mesophytes spinach (Coughlan and Wyn Jones, 1980; Weigel *et al.*, 1986) and barley (Hitz *et al.*, 1982) and the halophytes *Atriplex spongiosa* (Hanson, 1992) and *Limonium* (Hanson *et al.*, 1991), glycine betaine or β-alanine betaine (for *Limonium*) accumulation correlated with the measured decrease in leaf or root osmotic potentials.

β-Alanine Betaine and Proline Betaine

The biosynthesis of β-alanine betaine and proline betaine have not been well studied. In the *Limonium* species (Rathinasabapathi and Hanson, 1994a), β-alanine betaine is synthesized by three *S*-adenosyl-L-methionine-dependant *N*-methylations of β-alanine and synthesis does not require choline or oxygen (Hanson *et al.*, 1991). The enzyme catalysing the first two *N*-methylations is distinct from the enzyme catalyzing the last *N*-methylation (Rathinasabapathi and Hanson, 1994a). To date, all of the species which accumulate β-alanine

betaine also accumulate choline-*O*-sulphate (Hanson *et al.*, 1994a).

Proline betaine is synthesized from three sequential methylations of proline, via the intermediate *N*-methylproline in alfalfa (Essery *et al.*, 1962). The large initial increase in the osmoprotectant proline due to osmotic stress in some species may be followed by its conversion to the more potent osmoprotectants proline betaine and hydroxyproline betaine (Hanson *et al.*, 1994a).

Glycine Betaine

Glycine betaine (betaine) has been found to accumulate as an osmoprotectant in response to water deficits in the higher plant families Chenopodiaceae and Amaranthaceae of the order Chenopodiales and some members of the Gramineae family of the order Poales (Wyn Jones and Storey, 1981) and among certain species in many other families, including, Convolvulaceae of the order Polemoniales, Solanaceae of the order Scrophulariales and Asteraceae of the order Asterales (Weretilnyk *et al.*, 1989; Ishitani *et al.*, 1993). Interestingly, other species in the same families synthesize betaine at roughly 1000-fold lower levels (Weretilnyk *et al.*, 1989). Betaine levels in these plants were determined by fast atom bombardment mass spectrometry. Betaine accumulates in the leaves and especially the roots of sugar beet (even though synthesis occurs primarily in the leaves) and is phloem mobile (Hanson and Wyse, 1982; Hanson and Rhodes, 1983). Inside a chenopod leaf cell salinized with up to 300 mM NaCl, betaine accumulates up

to 300 mM in the extravacuolar compartments while Na^+ and Cl^- accumulate to 200 mM and 150 mM, respectively, within the vacuole (Hanson, 1992).

In addition to functioning as an osmoprotectant, betaine is implicated in other roles. Betaine has been shown to stabilize enzyme structure and function by excluding perturbing solutes from the protein surface and immediate hydration sphere (Low, 1985). Specifically, in the halophytic cyanobacterium *Apthanothece halophytica* the presence of betaine in *in vitro* assays promoted ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) activity, relieved inhibition of Rubisco by KCl and protected Rubisco from heat and cold inactivation (Incharoensakdi *et al.*, 1986). Betaine can also protect enzymes from heat inactivation *in vitro* (Paleg *et al.*, 1981) and alter membrane properties to convey increased membrane stability due to heat and freezing conditions (Coughlan and Heber, 1982; Jolivet *et al.*, 1982; Rudolph *et al.*, 1986).

GLYCINE BETAINE (BETAINE) BIOSYNTHESIS

For all plants studied to date, betaine biosynthesis proceeds by the pathway choline → betaine aldehyde → betaine (Fig. 1). This pathway has been determined in the chenopods, spinach (Coughlan and Wyn Jones, 1982) and sugarbeet (Hanson and Rhodes, 1983) and members of the Gramineae, barley (Hitz *et al.*, 1981) and wheat (McDonnell and Wyn Jones, 1988). The two-step synthesis of betaine from choline takes place in the chloroplasts of spinach

FIGURE 1. *Choline and Betaine Biosynthetic Pathway*

The betaine biosynthetic pathway and a grid showing the various possible routes of choline (Cho) synthesis from ethanolamine (EA) is given. The phosphobase route is represented by the P (phosphate) prefix in front of the various *N*-methylated EA products, the CDP prefix represents cytidine diphosphate nucleotide and the Ptd prefix represents the phosphatidyl *N*-methylated EA products. The solid arrows represent the phosphobase route of choline synthesis observed in spinach, *Lemna paucicostata* and sugar beet (Summers and Weretilnyk, 1993; Mudd and Datko, 1986; Datko and Mudd, 1988ab; Hanson and Rhodes, 1983). The dashed arrows highlight all other possible reactions. For simplicity, all arrows are uni-directional but does not imply that the possibility of reverse reactions can be ruled out. The enzymes of the phosphobase route have been numbered:

1 - Ethanolamine kinase

2 - PEA *N*-methyltransferase (PEAMeT)

3 - PMEA *N*-methyltransferase (PMEAMeT)

4 - PDEA *N*-methyltransferase (PDEAMeT)

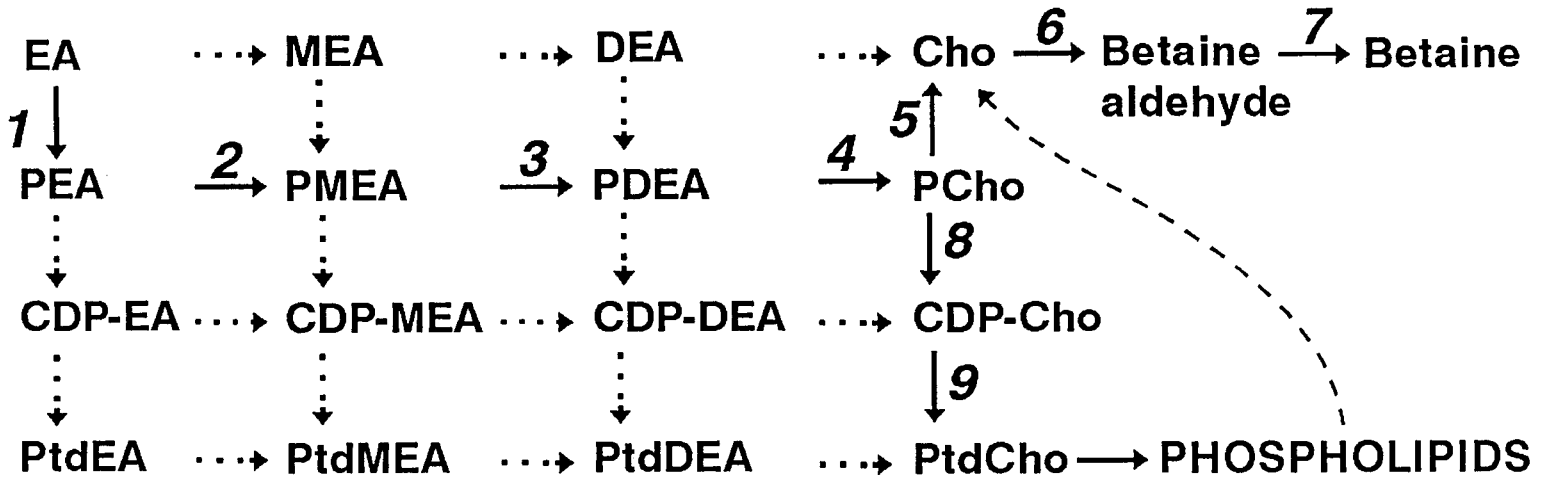
5 - PCho phosphatase

6 - Choline monooxygenase

7 - Betaine aldehyde dehydrogenase (BADH)

8 - PCho cytidylyltransferase

9 - CDP-choline:1,2-diacylglycerol PCho transferase


EA
MEA
DEA
Cho
6
Betaine aldehyde
7
Betaine
1
5
2
3
4
PEA
PMEA
PDEA
PCho
8
CDP-EA
CDP-MEA
CDP-DEA
CDP-Cho
9
PtdEA
PtdMEA
PtdDEA
PtdCho
PHOSPHOLIPIDS

and sugarbeet (Hanson *et al.*, 1985; Weigel *et al.*, 1986; Weigel *et al.*, 1988). The first reaction involves the oxidation of choline to betaine aldehyde catalyzed by a stromal, ferredoxin (hence light) dependant choline monooxygenase from spinach (Brouquisse *et al.*, 1989). Choline monooxygenase activity increases two- to three-fold in salinized spinach leaves (Brouquisse *et al.*, 1989) and has been purified and characterized in spinach (Burnet *et al.*, 1995). In *Limonium* species which accumulate both betaine and choline-*O*-sulphate, choline sulfotransferase (choline → choline-*O*-sulphate) may compete for available choline with choline monooxygenase (Rivoal and Hanson, 1994). However, in *Limonium* species which accumulate betaine, choline-*O*-sulphate and β-alanine betaine, the synthesis of β-alanine betaine could eliminate the metabolic competition for choline between glycine betaine and choline-*O*-sulphate.

Betaine aldehyde is then converted to betaine (with NAD or NADPH) via the stromal enzyme, betaine aldehyde dehydrogenase (BADH) (Weretilnyk and Hanson, 1988; Weretilnyk and Hanson, 1989; Arakawa *et al.*, 1990). Stromal BADH is encoded by a single nuclear gene with two alleles, designated slow (S) and fast (F), where the products of these two alleles can hybridize to form homodimers or heterodimers (Weretilnyk and Hanson, 1988). BADH has been purified to apparent homogeneity from spinach leaves (Arakawa *et al.*, 1987; Weretilnyk and Hanson, 1989) and more recently from *Amaranthus hypochondriacus* L. leaves (Valenzuela-Soto and Munoz-Clares, 1994). The

gene encoding BADH has been cloned from spinach (Weretilnyk and Hanson, 1990) and sugarbeet (McCue and Hanson, 1992). In spinach plants salinized to 200 mM NaCl, BADH activity, BADH protein levels (Weretilnyk and Hanson, 1989) and BADH mRNA levels (Weretilnyk and Hanson, 1990) all increased. BADH activity, BADH protein levels and BADH mRNA levels in leaves and roots of sugar beet plants salinized to 500 mM NaCl also increased (McCue and Hanson, 1992).

Biochemical evidence put forth to date has provided a means to use genetic evidence to ask questions regarding the role of glycine betaine in osmotic stress tolerance. Mutant *Zea mays* iso-populations were generated, which are glycine betaine-deficient due to a block in choline oxidation to betaine aldehyde (Yang *et al.*, 1995). Wild-type (*Bet1/Bet1*) betaine-accumulating *Zea mays* plants showed less shoot growth inhibition, had a higher leaf water content and greater turgor than the mutant (*bet1/bet1*) betaine-deficient plants (Saneoka *et al.*, 1995). These wild-type traits suggest that the product of the *Bet* locus allows for glycine betaine accumulation and hence osmotic adjustment so that the plant can adapt to drought or saline conditions.

CHOLINE BIOSYNTHESIS IN PLANTS

Choline must be synthesized in all plants in the presence or absence of water stress. Choline makes up part of the head group of a major phospholipid, phosphatidylcholine (PtdCho) (Moore, 1982). Choline is not an osmolyte (Le

Rudulier *et al.*, 1984), but is a precursor required for choline-*O*-sulphate and betaine synthesis.

Choline is synthesized from ethanolamine (EA). EA is synthesized by either the decarboxylation of serine, as in *Lemna paucicostata* (Mudd and Datko, 1989c), sugar beet (Hanson and Scott, 1980) and spinach (Coughlan and Wyn Jones, 1982)) or by a more complex route involving serine → phosphatidylserine → phosphatidylethanolamine (PtdEA) → EA, as reported for castor bean endosperm and pea seedlings (Moore, 1982).

The synthesis of choline from EA can, in theory, occur at four different levels: via sequential *N*-methylations of EA (free-base route), PEA (phosphobase route), CDP-EA (nucleotide route), PtdEA (phosphatidylbase route) or any combination thereof (Fig. 1). In plants, evidence for the operation of the free-base, phosphobase and phosphatidylbase routes has been found. Given the metabolic grid of Figure 1, the choline biosynthetic pathway is potentially very complicated. The actual pathway of choline biosynthesis along the grid that is actually used by specific plants appears to vary considerably between species. Elucidation of these biochemical routes has been experimentally determined by two methods. One method involves *in vivo* radiolabelling experiments, where tracer quantities of radioactivity originating from a radiolabelled precursor are measured among the putative metabolites of the precursor as a function of time. The other method, which provides corroborative evidence, is to determine if the enzyme activity responsible for

a hypothesized reaction can be detected in the tissue or extracts prepared from the tissue.

Support for the existence of a free-base route of choline biosynthesis comes primarily from studies using endosperm of castor bean, a betaine non-accumulating plant (Prud'homme and Moore, 1992ab). *In vivo* radiolabelling studies using either radiolabelled *S*-adenosyl-L-methionine (SAM), EA, PEA or phosphoserine showed that the freebase, phosphobase and phosphatidylbase routes of choline synthesis were operating (Prud'homme and Moore, 1992a). Further evidence of a freebase route was shown by the detection and characterization of an enzyme which catalyses the first reaction of the free-base route, EA → MEA (Prud'homme and Moore, 1992b). The existence of enzyme activities operating along the phosphobase and phosphatidylbase routes cannot be ruled out since enzyme activities along these two routes were not tested. An earlier study using radiolabelling experiments in detached spinach leaves which also gave evidence of a freebase route of choline synthesis (Coughlan and Wyn Jones, 1982) was shown to be erroneous by Summers and Weretilnyk (1993).

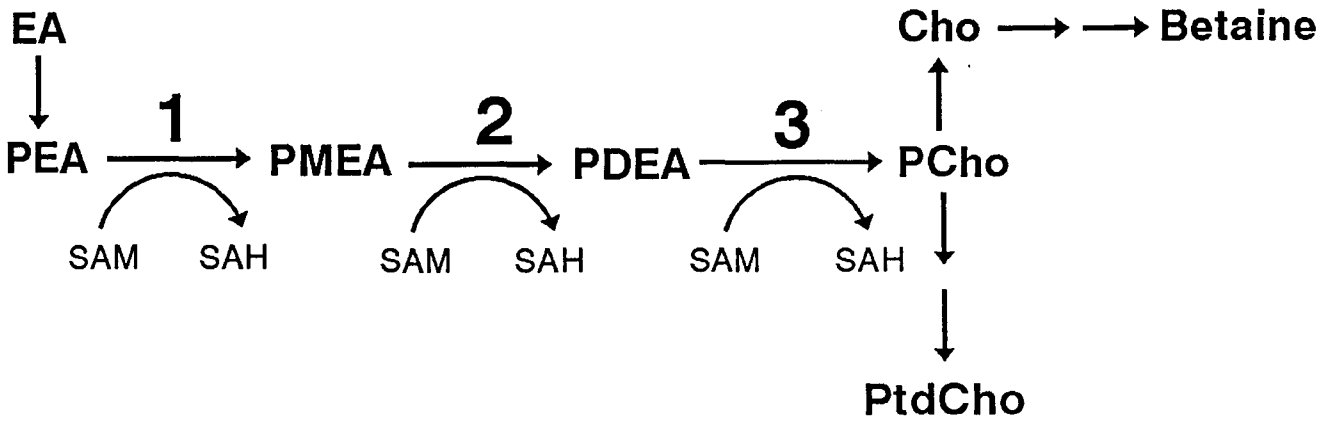
Evidence for the phosphobase route is more extensive, having been determined in salinized sugar beet leaf discs using radiotracer analysis (Hanson and Rhodes, 1983), spinach leaf discs using radiotracer and enzyme activity analysis (Summers and Weretilnyk, 1993), and in the betaine non-accumulator *Lemna paucicostata* using radiotracer analysis (Mudd and Datko, 1986) and

enzyme activity analysis (Datko and Mudd, 1988b). In the phosphobase route, phosphoethanolamine (PEA) is synthesized from EA by EA kinase. As shown in Figure 2, PEA is sequentially *N*-methylated three times using SAM as a methyl donating substrate to form phosphocholine (PCho). PCho is converted to choline by a PCho phosphatase (Hanson and Rhodes, 1983) or PCho is metabolized to PtdCho for lipids via PCho cytidylyltransferase and CDP-choline:1,2-diacylglycerol PCho transferase (Moore, 1982; Mudd and Datko, 1986; Datko and Mudd, 1988a). This pathway is not the only way to supply choline for PtdCho or betaine synthesis. In *Raphanus sativus* seedlings, choline for PtdCho is supplied by degradation of the seed constituent sinapine (Strack, 1981). In water stressed barley (Hitz *et al.*, 1981) and wheat (McDonnell and Wyn Jones, 1988) PtdCho turnover from membranes, presumably by an enzyme with phospholipase D-type activity, is able to release choline for betaine synthesis.

The route of choline synthesis in other species show some variation. In tissue cultures of carrot (a betaine non-accumulator), PtdCho was found to be synthesized simultaneously along both the phosphobase and phosphatidylbase routes, while in tissue cultures of soybean (also a betaine non-accumulator), PtdCho was found to be synthesized primarily along the phosphatidylbase route (Datko and Mudd, 1988ab). Also, from studies using betaine-accumulators, such as water stressed barley (Hitz *et al.*, 1981) and wheat (McDonnell and Wyn Jones, 1988), it was shown that PtdCho could be synthesized from either

FIGURE 2. *Phosphobase N-methyltransferases of the Choline and Betaine Biosynthetic Pathway*

The phosphobase route of choline synthesis is shown indicating the reactions catalyzed by the three phosphobase *N*-methyltransferases.



ENZYMES

1. *S*-adenosyl-L-methionine: phosphoethanolamine *N*-methyltransferase (PEAMeT)
2. *S*-adenosyl-L-methionine: phosphomethylethanolamine *N*-methyltransferase (PMEAMeT)
3. *S*-adenosyl-L-methionine: phosphodimethylethanolamine *N*-methyltransferase (PDEAMeT)

SUBSTRATES

EA = ethanolamine
PEA = phosphoethanolamine
PMEA = phosphomonomethylethanolamine
PDEA = phosphodimethylethanolamine
Cho = choline
PCho = phosphocholine
PtdCho = phosphatidylcholine
SAM = *S*-adenosyl-L-methionine
SAH = *S*-adenosyl-L-homocysteine

the phosphobase or phosphatidylbase routes. In soybean and barley, the first *N*-methylation occurs by the phosphobase route (PEA → PMEA) and subsequent *N*-methylations occur by the phosphatidylbase route for soybean and both the phosphobase and phosphatidylbase routes for barley. It is not known whether these differences in routes of choline synthesis are due to various environmental conditions under which the plants have been grown or reflect species-, developmental-, or tissue-specific differences in metabolism (Weretilnyk and Summers, 1992).

As discussed in the paragraph above, for the plants studied to date with the exception of castor bean, the first *N*-methylation occurs at the phosphobase level (Hitz *et al.*, 1981; Hanson and Rhodes, 1983; Mudd and Datko, 1986; Datko and Mudd, 1988ab; McDonnell and Wyn Jones, 1988; Summers and Weretilnyk, 1993) and this is shown in Figure 2. This reaction is catalysed by the enzyme, *S*-adenosyl-L-methionine:PEA *N*-methyltransferase (PEAMeT) (Datko and Mudd, 1988ab; Weretilnyk and Summers, 1992). PEAMeT is localized in the cytoplasm of spinach leaves (Weretilnyk *et al.*, in press) and utilizes *S*-adenosyl-L-methionine (SAM) as a methyl donating substrate for the *N*-methylation of PEA (Mudd and Datko, 1989ab). All *N*-methyltransferases require *S*-adenosyl-L-methionine (SAM) as a methyl-donating substrate (Poulton, 1981). *S*-adenosyl-L-methionine is synthesized from methionine and ATP by ATP: *L*-methionine *S*-adenosyltransferase (or SAM synthetase) (Cossins, 1980).

Using salinized sugar beet leaf discs, *in vivo* PCho synthesis was found to

be inhibited by PCho (Hanson and Rhodes, 1983). Experiments with carrot and soybean cell cultures (Mudd and Datko, 1989b) and *Lemna paucicostata* (Mudd and Datko, 1989a) showed that choline synthesis is inhibited when exogenous choline is added to the growth medium. In spinach, the activities of EA kinase and the three *N*-methyltransferases all increase in response to salinity, with the largest increase (approximately three-fold) found for PEAMeT activity (Weretilnyk and Summers, 1992; Summers and Weretilnyk, 1993). PEAMeT activity is up-regulated in spinach plants exposed to light and/or salinity and down-regulated in spinach plants exposed to darkness (Weretilnyk *et al.*, in press).

The remaining two *N*-methylation reactions are performed by enzyme(s) designated PMEAMeT and PDEAMeT, respectively (Weretilnyk and Summers, 1992). To date, these two *N*-methyltransferase enzymes have not been purified nor have their genes been cloned from any organism. In spinach, both PMEAMeT and PDEAMeT activities have similar leaf to root ratios, whereas PEAMeT activity is only detected in the leaves (Weretilnyk and Summers, 1992). Also, in spinach exposed to 200 mM NaCl, the fold-induction of PMEAMeT and PDEAMeT activities is similar but lower than the induction of PEAMeT activity (Weretilnyk and Summers, 1992). Therefore, based on this biochemical evidence, PMEAMeT and PDEAMeT in spinach could be a single *N*-methyltransferase which is distinct from PEAMeT.

CHOLINE SYNTHESIS IN OTHER ORGANISMS

Bacteria

Bacteria do not synthesize PtdCho or Cho, but can take up betaine or choline from the environment (Csonka and Hanson, 1991). In *Escherichia coli* and *Salmonella typhimurium*, ProU, a betaine binding/transport protein, transports betaine from the environment to the cytoplasm (Cairney *et al.*, 1985; May *et al.*, 1986). *Escherichia coli* can synthesize betaine from exogenous choline in the two step pathway (choline → betaine aldehyde → betaine) mediated by the BetA and BetB gene products, respectively (Landfald and Strom, 1986). Choline uptake is mediated by two transport systems, one encoded by the *betT* gene and other encoded by an unknown structural gene (Csonka, 1989).

Neurospora and Yeast

In *Neurospora crassa* (Scarborough and Nyc, 1967) and *Saccharomyces cerevisiae* (Yamashita *et al.*, 1982), PtdCho is synthesized along the phosphatidylbase route (see Fig. 1). Two enzymes are involved in synthesizing PtdCho from PtdEA. One enzyme catalyses the first *N*-methylation of PtdEA to PtdMEA while the second catalyses the remaining two *N*-methylations (PtdMEA → PtdDEA → PtdCho) (Yamashita *et al.*, 1982; Gaynor and Carman, 1990). In yeast, the genes for both of these enzymes have been cloned (Summers *et al.*, 1988; McGraw and Henry, 1989).

Animals

In *Drosophila melanogaster*, PtdCho is synthesized from PtdEA with a strong possibility that only one enzyme is responsible for all three *N*-methylations (De Sousa *et al.*, 1988). In mammalian tissues, including rat liver, rat heart, rat erythrocytes, mouse thymus, rat pituitary and dog lung, PtdCho is synthesized along the phosphatidylbase route (Vance and Ridgway, 1988). In a human neuronal cell line, choline can then be synthesized by the hydrolysis of PtdCho by a phospholipase D-type enzyme activity (Lee *et al.*, 1993). To date it is not known whether all choline-synthesizing animal tissues generate choline by this route or if choline could also be synthesized by the series of reactions: PtdCho → CDP-choline → PCho → Cho. In rat liver, a single enzyme catalyzing all three *N*-methylations of PtdEA has been purified and found to be an 18.3 kDa membrane-bound enzyme (Ridgway and Vance, 1987). Activity of this enzyme, SAM: PtdEA *N*-methyltransferase, is stimulated by the presence of PtdEA, inhibited by *S*-adenosyl-L-homocysteine (Ridgway *et al.*, 1989) and may be activated by phosphorylation (Vance and Ridgway, 1988). In cultured rat liver cells, choline deprivation was shown to cause an increase in PtdEA methylation (Datko *et al.*, 1990).

In animals, the most important source of choline is through the diet (Wecker and Trammer, 1984) and the other known source is by catabolism of PtdCho by phospholipase-D. In cultured chick neurons and rat brain, evidence has been found for a phosphobase route of choline synthesis (Andriamampandry *et al.*,

1989). Enzymes converting PEA → PMEA → PDEA → PCho were found in rat brain cytoplasm (Andriamampandry *et al.*, 1990) but not in rat liver cytoplasm (Datko *et al.*, 1990; Andriamampandry *et al.*, 1992). Also, in rat brain or chick brain, PtdEA *N*-methyltransferase activity was not present (Andriamampandry *et al.*, 1989; Andriamampandry *et al.*, 1991). Recently, the partial purification and characterization of PDEAMeT (PDEA → PCho) from rat brain cytosol has shown that at least two enzymes catalyse the three phosphobase *N*-methylations in this tissue (Andriamampandry *et al.*, 1992). PDEAMeT was found to be induced by 500 μM $CaCl_2$ and inhibited 100% by 500 μM *S*-adenosyl-L-homocysteine (Andriamampandry *et al.*, 1992).

GENETIC ENGINEERING OF SALT TOLERANCE

Drought conditions and saline soils are significant agricultural problems around the world. In the United States, approximately 45% of the arable soil is exposed to low water availability (Boyer, 1982). Conventional plant breeding methods have increased plant agricultural yields in these regions but overall progress is slow (Boyer, 1982), indicating that genetic engineering of salt-tolerance may be a viable alternative. Most morphological, structural and developmental mechanisms of drought resistance are hard to manipulate because of lack of knowledge of how these traits are defined genetically (McCue and Hanson, 1990). Biochemical or metabolic traits which confer drought resistance are much more amenable to genetic engineering since many

of the gene products have been identified and some of the genes have been cloned (McCue and Hanson, 1990).

Very few attempts at genetic engineering of drought resistance have been done. The two major attempts involve the introduction of the capacity to accumulate compatible solutes in plants which previously did not have the capacity to do so. Tobacco plants were used for both studies since they lack any significant salt tolerance mechanisms and are easily transformable. The first attempt at genetic engineering involved the introduction of mannitol biosynthesis to tobacco. The bacterial mannitol-1-phosphate gene (*mtlD*) was introduced into tobacco protoplasts via *Agrobacterium tumefaciens*-mediated transformation resulting in *mtlD* gene expression, functional enzyme synthesis and finally the accumulation of mannitol in second generation tobacco plant transformants (Tarczynski *et al.*, 1992). By comparing morphological characteristics of control tobacco plants without the *mtlD* gene to the mannitol-producing transformants, it was shown that the transformants had a greater ability to tolerate salinity (Tarczynski *et al.*, 1993). It remains to be seen if mannitol production can be introduced into other agricultural plants and if mannitol accumulation will allow prolonged salt tolerance under field conditions.

The second attempt at genetic engineering of salt tolerance involved the introduction of betaine synthesis in tobacco. All dicot plants tested to date have the ability to synthesize betaine, but do not necessarily have the ability to generate large quantities of betaine (Weretilnyk *et al.*, 1989). In order to

genetically engineer betaine accumulation into betaine non-accumulating species, betaine synthesis must function in a similar way as it does in betaine-accumulators, in that betaine accumulation must be stress inducible and the two enzymes must be localized in the chloroplast (McCue and Hanson, 1990). BADH cDNAs from spinach and sugar beet were transformed into tobacco plants, resulting in BADH enzyme production in chloroplasts (Rathinasabapathi *et al.*, 1994b). Transformation of BADH into these plants did not confer salt tolerance because choline monooxygenase in addition to BADH is necessary for betaine synthesis from choline. However, the plants were able to convert exogenously supplied betaine aldehyde into betaine. The genetic engineering of betaine accumulation into non-accumulators must therefore involve the introduction of both betaine biosynthetic enzymes, choline monooxygenase and BADH. In addition, adequate levels of choline must be present for betaine synthesis. Therefore the capacity to up-regulate choline biosynthesis may also have to be introduced into these betaine-accumulating transformants. Alternatively, since all dicot plants may synthesize betaine, up-regulation of the prevailing choline or betaine biosynthetic pathways could be all that is needed to generate higher levels of betaine.

MATERIALS AND METHODS

CHEMICALS AND RADIOISOTOPES

All chemicals and enzymes were purchased from Sigma unless otherwise stated. *S*-[methyl-^{3}H]adenosyl-L-methionine ([^{3}H]SAM) was purchased from New England Nuclear. The [^{3}H]SAM had a specific activity ranging from 64.6 to 85.0 Ci$\cdot$mmol^{-1} (0.55 mCi$\cdot$mL^{-1}) and a SAM concentration ranging from 0.0065 to 0.0085 μmol$\cdot$mL^{-1}. Upon receipt, [^{3}H]SAM was dispensed into 10-μL aliquots and then stored at -20°C.

SAM (Boehringer Mannheim Canada) was dissolved in 0.01 N H_2SO_4:ethanol (9:1, v/v). The concentration of SAM was determined using the molar extinction coefficient of 15 M$^{-1}\cdot$cm^{-1} at 257 nm (Eloranta *et al.*, 1976), made to 12 mM, dispensed into 25-μL aliquots then stored at -20°C.

PEA substrate was generated by dissolving PEA in 0.1 N HCl to a final concentration of 7.5 mM and then stored at -20°C. PMEA and PDEA substrates were prepared by Dr. Peter Summers or Sharon Dhadialla by phospholipase C (Type XI from *Bacillus cereus*) treatment of PtdMEA or PtdDEA as outlined by Datko and Mudd (1988b). Newly synthesized PMEA and PDEA concentrations ranged between 30 and 75 mM and each was diluted to 7.5 mM with 0.1 N HCl and stored at -20°C.

Dowex 50W(H^+) 50X8-200 resin (Sigma) was regenerated prior to use. The resin was first hydrated with two volumes of distilled water. In order to protonate the resin, 250 g of the resin was mixed with two volumes of 1 N HCl, stirred, allowed to settle and the supernatant was decanted. This procedure was repeated twenty times. To remove the excess acid, the resin was suspended in two volumes of distilled water, stirred, allowed to settle and the water was poured off. This washing procedure was repeated twenty times or until the pH of the water which was decanted was between 5 and 7.

PLANT GROWTH CONDITIONS

Spinach (*Spinacea oleracea* L.) seeds cv Savoy Hybrid 612 (Harris Moran Seeds, Rochester, New York) were planted about 1 cm deep in moist, coarse vermiculite with no additional water for 3 days. Trays with seeds were then watered for 4 days and one-week old seedlings were transplanted to individual 350-mL plastic pots filled with vermiculite.

Spinach plants were grown in growth chambers (Conviron E15) under the following conditions: 8 h, 24°C day, 325 μmol m^{-2} s^{-1} PAR/ 16 h, 19°C night. Spinach plants were watered daily with half-strength Hoagland's solution (Hoagland and Arnon, 1939). All spinach plants used for experiments were between 4 to 8 weeks old from seeding. Prior to enzyme extraction, the spinach plants were exposed to a continuous 24 h period of light at a temperature of 24°C.

ENZYME ASSAYS

Phosphobase *N*-Methyltransferase Assays

The assay of the phosphobase-*N*-methyltransferases (PEAMeT, PMEAMeT or PDEAMeT) was modified from that described by Datko and Mudd (1988). Each of the above enzymes were assayed with their appropriate phosphobase substrate PEA, PMEA or PDEA, respectively. The standard phosphobase *N*-methyltransferase assay contained 100 mM Hepes-KOH (pH 7.8, 22°C), 1 mM Na_2-EDTA, 250 μM phosphobase (PEA, PMEA or PDEA), 200 μM SAM, 40 nM [^{3}H]SAM (0.55 μCi), distilled water and sample (25 μL) to a final volume of 150 μL. For controls, 25 μL of enzyme extract was used but water replaced the phosphobase substrate. The assay mixtures were then incubated at 30°C for 30 min. The reaction was stopped by the addition of 1 mL cold water, the diluted mixture was vortexed and then placed on ice. A 1-mL aliquot of this mixture was then added to an Evergreen disposable column (Diamed, catalogue # 208-3384-060) containing a 1-mL bed volume of regenerated Dowex 50W X8-200 (H^+) resin. The column contents were washed two times with 0.5 mL of water and these washes were pooled and discarded. The unlabelled and radiolabelled phosphobases were eluted from the column with 10 mL of 0.1 N HCl and 1 mL of this eluate was then mixed with 10 mL of Formula 989 liquid scintillation fluor (DuPont) or 5 mL Ready Safe fluor (Beckman) in a scintillation vial, shaken and the radioactivity quantified using a Beckman LS 1801 scintillation counter. The estimated counting efficiency using Formula 989 was

40% and using Ready Safe was 41.6%.

All PEAMeT assays to which additions were made included all of the standard assay components except the distilled water, or a portion thereof, which was replaced by a solution containing the additive to be tested. Also, if samples had to be diluted for assay, this dilution was done before the assay using 100 mM Hepes-KOH (pH 7.8, 22°C). Frequently samples had to be diluted to yield a protein concentration per assay of 2 μg or less.

For examining the effect of pH on PEAMeT activity, various buffering agents were used to substitute for the 100 mM Hepes-KOH (pH 7.8, 22°C) in the PEAMeT assay. All of the buffers were used at a final assay concentration of 100 mM and all were adjusted to the desired pH at 22°C. These buffers included: Bis-Tris-HCl (pH 6, 6.5 and 7), Bis-Tris-Propane-HCl (pH 6.5, 7, 7.5, 8, 8.5, 9 and 9.5), CAPS-KOH (pH 10 and 11), Tris-HCl (pH 7, 7.5, 8, 8.5 and 9), Hepes-KOH (pH 7, 7.5 and 8) and CHES-KOH (pH 9, 9.5 and 10).

Phosphobase Phosphatase Assay

A 50-μL volume of enzyme sample, diluted with 100 mM Hepes-KOH (pH 7.8, 22°C) to the same concentration of protein that was used for the PEAMeT assay, was incubated with 350 μL buffer/substrate mixture for 30 min at 30°C. The assay contained 5 mM PEA, 100 mM Hepes-KOH (pH 7.8, 22°C) and 1 mM Na_2-EDTA. The reaction was stopped with 100 μL 7.5% (w/v) trichloroacetic acid (TCA) after 30 min. For control incubations, 100 μL 7.5%

(w/v) TCA was added prior to the incubation. The phosphobase phosphatase reaction mixtures stopped with 7.5% (w/v) TCA were centrifuged in an Eppendorf 5415C microcentrifuge for two min at 14,000 rpm, 22°C. The supernatant was removed and diluted with distilled water to a final volume of 500 μL.

Determination of inorganic phosphate (Pi) cleaved from PEA was taken from a procedure used by Martin and Tolbert, (1983). Pi standards were prepared from dilutions of a 1 mM KH_2PO_4 stock solution to 0.5, 2.5, 5, 10, 15, 20 and 25 nmol Pi in a 500 μL volume. Each of these Pi standards and each of the diluted supernatants of the phosphobase phosphatase reactions were incubated with 500 μL ammonium molybdate solution (4 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 114 mM L-ascorbic acid) in microfuge tubes for 90 min at 37°C. After a 90 min incubation period, the absorbance of each reaction mixture was measured at 820 nm using a UVIKON 930 spectrophotometer. A Pi standard curve (absorbance at 820 nm versus nmoles Pi) was generated using the Pi standards. The amount of Pi (nmoles) present in each of the phosphobase phosphatase reaction mixture was determined from this standard curve. Phosphobase phosphatase activity was expressed in nmol Pi released $\cdot mL^{-1}min^{-1}$.

Protein and Chlorophyll Determination

Protein concentration of the enzyme samples was determined

colorimetrically by the method of Bradford (1976) using BSA as a standard. Protein was detected following the Bio-Rad Micro Assay protocol using the Bio-Rad protein assay dye reagent concentrate.

To determine the concentration of chlorophyll, 25 μL of the crude extract was mixed with 3 mL 80% (v/v) acetone in 13 X 100 mm test tubes covered with foil to exclude light. The mixture was vortexed, centrifuged at step 7 in an IEC clinical centrifuge for 3-4 minutes and then the absorbance of the supernatant was measured at 700 nm, 663 nm and 645 nm using a UVIKON 930 spectrophotometer. The concentration of chlorophyll was then determined using the absorption coefficients of Arnon (1949). The formula used was: $20.2(A_{645}-A_{700}) + 8.02(A_{663}-A_{700})$ giving the chlorophyll concentration in $\mu g \cdot mL^{-1}$.

ENZYME EXTRACTION AND PROTEIN PURIFICATION

Enzyme Extraction

All procedures were carried out on ice or at 4°C. Spinach leaves (1 to 3 kg batches) were deveined, coarsely chopped with a razor blade, weighed and homogenized in a Waring blender with two volumes of extraction buffer containing 100 mM Tris-HCl (pH 7.8, 4°C), 2 mM Na_2-EDTA and 5 mM dithiothreitol (DTT, ICN Biochemical). The brei was filtered through four layers of cheesecloth plus one layer of Miracloth (Calbiochem). A 25-μL aliquot of the filtrate was used for chlorophyll determination and the remainder was

centrifuged for 10 min at 10,000*g*, 4°C using a Sorvall GSA rotor. One-mL aliquots of the crude supernatant and pellet (resuspended in extraction buffer) were reserved for determination of PEAMeT activity and protein concentration. These aliquots had to be desalted prior to determination of phosphobase *N*-methyltransferase activity and protein concentration. Desalting was performed by centrifugation through 1.2-mL columns of Sephadex G-25-medium (Pharmacia) equilibrated with 100 mM Hepes-KOH (pH 7.8, 4°C), 1 mM Na_2-EDTA and 5 mM DTT (Weigel *et al.*, 1986). The resuspended pellet had no detectable PEAMeT activity and was discarded.

The crude supernatant was raised to 1.8 M $(NH_4)_2SO_4$ by additions of solid $(NH_4)_2SO_4$ using the tables of Wood (1976), stirred for 30 min then centrifuged for 10 minutes at 10,000*g* using a Sorvall GSA rotor. The pellet was resuspended with extraction buffer and a 1-mL aliquot was removed, desalted and used for determination of PEAMeT activity and protein concentration. This resuspended pellet had no PEAMeT activity and was discarded. The supernatant was raised from 1.8 to 2.6 M $(NH_4)_2SO_4$, stirred for 30 min and then centrifuged for 10 minutes at 10,000*g* using a Sorvall GSA rotor. A 1-mL aliquot of the supernatant was desalted and used for determination of PEAMeT activity and protein concentration. This supernatant had no PEAMeT activity and was discarded. The pellet of the 1.8 to 2.6 M $(NH_4)_2SO_4$ fraction was resuspended in a minimal volume (100 to 200 mL) of dialysis buffer containing 20 mM Tris-HCl (pH 7.8, 4°C), 1 mM Na_2-EDTA and 5 mM DTT. A 1-mL

aliquot was removed prior to dialysis, desalted and used for determination of PEAMeT activity and protein concentration. The remainder of this fraction was transferred to dialysis tubing with a 12,000 to 14,000 molecular weight cut-off and dialysed against 2 L of dialysis buffer. Dialysis was carried out overnight and the following day and involved four, 2 L changes of buffer. A 1-mL aliquot of the 1.8 to 2.6 M $(NH_4)_2SO_4$ fraction (designated the "1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed sample") was removed after dialysis for determination of PEAMeT activity and protein concentration and the remainder of the fraction was flash frozen in liquid N_2 and stored at -80°C in 50 mL (approximate volume) aliquots. The initial extraction steps outlined above were carried out either by Dr. P.S. Summers or myself.

Column Chromatography Procedures

For protein purification, Gilson Model 740 ProTech system controller software was used with the two Gilson Minipuls 3 pumps, Gilson 112 UV/VIS detector, Gilson 506B system interface and Gilson FC 204 fraction collector. This system was used for all low pressure liquid chromatographic procedures. A Waters 625 LC System, Waters 486 tunable absorbance detector, Waters Fraction Collector and Baseline 810 software were used for all high performance liquid chromatography (HPLC) steps.

Following many of the column chromatography steps, the enzyme samples required concentration and dialysis by ultrafiltration. Substantial losses of

PEAMeT activity were initially experienced during these procedures. It was discovered later that these losses could be alleviated by passivation of the ultrafiltration equipment and plasticware prior to use. The passivation procedure prevents the binding of protein from the sample to the surface of the plastic components of the ultrafiltration equipment and was completed following Publication 334, "Passivation of Centricon Concentrators for Improved Recovery" by Amicon. The Amicon 8050 stirred cell containing a YM-30 membrane, Centricon-10 concentrators and retentate cups or Centrex UF-0.5 microconcentrators were filled with 6% (w/v) polyethylene glycol compound MW 15,000-20,000 and soaked overnight at 22°C. The 6% polyethylene glycol solution was then rinsed thoroughly from the equipment using distilled water. The ultrafiltration devices were assembled and then filled with buffer A, consisting of 20 mM Tris-HCl (pH 7.8, 4°C), 1 mM Na_2-EDTA, 10% (v/v) glycerol, 5 mM DTT. Half the quantity of buffer A was passed through the exclusion membrane and the excess buffer was discarded. This was repeated before the sample containing the enzyme was added or the equipment was stored containing buffer A overnight at 4°C until it was used.

Since the various purification steps entailed the packing and equilibration of columns, samples usually had to be stored for a period of time between the various steps. Unless otherwise specified, column fractions containing PEAMeT activity were pooled, frequently concentrated and dialysed, then flash frozen in liquid N_2 and stored at -80°C between steps.

DEAE Sepharose (Anion Exchange Chromatography)

DEAE Sepharose CL-4B (Sigma) anion exchange matrix was poured into a column (5 cm X 20 cm, 325-mL bed volume) and equilibrated with 3 L of buffer A. The flow rate was maintained at 1 $mL \cdot min^{-1}$. The 1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed sample was thawed and then loaded onto the equilibrated DEAE Sepharose matrix. To remove non-adsorbed proteins, the column was washed with 3 L of buffer A. Protein adsorbed to the matrix was then eluted with a 1-L linear gradient of either 0 to 300 or 0 to 500 mM NaCl in buffer A and the eluate was collected in 5 mL fractions. Proteins which were washed or eluted from the column were monitored continuously at an absorbance of 280 nm and PEAMeT activity was assayed from all column washes and selected gradient fractions. Fractions containing PEAMeT activity were pooled and an aliquot was removed from the pooled sample and used for the determination of PEAMeT activity and protein concentration. The remainder of this pooled sample was frozen and stored.

Phenyl Sepharose (Hydrophobic Interaction Chromatography)

Phenyl Sepharose CL-4B (Sigma) hydrophobic interaction matrix was poured into a column (2.5 cm X 20 cm, 100 mL bed volume) and equilibrated with 1 L of buffer A containing 25% (w/v) $(NH_4)_2SO_4$. The flow rate was maintained at 1 $mL \cdot min^{-1}$. The pooled sample from the separation on DEAE Sepharose was thawed, the concentration of $(NH_4)_2SO_4$ in the sample was made 25% (w/v)

(Wood, 1976) and the sample was then loaded onto the equilibrated phenyl Sepharose matrix. Non-adsorbed protein was washed from the column with 1 L of buffer A containing 25% (w/v) $(NH_4)_2SO_4$ followed by a second wash with 200 mL buffer A containing 15% (w/v) $(NH_4)_2SO_4$. The protein which remained adsorbed to the matrix was then eluted with a 300 mL linear gradient of 15% to 5% (w/v) $(NH_4)_2SO_4$ in buffer A, followed by a wash with 200 mL of buffer A containing 5% (w/v) $(NH_4)_2SO_4$. Finally, remaining adsorbed protein was eluted from the matrix with a 1-L linear gradient of buffer A with 0% (v/v) ethylene glycol/5% (w/v) $(NH_4)_2SO_4$ to 50% (v/v) ethylene glycol/0% (w/v) $(NH_4)_2SO_4$ and the eluate was collected in 5-mL fractions. The proteins which were washed or eluted from the column were monitored continuously at an absorbance of 280 nm and PEAMeT activity was assayed from all column washes and selected gradient fractions. Fractions containing PEAMeT activity were pooled, concentrated to less than 30 mL, dialysed 1000-fold with buffer A and a 250-μL aliquot was removed for determination of PEAMeT activity and protein concentration. The remainder of the concentrated and dialysed sample was frozen and stored.

ω-Aminohexyl Agarose (Ionic Exchange Chromatography)

ω-Aminohexyl Agarose (Sigma) chromatographic matrix was used as an anionic exchange medium. This matrix was poured into a column (1.5 cm X 20 cm, 28-mL bed volume) and equilibrated with 500 mL of buffer A. The flow

rate was maintained at 1 mL·min^{-1}. The concentrated and dialysed sample prepared using phenyl Sepharose was thawed and then loaded onto the equilibrated ω-aminohexyl agarose matrix. The non-adsorbed protein was washed from the matrix with 600 mL of buffer A. Adsorbed protein was eluted from the matrix with a 500-mL linear gradient of 0 to 300 mM NaCl in buffer A and the eluate collected in 5-mL fractions. The proteins which were washed or eluted from the column were monitored continuously at an absorbance of 280 nm and PEAMeT activity was assayed from all column washes and selected gradient fractions. Fractions containing PEAMeT activity were pooled, concentrated to less than 20 mL, dialysed 1000-fold with buffer A and a 250 μL aliquot was removed for determination of PEAMeT activity and protein concentration. The remainder of the concentrated and dialysed sample was frozen and stored.

Hydroxylapatite Chromatography

Hydroxylapatite Biogel HTP beads (Biorad) (10 g) were equilibrated in buffer A and transferred to a 1.5 cm X 20 cm column to yield a 28-mL bed volume. The matrix was equilibrated with 500 mL buffer A. The flow rate was maintained at 0.25 mL·min^{-1}. The concentrated and dialysed sample from ω-aminohexyl agarose chromatography was thawed and then loaded onto the equilibrated hydroxylapatite matrix. Non-adsorbed protein was washed from the column with 300 mL buffer A. The protein adsorbed to the matrix was

then eluted with a 150-mL linear gradient of 0 to 4 M NaCl in buffer A followed by a 150-mL wash with 4 M NaCl in buffer A. Next, a 75-mL linear gradient of 4 to 0 M NaCl in buffer A was used to remove NaCl from the column. Proteins still adsorbed to the matrix were then eluted with a 150-mL linear gradient of 0 to 300 mM NaF in buffer A followed by a 150-mL wash with 300 mM NaF in buffer A. A 75-mL linear gradient of 300 to 0 mM NaF in buffer A was then used to remove NaF from the column. Finally, a linear 150-mL gradient of 0 to 500 mM NaH_2PO_4 in buffer A followed by a wash with 150 mL of 500 mM NaH_2PO_4 in buffer A was used to elute the proteins still adsorbed to the matrix. Eluates from the 0 to 300 mM NaF and 0 to 500 mM NaH_2PO_4 gradients were collected into 5 mL fractions. The proteins which were washed or eluted from the column were monitored continuously at an absorbance of 280 nm and PEAMeT activity was assayed from all column washes and selected fractions from all gradients. Fractions from the 0 to 300 mM NaF and 0 to 500 mM NaH_2PO_4 gradients containing PEAMeT activity were pooled separately, each pooled sample was concentrated to less than 15 mL, dialysed up to 1,000,000-fold with buffer A and a 250-μL aliquot was removed from each sample for determination of PEAMeT activity and protein concentration. The remainder of the concentrated and dialysed sample was frozen and stored.

HPLC-Mono Q (Anion Exchange Chromatography)

The Mono Q HR 5/5 chromatographic column (1-mL bed volume,

Pharmacia) used with the HPLC system was equilibrated with 100 mL of buffer A. The flow rate was maintained at 0.5 $mL \cdot min^{-1}$. All buffers were filtered through Nucleopore filters with a diameter of 47 mm and a pore size of 0.22 μm before running on the HPLC system. The concentrated and dialysed sample prepared from either hydroxylapatite, phenyl Sepharose or ω-aminohexyl agarose was thawed, centrifuged for 10 minutes at 10,000*g*, 4°C using a Sorvall SS34 rotor to remove particulates and then the supernatant was loaded onto the equilibrated column. Non-adsorbed proteins were washed from the column with 40 mL buffer A. For some columns, adsorbed protein was eluted from the column with a 40 mL linear gradient of 0 to 1 M NaCl in buffer A. For other separations, adsorbed protein was eluted from the column with a 40-mL linear gradient of 150 to 400 mM NaCl in buffer A which was held at 180 mM NaCl until the absorbance at 280 nm dropped to less than 0.25 whereupon the gradient to 400 mM was restarted. In either case, the eluate was collected in 1 mL fractions. The proteins which were washed or eluted from the column were monitored continuously at an absorbance of 280 nm and PEAMeT activity was assayed from all column washes and selected fractions from both types of gradients. Fractions containing PEAMeT activity were pooled, concentrated to less than 1 mL, dialysed 200-fold with buffer A and a 50-μL aliquot was removed for determination of PEAMeT activity and protein concentration. The remainder of the concentrated and dialysed sample was frozen and stored.

HPLC-Phenyl Superose (Hydrophobic Interaction Chromatography)

The phenyl Superose HR 5/5 hydrophobic interaction column (1 mL bed volume) (Pharmacia) used with the HPLC system was equilibrated with 100 mL of buffer A containing 25% (w/v) $(NH_4)_2SO_4$. The flow rate was maintained at 0.5 $mL \cdot min^{-1}$. All buffers were filtered through Nucleopore filters with a diameter of 47 mm and a pore size of 0.22 μm before running on the HPLC system. The concentrated and dialysed sample prepared from the Mono Q separation was thawed, the concentration of $(NH_4)_2SO_4$ in the sample was made 25% (w/v) (Wood, 1976) and then the sample was centrifuged for 10 minutes at 14,000 rpm at 4°C using an Eppendorf microcentrifuge 5415 C to remove particulates. The supernatant was then loaded onto the equilibrated column. Non-adsorbed protein was washed from the column with 40-mL buffer A containing 25% (w/v) $(NH_4)_2SO_4$. Adsorbed protein was eluted from the column with a 30 mL linear gradient of buffer A containing 25% to 5% (w/v) $(NH_4)_2SO_4$ followed by 10-mL linear gradient of buffer A with 0% (v/v) ethylene glycol/5% (w/v) $(NH_4)_2SO_4$ to 50% (v/v) ethylene glycol/0% (w/v) $(NH_4)_2SO_4$. Eluate from both gradients were collected in 1-mL fractions. The proteins which were washed or eluted from the column were monitored continuously at an absorbance of 280 nm and PEAMeT activity was assayed from all column washes and selected fractions from both gradients. Those fractions containing PEAMeT activity were pooled, concentrated to less than 1 mL, dialysed 50-fold with buffer A and a 50-μL aliquot was removed for determination of PEAMeT

activity and protein concentration. The remainder of the concentrated and dialysed sample was frozen and stored.

Adenosine Agarose (Affinity Chromatography)

The preparation of adenosine agarose affinity matrix was modified from a procedure recommended by J. Attieh and D. Saini from the Institut de Recherche en Biologie Végétale, Université de Montréal. An adenosine agarose affinity matrix was prepared by the cleavage of the phosphate group from adenosine 2'-monophosphate (2'AMP) attached to a 4% agarose bead (Sigma). The 2'AMP was attached to the agarose by a C-8 linkage with a nine atom spacer. Prior to cleavage, 5 mL of the 2'AMP agarose was transferred to a glass funnel fitted with a sintered glass filter and repeatedly washed with distilled water. The washed 2'AMP agarose was transferred to a 15-mL polypropylene tube. The dephosphorylation reaction was performed using a ratio of 1 mL 2'AMP agarose:200 units type VII-S bovine intestinal mucosa alkaline phosphatase (Sigma, catalogue # P5521):1.5 mL dephosphorylation buffer (50 mM Tris-HCl, pH 9, 22°C and 1 mM $MgCl_2$). Dephosphorylation of the 2'AMP agarose was carried out at 37°C with constant shaking for 12 h. After the incubation, the dephosphorylation mixture was poured into a column (1 cm X 10 cm) and the contents were washed with 2 X 5 mL of buffer A containing 2 M NaCl followed by 2 X 10 mL of buffer A containing 0.02% NaN_3 and finally 50 mL distilled water. Pi content of the washes was measured

as reported for the phosphobase phosphatase assay. The dephosphorylation was repeated until most of the Pi was cleaved. The percentage of Pi cleaved was determined by comparing the amount of Pi cleaved from the matrix and the known original amount of Pi on the matrix (2.4 μmoles$\cdot$mL^{-1} matrix). A 7-mL adenosine agarose affinity matrix was prepared from which greater than 80% of the original Pi had been cleaved.

The adenosine agarose affinity matrix was poured into a column (1 cm X 20 cm, 7-mL bed volume) and equilibrated with 200 mL of buffer A. The flow rate was maintained at 0.25 mL$\cdot$min^{-1}. The concentrated and dialysed sample prepared from the phenyl Superose, Mono Q or hydroxylapatite separation was thawed and then loaded onto the equilibrated column. Non-adsorbed proteins were washed from the matrix with 40 mL buffer A. Adsorbed proteins were eluted from the column with a 20 mL-linear gradient of 0 to 2 M NaCl in buffer A followed by a wash with 40 mL of 2 M NaCl in buffer A. Eluate from the 0 to 2 M NaCl gradient and 2 M NaCl wash were collected in 2-mL fractions. The proteins which were washed or eluted from the column were monitored continuously at an absorbance of 280 nm and PEAMeT activity was assayed from all column washes and selected gradient fractions. Fractions containing PEAMeT activity were pooled, concentrated to less than 1 mL, dialysed 200-fold with buffer A and a 50 μL aliquot was removed for determination of PEAMeT activity and protein concentration. The remainder of the concentrated and dialysed sample was frozen and stored.

NATIVE MOLECULAR WEIGHT DETERMINATION

The molecular weight (MW) of PEAMeT was estimated by gel filtration chromatography using a Protein Pak 300 SW HPLC gel filtration chromatographic column (7.5 mm X 30 cm, 13.3-mL bed volume) (Waters) equilibrated with 50 mL buffer B consisting of 50 mM Tris-HCl (pH 7.4, 4°C), 100 mM KCl, 1 mM DTT and 5% (v/v) glycerol. Known MW standards (Sigma MW-GF-200 Kit) consisted of horse heart cytochrome *c* (12.4 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), BSA (132 kDa), yeast alcohol dehydrogenase (150 kDa) and sweet potato β-amylase (200 kDa). Each standard was dissolved in buffer B. The final concentration of each of the standards was made to 10 $mg \cdot mL^{-1}$ BSA, 5 $mg \cdot mL^{-1}$ alcohol dehydrogenase, 4 $mg \cdot mL^{-1}$ β-amylase, 3 $mg \cdot mL^{-1}$ carbonic anhydrase and 2 $mg \cdot mL^{-1}$ cytochrome *c*. A sample for injection was prepared by mixing a 200-μL aliquot of each dissolved standard together. This mixture of standards was filtered through a disposable, sterile Acrodisc (0.45 μm pore size, Gelman) and a 50-μL aliquot of the filtered mixture was injected onto the column. The fraction collector was set to start collecting 100-μL fractions at the same time the sample was injected onto the column. This procedure was carried out at 4°C using a flow rate of 0.1 $mL \cdot min^{-1}$ and the detection of proteins eluted from the column was completed by monitoring the absorbance of the eluate at 280 nm. The elution volume (V_e) of each standard was determined as the total volume of buffer B collected when the standard was detected by absorbance at 280

nm. The void volume (V_o) for each injected sample was determined as the total volume of buffer B collected before the first peak of absorbance at 280 nm was detected. A standard curve of V_e/V_o versus log MW was generated for each injection of the standards.

After the standards were run, the matrix was equilibrated with 50 mL of buffer B. A concentrated and dialysed hydroxylapatite or adenosine agarose sample enriched with PEAMeT activity was centrifuged at 14,000 rpm, 4°C for 2 min on an Eppendorf microcentrifuge 5415 C and then a 50-μL volume of the supernatant was injected onto the column. A portion of the remaining supernatant was then mixed with the internal standards to give a final concentration of standards of 3 $mg \cdot mL^{-1}$ carbonic anhydrase and 5 $mg \cdot mL^{-1}$ alcohol dehydrogenase. A 50-μL volume of this mixture was also injected onto the column in a separate run. Conditions of flow rate, protein detection and fraction collection for both runs containing sample were the same as for the standards alone. Selected fractions were assayed for PEAMeT activity. The V_e for PEAMeT was determined as the total volume of buffer B collected up to the fraction which contained the highest PEAMeT activity. Elution positions (V_e values) of the internal standards were also determined. The V_o values from the sample injected alone and sample mixed with internal standards were determined as for the standards alone.

POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS

Protein samples were analyzed electrophoretically using a 7.5 to 15% SDS-polyacrylamide gradient gel with the buffer system of Neville (1971). The protein samples were diluted in SDS-solubilizing buffer (60 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 1% (w/v) DTT and 0.002% (w/v) bromophenol blue) and heated at 90°C for 3 min prior to loading (Merrick, 1983). Polypeptides of known MW (Bio-Rad SDS-PAGE low molecular standard kit, catalogue # 161-0304) were also diluted with SDS-solubilizing buffer, heated at 90°C for 3 min and loaded (10 μL/lane) as recommended by Bio-Rad. Electrophoresis was carried out at constant current (15 mamp/1.5 mm thick gel) at 15°C for approximately 5 hours until the dye from the SDS-solubilizing buffer ran off the bottom of the gel.

After electrophoresis, gels were stained with silver reagent or a Coomassie dye solution. Silver-staining was performed using the method of Wray *et al.*, (1981). Gels stained with the Coomassie based protein stain (0.1% (w/v) Coomassie Brilliant Blue R250, 25% (v/v) isopropanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) cupric acetate) were then destained using 40% (v/v) methanol and 7% (v/v) glacial acetic acid and gentle agitation. Silver-stained or the Coomassie-stained and destained gels were then put into a gel drying solution consisting of destain solution plus 3% (v/v) glycerol. For fluorography, the Coomassie-stained gels were then exposed to a fluorographic reagent, Amplify (Amersham), for 15-30 min with gentle agitation. Gels were then dried

between two sheets of cellophane (Bio-Rad) in front of a fan (Wallevik and Jensenius, 1982). After drying, the position of the protein standards were marked using [^{14}C]choline-spiked ink spotted onto the dried gel. The dried gels were exposed to X-ray film (X-OMAT AR; 20.3 X 25.4 cm) at -80°C for varying periods of time. X-rays were then developed using a Kodak M35A X-OMAT Diagnostic Imaging Processor.

PHOTOAFFINITY CROSS-LINKING OF [^{3}H]SAM TO PEAMET

Aliquots (100 μL) of a 60-fold purified sample (14.3 nmol$\cdot$mL$^{-1}\cdot$mg^{-1} protein) were dispensed into wells of a Falcon 3911 Microtest III flexible assay plate (Becton Dickinson) which was kept on ice. SAM was added to the sample in each well to a final concentration of 200 μM and the contents of the well were mixed. A Mineralight UVS-54 lamp (short-wave, Ultraviolet Products Inc.) was placed 1.5 cm above the assay plate and the enzyme/SAM mixture was exposed to the UV-light for 5 min or 1 h. After the incubation, PEAMeT activity was assayed in these samples. PEAMeT activity was also assayed for samples which were incubated with 200 μM SAM in the absence of UV-light and for samples which were not incubated with SAM, but exposed to UV-light. Control PEAMeT activity for samples which remained on ice for 60 min without incubation with SAM or UV-light exposure was also determined.

In order to identify the position of the band corresponding to PEAMeT on SDS-polyacrylamide gels, the [^{3}H]SAM substrate was photoaffinity cross-linked

to the enzyme in the presence of UV-light (Som and Friedman, 1991). Aliquots of various enzyme preparations were dispensed into wells of a flexible assay plate which was kept on ice. [^{3}H]SAM (9:1 ratio of extract to [^{3}H]SAM) was then added to each well containing sample and the contents of the well were mixed. A UV-lamp (short-wave) was placed 1.5 cm above the assay plate and the enzyme/[^{3}H]SAM mixture was exposed to the UV-light for 1 h. The cross-linking reaction was stopped by the addition of SDS-solubilizing buffer equal to the sample volume or greater. The SDS-treated/UV-crosslinked enzyme mixture was removed from the assay plate, transferred to a microfuge tube and then heated (90°C for 3 min) before loading onto a 7.5% to 15% SDS-polyacrylamide gradient gel.

THIN LAYER CHROMATOGRAPHY

[^{3}H]-labelled phosphobases, eluted by 0.1 N HCl from the Dowex 50W columns of PEAMeT assays were pooled together and stored at -20°C. The enzyme samples used to generate the [^{3}H]phosphobase samples were from separations on hydroxylapatite chromatography columns. The Dowex eluates contained radioactivity of 1000 cpm/mL or higher. The frozen [^{3}H]phosphobase samples were then concentrated by freeze drying using a freeze drier (Thermovac Industries Inc.) or evaporated under nitrogen to dryness using a Meyer N-evap analytical evaporator (Organomation). These dried samples were dissolved in 150 μL of 0.1 N HCl. Finally, all [^{3}H]phosphobase samples

prepared in this manner were pooled together, evaporated under nitrogen to dryness then resuspended in 50 μL of 0.1 N HCl. An aliquot of this [^{3}H]phosphobase sample was also mixed with phosphobase standards consisting of 116 mM PCho, 52.5 mM PDEA, 78.3 mM PMEA and 213 mM PEA. A 2-μL volume of each standard along with 2 μL of the [^{3}H]phosphobase sample were mixed together, evaporated under nitrogen to dryness then resuspended in 4 μL of 0.1 N HCl.

Polygram silica G plates (Machenen-Nagel) were activated by heating in a 40°C oven for at least 24 hours. To equilibrate the TLC tanks, a silica G plate and two 26 cm X 27 cm 3 MM chromatography papers (Whatman) were placed inside a tank containing 120 mL equilibration solvent (42% 1-butanol, 42% methanol, 8% concentrated HCl, 8% distilled water, v/v/v/v) and left overnight. The plate was then removed and allowed to dry in the fume hood for 2 h at 22°C. The equilibration solvent was removed from the tank and replaced with 110 mL running solvent (45% 1-butanol, 45% methanol, 5% concentrated HCl, 5% distilled water, v/v/v/v) and allowed to equilibrate for 1 hour. After the TLC plate was dry, a thin line was drawn with a pencil 1.5 cm from the bottom of the TLC plate. Nineteen evenly spaced dots (1 cm apart) were drawn along this line, indicating the positions for application of the various samples. A 0.5 μL volume of each sample was applied to the dried TLC plate and was allowed to dry in the fumehood. This step was repeated two more times until 1.5 μL of sample were applied at the same position on the TLC plate. The TLC plate

with applied samples was placed into the tank and the solvent was allowed to move up the TLC plate for 6 h. After 6 h, the TLC plate was removed, dried in a fume hood and exposed to I_2 vapours in a sealed plastic container overnight. The TLC plate was then removed from the container. Although this is a convenient way to visualize organic compounds, I_2 was bound all over the plate and the positions to which the samples migrated were not distinct above the background I_2 staining. The TLC plate was placed in a fume hood for 2 h so that the I_2 bound to plate was allowed to dissipate. The TLC plate was then sprayed with an ammonium molybdate spray (5 mL distilled water, 0.5 g ammonium molybdate, 0.38 mL concentrated HCl, 2.5 mL 70% (v/v) perchloric acid, 42 mL acetone) and the sprayed plate was exposed to UV-light for 1 h, using the Mineralight UVS-54 (short wave) lamp placed 10 cm above the plate. Distinct purple spots were detected for the standards and for the [^{3}H]phosphobase sample containing internal standards, but no spots were detected for the [^{3}H]phosphobase sample applied without internal phosphobase standards. Using the position of the spots for the standards, the identity of the spots for the [^{3}H]phosphobase sample containing internal standards could be determined. For samples not containing internal standards, relative positions of the spots on the template were deduced by the migration position of the standards and marked on the plate with a pencil.

To quantify the radioactivity associated with each of the spots, the plate was sectioned into separate lanes with a razor blade, the individual purple spots

for the [^{3}H]phosphobase sample containing standards were moistened with a drop of distilled water and scraped off of the plate into a scintillation vial containing 10 mL of Ready Safe fluor (Beckman). The same procedure was performed for the [^{3}H]phosphobase sample without internal standards. Also, to determine the amount of radioactivity loaded onto the TLC plate corresponding to the [^{3}H]phosphobase sample, a 10-μL aliquot from this sample was transferred to a scintillation vial containing 10 mL of Ready Safe fluor. All vials were then shaken and radioactivity was counted in a Beckman LS 1801 liquid scintillation counter.

RESULTS

PEAMeT PURIFICATION PROCEDURE

General Comments

The partial purification of PEAMeT from spinach has been performed using seven different crude leaf extract preparations. Tables I to VII outline the purification steps used for each of the seven crude extracts. A composite or summary of all seven attempts is provided by Table VIII. These tables show that the order of the steps used and the separation strategy used varies between the seven trials. However, in all trials (Tables I to VII), spinach plants were exposed to 24 h of light prior to enzyme extraction. Prolonged light exposure was carried out to increase PEAMeT activity in the leaves since PEAMeT activity is higher in spinach leaves exposed to 24 h continuous light than in spinach leaves exposed to the 8 h light period used to grow the plants (Weretilnyk *et al.*, in press).

The recoveries of protein and PEAMeT activity varied for each of the purification steps (Tables I to VII). Indeed, even the recoveries of PEAMeT activity and protein reported for seemingly identical or at least comparable procedures repeated in various trials were considerably different. This apparent lack of reproducible performance for the various trials was, particularly at the

TABLE I. *PEAMeT Purification Summary #1*

Purification Step[a]	Protein (% Crude)[b]	Activity (% Crude)[b]	Specific Activity	Fold Pure
	mg	*units*[c]	*units·mg*$^{-1}$ *protein*	
Crude	11446 (100)	1661 (100)	0.145	1
1.8 to 2.6 M Ammonium Sulphate Dialysed	1796 (16)	1767 (106)	0.980	7
DEAE Sepharose[d]	1581 (14)	515 (31)	0.326	2
Phenyl Sepharose	40 (0.3)	164 (10)	4.100	28
HPLC-Mono Q[e]	5 (0.04)	59 (4)	11.800	81
HPLC-Phenyl Superose	2 (0.02)	32 (2)	19.198	132

[a] Refer to Materials and Methods for elution conditions for each column.
[b] Values in parentheses are the % recoveries relative to that determined for the crude leaf extract.
[c] units = nmol·min^{-1}
[d] Column eluted with a 0 to 300 mM NaCl gradient in buffer A.
[e] Column eluted with a 150 to 400 mM NaCl gradient in buffer A held at 180 mM NaCl for 11 min.

TABLE II. *PEAMeT Purification Summary #2*

Purification Step[a]	Protein (% Crude)[b]	Activity (% Crude)[b]	Specific Activity	Fold Pure
	mg	*units*[c]	*units·mg*$^{-1}$ *protein*	
Crude	7328 (100)	1697 (100)	0.232	1
1.8 to 2.6 M Ammonium Sulphate Dialysed	4187 (57)	2436 (144)	0.582	3
DEAE Sepharose[d]	2409 (33)	1114 (66)	0.462	2
Phenyl Sepharose	90 (1.2)	331 (20)	3.678	16
ω-Aminohexyl Agarose[e]	27 (0.4)	204 (12)	7.556	33
HPLC-Mono Q	1.9 (0.03)	37 (2)	19.474	84
HPLC-Phenyl Superose	1.2 (0.02)	34 (2)	29.153	126
Adenosine Agarose	0.007 (0)	0.756 (0.0004)	104.68	451

[a] Refer to Materials and Methods for elution conditions for each column.
[b] Values between parentheses are the % recoveries relative to that determined for the crude leaf extract.
[c] units = nmol·min^{-1}
[d] Column eluted with a 0 to 300 mM NaCl gradient in buffer A.
[e] Column eluted with a 150 to 400 mM NaCl gradient in buffer A held at 180 mM for 36 min.

TABLE III. *PEAMeT Purification Summary #3*

Purification Step[a]	Protein (% Crude)[b]	Activity (% Crude)[b]	Specific Activity	Fold Pure
	mg	*units*[c]	*units·mg^{-1} protein*	
Crude	19003 (100)	667 (100)	0.035	1
1.8 to 2.6 M Ammonium Sulphate Dialysed	2475 (13)	1264 (189)	0.511	15
DEAE Sepharose[d]	1587 (8)	466 (70)	0.293	8
Phenyl Sepharose	109 (0.6)	229 (34)	2.101	60
ω-Aminohexyl Agarose	20 (0.1)	79 (12)	3.992	114
HPLC-Mono Q[e]	3 (0.02)	58 (9)	18.719	535
Adenosine Agarose	0.051 (0.0003)	10 (1.4)	189.101	5403

[a] Refer to Materials and Methods for elution conditions for each column.
[b] Values in parentheses are the % recoveries relative to that determined for the crude leaf extract.
[c] units = nmol·min^{-1}
[d] Column eluted with a 0 to 300 mM NaCl gradient in buffer A.
[e] Column eluted with a 150 to 400 mM NaCl gradient in buffer A held at 180 mM for 36 min.

TABLE IV. *PEAMeT Purification Summary #4*

Purification Step[a]	Protein (% Crude)[b]	Activity (% Crude)[b]	Specific Activity	Fold Pure
	mg	*units*[c]	*units·mg*$^{-1}$ *protein*	
Crude	18533 (100)	1187 (100)	0.064	1
1.8 to 2.6 M Ammonium Sulphate Dialysed	3127 (17)	1654 (139)	0.529	8
DEAE Sepharose[d]	2223 (12)	1971 (166)	0.887	14
Phenyl Sepharose	182 (1)	541 (46)	2.973	46
ω-Aminohexyl Agarose	23 (0.1)	224 (19)	9.739	152
HPLC-Mono Q[e]	1.5 (0.008)	75 (6)	49.792	778

[a] Refer to Materials and Methods for elution conditions for each column.
[b] Values in parentheses are the % recoveries relative to that determined for the crude leaf extract.
[c] units = nmol·min^{-1}
[d] Column eluted with a 0 to 300 mM NaCl gradient in buffer A.
[e] Column eluted with a 150 to 400 mM NaCl gradient in buffer A held at 180 mM for 36 min.

TABLE V. *PEAMeT Purification Summary #5*

Purification Step[a]	Protein (% Crude)[b]	Activity (% Crude)[b]	Specific Activity	Fold Pure
	mg	*units*[c]	*units·mg*$^{-1}$ *protein*	
Crude	10815 (100)	4783 (100)	0.442	1
1.8 to 2.6 M Ammonium Sulphate Dialysed	4633 (43)	3086 (65)	0.666	1.5
DEAE Sepharose	3280 (30)	1996 (42)	0.609	1.4
Phenyl Sepharose	158 (1.5)	793 (17)	5.009	11
ω-Aminohexyl Agarose	72 (0.7)	695 (15)	9.653	22
Hydroxylapatite[d]	8.8 (0.08)	321 (3)	36.478	83
HPLC-Mono Q[e]	6.6 (0.06)	165 (0.5)	25.050	57
Adenosine Agarose	0.8 (0.007)	52 (1.1)	67.927	154

[a] Refer to Materials and Methods for elution conditions for each column.
[b] Values in parentheses are the % recoveries relative to that determined for the crude leaf extract.
[c] units = nmol·min^{-1}
[d] Column was eluted with a 0 to 300 mM NaH_2PO_4 gradient in buffer A.
[e] Column was eluted with a 0 to 2 M NaCl gradient in buffer A.

TABLE VI. *PEAMeT Purification Summary #6*

Purification Step[a]	Protein (% Crude)[b]	Activity (% Crude)[b]	Specific Activity	Fold Pure
	mg	*units*[c]	*units·mg*$^{-1}$ *protein*	
Crude	53033 (100)	8895 (100)	0.168	1
1.8 to 2.6 M Ammonium Sulphate Dialysed	12866 (24)	9283 (104)	0.722	4
DEAE Sepharose	5191 (10)	6660 (75)	1.283	8
Phenyl Sepharose	563 (1.1)	2663 (30)	4.730	28
ω-Aminohexyl Agarose[d]	499 (0.9)	2216 (25)	4.441	26
Hydroxylapatite A	31 (0.06)	618 (7)	19.935	119
Hydroxylapatite B	36 (0.07)	394 (4)	10.944	65
Adenosine Agarose A	3 (0.006)	146 (1.6)	49.714	296
Adenosine Agarose B	1.8 (0.003)	121 (1.4)	68.693	409

[a] Refer to Materials and Methods for elution conditions for each column.
[b] Values in parentheses are the % recoveries relative to that determined for the crude leaf extract.
[c] units = nmol·min^{-1}
[d] Purified preparation eluted from ω-aminohexyl agarose matrix was divided into two volumes (A & B).

TABLE VII. *PEAMeT Purification Summary #7*

Purification Step[a]	Protein (% Crude)[b]	Activity (% Crude)[b]	Specific Activity	Fold Pure
	mg	*units*[c]	*units·mg*$^{-1}$ *protein*	
Crude	26592 (100)	2642 (100)	0.099	1
1.8 to 2.6 M Ammonium Sulphate Dialysed	5686 (21)	1644 (62)	0.289	3
DEAE Sepharose	4680 (18)	3016 (114)	0.644	7
Phenyl Sepharose	328 (1.2)	924 (35)	2.817	28
ω-Aminohexyl Agarose	207 (0.8)	788 (30)	3.807	38
Hydroxylapatite[d]	51 (0.2)	396 (15)	7.765	78
Adenosine Agarose	2.9 (0.01)	149 (6)	51.74	523

[a] Refer to Materials and Methods for elution conditions for each column.
[b] Values in parentheses are the % recoveries relative to that determined for the crude leaf extract.
[c] units = nmol·min^{-1}
[d] Column was eluted with a 0 to 300 mM NaH_2PO_4 gradient in buffer A.

TABLE VIII. *Summary of PEAMeT Purifications*

Purification	1	2	3	4	5	6	7
Purification Steps[ab]	DEAE P Seph Mono Q P Sup	DEAE P Seph ω-AH Mono Q P Sup Ad Ag	DEAE P Seph ω-AH Mono Q Ad Ag	DEAE P Seph ω-AH Mono Q	DEAE P Seph ω-AH OH-Apt Mono Q Ad Ag	DEAE P Seph ω-AH OH-Apt Ad Ag	DEAE P Seph ω-AH OH-Apt Ad Ag
Protein (mg)	2	0.007	0.051	1.5	0.8	3 1.8	2.9
PEAMeT Activity (units)[c]	32	0.756	10	75	52	146 121	149
Specific Activity (units·mg^{-1}protein)	19.20	104.69	189.10	49.79	67.93	49.71 68.69	51.74
Purification (fold)	132	451	5403	778	154	296 409	523

[a] All samples previous to DEAE-Sepharose were 1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed preparations.
[b] DEAE = DEAE Sepharose, P Seph = phenyl Sepharose, Mono Q = HPLC-Mono Q, ω-AH = ω-aminohexyl agarose P Sup = HPLC-phenyl Superose, OH-Apt = hydroxylapatite, Ad Ag = adenosine agarose
[c] units = nmol·min^{-1}

onset of these studies, difficult to reconcile. Rather, as more extensive work on purification was carried out, factors such as column performance for the separations were found to be reproducible and representative results are provided in this section of the thesis. Where variability arose seemed to be in the post-column processing of the samples. That is, variable recoveries of PEAMeT activity and, more importantly, irrevocable losses of PEAMeT activity, could almost exclusively be attributed to periods during which the samples were concentrated and dialysed by ultrafiltration. These losses were overcome by passivation of the equipment following the method outlined in the "Materials and Methods" section. Therefore any conclusions regarding the efficacy of the column chromatography steps given in this section of the results are only drawn when passivation procedures were used to minimize post-column losses.

Enzyme Extraction and Fractionation

All purification trials began with $(NH_4)_2SO_4$ fractionation of the crude leaf extract. Since PEAMeT activity was only found in the 1.8 to 2.6 M $(NH_4)_2SO_4$ fraction (see Materials and Methods), only PEAMeT activity of this fraction following dialysis is given in Tables I to VII. The 1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed sample from Table III provides an example of how well this procedure could work as an initial step. That is, this sample had a 189% recovery of PEAMeT activity, a 13% recovery of protein and a 15-fold improvement of specific activity over the crude extract. However, the average 1.8 to 2.6 M

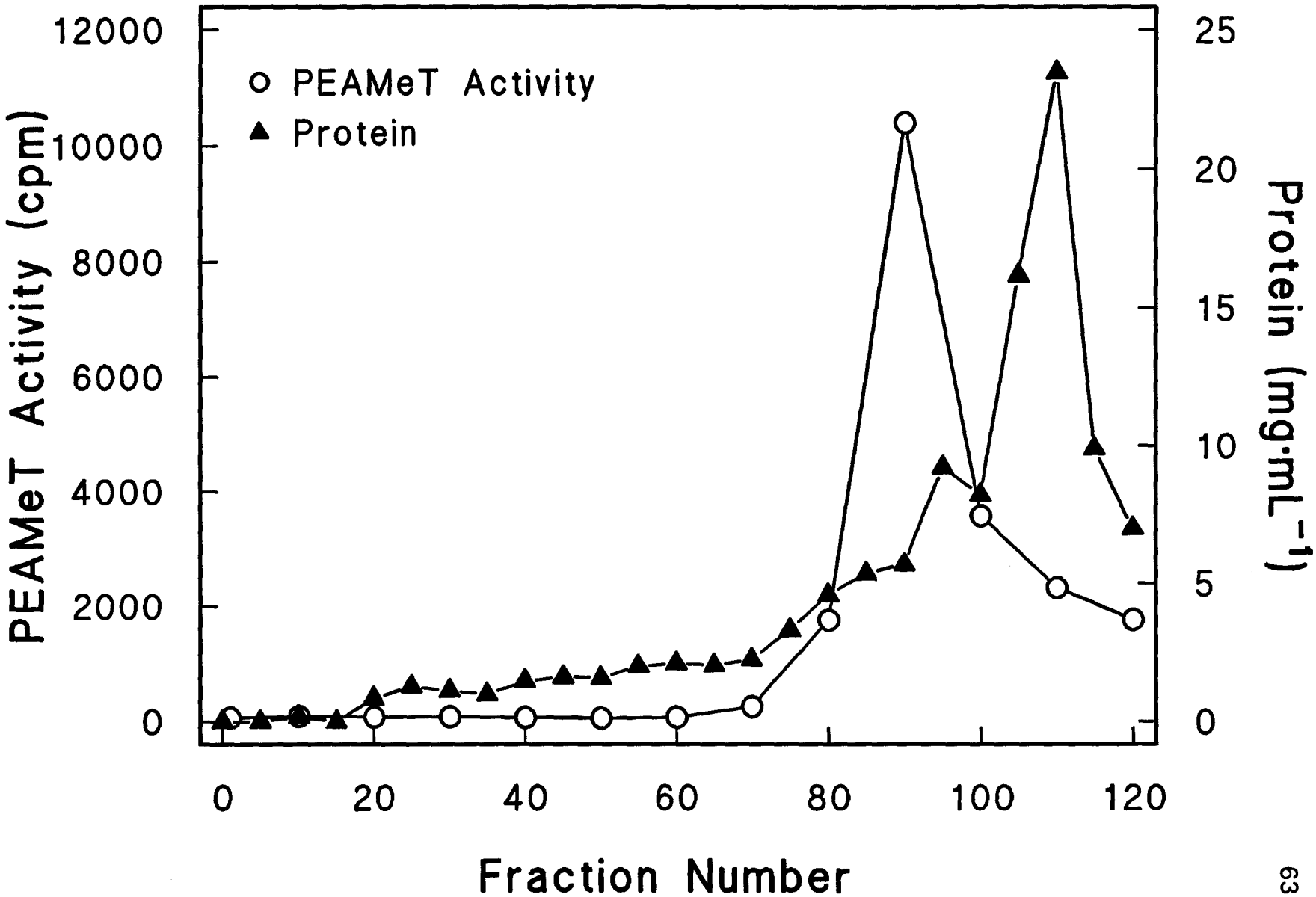
$(NH_4)_2SO_4$ dialysed sample gave a PEAMeT activity recovery of 116%, a protein recovery of 27% and a 6-fold improvement of specific activity over the crude extract.

DEAE Sepharose Chromatography

The 1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed samples were loaded onto the anion exchange matrix DEAE Sepharose. Typically, 10 to 25% of the protein loaded onto the DEAE Sepharose column did not adsorb to the matrix. However, no PEAMeT activity was detected in the non-adsorbed material. Proteins bound to the column were then eluted with a 0 to 300 or a 0 to 500 mM NaCl gradient. Figure 3 shows the typical elution profile for protein and PEAMeT activity determined using selected fractions from a DEAE Sepharose matrix eluted with a 0 to 300 mM NaCl gradient. PEAMeT activity eluted from the matrix at a position preceding the peak of protein. The DEAE Sepharose purified sample described in Table VII showed a 184% recovery of PEAMeT activity, a 86% recovery of protein and a 2.3-fold improvement of specific activity over the 1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed sample loaded. The average DEAE Sepharose purified sample gave a 194% recovery of PEAMeT activity, a 79% recovery of protein and a 1.2-fold improvement in specific activity over the 1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed sample loaded. There was no correlation between recoveries of PEAMeT activity, protein or specific activities achieved whether a 0 to 300 or 0 to 500 mM NaCl gradient was used to elute PEAMeT

FIGURE 3. *DEAE Sepharose Elution Profile.*

Representative elution profile of a 0 to 300 mM NaCl gradient from a chromatographic separation using DEAE Sepharose (see Materials and Methods for column elution conditions). Protein concentration (▲) and PEAMeT activity (○) for selected fractions along the gradient are shown in the figure. PEAMeT activity is reported as cpm per 25-μL aliquot of the selected fraction and protein is reported as $mg \cdot mL^{-1}$.


PEAMeT Activity
Protein
PEAMeT Activity (cpm)
Protein (mg·mL⁻¹)
Fraction Number
0
2000
4000
6000
8000
10000
12000
0
5
10
15
20
25
0
20
40
60
80
100
120

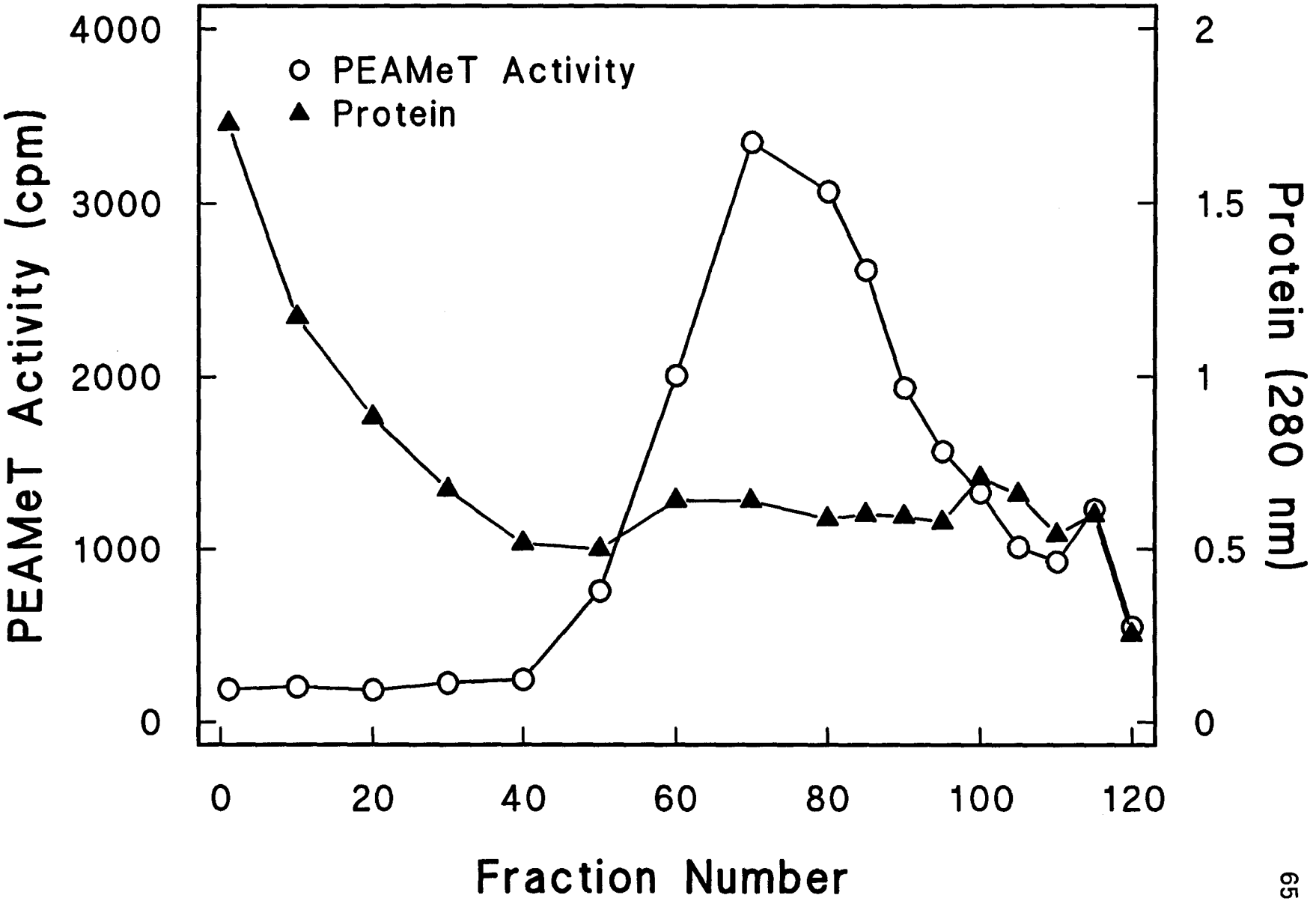
activity from the DEAE-Sepharose column. However, PEAMeT activity eluted from the column with a sharper peak and in fewer fractions using the 0 to 500 mM NaCl gradient.

Phenyl Sepharose Chromatography

The concentration of $(NH_4)_2SO_4$ in the pooled DEAE Sepharose preparation was made 25% (w/v) with respect to $(NH_4)_2SO_4$ in order to maximize adsorption of the protein in the sample to the phenyl Sepharose hydrophobic interaction matrix. Greater than 90% of the protein adsorbed to the hydrophobic matrix under the equilibration conditions used and then 30 to 50% of this adsorbed protein was eluted from the matrix with a 15% to 5% (w/v) $(NH_4)_2SO_4$ gradient followed by a 5% (w/v) $(NH_4)_2SO_4$ wash. However, no PEAMeT activity was detected in either this gradient or the subsequent wash. Remaining adsorbed protein was eluted from the matrix with a 5% (w/v) $(NH_4)_2SO_4$/0% (v/v) ethylene glycol to 0% (w/v) $(NH_4)_2SO_4$/50% (v/v) ethylene glycol gradient. Figure 4 shows the typical elution profile for protein and PEAMeT activity determined using selected fractions from the phenyl Sepharose matrix under these gradient conditions. PEAMeT activity was eluted from the matrix at a position which followed the peak of the protein. The phenyl Sepharose purified sample described in Table I provides an example of how well this procedure could work. That is, this sample had a 32% recovery of PEAMeT activity, a 2.1% recovery of protein and a 14-fold improvement of

FIGURE 4. *Phenyl Sepharose Elution Profile.*

Representative elution profile highlighting the results using the 5% (w/v) $(NH_4)_2SO_4$/0% (v/v) ethylene glycol to 0% (w/v) $(NH_4)_2SO_4$/50% (v/v) ethylene glycol linear gradient from a chromatographic separation on phenyl Sepharose. Complete description of elution conditions are outlined in Materials and Methods. PEAMeT activity (○) is reported as cpm per 25-μL aliquot of the selected fraction and protein (▲) is reported as absorbance at 280 nm.


PEAMeT Activity
Protein
PEAMeT Activity (cpm)
Protein (280 nm)
Fraction Number
4000
3000
2000
1000
0
2
1.5
1
0.5
0
0
20
40
60
80
100
120

specific activity over the DEAE Sepharose preparation loaded. However, the average phenyl Sepharose purified sample gave a 36% recovery of PEAMeT activity, a 6.3% recovery of protein and a 6.9-fold improvement of specific activity over the DEAE Sepharose sample loaded.

ω-Aminohexyl Agarose Chromatography

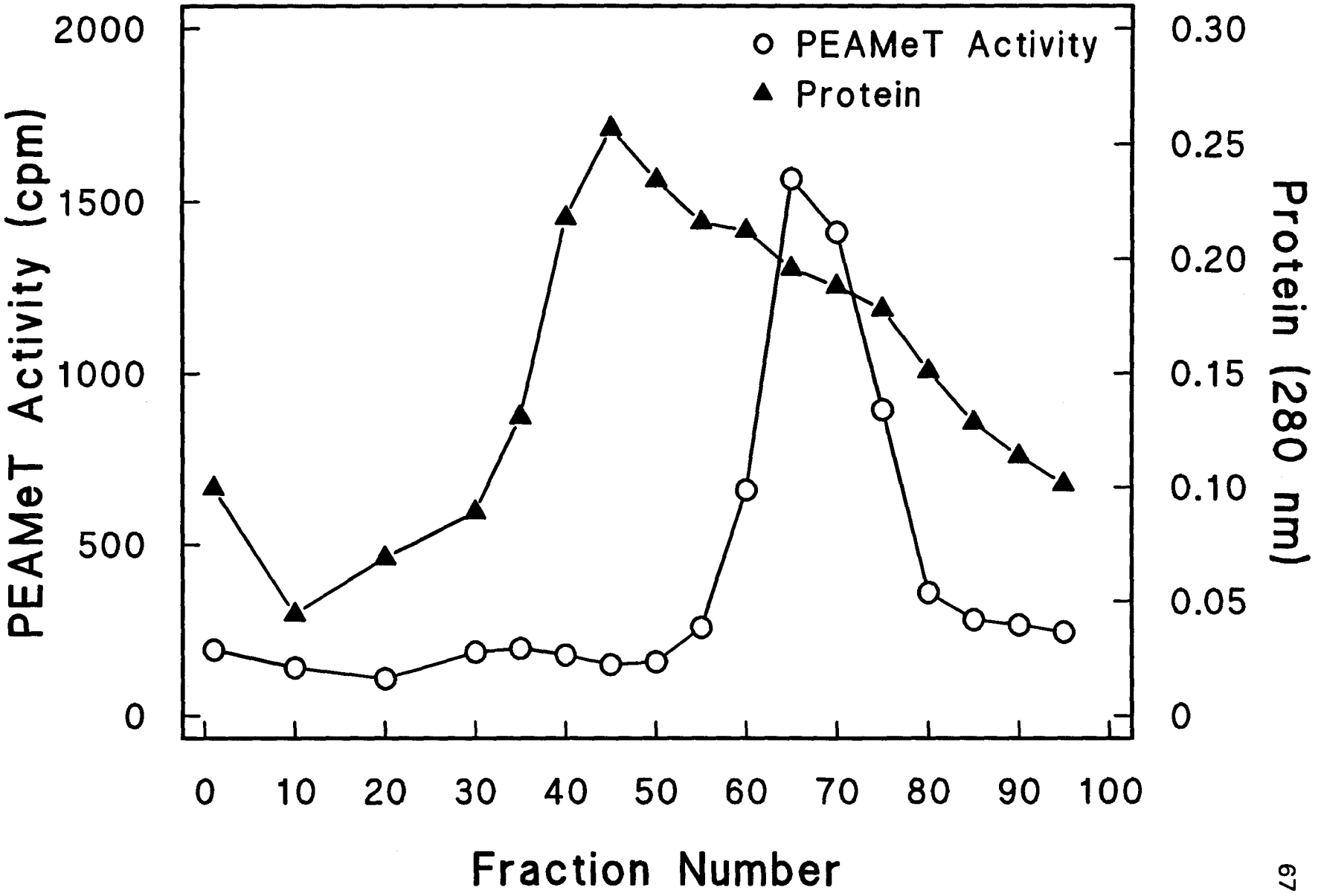
Concentrated and dialysed preparations separated by chromatography on phenyl Sepharose were loaded onto ω-aminohexyl agarose. Greater than 90% of the protein loaded onto the ω-aminohexyl agarose column was absorbed to the matrix and no PEAMeT activity was detected in the non-adsorbing fraction. The adsorbed protein was eluted from the matrix by a 0 to 300 mM NaCl gradient and PEAMeT activity was detected among the column fractions. Figure 5 shows the typical elution profile for protein and PEAMeT activity determined using aliquots of selected fractions from the ω-aminohexyl agarose separation under these gradient conditions. PEAMeT activity was eluted from the matrix at a position following the major peak of protein. The ω-aminohexyl agarose purified sample described in Table IV showed a 41% recovery of PEAMeT activity, a 8.3% recovery of protein and a 3.3-fold improvement of specific activity over the phenyl Sepharose purified sample loaded.

Hydroxylapatite Chromatography

Concentrated and dialysed preparations which were separated by

FIGURE 5. *ω-Aminohexyl Agarose Elution Profile.*

Representative elution profile of a 0 to 300 mM NaCl gradient from a chromatographic separation using ω-aminohexyl agarose (see Materials and Methods for column elution conditions). PEAMeT activity (○) is reported as cpm per 25-μL aliquot of the selected fraction and protein (▲) is reported as absorbance at 280 nm.


PEAMeT Activity
Protein
PEAMeT Activity (cpm)
Protein (280 nm)
Fraction Number
2000
1500
1000
500
0
0.30
0.25
0.20
0.15
0.10
0.05
0
0
10
20
30
40
50
60
70
80
90
100

chromatography on ω-aminohexyl agarose (Tables V, VI and VII) were loaded onto the hydroxylapatite column. Approximately 40% of the protein loaded onto the column did not adsorb to the matrix under the conditions used. Some PEAMeT activity could be detected in the non-adsorbed material but this accounted for less than 10% of the estimated PEAMeT activity loaded. The adsorbed basic proteins were eluted from the PO_4^{n-} sites on the hydroxylapatite matrix by 4 M NaCl while the more acidic proteins were eluted from the Ca^{2+} sites on the matrix with a 0 to 300 mM NaF gradient (Gorbunoff, 1985). Figure 6 shows the typical elution profile for protein and PEAMeT activity determined using selected fractions from the hydroxylapatite matrix eluted with a 0 to 300 mM NaF gradient. Approximately 25% to 97% of the protein and less than 10% of the PEAMeT activity which was absorbed to the matrix co-eluted under these conditions. All remaining adsorbed proteins were eluted from the matrix with a 0 to 300 or 0 to 500 mM NaH_2PO_4 gradient. Figure 7 shows the elution profile for protein and PEAMeT activity determined using selected fractions from the hydroxylapatite matrix eluted with a 0 to 500 mM NaH_2PO_4 gradient. PEAMeT activity elutes in fractions following those containing the first peak of protein and co-elutes in fractions containing the second peak of protein. The hydroxylapatite purified sample outlined in Table VI showed a 16% recovery of PEAMeT activity, a 6.6% recovery of protein and a 4.5-fold improvement of specific activity over the ω-aminohexyl agarose purified sample loaded. There was no correlation between recoveries of

FIGURE 6. *Hydroxylapatite Elution Profile A.*

Representative elution profile highlighting the results using the 0 to 300 mM NaF linear gradient from a chromatographic separation on hydroxylapatite. Complete description of elution conditions are outlined in Materials and Methods. PEAMeT activity (○) is reported as cpm per 25-μL aliquot of the selected fraction and protein (▲) is reported as absorbance at 280 nm.

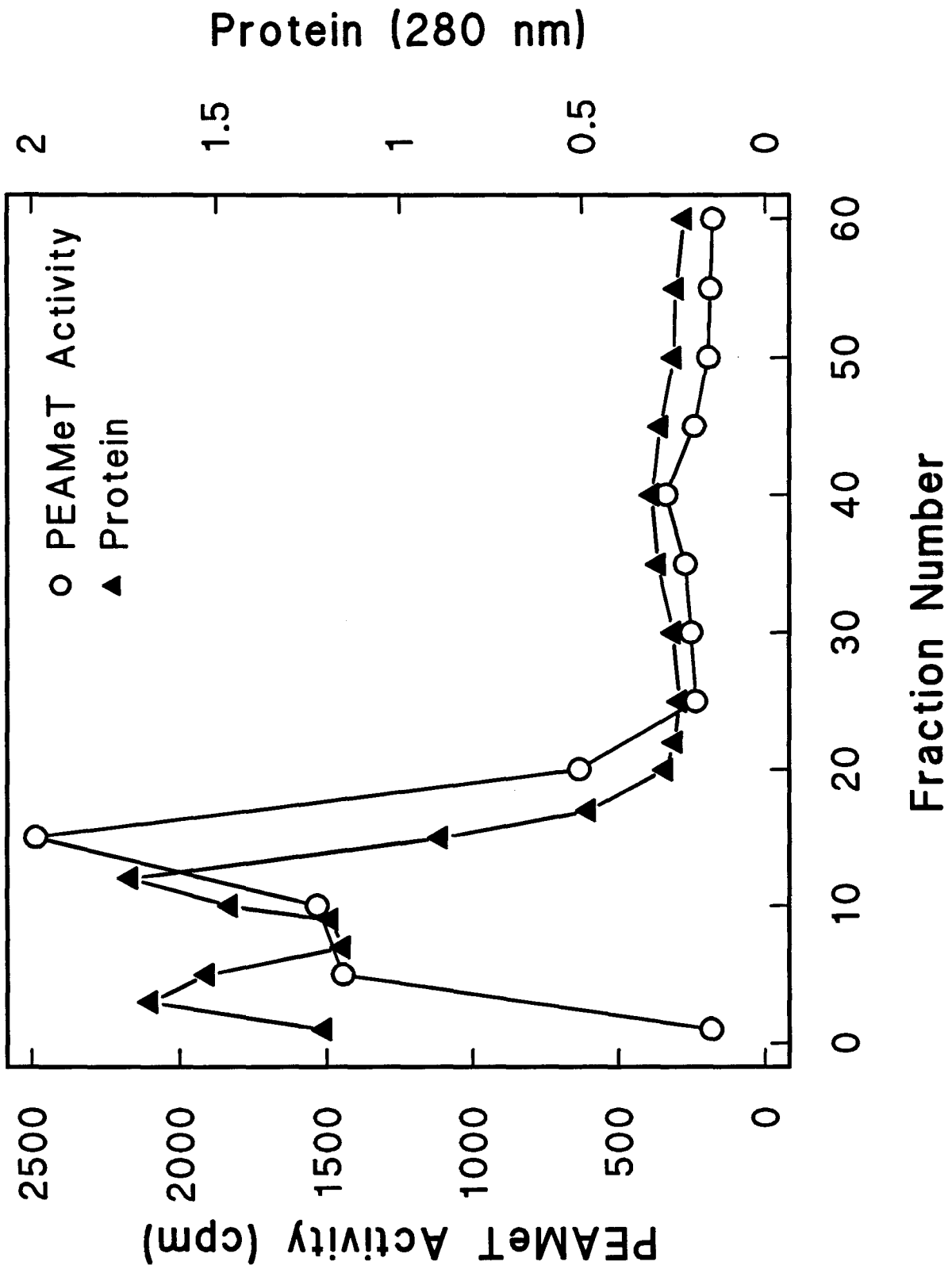
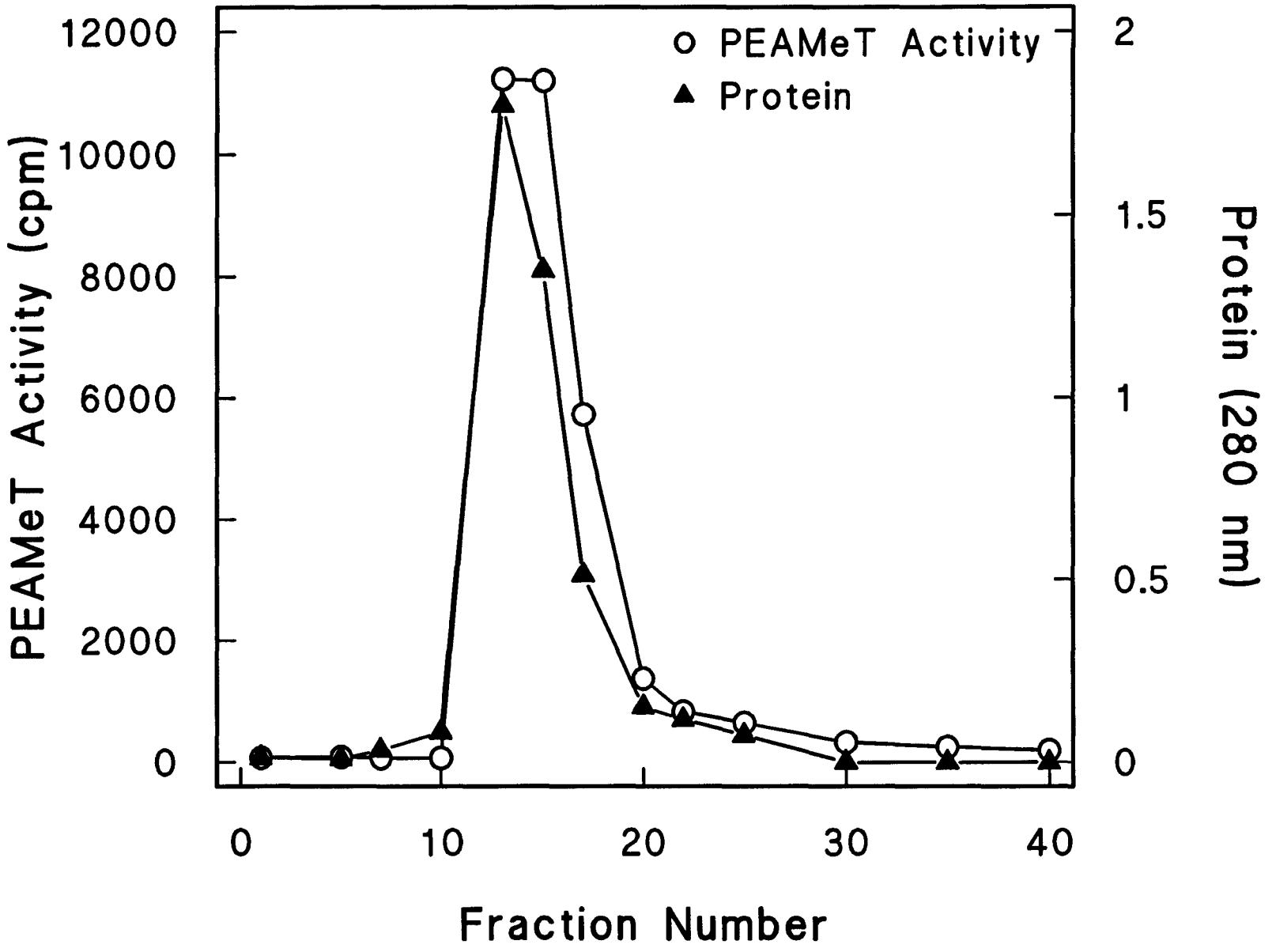

Protein (280 nm)
2
1.5
1
0.5
0
o PEAMeT Activity
▲ Protein
2500
2000
1500
1000
500
0
PEAMeT Activity (cpm)
0
10
20
30
40
50
60
Fraction Number

FIGURE 7. *Hydroxylapatite Elution Profile B.*

Representative elution profile highlighting the results using the 0 to 500 mM NaH_2PO_4 linear gradient from a chromatographic separation on hydroxylapatite. Complete description of elution conditions are outlined in Materials and Methods. PEAMeT activity (○) is reported as cpm per 25-μL aliquot of the selected fraction and protein (▲) is reported as absorbance at 280 nm.


PEAMeT Activity
Protein
PEAMeT Activity (cpm)
Protein (280 nm)
Fraction Number
12000
10000
8000
6000
4000
2000
0
2
1.5
1
0.5
0
0
10
20
30
40

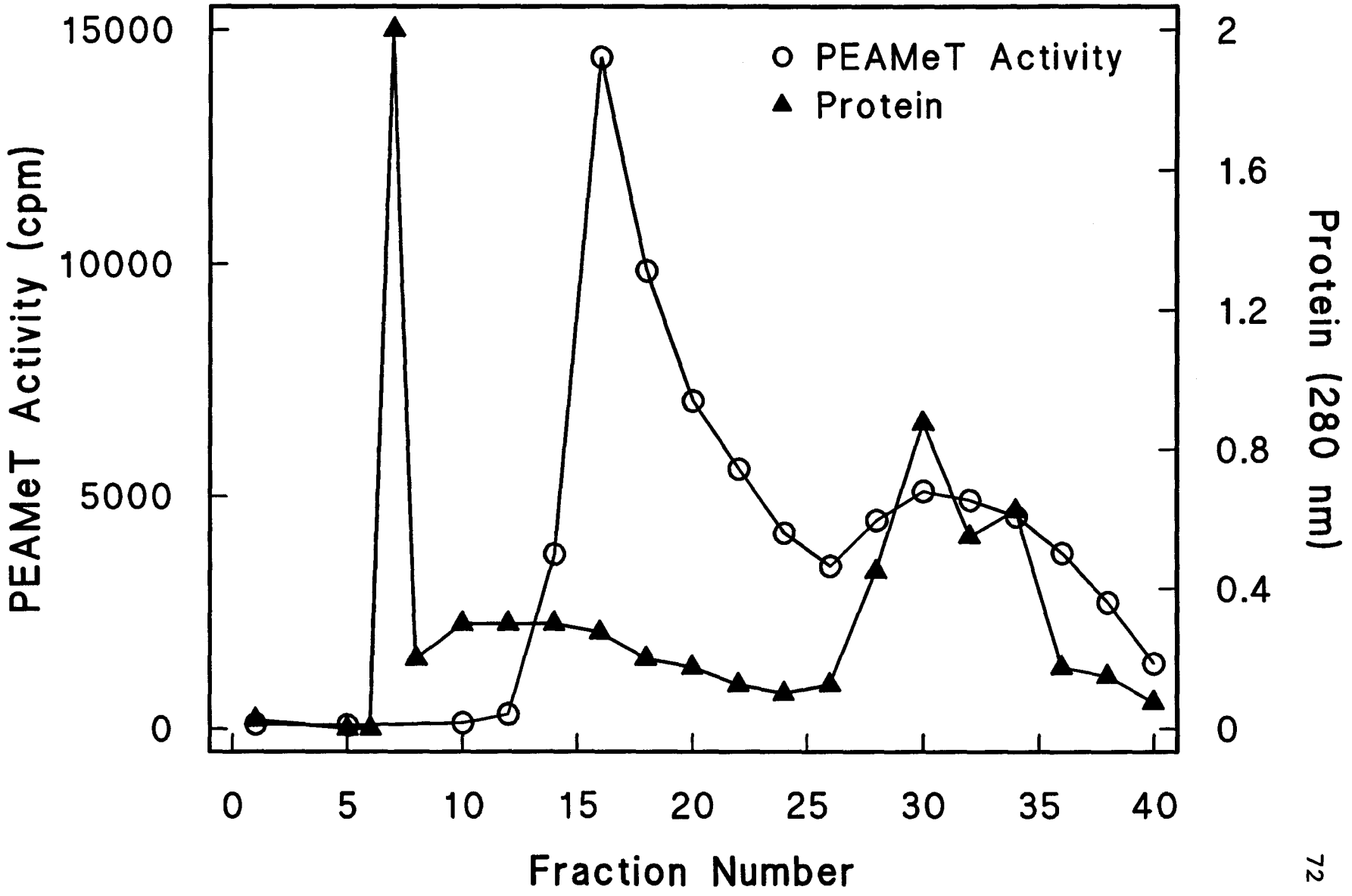
PEAMeT activity, protein or specific activity whether a 0 to 300 or 0 to 500 mM NaH_2PO_4 gradient was used to elute PEAMeT activity from the hydroxylapatite matrix.

HPLC-Mono Q Chromatography

Concentrated and dialysed preparations separated by chromatography on phenyl Sepharose (Table I), ω-aminohexyl agarose (Tables II, III and IV) or hydroxylapatite (Table V) were loaded onto the Mono Q column. In each case, all of the sample protein was adsorbed to this anion exchange matrix. Adsorbed protein and all of the PEAMeT activity were eluted from the matrix with either a 150 to 400 mM NaCl gradient (Tables I, II, III and IV) or a 0 to 1 M NaCl gradient (Table V). Figure 8 shows the typical elution profile for protein and PEAMeT activity determined using selected fractions from a Mono Q matrix eluted with a 150 to 400 mM NaCl gradient held at 180 mM for 36 min. PEAMeT activity was eluted from the matrix in two peaks. The first peak of PEAMeT activity corresponds to fractions collected between those containing the highest concentrations of protein and the second peak of PEAMeT activity co-eluted with the second protein peak. Although samples from different purification steps were loaded onto the Mono Q matrix, the Mono Q purified sample described in Table IV provides an example of the best separation achieved. That is, this sample had a 32% recovery of PEAMeT activity, an 8% recovery of protein and a 5.1-fold improvement of specific activity over the ω-

FIGURE 8. *HPLC-Mono Q Elution Profile A.*

Representative elution profile highlighting the results using the 150 to 400 mM NaCl gradient held at 180 mM NaCl for 36 min from a chromatographic separation on Mono Q. Complete description of elution conditions are outlined in Materials and Methods. PEAMeT activity (○) is reported as cpm per 25-μL aliquot of the selected fraction and protein (▲) is reported as absorbance at 280 nm.


PEAMeT Activity
Protein
PEAMeT Activity (cpm)
Protein (280 nm)
Fraction Number
15000
10000
5000
0
2
1.6
1.2
0.8
0.4
0
0
5
10
15
20
25
30
35
40

aminohexyl agarose purified sample loaded.

Figure 9 shows the typical elution profile for protein and PEAMeT activity determined using selected fractions from a Mono Q matrix eluted with a 0 to 1 M NaCl gradient. PEAMeT activity and protein co-eluted from the matrix. The Mono Q purified sample described in Table V showed a 50% recovery of PEAMeT activity, a 75% recovery of protein and a 0.7-fold improvement of specific activity over the hydroxylapatite purified sample loaded. The lower fold-purification obtained using a 0 to 1 M NaCl gradient to elute adsorbed proteins from the Mono Q matrix indicates that this gradient does not separate PEAMeT from the bulk of the adsorbed protein as well as the 150 to 400 mM NaCl gradient held at 180 mM for 36 min.

HPLC-Phenyl Superose Chromatography

The concentration of $(NH_4)_2SO_4$ in the concentrated and dialysed Mono Q preparation (Tables I and II) was made 25% (w/v) with respect to $(NH_4)_2SO_4$ in order to maximize adsorption conditions for proteins in the sample to the phenyl Superose hydrophobic interaction matrix. All of the protein was adsorbed to the column under the equilibration conditions used and no PEAMeT activity was detected in the column wash fractions. Adsorbed protein was eluted from the matrix with a linear gradient of 5% (w/v) $(NH_4)_2SO_4$/0% (v/v) ethylene glycol to 0% (w/v) $(NH_4)_2SO_4$/50% (v/v) ethylene glycol. Figure 10 shows the typical

FIGURE 9. *HPLC-Mono Q Elution Profile B.*

Representative elution profile highlighting the results using the linear 0 to 1 M NaCl gradient from a chromatographic separation on Mono Q (Table V). Complete description of elution conditions are outlined in Materials and Methods. PEAMeT activity (○) is reported as cpm per 25-μL aliquot of the selected fraction and protein (▲) is reported as absorbance at 280 nm.

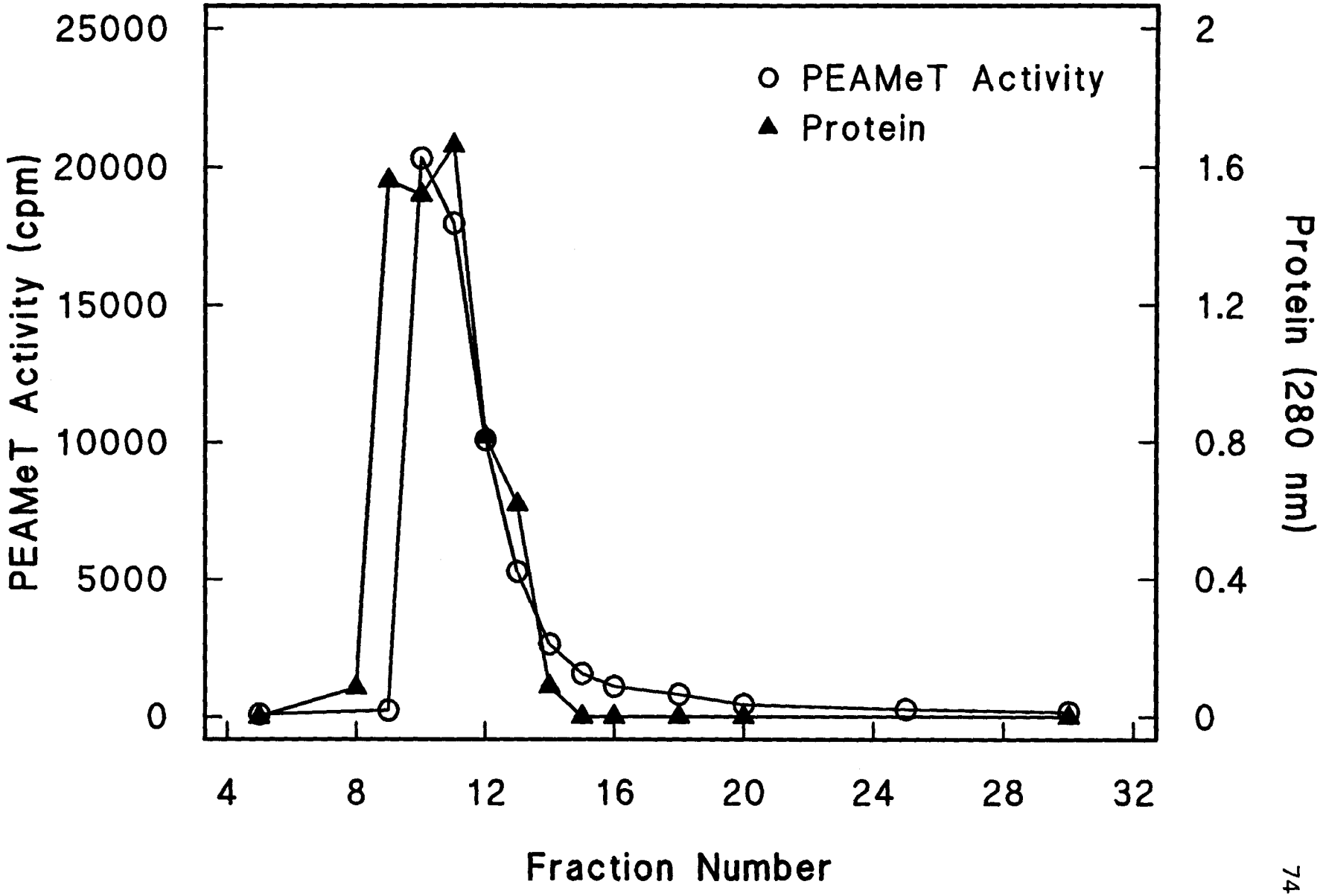
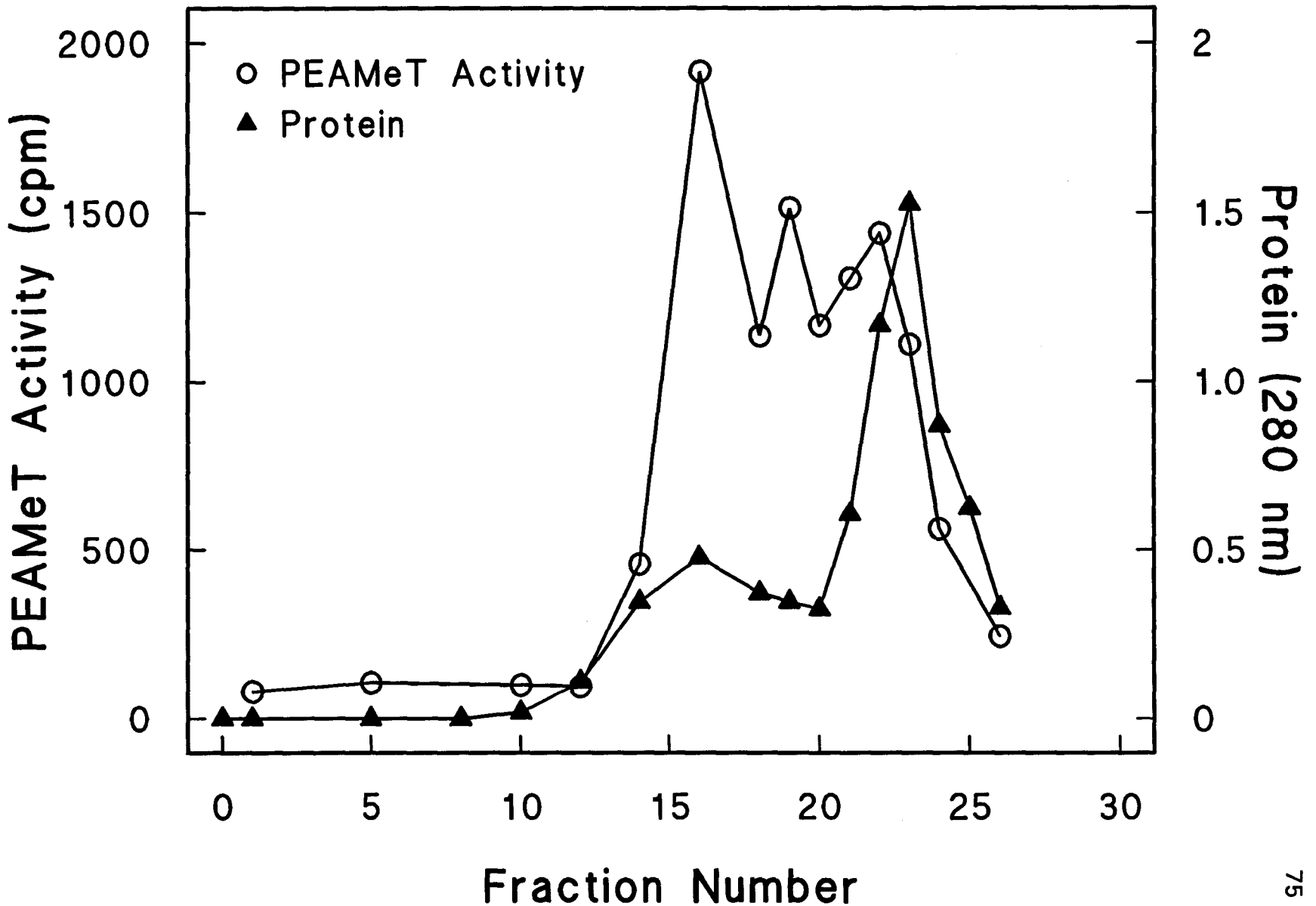

PEAMeT Activity (cpm)
25000
20000
15000
10000
5000
0
Protein (280 nm)
2
1.6
1.2
0.8
0.4
0
PEAMeT Activity
Protein
4
8
12
16
20
24
28
32
Fraction Number

FIGURE 10. *HPLC-Phenyl Superose Elution Profile.*

Representative elution profile highlighting the results using the 5% (w/v) $(NH_4)_2SO_4$/0% (v/v) ethylene glycol to 0% (w/v) $(NH_4)_2SO_4$/50% (v/v) ethylene glycol linear gradient from a chromatographic separation on phenyl Superose. Complete description of elution conditions are outlined in Materials and Methods. PEAMeT activity (○) is reported as cpm per 25-μL aliquot of the selected fraction and protein (▲) is reported as absorbance at 280 nm.


PEAMeT Activity
Protein
PEAMeT Activity (cpm)
Protein (280 nm)
Fraction Number
2000
1500
1000
500
0
2
1.5
1.0
0.5
0
0
5
10
15
20
25
30

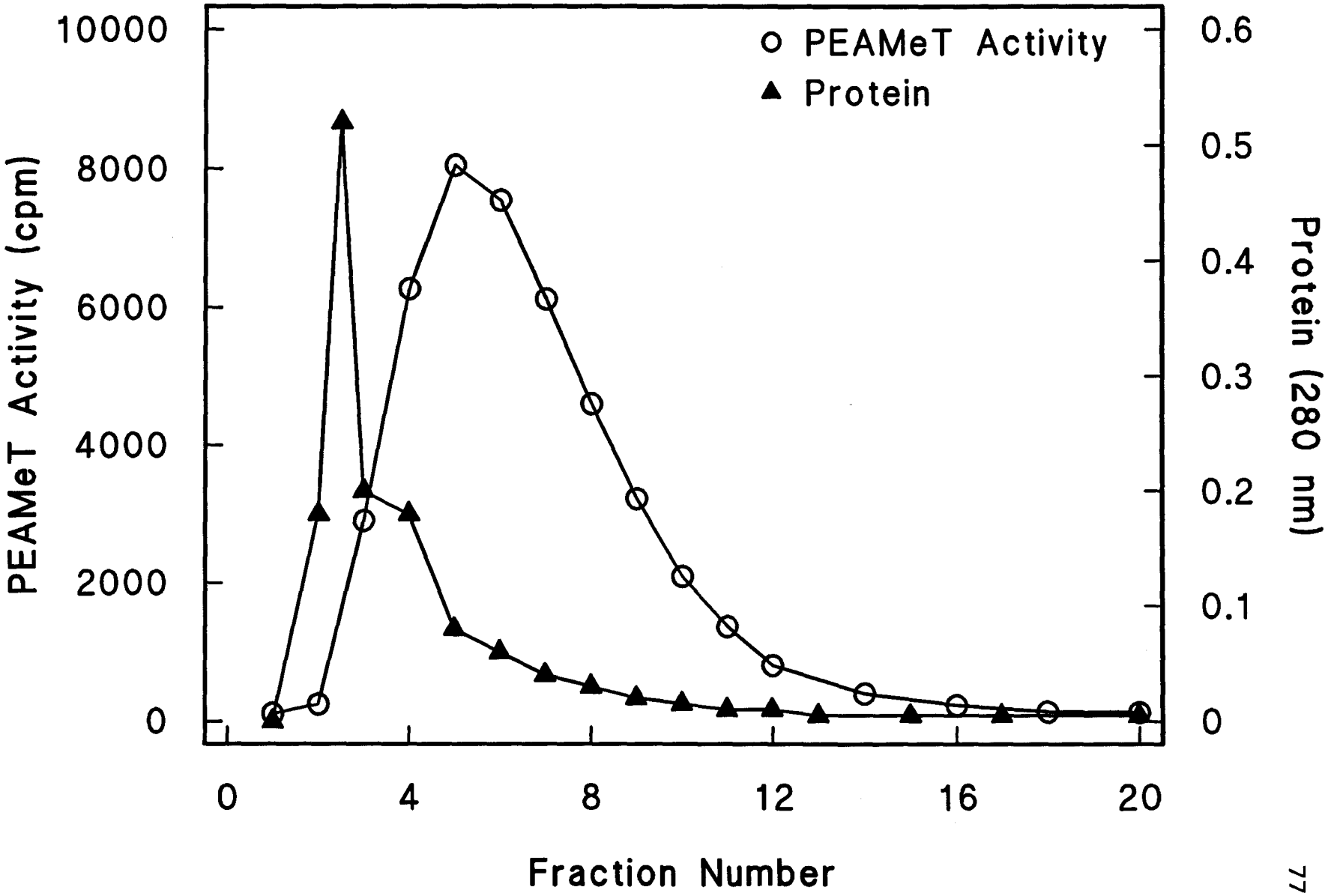
elution profile for protein and PEAMeT activity determined using selected fractions from the phenyl Superose matrix under these gradient conditions. PEAMeT activity eluted from the matrix in fractions following those containing the largest concentrations of protein. The phenyl Superose purified sample described in Table II showed that a 92% recovery of PEAMeT activity, a 67% recovery of protein and a 1.5-fold improvement of specific activity over the Mono Q purified sample loaded.

Adenosine Agarose Chromatography

Concentrated and dialysed preparations which were separated by chromatography on either the phenyl Superose (Table II), Mono Q (Tables III and V) or hydroxylapatite (Tables VI and VII) matrix were loaded onto the adenosine agarose affinity matrix. Approximately 59 to 99% of the protein loaded onto the column did not adsorb to the matrix under the conditions used. Some PEAMeT activity could be detected in the non-adsorbed material but on average this comprised less than 2% of the estimated PEAMeT activity loaded. Adsorbed protein was eluted from the affinity matrix with a 0 to 2 M NaCl gradient. Figure 11 shows the elution profile for protein and PEAMeT activity determined using selected fractions from the adenosine agarose matrix eluted with this gradient. PEAMeT activity was found in fractions following those containing the highest concentrations of protein. Although samples from different purification steps were loaded onto the adenosine agarose matrix, the

FIGURE 11. *Adenosine Agarose Elution Profile.*

Representative elution profile of a 0 to 2 M NaCl gradient from a chromatographic separation using adenosine agarose. Complete description of elution conditions are outlined in Materials and Methods. PEAMeT activity (○) is reported as cpm per 25-μL aliquot of the selected fraction and protein (▲) is reported as absorbance at 280 nm.


PEAMeT Activity
Protein
PEAMeT Activity (cpm)
Protein (280 nm)
Fraction Number
10000
8000
6000
4000
2000
0
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
8
12
16
20

adenosine agarose purified sample described in Table III exhibited the best separation. For this sample a 17% recovery of PEAMeT activity and a 1.7% recovery of protein was achieved giving a 10-fold improvement of specific activity over the Mono Q purified sample loaded.

PHOTOAFFINITY CROSS-LINKING AND ELECTROPHORETIC ANALYSIS

Enzyme Data

Table IX shows that PEAMeT activity of a 60-fold partially purified preparation was not significantly inhibited by a 60 min incubation with 200 μM SAM or a 60 min exposure to UV-light. However, when this preparation was incubated with 200 μM SAM under UV-light for 60 min, PEAMeT activity was reduced to 55% of the control activity (Table IX). Therefore, when PEAMeT is incubated with 200 μM SAM in the presence of UV-light for 60 min, PEAMeT becomes photoaffinity cross-linked to SAM resulting in a loss in the activity of PEAMeT.

SDS-Polyacrylamide Gel Electrophoresis

If PEAMeT is photoaffinity cross-linked to [^{3}H]SAM, the radiolabelled PEAMeT protein can then be identified on fluorographs of Coomassie-stained, SDS-polyacrylamide gels (see Materials and Methods). Such an analysis using crude leaf extracts has been done as part of a previous study (Smith, 1993) and shows that many polypeptides are capable of photoaffinity cross-linking to

TABLE IX. ***Inhibition of PEAMeT Activity by Photoaffinity Cross-Linking to [^{3}H]SAM.***

Treatment	Incubation Time (min)	PEAMeT Activity (% Control)[a,b]
200 μM SAM	5	102 ± 0.3
	60	87 ± 2
0 μM SAM + UV exposure	5	97 ± 8
	60	84 ± 5
200 μM SAM + UV exposure	5	98 ± 2
	60	55 ± 2

[a] Control activity for sample which remained on ice for 60 min without incubation with SAM or UV-light exposure was 14.3 ± 1.6 nmol·mL^{-1}·mg^{-1} protein.

[b] Duplicate measurements ± SE.

[^{3}H]SAM in a crude preparation. However, the intensity of one band corresponding to a MW of 57 kDa correlated with the rise and fall of PEAMeT activity detected from spinach plants exposed to various light periods and salt treatments. In this thesis, a 5403-fold purified sample prepared by adenosine agarose affinity chromatography (Table III) was subjected to SDS-PAGE and either silver-stained (Fig. 12, lane 1) or the sample was photoaffinity cross-linked first to [^{3}H]SAM and the labelled polypeptide(s) visualized by fluorography (Fig. 12, lane 2). The fluorograph (Fig. 12, lane 2) shows that only one polypeptide is specifically labelled by [^{3}H]SAM, despite a prolonged (51 day) exposure to X-ray film. This band corresponds to an estimated monomer MW of approximately 57 kDa as determined using a standard curve shown in Figure 13.

With specific reference to lane 2 of Figure 12, it is apparent that even a 5403-fold purified sample was composed of many polypeptides that stained with silver reagent. Detection of polypeptides with silver is very sensitive (Wray *et al.*, 1981). Thus the 10.6 μg protein loaded into this lane is probably too much to visually resolve the protein bands. Despite this overloading of the sample, the band (Fig. 12, lane 1) which stains most intensely (indicated by the arrow) has a MW of 54 kDa as determined from a standard curve shown in Figure 14. The only polypeptide which is specifically labelled by [^{3}H]SAM, corresponds to the same position (within measurement error) as the most intensely stained band revealed by the silver reagent. Taken together, the

FIGURE 12. *SDS-PAGE Analysis of PEAMeT Prepared by Adenosine Agarose Affinity Chromatography.*

The 5403-fold purified sample prepared using adenosine agarose affinity chromatography (Table III) was subjected to SDS-PAGE. The sample (approximately 10.6 μg) was treated with sample solubilizing buffer, electrophoresed and the gel silver-stained (lane 1) or photoaffinity cross-linked to [^{3}H]SAM, treated with sample solubilizing buffer, electrophoresed, the gel stained with Coomassie and the dried gel exposed to X-ray film (lane 2). The MW corresponding to protein standards is indicated on right. The band corresponding to PEAMeT (see Results) is denoted by the arrow.

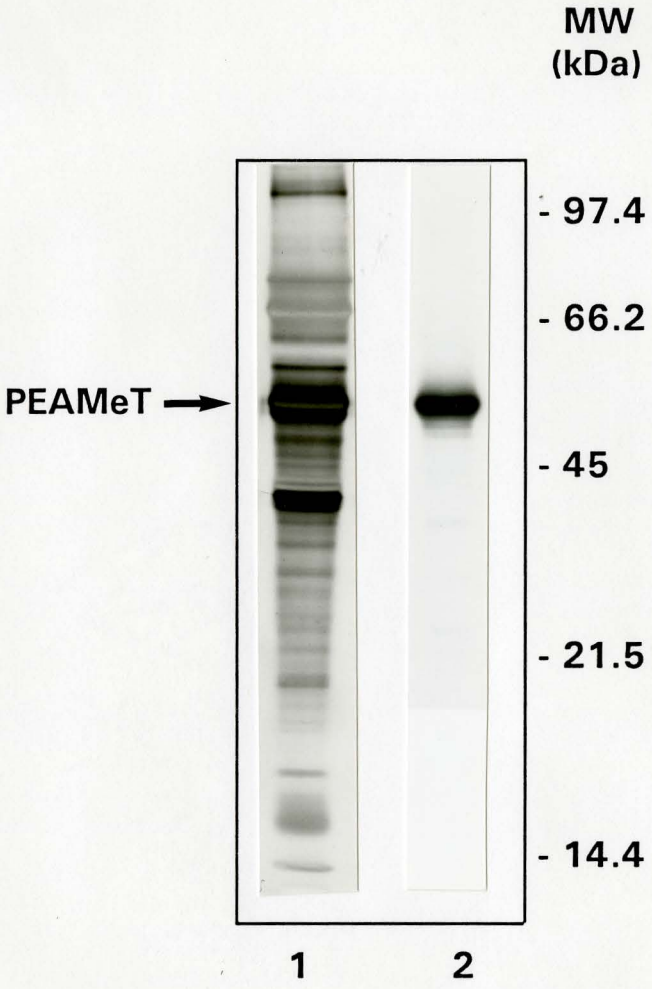

MW
(kDa)
- 97.4
- 66.2
PEAMeT
- 45
- 21.5
- 14.4
1
2

FIGURE 13. ***Estimation of PEAMeT Molecular Weight From a Fluorograph of a Coomassie Stained Gel Containing [^{3}H]SAM Photoaffinity Cross-Linked Proteins.***

This standard curve was constructed using the R_f of protein standards (●) from a SDS-polyacrylamide gel stained with Coomassie, dried and exposed to X-ray film. Regression analysis of the standard curve yielded the equation $y = -0.71x + 3.84$ and a fit of $r^2 = 0.986$. This standard curve was used to determine the estimated MW of [^{3}H]SAM photoaffinity cross-linked PEAMeT from a 5403-fold purified adenosine agarose preparation (Table III). This standard curve predicted a MW of 57 kDa for [^{3}H]SAM photoaffinity cross-linked PEAMeT (◇).

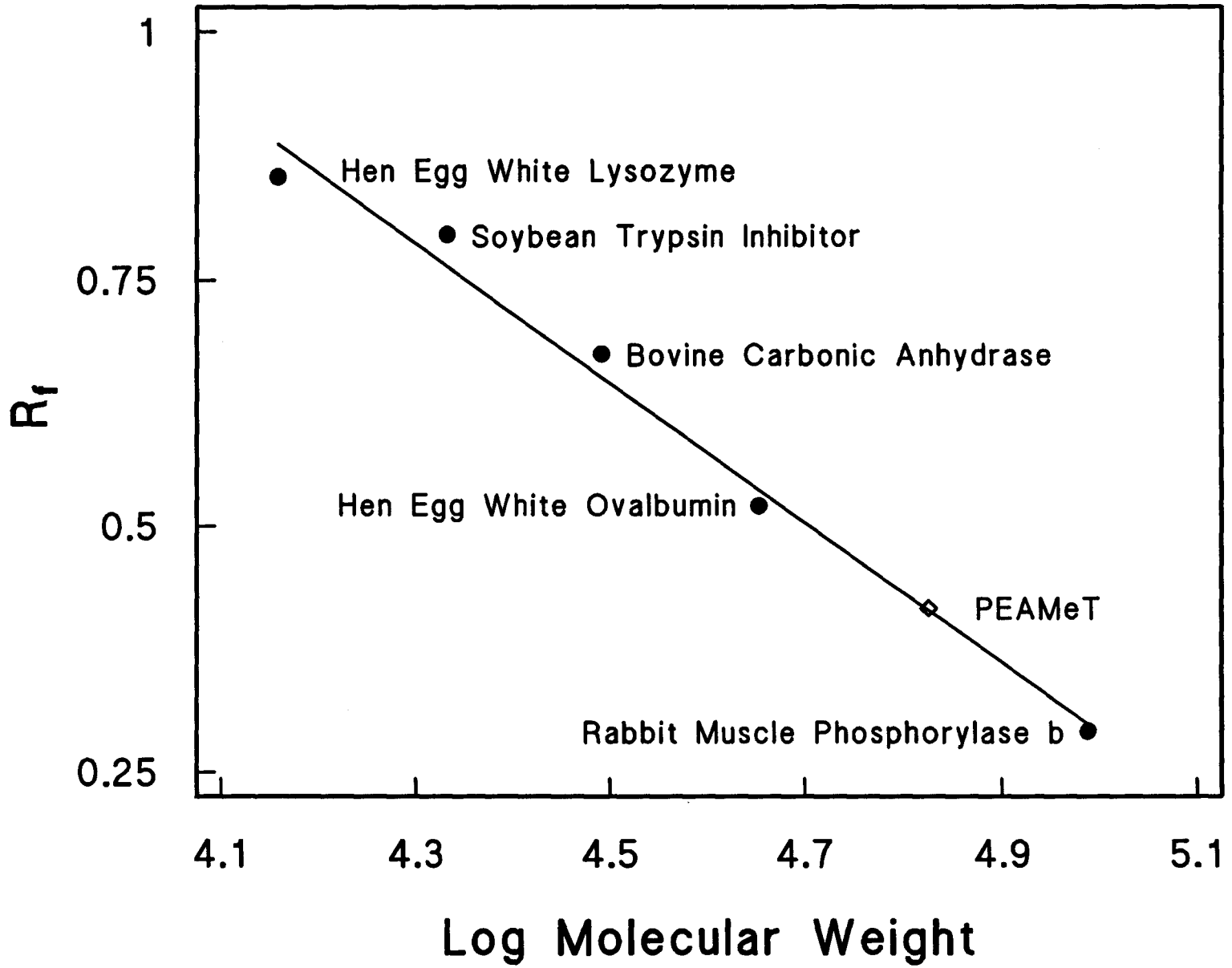
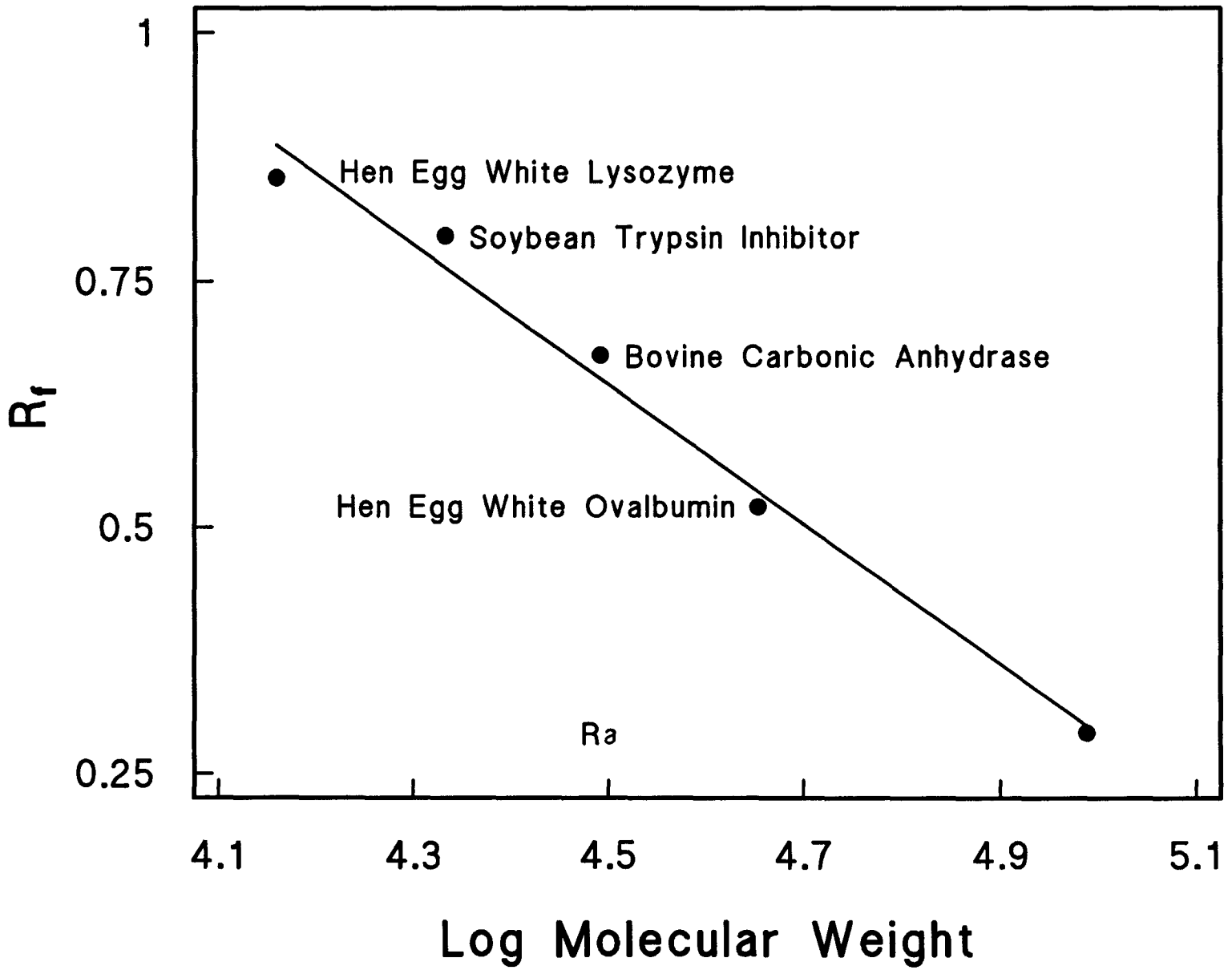

1
0.75
0.5
0.25
R_f
Hen Egg White Lysozyme
Soybean Trypsin Inhibitor
Bovine Carbonic Anhydrase
Hen Egg White Ovalbumin
PEAMeT
Rabbit Muscle Phosphorylase b
4.1
4.3
4.5
4.7
4.9
5.1
Log Molecular Weight

FIGURE 14. *Estimation of PEAMeT Molecular Weight From a Silver-Stained SDS-Polyacrylamide Gel.*

This standard curve was constructed using the R_f of protein standards (●) run on a SDS-polyacrylamide gel stained with silver reagent. Regression analysis of the standard curve yielded the equation $y = -0.67x + 3.62$ and a fit of $r^2 = 0.988$. This standard curve was used to determine the estimated MW of PEAMeT from a 5403-fold purified adenosine agarose preparation (Table III). This standard curve predicted a MW of 54 kDa for PEAMeT (◇).


1
0.75
0.5
0.25
R_f
Hen Egg White Lysozyme
Soybean Trypsin Inhibitor
Bovine Carbonic Anhydrase
Hen Egg White Ovalbumin
Ra
4.1
4.3
4.5
4.7
4.9
5.1
Log Molecular Weight

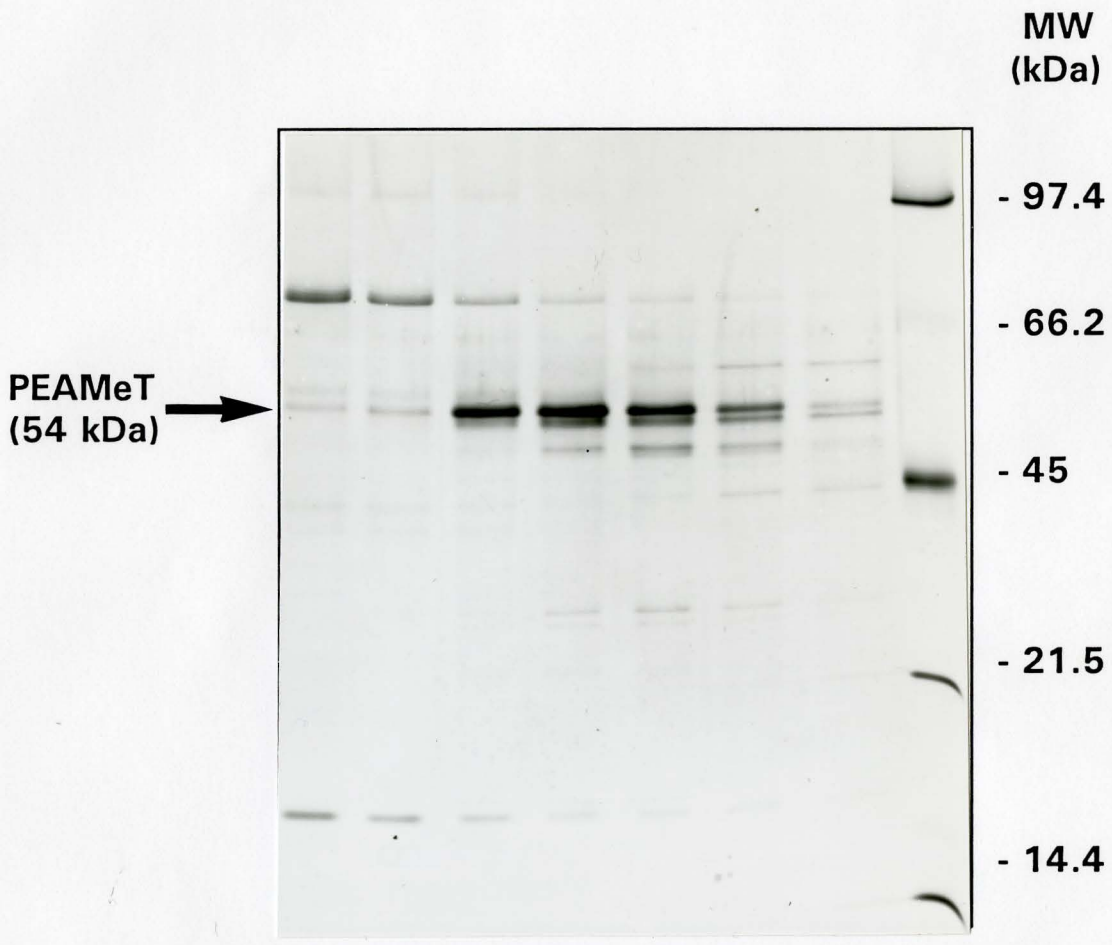
enzyme activity measurements in the presence of SAM and UV-light, [^{3}H]SAM photoaffinity cross-linking fluorographs and silver-stained products of the most highly purified PEAMeT preparation all strongly implicate a 54 kDa (57 kDa for [^{3}H]SAM photoaffinity cross-linked PEAMeT) polypeptide as being a SAM-binding component of PEAMeT. This does not , however, preclude the presence of other component subunit(s) for the PEAMeT native enzyme.

Gel Filtration Chromatography

Figure 15 shows a silver-stained SDS-polyacrylamide gel of aliquots from fractions containing PEAMeT activity eluted by gel filtration chromatography. The sample used was a 409-fold purified adenosine agarose preparation (Table VI). The intensity of the band indicated by the arrow was the only band which correlated with the rise and fall of PEAMeT activity determined for the fractions eluting from this column. Two other prominent bands migrate to positions below this band but the changes in intensity with which they stained with the silver reagent do not correspond to changes in PEAMeT activity. Indeed, no other bands in these lanes show comparable changes in staining intensity with PEAMeT activity. These observations are consistent with PEAMeT being composed of a 54 kDa polypeptide, in agreement with the photoaffinity cross-linking and silver-staining studies.

FIGURE 15. *SDS-PAGE Analysis of Fractions Containing PEAMeT Activity Obtained by Gel Filtration Chromatography.*

A 409-fold purified adenosine agarose preparation (Table VI) was subjected to HPLC-gel filtration chromatography. A 20-μL volume from each fraction in which PEAMeT activity was detected was loaded onto an SDS-polyacrylamide gel, electrophoresed and then stained with silver reagent. The total PEAMeT activity (pmol$\cdot$min^{-1}) loaded into each lane of the gel is given. The position of the MW standards is indicated to the right of the gel. The position of the band corresponding to PEAMeT (see Results) is denoted by the arrow.



Lane #	1	2	3	4	5	6	7	8
PEAMeT Activity ($pmol \cdot min^{-1}$)	36	50	222	269	180	120	48	-

NATIVE MOLECULAR WEIGHT OF PEAMeT

The native MW of PEAMeT was determined using a comparable gel filtration procedure as described above. For this analysis, the elution volume (V_e) was compared to the void volume (V_o) of samples relative to the V_e/V_o values of protein standards. The native MW of PEAMeT was estimated using standard curves generated from two separate runs of the protein standards. Also, for both of these runs, internal standards were included with the sample injected. When calculations were made to estimate the MW of the standard proteins based on V_e/V_o, the values for carbonic anhydrase (MW 29 kDa) and alcohol dehydrogenase (MW 150 kDa) came within 92 and 99% of their actual values (as reported by Sigma). Therefore, the estimate determined for PEAMeT native MW could deviate from the actual MW in the range of 1 to 8%, however this deviation may be larger if the migration of PEAMeT on this gel filtration column is anomolous. Analysis of a 82-fold purified hydroxylapatite preparation (Table V) provided an estimated native PEAMeT MW of 74.0 ± 1.2 kDa (Fig. 16) and that of 409-fold purified adenosine agarose preparation (Table VI) a value of 80.7 ± 2.8 kDa (standard curve not shown). Therefore the average estimated native MW from these two separate determinations is 77 ± 3 kDa.

ANALYSIS OF PHOSPHOBASE *N*-METHYLTRANSFERASE ACTIVITIES

Table X gives the ratios of PEAMeT:PMEAMeT:PDEAMeT activities, relative to PEAMeT activity, at each step in the purification of PEAMeT outlined on

FIGURE 16. ***Estimation of PEAMeT Native Molecular Weight by Gel Filtration Chromatography.***

This standard curve was constructed using the average V_e/V_o from two runs of protein standards (●) eluted from a gel filtration column. Regression analysis of the standard curve yielded the equation $y = -0.69x + 4.9$ and a fit of $r^2 = 0.995$. Standard error bars are not shown as they do not extend beyond the margins of the symbol(s) used. This standard curve was used to estimate the native MW of PEAMeT from a 82-fold purified hydroxylapatite preparation (Table V). This standard curve predicts a PEAMeT (◇) native MW of 74.0 ± 1.2 kDa.

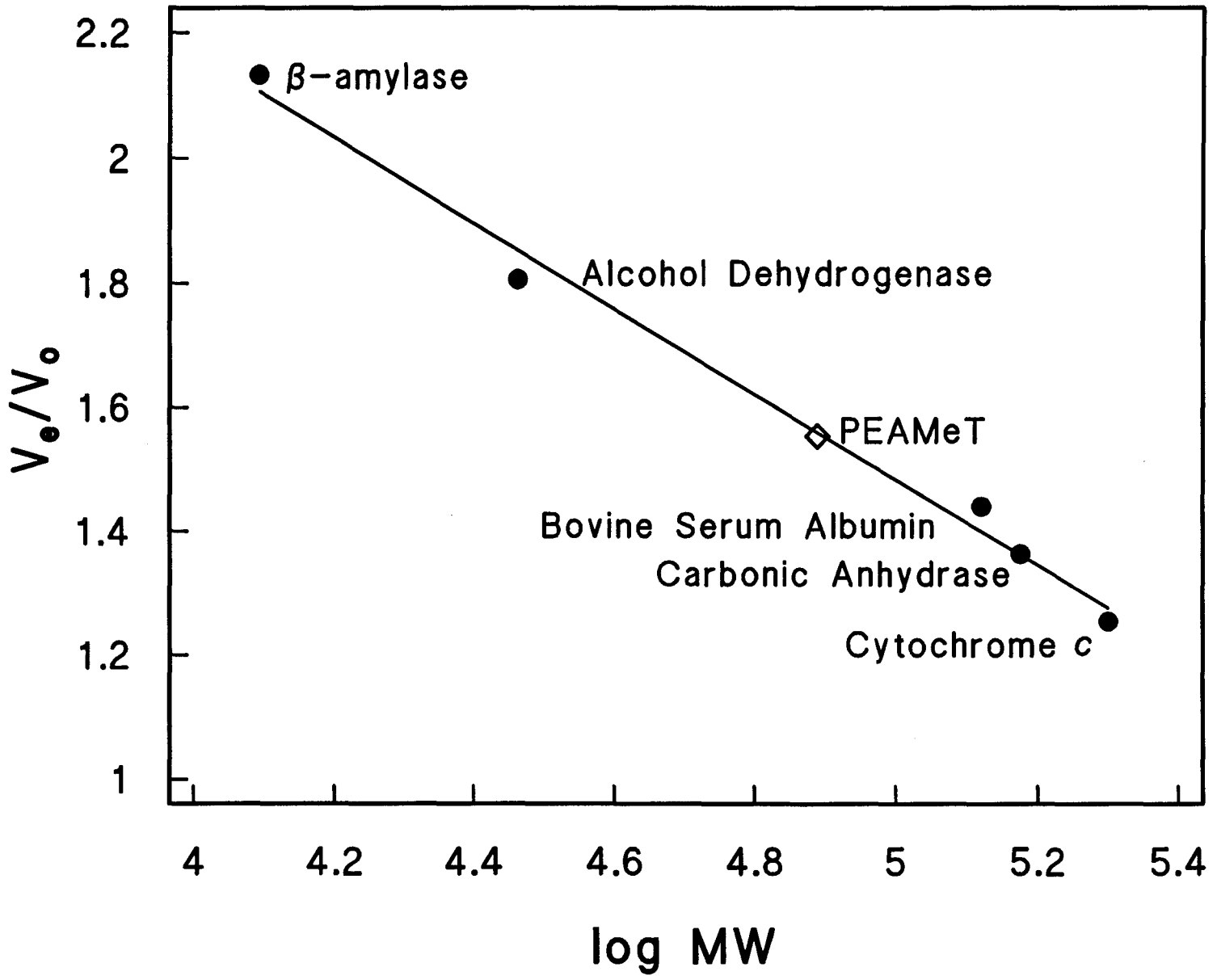

2.2
2
1.8
1.6
1.4
1.2
1
V_e/V_o
β−amylase
Alcohol Dehydrogenase
PEAMeT
Bovine Serum Albumin
Carbonic Anhydrase
Cytochrome c
4
4.2
4.4
4.6
4.8
5
5.2
5.4
log MW

TABLE X. *Ratios of Phosphobase N-Methyltransferase Activity From PEAMeT Purification Steps*

Purification Step	Ratio of Activities Relative to PEAMeT[a]		
	PEAMeT	PMEAMeT	PDEAMeT
Crude[b]	1	1.45 ± 0.04	1.24 ± 0.04
1.8 to 2.6 M Ammonium Sulphate Dialysed	1	1.32 ± 0.01	1.00 ± 0.02
DEAE Sepharose	1	2.31 ± 0.07	1.73 ± 0.01
Phenyl Sepharose	1	0.91 ± 0.02	0.72 ± 0.03
ω-Aminohexyl Agarose	1	0.92 ± 0.02	0.63 ± 0.06
Hydroxylapatite	1	1.05 ± 0.01	0.60 ± 0.02
Adenosine Agarose	1	0.91 ± 0.02	0.74 ± 0.01

[a] Duplicate measurements ± SE.
[b] Crude sample and purification steps given in Table VII

Table VII (purification #7). Initially, in the crude extract (1:1.45:1.24), the activities of all three phosphobase *N*-methyltransferases could be ranked (from highest to lowest *in vitro* activity): PMEAMeT > PDEAMeT > PEAMeT. PMEAMeT and PDEAMeT activities remained higher relative to PEAMeT activity until the chromatographic separation on phenyl Sepharose. For all purified samples after this step, except after the separation on hydroxylapatite shown in Table VII, PEAMeT activity was higher relative to PMEAMeT and PDEAMeT activities, yielding an average overall ratio of 1:0.95:0.67 relative to PEAMeT activity. This ratio was not specific to purification #7. Table XI shows the ratio of the phosphobase *N*-methyltransferase activities from the final purification step of each of the seven purifications (Tables I to VII). These preparations gave an average ratio of PEAMeT:PMEAMeT:PDEAMeT activity of 1:0.93:0.7. With the exception of purification #1 (Table I), PMEAMeT activity approaches the same level as PEAMeT activity, while PDEAMeT activity was approximately 70% of PEAMeT and PMEAMeT activities.

PEAMET ACTIVITY: BIOCHEMICAL CHARACTERIZATION

The recoveries of PEAMeT activity from the various preparations were low. Consequently, different sample preparations were used for various studies and the identity of the sample used is reported along with the findings.

TABLE XI. *Ratios of Phosphobase N-Methyltransferase Activity in Partially Purified PEAMeT Preparations*

Purification[a]	Final Purification Step[b]	Fold Pure	Ratio of Activities Relative to PEAMeT[c]		
			PEAMeT	PMEAMeT	PDEAMeT
1	P Sup	132	1	0.78 ± 0.04	0.57[d]
2	Ad Ag	451	1	0.93 ± 0.06	0.89 ± 0.04
3	Ad Ag	5403	1	0.87[d]	0.56[d]
4	Mono Q	778	1	0.91[d]	0.70[d]
5	Ad Ag	154	1	1.01 ± 0.01	0.68 ± 0.01
6	Ad Ag[e]	296	1	0.97 ± 0.07	0.78 ± 0.01
		409		1.02 ± 0.04	0.67 ± 0.05
7	Ad Ag	523	1	0.91 ± 0.02	0.74 ± 0.01
Average ± SE			1	0.93 ± 0.03	0.70 ± 0.04

[a] As shown in Tables I to VII.
[b] Mono Q = HPLC-Mono Q, P Sup = HPLC-phenyl Superose, Ad Ag = adenosine agarose
[c] Duplicate measurements ± SE.
[d] Single measurement.
[e] Two separate final partially purified samples (see Table VI).

ASSESSMENT OF ASSAY CONDITIONS

Phosphobase Phosphatase Activity

The presence of phosphobase phosphatase activity in any of the samples assayed for phosphobase *N*-methyltransferase activity would lead to an underestimate of the measured phosphobase *N*-methyltransferase activity. Specific or non-specific phosphatases present during the assay incubation could cleave phosphate from PEA leaving less substrate for the reaction. Phosphobase phosphatases could also cleave phosphate from the radiolabelled ([^{3}H]PMEA, [^{3}H]PDEA or [^{3}H]PCho) and non-radiolabelled phosphobase products (PMEA, PDEA or PCho) leaving radiolabelled ([^{3}H]MEA, [^{3}H]DEA and [^{3}H]Cho) and non-radiolabelled freebases (MEA, DEA and Cho). Phosphobase phosphatase activity was analyzed at each step in the purification of PEAMeT outlined on Table VII (purification #7) using PEA as the substrate for the phosphobase phosphatase reaction and using essentially the same components and conditions of the PEAMeT assay. Table XII shows that a significant amount of phosphobase phosphatase activity capable of dephosphorylating PEA was present in the crude leaf extract. These crude samples are therefore unsuitable for the biochemical characterization of PEAMeT. After separation on phenyl Sepharose, phosphobase phosphatase activity was no longer detected. A sample with no detectable phosphobase phosphatase activity was considered suitable for use in biochemical characterizations of PEAMeT. However, for future studies, the phosphobase phosphatase assays should be

TABLE XII. *Phosphobase Phosphatase Activity Associated With PEAMeT Purification Steps*

Purification Step	Phosphatase Activity[a] ($nmol \cdot min^{-1} \cdot mg^{-1}$protein)
Crude	8.30 ± 0.26
1.8 to 2.6 M Ammonium Sulphate Dialysed	0.01 ± 0
DEAE Sepharose	0.02 ± 0
Phenyl Sepharose	0
ω-Aminohexyl Agarose	0
Hydroxylapatite	0
Adenosine Agarose	0

[a] Duplicate measurements ± SE.

repeated using PMEA, PDEA and PCho as substrates in order to determine if any phosphatases able to cleave these metabolites are present in any of the preparations.

PEAMeT Activity and Protein Concentration

PEAMeT activity was assayed as a function of various dilutions using a sample prepared by adenosine agarose affinity chromatography (Table VII). This sample was purified 523-fold for PEAMeT activity. Figure 17 shows PEAMeT activity versus μg protein used per assay. This plot is non-linear at protein concentrations above 2 μg per assay. The PEAMeT activity at 2 μg per assay corresponds to roughly 2500 cpm$\cdot$mL^{-1} Dowex eluate or less than 60 pmol$\cdot$min^{-1} per assay. When counts present in the Dowex eluate exceeded 2,500 cpm$\cdot$mL^{-1}, samples containing PEAMeT activity were usually diluted to reduce counts to this range. For most purified preps, a value of 2 μg protein or less per assay usually provides acceptable cpm$\cdot$mL^{-1}.

PEAMeT Activity and Incubation Time

PEAMeT activity was measured after various lengths of PEAMeT assay incubation time for a sample prepared by adenosine agarose affinity chromatography (Table VI). This sample is 409-fold purified for PEAMeT. Figure 18 shows PEAMeT activity versus time is linear up to 30 min after which the slope decreased and PEAMeT activity was non-linear with time. The

FIGURE 17. *PEAMeT Activity as a Function of Protein Concentration.*

A 523-fold purified sample (Table VII) was serially diluted and variable quantities of protein used in the PEAMeT standard assay. This experiment was repeated twice with similar results. The data shown are the means of duplicate PEAMeT activity measurements. The solid line ($y = 30.45x + 3.21$, $r^2 = 0.968$) represents a line of best fit through data points within the linear range for the PEAMeT assay. Standard error bars are not shown as they do not extend beyond the margins of the symbol(s) used.

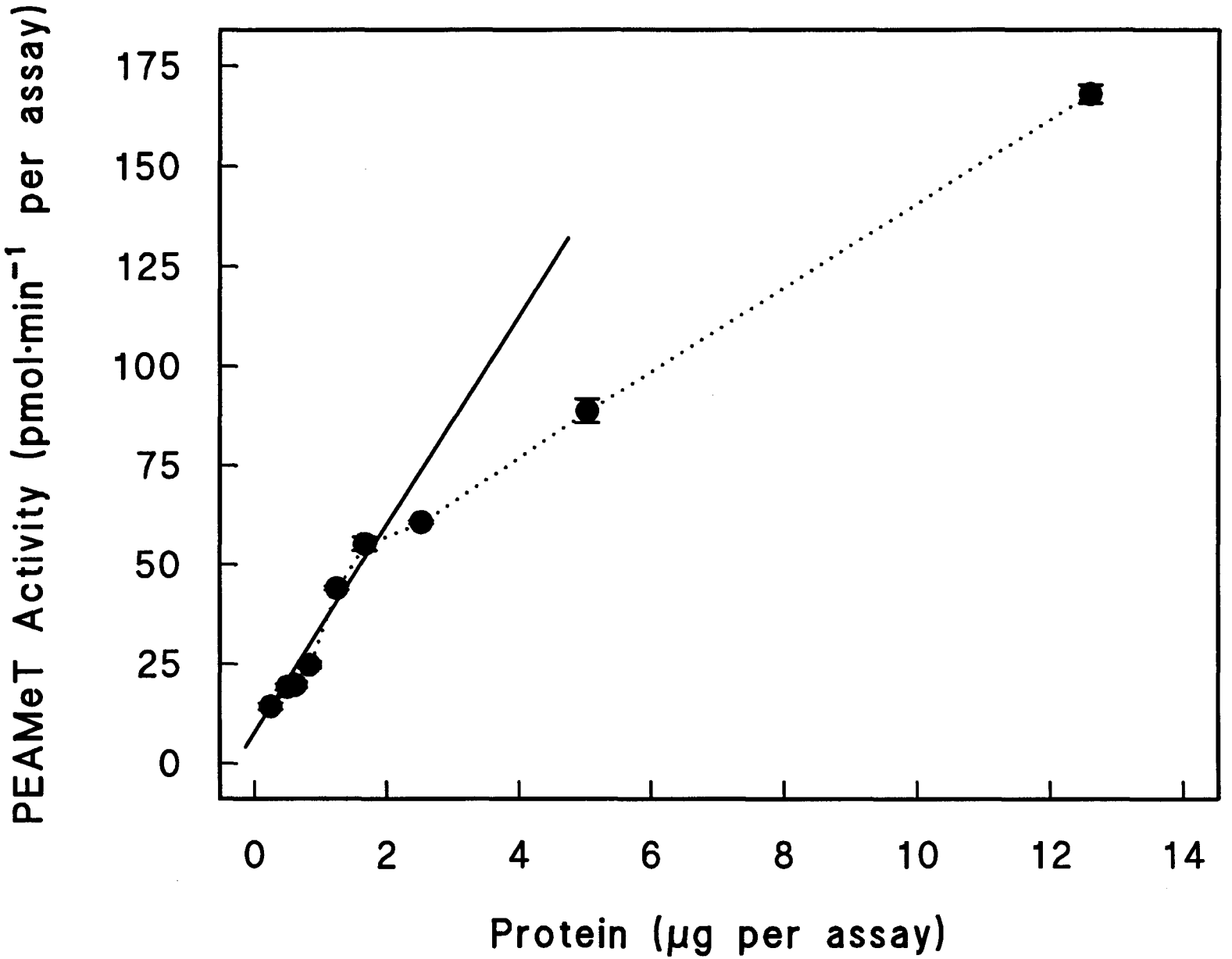
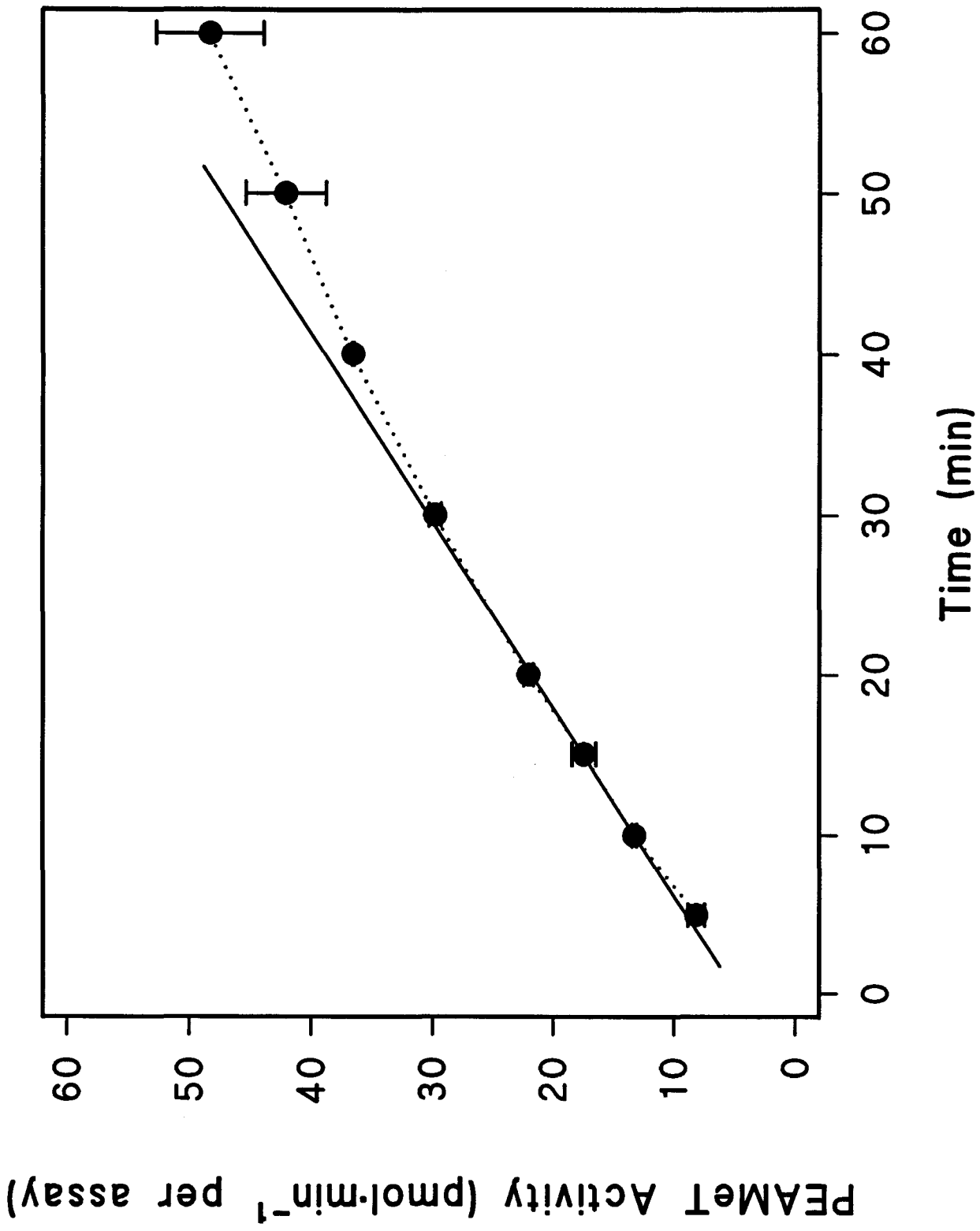

PEAMeT Activity (pmol·min⁻¹ per assay)
175
150
125
100
75
50
25
0
0
2
4
6
8
10
12
14
Protein (µg per assay)

FIGURE 18. *PEAMeT Activity as a Function of Assay Incubation Time*

A 409-fold purified preparation (Table VI) was used in the PEAMeT standard assay where incubated at 30 °C for various lengths of time. This experiment was repeated twice with similar results. The data are the means of duplicate PEAMeT activity measurements. The line of best fit through data points from 0 to 30 min generates the equation $y = 0.86x + 4.34$; $r^2 = 0.997$.


PEAMeT Activity (pmol·min⁻¹ per assay)
0
10
20
30
40
50
60
0
10
20
30
40
50
60
Time (min)

line of best fit representing the first five data points which are linear with time (0 to 30 min) has a slope of 0.86 pmol • min^{-1} per assay. For all PEAMeTassays reported in this thesis with the exception of this experiment, the assay incubation period was 30 min.

pH Profile Using Overlapping pH's

PEAMeT activity as a function of assay pH is given in Figure 19. This profile shows a unimodal peak of activity with an apparent sharp pH optimum at 7.8 using Hepes-KOH buffer. PEAMeT activity was not detected in PEAMeT assays buffered at a pH of less than 6.5 or greater than 9.5. Phosphate buffers were not used as they proved inhibitory towards PEAMeT activity (P.S. Summers, unpublished results).

EFFECT OF METABOLITES AND CATIONS ON PEAMeT ACTIVITY

Metabolites

The effect of various metabolites were tested for their effect on *in vitro* PEAMeT activity (recall pathway in Fig. 1). These results are summarized in Tables XIII and XIV. It should be noted that the pH at the beginning and end of the PEAMeT assay was not altered by the addition of any of the metabolites tested. For Table XIII, the concentrations of the metabolites tested were either equivalent to the concentration of PEA (0.25 mM) in the PEAMeT assay or a multiple thereof. EA, MEA, DEA, choline and betaine at 0.5 or 1 mM per assay

FIGURE 19. *PEAMeT Activity as a Function of pH.*

A series of buffers of overlapping pH were used to assay PEAMeT activity from a 778-fold purified preparation (Table IV). All buffers were used at a final assay concentration of 100 mM. Data shown are means of duplicate PEAMeT activity measurements. This experiment was repeated twice with similar results.

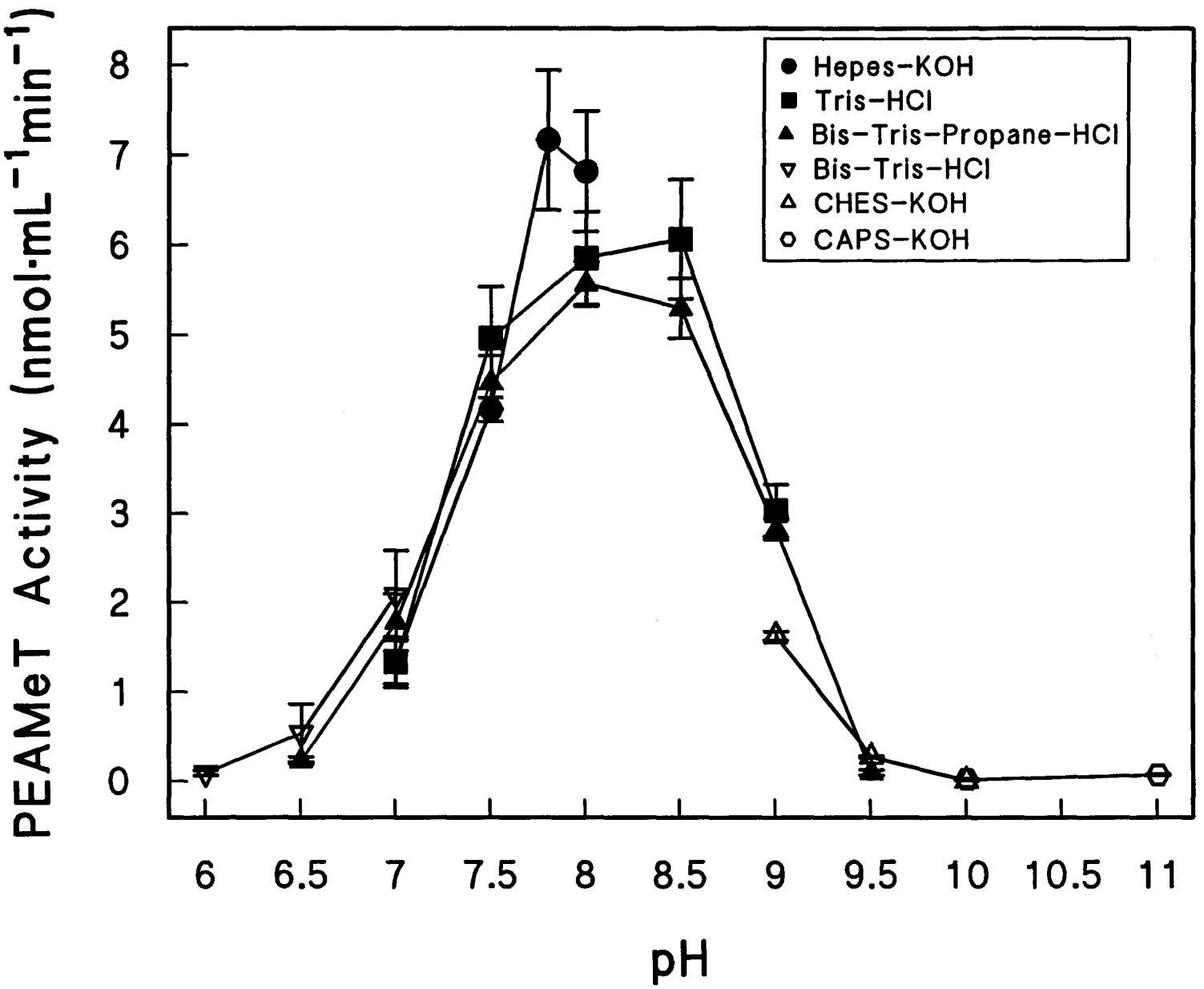

PEAMeT Activity (nmol·mL⁻¹min⁻¹)
0
1
2
3
4
5
6
7
8
6
6.5
7
7.5
8
8.5
9
9.5
10
10.5
11
pH
Hepes-KOH
Tris-HCl
Bis-Tris-Propane-HCl
Bis-Tris-HCl
CHES-KOH
CAPS-KOH

TABLE XIII. *Effect of Metabolites on PEAMeT Activity*

Metabolite[a]	Concentration[b] (mM)	PEAMeT Activity (% Control)[c]
EA	0.5	90 ± 1
	1	92 ± 3
MEA	0.5	102 ± 4
	1	103 ± 3
DEA	0.5	93 ± 6
	1	97 ± 2
Choline	0.5	99 ± 3
	1	93 ± 7
Betaine	0.5	96 ± 13
	1	88 ± 1
PCho (Na-salt)	0.125	69 ± 4
	0.25	61 ± 5
	0.5	45[d]
	1	48[d]
Phosphate (NaH_2PO_4)	0.25	76 ± 3
	0.5	80 ± 3
	5	63 ± 3
	10	54 ± 3

[a] Assayed using 409- or 778-fold purified preparations with control activities between 43 to 80 $nmol \cdot min^{-1} \cdot mg^{-1}$protein.
[b] Final concentration in assay.
[c] Duplicate measurements ± SE.
[d] Single measurement.

TABLE XIV. *Effect of SAH on PEAMeT Activity*

Concentration[a] (mM)	PEAMeT Activity (% Control)[bc]
0.01	53 ± 5
0.025	34 ± 2
0.1	20 ± 4
0.2	11 ± 0

[a] Final concentration in assay.
[b] Duplicate measurements ± SE.
[c] Assayed using a 409-fold purified preparation with control activity of 77.7 ± 3.0 nmol·min^{-1}·mg^{-1}protein

had no effect upon PEAMeT activity. PCho, however, at 0.125 mM reduced PEAMeT activity to 69% of control activity and a further 4-fold increase in PCho per assay reduced PEAMeT activity to 45% of control activity. Phosphate (NaH_2PO_4) at 0.25 mM reduced PEAMeT activity to 76% of control and a further 20-fold increase in phosphate per assay reduced PEAMeT activity to 63% of control. Since phosphate inhibits PEAMeT activity and choline does not, it is likely that the phosphate and not the choline moiety of PCho is involved in the inhibition of PEAMeT activity.

SAH was also tested for its effect on PEAMeT activity (Table XIV). SAH at a final assay concentration of 10 μM reduced *in vitro* PEAMeT activity by 50%. It is important to emphasize that this PEAMeT assay also contains 200 μM SAM. At an SAH concentration equal to 200 μM, only 11% of the level of control PEAMeT activity was detected.

Cations

Various divalent metal cations were tested for their effect on *in vitro* PEAMeT activity. All PEAMeT assays containing metal ions were performed using PEAMeT assay buffer that did not include Na_2-EDTA due to its chelating effect on metal ions. PEAMeT activity in the absence of EDTA was not significantly different from PEAMeT activity in the presence of EDTA. To minimize counter ion effects, all of the divalent metal ions were added to the PEAMeT assay as Cl^- salts. No inhibition of PEAMeT activity was found by

additions of NaCl or $MgCl_2$ at the concentrations tested (Table XV). Some inhibitory effects were found with the remaining cations tested and the degree of inhibition ranked (from lowest to highest) $Ca^{2+} < Mn^{2+} < Co^{2+}$ for all the concentrations tested (Table XV). These findings suggest that inclusion of EDTA in assays as a contaminant heavy-metal chelator is potentially a valuable precautionary measure for maximizing *in vitro* PEAMeT activity.

THIN LAYER CHROMATOGRAPHY OF PHOSPHOBASES PRODUCED FROM THE PEAMeT ASSAY

Our measurement of PEAMeT activity is based upon the premise that PEAMeT transfers a [^{3}H]methyl group from SAM to PEA, producing [^{3}H]PMEA and SAH as products. Since all studies to date have been done using crude preparations containing PEAMeT, PMEAMeT and PDEAMeT activities, we could not establish whether or not PEAMeT could catalyze more than just the first *N*-methyl transfer. Consequently, a preparation enriched between 22- and 38-fold for PEAMeT activity was selected to identify and quantify the phosphobase *N*-methyltransferase reaction products. Aside from being partially purified, this preparation also exhibited the PEAMeT:PMEAMeT:PDEAMeT ratio of 1:0.92:0.63, a ratio characteristic of the most pure preparations (Table XI). In order to determine if PEAMeT could also transfer a [^{3}H]methyl group from SAM to both PMEA and PDEA producing [^{3}H]PDEA, [^{3}H]PCho and SAH as products, the [^{3}H]phosphobases produced by PEAMeT during the PEAMeT assay were

TABLE XV. *Effects of Divalent Metal Ions on PEAMeT Activity*

Ion[a]	PEAMeT Activity (% of Control)[b]
CONTROL[c]	100
+ 1 mM NaCl	105 ± 1
+ 10 mM NaCl	105 ± 1
+ 1 mM $MgCl_2$	102 ± 2
+ 10 mM $MgCl_2$	96 ± 2
+ 1 mM $CaCl_2$	93 ± 0.3
+ 10 mM $CaCl_2$	73 ± 5
+ 1 mM $MnCl_2$	74 ± 8
+ 10 mM $MnCl_2$	54 ± 1
+ 1 mM $CoCl_2$	0
+ 10 mM $CoCl_2$	0

[a] Concentrations listed are the final concentrations used in assay.

[b] Duplicates measurements ± SE.

[c] All assays were performed using a 778-fold purified preparation with a control activity of 71.3 ± 3 nmol·min^{-1}·mg^{-1}protein without the addition of 1 mM Na_2-EDTA to the assay mixture. PEAMeT activity in assays with the addition of 1 mM Na_2-EDTA were 95 ± 7% of the PEAMeT activity in the assays without.

eluted from a Dowex 50 W H^+ cation exchange column using 0.1 N HCl, the eluate was concentrated and analyzed by TLC. Identification of products was made by using non-radioactive phosphobase standards (PEA, PMEA, PDEA and PCho) as described in the Materials and Methods. Identification of the purple spots on the TLC plate corresponding to the [^{3}H]phosphobases showed that [^{3}H]PMEA, [^{3}H]PDEA and [^{3}H]PCho were all produced during the 30 min PEAMeT assay period with only PEA present as a phosphobase substrate. Once identified, the radiolabelled spots were scraped from the TLC plate and the radioactivity was counted (Table XVI). The amount of each [^{3}H]phosphobase produced in the PEAMeT assay is proportional to the radioactivity (cpm) present in each spot on the TLC plate corresponding to the specific [^{3}H]phosphobase. For quantification, the cumulative amount of [^{3}H]PMEA produced in the PEAMeT assay includes the [^{3}H]PMEA scraped from the PMEA spot, half of the [^{3}H]PDEA scraped from the PDEA spot and one-third of the [^{3}H]PCho scraped from the PCho spot, the cumulative amount of [^{3}H]PDEA produced in the PEAMeT assay includes the [^{3}H]PDEA scraped from the PDEA spot and one-third [^{3}H]PCho scraped from the PCho spot while the cumulative amount of [^{3}H]PCho produced in the PEAMeT assay only includes the [^{3}H]PCho scraped from the PCho spot (Audubert and Vance, 1983). Using these considerations, PEAMeT assay products can be divided into 77% [^{3}H]PMEA, 17% [^{3}H]PDEA and 6% [^{3}H]PCho, when PEA and no other phosphobase is supplied as the substrate (Table XVI).

TABLE XVI. *Detection of Phosphobases Produced In PEAMeT Assay By Thin Layer Chromatography*

Phosphobase Product	Amount of [^{3}H]Phosphobase Produced (cpm)[a]	Amount of [^{3}H]Phosphobase Produced (% Total)
PMEA	6327 ± 272	77 ± 3
PDEA	1412 ± 6	17 ± 0.1
PCho	483 ± 27	6 ± 0.3
Total[b]	8221 ± 272	100

[a] cpm in spot scraped from silica plate. Average of [^{3}H]phosphobase sample and [^{3}H]phosphobase sample plus standards ± SE.

[b] Total amount of radioactivity (cpm) recovered from TLC scrapings. Total amount of radioactivity (cpm) in [^{3}H]phosphobase sample loaded onto TLC plate was estimated to be 5880 cpm.

DISCUSSION

PHOSPHOBASE *N*-METHYLTRANSFERASE ACTIVITIES

The number of phosphobase *N*-methyltransferases needed to catalyse the reaction sequence, PEA → PMEA → PDEA → PCho is uncertain. For simplicity, the enzymes reported to catalyse the sequence PEA → PMEA → PDEA → PCho have been designated PEAMeT (PEA → PMEA), PMEAMeT (PMEA → PDEA) and PDEAMeT (PDEA → PCho).

Even though all three *N*-methylating activities have been detected in spinach leaves (Weretilnyk and Summers, 1992), it is not known whether one, two or three phosphobase *N*-methyltransferases are responsible. These phosphobase *N*-methyltransferase activities have been compared between spinach roots and leaves. Negligible PEAMeT activity is detected in spinach roots, whereas PMEAMeT and PDEAMeT activities can be detected in both spinach leaves and roots (Weretilnyk and Summers, 1992). This indicates that PEAMeT (PEA → PMEA), is distinct from the enzymes PMEAMeT (PMEA → PDEA) and PDEAMeT (PDEA → PCho). These authors reported that PMEAMeT:PDEAMeT activities in leaves and roots of spinach were present in a ratio approximating 1:1, indicating that one enzyme may catalyse both reactions.

A comparision of spinach leaf extracts from unsalinized plants and plants exposed to 200 mM NaCl showed that in salinized plants, PEAMeT activity is up-regulated 2.9-fold whereas PMEAMeT and PDEAMeT activities are only up-regulated 1.7- and 1.5-fold, respectively (Weretilnyk and Summers, 1992). This difference in fold-induction of activity also indicates that PEAMeT (PEA → PMEA), is distinct from the enzymes PMEAMeT (PMEA → PDEA) and PDEAMeT (PDEA → PCho) and since PMEAMeT and PDEAMeT activities are up-regulated by a similar amount, one enzyme may catalyse both reactions.

PEAMeT activity was found to be diurnally regulated, in that, PEAMeT activity is highest at the end of an 8 h day and lowest at the end of a 16 h night, whereas PMEAMeT and PDEAMeT activities do not change over the same period (Weretilnyk *et al.*, in press). This difference in regulation of PEAMeT versus PMEAMeT and PDEAMeT activities shows that PEAMeT (PEA → PMEA), is distinct from the enzymes PMEAMeT (PMEA → PDEA) and PDEAMeT (PDEA → PCho).

Additional evidence in support of a two *N*-methyltransferase pathway comes from studies of betaine and PtdCho biosynthesis in various plant species. The reaction PEA → PMEA is conserved in *Lemna paucicostata* (Mudd and Datko, 1986), soybean and carrot (Datko and Mudd, 1988ab), barley (Hitz *et al.*, 1981), sugarbeet (Hanson and Rhodes, 1983) and spinach (Weretilnyk and Summers, 1992; Summers and Weretilnyk, 1993). The remaining two *N*-methyl transfers can occur at the phosphobase level in *Lemna paucicostata*

(Mudd and Datko, 1986), carrot (Datko and Mudd, 1988ab), sugarbeet (Hanson and Rhodes, 1983) and spinach (Weretilnyk and Summers, 1992; Summers and Weretilnyk, 1993) or at the phosphatidylbase level in soybean and carrot (Datko and Mudd, 1988ab) and barley (Hitz *et al.*, 1981). Taken together, the information presented in this section shows that PEAMeT (PEA → PMEA) is distinct from PMEAMeT (PMEA → PDEA) and PDEAMeT (PDEA → PCho) enzymes and the reaction sequence, PMEA → PDEA → PCho, may be catalysed by a single enzyme.

Evidence which suggests that PEAMeT (PEA → PMEA) can also catalyse the reaction PMEA → PDEA, in spinach, comes from analysis using DEAE Sepharose chromatography. This data is assembled, in part, from work done towards the purification of PEAMeT as part of this thesis (Fig. 3) and as part of work recently started by Ms. Sharon Dhadialla on the purification of enzyme(s) exhibiting PMEAMeT and PDEAMeT activities. These chromatographic separations (compiled for comparison in Fig. 20), were completed using 1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed samples from leaves of spinach plants exposed to prolonged light or dark periods prior to harvest. The significance of these light/dark treatments relates to the phosphobase *N*-methyltransferase activities observed in extracts of these tissues (Weretilnyk *et al.*, in press). Specifically, extracts from light-exposed plants ('light') catalyse PEA → PMEA → PDEA → PCho but those from dark-adapted plants ('dark') only catalyse PMEA → PDEA → PCho.

FIGURE 20. ***DEAE Sepharose Chromatography Analysis of Phosphobase N-Methyltransferase Activities of Samples from Light-Exposed and Dark-Adapted Spinach.***

PEAMeT (PEA → PMEA) and PMEAMeT (PMEA → PDEA) activities were measured among selected fractions eluted from two separate DEAE Sepharose columns packed, equilibrated and eluted under identical conditions. Samples loaded onto the column were 1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed samples of extracts from spinach plants exposed to 24 h light ('light') or 48 h dark ('dark') prior to harvest. A linear 0 to 300 mM NaCl gradient was used to elute adsorbed proteins from the column in both cases. The elution profiles of 'light' PEAMeT (○), 'light' PMEAMeT (△) and 'dark' PMEAMeT (▲) are shown in the figure. PEAMeT and PMEAMeT activities are shown as $nmol \cdot mL^{-1} \cdot min^{-1}$ for selected fractions eluted from the DEAE Sepharose column.

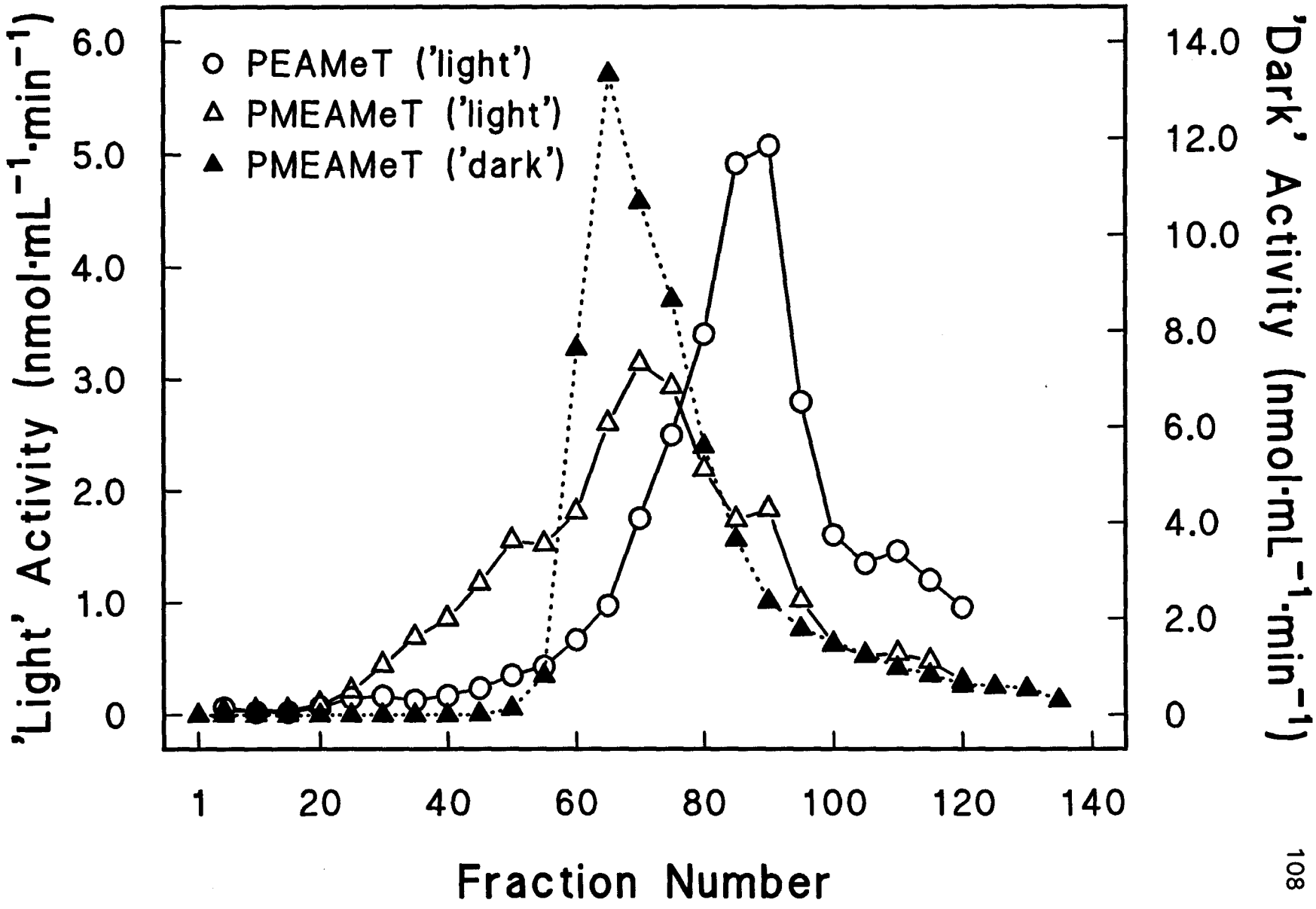

PEAMeT ('light')
PMEAMeT ('light')
PMEAMeT ('dark')
'Light' Activity (nmol·mL⁻¹·min⁻¹)
'Dark' Activity (nmol·mL⁻¹·min⁻¹)
Fraction Number
0
1.0
2.0
3.0
4.0
5.0
6.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
1
20
40
60
80
100
120
140

Figure 20 shows the elution profile for 'light' PEAMeT (PEA → PMEA) and 'light' PMEAMeT (PMEA → PDEA) activities and compares it to that for 'dark' PMEAMeT (PMEA → PDEA) activity. The peak of 'light' PEAMeT activity elutes after the most prominent peak corresponding to 'light' PMEAMeT or 'dark' PMEAMeT activities, showing that PEAMeT is a different enzyme than PMEAMeT. However, 'light' PMEAMeT activity elutes with a bimodal profile, where the first peak of activity eluted from the column is higher than the second peak. This profile suggests that two enzymes possessing PMEAMeT activity are present in extracts from light-exposed plants. Since the smaller peak of 'light' PMEAMeT activity corresponds to fractions containing PEAMeT activity, PEAMeT may catalyse the reaction PMEA → PDEA in addition to PEA → PMEA. An elution profile of PDEAMeT activity from light exposed tissue was not done, but this would be useful to determine the number of peaks corresponding to PDEA → PCho activity.

The analysis using DEAE Sepharose suggests that PEAMeT catalyses the reaction sequence PEA → PMEA → PDEA, however, evidence that PEAMeT may catalyse all three *N*-methylations (PEA → PMEA → PDEA → PCho) is demonstrated by comparing the ratios of PEAMeT:PMEAMeT:PDEAMeT activities in crude extracts with those of partially purified extracts. The ratios of PEAMeT:PMEAMeT:PDEAMeT activity (Table X), for crude (1:1.45:1.24), 1.8 to 2.6 M $(NH_4)_2SO_4$ dialysed (1:1.32:1) and DEAE Sepharose preparations (1:2.31:1.73) show that PMEAMeT > PDEAMeT > PEAMeT activity. The

ratio of PEAMeT:PMEAMeT:PDEAMeT activity remains relatively constant after the phenyl Sepharose step (1:0.95:0.67), and changes toward favouring PEAMeT activity, where PEAMeT > PMEAMeT > PDEAMeT activity. This change in ratio of activity indicates a reduction of PMEAMeT (PMEA → PDEA) and PDEAMeT (PDEA → PCho) activities (Tables X and XI). This constant ratio also indicates that the most highly purified PEAMeT preparations contain PEAMeT (PEA → PMEA), PMEAMeT (PMEA → PDEA) and PDEAMeT (PDEA → PCho) activities and is best explained if a single enzyme, PEAMeT, catalyses all three *N*-methylation reactions. However, since these preparations are not homogeneous for PEAMeT, these results cannot completely rule out the presence of PMEAMeT (PMEA → PDEA) or PDEAMeT (PDEA → PCho). However, such a contribution is highly unlikely since PMEAMeT or a putative PDEAMeT would have to co-purify with PEAMeT at every purification step following phenyl Sepharose.

If PEAMeT can catalyse all three *N*-methyl transfers, PMEA, PDEA and PCho should be detected as products of PEAMeT assays with PEA as the only substrate. Hydroxylapatite purified preparations with a PEAMeT:PMEAMeT:PDEAMeT activity ratio of approximately 1:0.95:0.67 (Table X) were used because this ratio reflects that given by preparations enriched for PEAMeT and those which are likely depleted of the other phosphobase *N*-methyltransferase(s). Using PEA as the only substrate, [^{3}H]PMEA (77%), [^{3}H]PDEA (17%) and [^{3}H]PCho (6%) were all generated

during the PEAMeT assay (Table XVI). This suggests that PEAMeT can catalyse the reaction sequence PEA → PMEA → PDEA → PCho.

To date, phosphobase *N*-methyltransferases have never been purified or characterized from any organism. The partial purification and preliminary characterization of an enzyme which catalyses the reaction PDEA → PCho has been reported for rat brain cytosol. This study indicates that at least two enzymes catalyse the three phosphobase *N*-methylations in this tissue (Andriamampandry *et al.*, 1992).

Other, somewhat similar *N*-methyltransferases which have been studied are the phosphatidylbase *N*-methyltransferases. Like the phosphobase *N*-methyltransferases, these phosphatidylbase *N*-methyltransferases *N*-methylate the PEA, PMEA or PDEA moiety of the phosphatidylbase using SAM as a methyl donating substrate. However, unlike the soluble, phosphobase *N*-methyltransferases, these phosphatidylbase *N*-methyltransferases are membrane associated and generate PtdCho and not PCho. In *Drosophila melanogaster* (De Sousa *et al.*, 1988) and rat liver (Ridgway and Vance, 1987), PtdCho is synthesized by phosphatidylbase intermediates and only one enzyme is responsible for all three phosphatidylbase *N*-methylations. In *Neurospora crassa* and *Saccharomyces cerevisiae*, PtdCho is synthesized by phosphatidylbase intermediates using two enzymes, PtdEA *N*-methyltransferase (PtdEA → PtdMEA) and PtdMEA *N*-methyltransferase (PtdMEA → PtdDEA → PtdCho) (Yamashita *et al.*, 1982).

PEAMeT PURIFICATION

For the purpose of this thesis, the presence of PEAMeT during all stages of purification was determined by the assay of PEA → PMEA. This was done since it was the sole catalytic feature by which we could distinguish PEAMeT from PMEAMeT. The phosphobase substrate specificities were only evaluated as a routine measure and not used as a criterion for purification. Soluble protein extracted from spinach leaves was subjected to a variety of different separation procedures in order to purify the enzyme responsible for PEAMeT activity from the remainder of the proteins present (Tables I to VII). Each crude leaf extract was $(NH_4)_2SO_4$ fractionated and subjected to a series of chromatographic matrices, often representing different protein binding properties, to determine a purification scheme which would result in the purification of PEAMeT. The types of chromatographic separations used included anion exchange chromatography, hydrophobic interaction chromatography, hydroxylapatite chromatography and affinity chromatography. Of these various matrices, the separation using the hydrophobic interaction with phenyl Sepharose and the affinity step on adenosine agarose, resulted in the highest fold-improvement of specific activity over the samples which were loaded onto the columns (Tables I to VII). Hydroxylapatite chromatography was also an effective purification step since it resulted in the loss of many contaminating acidic and basic proteins (Gorbunoff, 1985) which may not have separated from PEAMeT using any of the other chromatographic matrices (Tables I to VII). However, despite

all of these different combinations of protein separation methods, one of which resulted in a 5403-fold purified preparation (Table III), PEAMeT was not purified to homogeneity (Fig. 12, lane 1). Of all of the strategies used, a 409-fold purified preparation subjected to gel filtration chromatography provided the least complex profile of polypeptides (Fig. 15) and was likely the most pure sample of PEAMeT obtained.

The lack of a homogeneous PEAMeT preparation after all of the different protein separation procedures suggests that PEAMeT shares many properties with the other proteins still present in the preparations. Also, if one considers the large quantities of spinach leaves harvested, the final concentrations of protein in the partially purified preparations (Table VIII) and the number of proteins still present in the partially purified preparations separated by SDS-PAGE (Figs. 12 and 15), PEAMeT is probably not an abundant protein. Specifically, the total amount of protein in the partially purified preparations, a component of which is PEAMeT, is less than 0.01% of the total soluble protein extracted in the crude extract (Tables I to VII). This amount is similar to the low amount (0.01%) predicted for the choline oxidation enzymes (Hanson *et al.*, 1986). Prior to obtaining the technique for passivation of the ultrafiltration equipment, the low amount of protein present in the preparations created difficulties during concentration and dialysis. A large proportion of PEAMeT in the preparation bound to the plastic of the ultrafiltration equipment, resulting in even lower recoveries of PEAMeT activity in the final concentrated and

dialysed samples. In summary, low abundance, poor sample recoveries and few distinctive biochemical properties provided obstacles to the purification of PEAMeT. Therefore, further progress to this goal will likely require different strategies for separation.

Future attempts to purify PEAMeT might exploit its binding properties to SAM under UV-light. Recall that PEAMeT activity is inhibited by 45% when PEAMeT is incubated with SAM and UV-light for 60 min and not when PEAMeT is incubated with SAM or UV-light alone (Table IX). This result demonstrates that PEAMeT can be inactivated under specific conditions, namely with its substrate SAM and UV-light. Taking this result into consideration, the sample purified 5403-fold (Table III) was photoaffinity cross-linked to [^{3}H]SAM and subjected to SDS-PAGE. Only one polypeptide was photoaffinity cross-linked to [^{3}H]SAM (Fig. 12, lane 2). The band at this position corresponds to one which is salt and light regulated (Smith, 1993) and to the single band which corresponds to PEAMeT activity upon analysis of a gel filtration profile (Fig. 15).

The covalent photo-labelling of this *N*-methyltransferase is not a unique property of PEAMeT. The analysis of photoaffinity cross-linked enzymes by SDS-PAGE has also been demonstrated for many other methyltransferases such as EcoRII methyltransferase (Som and Friedman, 1990) the *S. typhimurium* CheR methyltransferase (Subbaramaiah and Simms, 1992), rat guanidinoacetate methyltransferase (Takata and Fujioka, 1992), *E. coli* Dam

methylase (Wenzel *et al.*, 1991) and vaccinia virus mRNA (guanine-7-)methyltransferase (Higman and Niles, 1994). Som and Friedman (1991) photoaffinity cross-linked [^{3}H]SAM to *Eco*RII methyltransferase and then subjected this labelled protein to proteolytic cleavage. HPLC gel filtration chromatography was then used to separate the [^{3}H]SAM-photolabelled polypeptides, which were eventually purified away from the remainder of the polypeptides. This technique could also be used for the separation of denatured, [^{3}H]SAM-photoaffinity cross-linked PEAMeT. Suitable preparations would contain only one photoaffinity cross-linked polypeptide, as viewed by fluorography and such a preparation would be the 5403-fold purified preparation (Fig. 12, lane 2).

PEAMeT ACTIVITY: BIOCHEMICAL CHARACTERIZATION

Molecular Weight of PEAMeT

Using SDS-PAGE, the estimated monomer MW of PEAMeT and [^{3}H]SAM-cross-linked PEAMeT were found to be approximately 54 kDa and 57 kDa, respectively (Figs. 13 and 14). The larger estimated MW of photoaffinity cross-linked PEAMeT may be due to the binding of [^{3}H]SAM to the enzyme. This anomalous estimate of MW could arise if the photoaffinity cross-linking of [^{3}H]SAM to PEAMeT altered the charge of the denatured enzyme causing PEAMeT to migrate slower through the gel. Alternatively, it is not known how many [^{3}H]SAM molecules bind to an enzyme after photoaffinity cross-linking

and therefore if the binding of [^{3}H]SAM (MW of 0.48 kDa) exceeds a 1:1 mole ratio of PEAMeT:SAM, the MW of this complex would be greater than 54 kDa.

The estimated native MW of PEAMeT was found to be approximately 77 kDa using HPLC gel filtration chromatography (Fig. 16). The discrepancy between the estimated native and monomer MW of PEAMeT is approximately 23 kDa. This suggests that PEAMeT is composed of at least two, non-identical subunits of 54 and 23 kDa. However, no 23 kDa band was found among fractions eluted from the gel filtration column containing PEAMeT activity (Fig. 15). Indeed, no polypeptide bands other than the one at 54 kDa, correlated with PEAMeT activity. Thus, this discrepancy in MW estimates may be due to the anomalous migration of PEAMeT either under conditions used for gel filtration or SDS-PAGE.

Both of the estimated MWs (monomer or native) of PEAMeT differ from the MWs of the phosphatidylbase *N*-methyltransferases such as the PtdEA *N*-methyltransferase (PtdEA → PtdCho) from rat liver which has a monomer MW of 18.3 kDa (Ridgway and Vance, 1987) and the PtdEA *N*-methyltransferase (PtdEA → PtdMEA) and the PtdMEA *N*-methyltransferase (PtdMEA → PtdDEA → PCho) from yeast which have MW's of 101.2 and 23.2 kDa, respectively (Summers *et al.*, 1988; McGraw and Henry, 1989). The MW of the enzyme from rat liver was estimated by SDS-PAGE while those from yeast were determined from the deduced amino acid sequence from the cloned genes.

Optimal PEAMeT Assay Conditions

Optimal assay conditions define the conditions under which enzyme activity is proportional to enzyme concentration over a period of time. Assay conditions such as pH, concentration of substrates, concentration of protein and assay incubation time are factors which can be adjusted to provide for such optimal assay conditions. PEAMeT activity was highest when enzyme preparations were assayed in Hepes-KOH buffer at a pH of 7.8 (Fig. 19), which agrees with the results obtained using *Lemna paucicostata* extracts (Mudd and Datko, 1988). Bligny *et al.* (1990) reported the pH of the spinach cytoplasm is 7.5. Thus, the optimum pH of PEAMeT activity *in vitro* is similar to the *in vivo* pH of the cytoplasm. Since PEAMeT activity was shown to be localized to the cytoplasmic fraction by the method of differential centrifugation (Weretilnyk *et al.*, in press), *in vivo*, PEAMeT is operating at a pH which likely allows for near optimum PEAMeT activity.

Ideally, PEAMeT activity in relation to protein concentration and assay incubation time should be determined using a homogeneous preparation containing only PEAMeT protein. In this thesis, the partially purified samples all contained different concentrations of protein with PEAMeT comprising a variable proportion of the preparation. Therefore, the determination of enzyme activity in relation to protein concentration or assay incubation time will vary according to the sample used and the results obtained cannot be directly related to all of the other samples assayed. For a 523-fold purified preparation

(Table VII), a maximal linear activity of 60 pmol·min^{-1} per assay was reached at 2 μg of protein per 30 min assay (Fig. 17). Also, for a 409-fold purified preparation, PEAMeT activity was linear with time up to a 30 min assay period, whereupon the reaction rate then decreased (Fig. 18). This result suggests that a shorter PEAMeT assay incubation time of 15 or 20 min should be used for the assay of purified samples. Shortening the assay time is impractical due to the number of manipulations necessary for each sample during the assay. Therefore, with respect to these findings, it is recommended that samples with high levels of PEAMeT activity be diluted to a protein concentration no greater than 2 μg or, alternatively, to a concentration which will give less than 2500 cpm/mL Dowex eluate.

One possible reason for the deviation from linearity could be due to limiting amounts of substrate (PEA or SAM). In a 30 min assay period using 2 μg protein per assay, approximately 1.8 nmol of PEA and SAM are consumed. Since the initial amounts of PEA and SAM in the assay are 37.5 nmoles and 30 nmoles, respectively, it is unlikely that these substrates would be in limited supply in assays for any of the samples tested. Another possible reason for deviation from linearity could be due to the accumulation of a product or by-product of the PEAMeT reaction which inhibits PEAMeT activity. Of the potential products, the effects of PCho and SAH on PEAMeT activity were tested (Tables XIII and XIV). Since PEAMeT generates the same amount of SAH as the amount of the substrate SAM utilized, SAH would accumulate to

12 μM in the assay after 30 min. PEAMeT activity is inhibited 50% by 10 μM SAH and therefore the 12 μM generated during an assay could certainly inhibit PEAMeT activity.

A second potential inhibitor is the product of the three *N*-methylations, PCho. If one assumes that all of the phosphobases generated after a 30 min PEAMeT assay period consisted of PCho, 1.8 nmol of PCho should be synthesized after 30 min. This amount of PCho corresponds to approximately 12 μM and is approximately 10-fold lower than the 125 μM concentration of PCho needed to inhibit PEAMeT activity by 30% (Table XIII). Therefore, it is most likely that the accumulation of SAH, and not PCho, is responsible for the deviation of PEAMeT activity from linearity with higher protein concentrations and longer time periods. More detailed kinetic studies using the substrates PEA and SAM and the products PCho and SAH should be carried out in the future to determine the precise concentrations of SAH and PCho necessary to inhibit PEAMeT activity *in vitro*.

Deviation from linearity with protein concentration and time could also be due to PEAMeT instability, SAM instability or a change in assay pH over time. Since it is known that PEAMeT binds to the plastic of the ultrafiltration equipment, it is possible that PEAMeT also binds to the plastic microfuge tubes used to assay PEAMeT activity, resulting in lower estimates of enzyme activity. At present, it is unknown whether SAM instability could affect PEAMeT activity under the assay conditions used, but as a precautionary measure, SAM and

[^{3}H]SAM were stored at -20°C in an acidic solution and kept for a maximum of two months. These precautions were taken because, under acidic conditions it was reported that SAM and [^{3}H]SAM are more stable at -20°C than -80°C (New England Nuclear NET-155H Technical Data Sheet). Under alkaline conditions SAM is unstable and degrades to adenine and pentosylmethionine (Audubert and Vance, 1983). With respect to assay pH, this is not likely responsible for deviation of PEAMeT activity from linearity with protein concentration and time since the pH of the assay mixture was 7.8 when tested at the beginning and at the end of the incubation.

PEAMeT activity of a 778-fold purified preparation was not affected by Mg^{2+} or Na_2-EDTA present during the assay incubation (Table XV). Many methyltransferases require Mg^{2+} for activity and are inhibited by EDTA, while others are inhibited or not affected by Mg^{2+} at all (Poulton, 1981). The PtdEA *N*-methyltransferase of yeast was inhibited by Mg^{2+} whereas the PtdMEA *N*-methyltransferase of yeast (Gaynor and Carman, 1990) and the EA *N*-methyltransferase of castor bean endosperm (Prud'homme and Moore, 1992) are activated by Mg^{2+}.

PEAMeT activity was inhibited to varying extents by the other divalent metal ions tested. The presence of these divalent metal ions during the assay incubation inhibited PEAMeT activity, where the strength of the inhibition was: $Co^{2+} > Mn^{2+} > Ca^{2+}$ (Table XV). This result showed that for crude extracts, the inclusion of the divalent metal ion chelator, Na_2-EDTA, in the PEAMeT

assay buffer may be a necessary precaution against possible contamination by inhibitory metal ions. Specifically, it is not known how metal ions inhibit the activity of enzymes. However, in general, heavy metals can complex with oxygen, creating an activated oxygen molecule. This activated oxygen can catalyse the formation of disulphide bonds (-S-S-), sulphinic acids (-S-OH) or sulphonic acids (-S-O_2H) from the reduced sulphydryl (-SH) groups of cysteine residues in proteins (Scopes, 1982; Eisenthal and Danson, 1992). Any of these modifications to sulphydryl groups due to the presence of metal ions could alter enzyme activity.

PEAMeT Activity and Metabolites

The freebases, EA, MEA, DEA and choline did not inhibit PEAMeT activity (Table XIII). Indeed, radiotracer studies using spinach leaf discs showed that label from [1,2-^{14}C]EA was not detected in the freebases EA, MEA and DEA and enzyme studies did not detect any EA *N*-methyltransferase activity in spinach (Summers and Weretilnyk, 1993). The lack of inhibition of PEAMeT activity by EA, MEA or DEA and the absence of evidence for the conversion of EA → MEA → DEA → Cho *in vivo*, all argue against PEAMeT using the freebases as substrates.

PEAMeT activity was found to be inhibited by the product SAH (Table XIV). SAH is an inhibitor of most SAM-dependant methyltransferases by competing with SAM for the active site on the methyltransferase (Poulton, 1981). This

suggests that *in vivo*, PEAMeT activity could be inhibited by SAH if the SAH concentration is elevated to inhibitory levels in the same subcellular compartment as PEAMeT. However, *in vivo*, the concentration of intracellular SAH could be decreased if SAH was quickly metabolized to adenosine and homocysteine by SAH hydrolase (Poulton and Butt, 1976) or SAH was compartmentalized away from PEAMeT, perhaps in the vacuole.

Currently it is not known whether PCho can feedback inhibit its own synthesis *in vivo*. Studies using a glycine betaine deficient mutant (*bet1/bet1*) of *Zea mays* with a block in choline oxidation to betaine aldehyde, provide evidence for *in vivo* feedback inhibition of choline synthesis (Yang *et al.*, 1995). Since these betaine deficient plants cannot convert choline to betaine, choline pool size was predicted to increase significantly. However, only a modest increase in choline pool size was observed. Therefore the authors suggested that choline (or a product of choline metabolism) could down-regulate its own synthesis.

In an earlier study using salinized sugar beet leaf discs, Hanson and Rhodes (1983) hypothesized that PCho may feedback inhibit choline and betaine synthesis. By using trapping pools of PCho, the authors found that there was less incorporation of label from [^{14}C]formate into choline and betaine. Although the results suggest that an elevated intracellular pool of PCho was responsible for the inhibition of choline and betaine synthesis, the authors did not identify which reaction in the choline or betaine biosynthetic pathway was inhibited.

The inhibition of any one of the phosphobase *N*-methyltransferase activities by PCho can potentially down-regulate PCho, choline and betaine synthesis. In this way, both the betaine biosynthetic pathway and the phospholipid biosynthetic pathway (Fig. 1) could be regulated by one metabolite, PCho. It has been hypothesized by computer modelling that there are two separate *in vivo* pools of both PCho and Cho in sugar beet (Hanson and Rhodes, 1983). For each, a metabolic pool and a storage pool were postulated and PCho and choline for betaine and phospholipid biosynthesis would only come from the small metabolically active pools (Hanson and Rhodes, 1983). It is not known whether PCho from the metabolic or storage pool (or both) is involved in the regulation of PEAMeT activity nor if the activity of PCho phosphatase could be involved in limiting the amount of PCho present for inhibition.

To address the identity of a committing and possible rate-regulating enzyme, Mudd and Datko (1989ab) examined enzymes of the choline biosynthetic pathway using *Lemna paucicostata* plants growing in the presence of 3 to 25 μM choline or using soybean and carrot cell cultures growing in the presence of 50 μM choline. Under these conditions, *in vivo* PCho levels increased for all three plant tissues in response to increases in choline levels. Mudd and Datko (1989ab) then showed that the reaction PEA → PMEA was inhibited in both *Lemna paucicostata* plants and carrot and soybean cell cultures exposed to choline. This suggests that choline and/or PCho may inhibit PEAMeT activity in these plants. To test this hypothesis, the effect of

choline and PCho on *in vitro* PEAMeT activity was determined. *In vitro* PEAMeT activity was measured using *Lemna paucicostata* extracts and was shown not to be inhibited by 400 μM choline but was inhibited 27% by 175 μM PCho (Mudd and Datko, 1989a). In this thesis it was shown that PEAMeT activity of purified spinach leaf extracts was not inhibited by choline or betaine at concentrations less than 1 mM, but was inhibited by PCho (Table XIII). Therefore, studies using these two plant species show that PEAMeT activity is feedback inhibited by PCho *in vitro*.

In order to determine whether feedback regulation of PEAMeT activity by PCho can occur *in vivo*, the concentration of intracellular PCho must be known. This concentration has been determined using ^{31}P NMR studies of spinach leaves (Bligny *et al.*, 1990). These authors showed that, *in vivo*, PCho is located in the cytoplasm at an approximate concentration of 3.3 mM. The presence of this *in vivo* concentration of PCho in the same subcellular compartment as PEAMeT could inhibit PEAMeT activity since 1 mM PCho inhibits *in vitro* PEAMeT activity by 52% (Table XIII). Therefore, *in vitro* assays and *in vivo* PCho measurements show that *in vivo*, PEAMeT activity can be feedback regulated by PCho. For future studies, it would be interesting to determine if *in vivo*, changes in the level of PCho could be involved in the up-regulation of PEAMeT activity in response to light and salinity.

LITERATURE CITED

Adams P, Thomas JC, Vernon DM, Bohnert HJ, Jensen RG (1992) Distinct cellular and organismic responses to salt stress. Plant Cell Physiol **33**: 1215-1223

Andriamampandry C, Freysz L, Kanfer JN, Dreyfus H, Massarelli R (1989) Conversion of ethanolamine, monomethylethanolamine and dimethylethanolamine to choline-containing compounds by neurons in culture and by the rat brain. Biochem J **264**: 555-562

Andriamampandry C, Massarelli R, Freysz L, Kanfer JN (1990) A rat brain cytosolic *N*-methyltransferase(s) activity converting phosphorylethanolamine into phosphorylcholine. Biochem Biophys Res Comm **171**: 758 763

Andriamampandry C, Massarelli R, Kanfer JN (1992) Properties of a partially purified phosphodimethylethanolamine methyltransferase from rat brain cytosol. Biochem J **288**: 267-272

Arakawa K, Takabe T, Sugiyama T, Akazawa T (1987) Purification of betaine aldehyde dehydrogenase from spinach leaves and preparation of its antibody. J Biochem **101**: 1485-1488

Arakawa K, Katayama M, Takabe T (1990) Levels of betaine aldehyde dehydrogenase activity in the green leaves, etiolated leaves and roots of barley. Plant Cell Physiol **31**: 797-803

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol **24**: 1-15

Audubert F, Vance DE (1983) Pitfalls and problems in studies on the methylation of phosphatidylethanolamine. J Biol Chem **258**: 10695-10701

Binzel ML, Hess FD, Bressan RA, Hasegawa PM (1988) Intracellular compartmentation of ions in salt adapted tobacco cells. Plant Physiol **86**: 607-614

Bligny R, Gardestrom P, Roby C, Douce R (1990) ^{31}P NMR studies of spinach leaves and their chloroplasts. J Biol Chem **265**: 1319-1326

Boggess SF, Aspinall D, Paleg LG (1976a) Stress metabolism IX. The significance of end-product inhibition of proline biosynthesis and of compartmentation in relation to stress-induced proline accumulation. Aust J Plant Physiol **3**: 513-525

Boggess SF, Stewart CR, Aspinall D, Paleg LG (1976b) Effect of water stress on proline synthesis from radioactive precursors. Plant Physiol **58**: 398-401

Boyer JS (1982) Plant productivity and environment. Science **218**: 443-448

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**: 248-254

Brouquisse R, Weigel P, Rhodes D, Yocum CF, Hanson AD (1989) Evidence for a ferredoxin-dependent choline monooxygenase from spinach chloroplast stroma. Plant Physiol **90**: 322-329

Brown LM, Hellebust JA (1978) Sorbitol and proline as intracellular osmotic solutes in the green alga *Stichococcus bacillaris*. Can J Bot **56**: 676-680

Burnet M, Lafontaine PJ, Hanson AD (1995) Assay, purification, and partial characterization of choline monooxygenase from spinach. Plant Physiol **108**: 581-588

Cairney J, Booth IR, Higgins CF (1985) Osmoregulation of gene expression in *Salmonella typhimurium: proU* encodes an osmotically induced betaine transport system. J Bacteriol **164**: 1224-1232

Charlson RJ, Lovelock JE, Andreae MO, Warren SG (1987) Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate. Nature **326**: 655-661

Christian JHB (1955a) The water relations of growth and respiration of *Salmonella oranienburg* at 30°C. Aust J Biol Sci **8**: 75-82

Christian JHB (1955b) The influence of nutrition on the water relations of *Salmonella oranienburg*. Aust J Biol Sci **8**: 490-497

Coughlan SJ, Wyn Jones RG (1980) Some responses of *Spinacia oleracea* to salt stress. J Exp Bot **31**: 883-893

Coughlan SJ, Heber U (1982) The role of glycinebetaine in the protection of thylakoids against freezing stress. Planta **156**: 62-69

Coughlan SJ, Wyn Jones RG (1982) Glycinebetaine biosynthesis and its control in detached secondary leaves of spinach. Planta **154**: 6-17

Csonka LN (1981) Proline over-production results in enhanced osmotolerance in *Salmonella typhimurium*. Mol Gen Genet **182**: 82-86

Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic stress. Microbiol Rev **53**: 121-147

Csonka LN, Hanson AD (1991) Prokaryotic osmoregulation: genetics and physiology. Annu Rev Microbiol **45**: 569-606

Dacey JWH, King GM, Wakeham SG (1987) Factors controlling emission of dimethylsulphide from salt marshes. Nature **330**: 643-645

Datko AH, Mudd HS (1988a) Phosphatidylcholine synthesis: differing patterns in soybean and carrot. Plant Physiol **88**: 854-861

Datko AH, Mudd HS (1988b) Enzymes of phosphatidylcholine synthesis in *Lemna*, soybean, and carrot. Plant Physiol **88**: 1338-1348

Datko AH, Aksamit RR, Mudd HS (1990) Phosphatidylcholine synthesis in the rat: the substrate for methylation and regulation by choline. Lipids **25**: 135-142

Delauney AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. Plant J **4**: 215-223

De Sousa SM, Krishnan KS, Kenkare UW (1988) Phospholipid methyltransferase from *Drosophila melanogaster*: purification and properties. Insect Biochem **18**: 377-388

Ditrich P, Korak A (1984) Novel biosynthesis of D-pinitol in *Simmondsia chinensis*. Phytochemistry **23**: 65-66

Eisenthal R, Danson MJ (1992) Enzyme Assays: A Practical Approach. IRL Press, Oxford, pp 265-266, 317-334

Eloranta TO, Kajander EO, Raina AM (1976) A new method for the assay of tissue *S*-adenosylhomocysteine and *S*-adenosylmethionine. Biochem J **160**: 287-294

Essery JM, McCaldin DJ, Marion L (1962) The biosynthesis of stachydrine. Phytochemistry **1**: 209-213

Ford CW (1984) Accumulation of low molecular weight solutes in water-stressed tropical legumes. Phytochemistry **23**: 1007-1015

Gaynor PM, Carman GM (1990) Phosphatidylethanolamine methyltransferase and phospholipid methyltransferase activities from *Saccharomyces cerevisiae*. Enzymological and kinetic properties. Biochim Biophys Acta **1045**: 156-163

Gorbunoff MJ (1985) Protein chromatography on hydroxylapatite columns. Methods Enzymol **117**: 370-380

Gorham J, Hughes L, Wyn Jones RG (1981) Low molecular weight carbohydrates in some salt-stressed plants. Physiol Plant **53**: 27-33

Gorham J, Wyn Jones RG (1983) Solute distribution in *Suaeda maritima*. Planta **157**: 344-349

Gorham J, Tomar OS, Wyn Jones RG (1988) Salinity-induced changes in the chemical composition of *Leucaena leucocephala* and *Sesbania bispinosa*. J Plant Physiol **132**: 678-682

Hanson AD, Scott NA (1980) Betaine synthesis from radioactive precursors in attached, water-stressed barley leaves. Plant Physiol **66**: 342-348

Hanson AD, Hitz WD (1982) Metabolic responses of mesophytes to plant water deficits. Annu Rev Plant Physiol **33**: 163-203

Hanson AD, Wyse R (1982) Biosynthesis, translocation, and accumulation of betaine in sugar beet and its progenitors in relation to salinity. Plant Physiol **70**: 1191-1198

Hanson AD, Rhodes D (1983) ^{14}C tracer evidence for synthesis of choline and betaine via phosphoryl base intermediates in salinized sugarbeet leaves. Plant Physiol **71**: 692-700

Hanson AD, May AM, Grumet R, Bode J, Jamieson GC, Rhodes D (1985) Betaine synthesis in chenopods: localization in chloroplasts. Proc Natl Acad Sci USA **82**: 3678-3682

Hanson AD, Hoffman NE, Samper C (1986) Identifying and manipulating metabolic stress-resistance traits. Hortscience **21**: 1313-1317

Hanson AD, Gage DA (1991) Identification and determination by fast atom bombardment mass spectrometry of the compatible solute choline-*O*-sulphate in *Limonium* species and other halophytes. Aust J Plant Physiol **18**: 317-327

Hanson AD, Rathinasabapathi B, Chamberlin B, Gage DA (1991) Comparative physiological evidence that β-alanine betaine and choline-*O*-sulphate act as compatible osmolytes in halophytic *Limonium* species. Plant Physiol **97**: 1199-1205

Hanson AD (1992) Compatible solute synthesis and compartmentation in higher plants. *In*, Somero G, Osmond CB, Bolis CL, eds, Water and Life. Springer-Verlag, Berlin, Heidelberg, pp 52-60

Hanson AD, Rathinasabapathi B, Rivoal J, Burnet M, Dillon MO, Gage DA (1994a) Osmoprotective compounds in the Plumbaginaceae: a natural experiment in metabolic engineering of stress tolerance. Proc Natl Acad Sci USA **91**: 306-310

Hanson AD, Rivoal J, Paquet L, Gage DA (1994b) Biosynthesis of 3-dimethylsulfoniopropionate in *Wollastonia biflora* (L.) DC.: Evidence that *S*-methylmethionine is an intermediate. Plant Physiol **105**: 103-110

Hatch MD (1987) C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim Biophys Acta **185**: 81-106

Higman MA, Niles EG (1994) Location of the *S*-adenosyl-L-methionine binding region of the vaccinia virus mRNA (guanine-7-)methyltransferase. J Biol Chem **269**: 14982-14987

Hitz WD, Rhodes D, Hanson AD (1981) Radiotracer evidence implicating phosphoryl and phosphatidyl bases as intermediates in betaine synthesis by water-stressed barley leaves. Plant Physiol **68**: 814-822

Hitz WD, Ladyman JAR, Hanson AD (1982) Betaine synthesis and accumulation in barley during field water-stress. Crop Sci **22**: 47-54

Hoagland DR, Arnon DI (1939) The water-culture method for growing plants without soil. Agric Exp Stn Berkeley, Cal. Circular 347

Hu CA, Delauney AJ, Verma DPS (1992) A bifunctional enzyme (Δ^1-pyrroline-5-carboxylate synthetase) catalyses the first two steps in proline biosynthesis in plants. Proc Natl Acad Sci USA **89**: 9354-9358

Incharoensakdi A, Takabe T, Akazawa T (1986) Effect of betaine on enzyme activity and subunit interaction of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Apanothece halophytica*. Plant Physiol **81**: 1044-1049

Ishitani M, Arakawa K, Mizuno K, Kishitani S, Takabe T (1993) Betaine aldehyde dehydrogenase in the Gramineae: levels in leaves of both betaine-accumulating and non-accumulating cereal plants. Plant Cell Physiol **34**: 493-495

Jones MM, Turner NC, Osmond CB (1981) Mechanisms of drought resistance. *In*, Paleg LG, Aspinall D, eds, The Physiology and Biochemistry of Drought Resistance in Plants. Academic Press, Sydney, Australia, pp 15-37

Jolivet Y, Larher F, Hamelin J (1982) Osmoregulation in halophytic higher plants: the protective effect of glycine betaine against the heat destabilization of membranes. Plant Sci Lett **25**: 193-201

Kagan RM, Clarke S (1994) Widespread occurrence of three sequence motifs in diverse *S*-adenosylmethionine-dependent methyltransferases suggests a common structure for these enzymes. Arch Biochem Biophys **310**: 417-427

Keiller DR, Paul MJ, Cockburn W (1987) Regulation of reserve carbohydrate metabolism in *Mesembryanthemum crystallinum* exhibiting C_3 and CAM photosynthesis. New Phytol **107**: 1-13

Kemble AR, MacPherson HT (1954) Liberation of amino acids in perennial rye grass during wilting. Biochem J **58**: 46-49

Kramer PJ (1988) Changing concepts regarding plant water relations. Plant Cell Environ **11**: 565-568

Landfald B, Strom AR (1986) Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. J Bacteriol **165**: 849-855

Lee HC, Fellenz-Maloney MP, Liscovitch M, Blusztajn JK (1993) Phospholipase D-catalyzed hydrolysis of phosphatidylcholine provides the choline precursor for acetylcholine synthesis in a human neuronal cell line. Proc Natl Acad Sci USA **90**: 10086-10090

Le Rudulier D, Bouillard L (1983) Glycine betaine, an osmotic effector in *Klebsiella pneumoniae* and other members of the *Enterbacteriaceae*. Appl Environ Microbiol **46**: 152-159.

Le Rudulier D, Strom R, Dandekar M, Smith LT, Valentine RC (1984) Molecular biology of osmoregulation. Science **224**: 1064-1068

Low PS (1985) Molecular basis of the biological compatibility of nature's osmolytes. *In*, R Gilles, M Gilles-Baillien eds, Transport Processes, Iono- and Osmoregulation. Springer-Verlag, Berlin, pp. 469-477.

Martin F, Tolbert NE (1983) Factors which affect the amount of inorganic phosphate, phosphorylcholine and phosphorylethanolamine in xylem exudate of tomato plants. Plant Physiol **73**: 464-470

May G, Faatz E, Villarejo M, Bremer E (1986) Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K12. Mol Gen Genet **20**: 225-233

McCue KF, Hanson AD (1990) Drought and salt tolerance: towards understanding and application. Trends Biotech **8**: 358-362

McCue KF, Hanson AD (1992) Salt-inducible betaine aldehyde dehydrogenase from sugar beet: cDNA cloning and expression. Plant Molec Biol **18**: 1-11

McDonnell E, Wyn Jones RG (1988) Glycinebetaine biosynthesis and accumulation in unstressed and salt-stressed wheat. J Exp Bot **39**: 421-430

McGraw P, Henry SA (1989) Mutations in the *Saccharomyces cerevisiae opi3* gene: effects on phospholipid methylation, growth and cross-pathway regulation of inositol synthesis. Genetics **122**: 317-330

Merrick WC (1983) Translation of exogenous mRNAs in reticulocyte lysates. Methods Enzymol **101**: 606-615

Moore TS (1982) Phospholipid biosynthesis. Annu Rev Plant Physiol **33**: 235-259

Morgan JM (1984) Osmoregulation and water stress in higher plants. Annu Rev Plant Physiol **35**: 299-319

Mudd SH, Datko AH (1986) Phosphoethanolamine bases as intermediates in phosphatidylcholine synthesis by *Lemna*. Plant Physiol **82**: 126-135

Mudd SH, Datko AH (1989a) Synthesis of methylated ethanolamine moieties: regulation by choline in *Lemna*. Plant Physiol **90**: 296-305

Mudd SH, Datko AH (1989b) Synthesis of methylated ethanolamine moieties: regulation by choline in soybean and carrot. Plant Physiol **90**: 306-310

Mudd SH, Datko AH (1989c) Synthesis of ethanolamine and its regulation in *Lemna paucicostata*. Plant Physiol **91**: 587-597

Neville Jr. DM (1971) Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J Biol Chem **246**: 6328-6334

Nguyen A, Lamant A (1988) Pinitol and myo-inositol accumulation in water-stressed seedlings of Maritime Pinc. Phytochemistry **27**: 3423-3427

Paleg LG, Douglas TJ, van Daal A, Keech DB (1981) Proline, betaine and other organic solutes protect enzymes against heat inactivation. Aust J Plant Physiol **8**: 107-114

Paquet L, Rathinasabapathi B, Saini H, Zamir L, Gage DA, Huang ZH, Hanson AD (1994) Accumulation of the compatible solute 3-dimethylsulfoniopropionate in sugarcane and its relatives, but not other Gramineous crops. Aust J Plant Physiol **21**: 37-48

Paul MJ, Cockburn W (1989) Pinitol, a compatible solute in *Mesembryanthemum crystallinum* L.? J Exp Bot **40**: 1093-1098

Poulton JE, Butt VS (1976) Purification and properties of *S*-adenosyl-L-homocysteine hydrolase from leaves of spinach beet. Arch Biochem Biophys **172**: 135-142

Poulton JE (1981) Transmethylation and demethylation reactions in the metabolism of secondary plant products. *In* DD Davies, ed, The Biochemistry of Plants Vol. 7. Academic Press, San Diego, pp 667-715

Prud'homme M, Moore TS (1992a) Phosphatidylcholine synthesis in castor bean endosperm: free bases as intermediates. Plant Physiol **100**: 1527-1535

Prud'homme M, Moore TS (1992b) Phosphatidylcholine synthesis in castor bean endosperm: occurrence of an *S*-adenosyl-L-methionine:ethanolamine *N*-methyltransferase. Plant Physiol **100**: 1536-1540

Rathinasabapathi B, Hanson AD (1994a) Characterization of *N*-methyltransferases involved in β-alanine betaine biosynthesis in the Plumbaginaceae. Plant Physiol Suppl **102**: 38

Rathinasabapathi B, McCue KF, Gage DA, Hanson AD (1994b) Metabolic engineering of glycine betaine synthesis: plant betaine aldehyde dehydrogenases lacking typical transit peptides are targeted to tobacco chloroplasts where they confer betaine aldehyde resistance. Planta **193**: 155-162

Rhodes D (1987) Metabolic Responses to Stress. *In* DD Davies, eds, The Biochemistry of Plants, Vol. 12. Academic Press, San Diego, pp 201-241

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulphonium compounds in higher plants. Annu Rev Plant Physiol Plant Mol Biol **44**: 357-384

Ridgway ND, Vance DE (1987) Purification of phosphatidylethanolamine *N*-methyltransferase from rat liver. J Biol Chem **262**: 17231-17239

Ridgway ND, Yao Z, Vance DE (1989) Phosphatidylethanolamine levels and regulation of phosphatidylethanolamine *N*-methyltransferase. J Biol Chem **264**: 1203-1207

Rivoal J, Hanson AD (1994) Choline-*O*-sulfate biosynthesis in plants. Plant Physiol **106**: 1187-1193

Rudolph AS, Crowe JH, Crowe LM (1986) Effects of three stabilizing agents - proline, betaine and trehalose - on membrane phospholipids. Arch Biochem Biophys **245**: 134-143

Saneoka H, Nagasaka C, Hahn DT, Yang W, Premachandra GS, Joly RJ, Rhodes D (1995) Salt tolerance of glycinebetaine-deficient and -containing maize lines. Plant Physiol **107**: 631-638

Scarborough GA, Nyc JF (1967) Methylation of ethanolamine phosphatides by microsomes from normal and mutant strains of *Neurospora crassa*. J Biol Chem **242**: 238-242

Scopes RK (1982) Protein Purification: Principles and Practice. Springer, New York. pp 195-197.

Sharma SK, Brown SA (1978) Affinity chromatography on immobilized *S*-adenosyl-L-homocysteine. Purification of a furanocoumarin *O*-methyltransferase from cell cultures of *Ruta graveolens* L. J Chromatogr **157**: 427-431

Singh TN, Aspinall D, Paleg LG (1972) Proline accumulation and varietal adaptability to drought in barley: a potential metabolic measure of drought resistance. Nature (New Biology) **236**: 188-190

Smith DD (1993) Light and salt regulation of a choline biosynthetic enzyme: *S*-adenosyl-L-methionine: phosphoethanolamine *N*-methyltransferase. Biology 4C09 Thesis. McMaster University, Hamilton

Som S, Friedman S (1990) Direct photolabeling of the *Eco*RII methyltransferase with S-adenosyl-L-methionine. J Biol Chem **265**: 4278-4283

Som S, Friedman S (1991) Identification of a highly conserved domain in the *Eco*RII methyltransferase which can be photolabelled with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. J Biol Chem **266**: 2937-2945

Storey R, Gorham J, Pitman MG, Hanson AD, Gage D (1993) Response of *Melanthera biflora* to salinity and water stress. J Exp Bot **44**: 1551-1560

Stack D (1981) Sinapine as a supply of choline for the biosynthesis of phosphatidylcholine in *Raphanus sativus* seedlings. Z Naturforsch **36c**: 215-221

Subbaramaiah K, Simms SA (1992) Photolabelling of CheR methyltransferase with *S*-adenosyl-L-methionine (AdoMet). J Biol Chem **267**: 8636-8642

Sugiura M, Kisumi M (1985) Osmoregulation in a proline-producing strain of *Serratia marcescens*. J Gen Microbiol **131**: 2525-2520

Summers EF, Lett VA, McGraw P, Henry SA (1988) *Saccharomyces cerevisiae cho2* mutants are deficient in phospholipid methylation and cross-pathway regulation of inositol synthesis. Genetics **120**: 909-922

Summers PS, Weretilnyk EA (1993) Choline synthesis in spinach in relation to salt stress. Plant Physiol **103**: 1269-1276

Takata Y, Fujioka M (1992) Identification of a tyrosine residue in rat guanidinoacetate methyltransferase that is photolabelled with *S*-adenosyl-L-methionine. Biochemistry **31**: 4369-4374

Tarczynski MC, Jensen RG, Bohnert HJ (1992) Expression of a bacterial *mtlD* gene in transgenic tobacco leads to production and accumulation of mannitol. Proc Natl Acad Sci USA **89**: 2600-2604

Tarczynski MC, Jensen RG, Bohnert HJ (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science **259**: 508-510

Ting IP (1985) Crassulacean acid metabolism. Annu Rev Plant Physiol **35**: 595-622

Valenzuela-Soto EM, Munoz-Clares RA (1994) Purification and properties of betaine aldehyde dehydrogenase extracted from detached leaves of *Amaranthus hypochondriacus* L. subjected to water deficit. J Plant Physiol **143**: 145-152

Vance DE, Ridgway ND (1988) The methylation of phosphatidylethanolamine. Prog Lipid Res **27**: 61-79

Vernon DM, Bohnert HJ (1992a) A novel methyltransferase induced by osmotic stress in the facultative halophyte *Mesembryanthemum crystallinum*. EMBO J **11**: 2077-2085

Vernon DM, Bohnert HJ (1992b) Induction of a novel methyltransferase by salt stress in *Mesembryanthemum crystallinum* differs from that for CAM-related genes. Plant Physiol **99**: 1695-1698

Wallevik K, Jensenius JC (1982) A simple and reliable method for the drying of polyacrylamide slab gels. J Biochem Biophys Methods **6**: 17-21

Wecker L, Trommer BA (1984) Effects of chronic (dietary) choline availability on the transport of choline across the blood-brain barrier. J Neurochem **43**: 1762-1765

Weigel P, Weretilnyk EA, Hanson AD (1986) Betaine aldehyde oxidation by spinach chloroplasts. Plant Physiol **82**: 753-759

Weigel P, Lerma C, Hanson AD (1988) Choline oxidation by intact spinach chloroplasts. Plant Physiol **86**: 54-60

Weretilnyk EA, Hanson AD (1988) Betaine aldehyde dehydrogenase polymorphism in spinach: genetic and biochemical characterization. Biochem Genet **26**: 143-151

Weretilnyk EA, Bednarek S, McCue KF, Rhodes D, Hanson AD (1989) Comparative biochemical and immunological studies of the glycine betaine synthesis pathway in diverse families of dicotyledons. Planta **178**: 342-352

Weretilnyk EA, Hanson AD (1990) Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. Proc Natl Acad Sci USA **87**: 2745-2749

Weretilnyk EA, Summers PS (1992) Betaine and choline metabolism in higher plants. *In*, Singh BK, Flores HE, Shannon JC, eds, Biosynthesis and Molecular Regulation of Amino Acids in Plants. American Society of Plant Physiologists, Rockville, Maryland, pp 89-97

Weretilnyk EA, Smith DD, Wilch GA, Summers PS (1995) Enzymes of choline synthesis in spinach. Response of phospho-base *N*-methyltransferase activities to light and salinity. Plant Physiol, in press

Wood WI (1976) Tables for the preparation of ammonium sulfate solutions. Anal Biochem **73**: 250-257

Wray W, Boulikas T, Wray VP, Hancock R (1981) Silver staining of proteins in polyacrylamide gels. Anal Biochem **118**: 197-203

Wyn Jones RG, Storey R (1981) Betaines. *In*, Paleg LG, Aspinall D, eds, The Physiology and Biochemistry of Drought Resistance in Plants. Academic Press, Sydney, Australia, pp 171-204

Wyn Jones RG, Gorham J (1983) Osmoregulation. *In*, Osmond CB, Ziegler DH, eds, Encyclopedia of Plant Physiology. Springer-Verlag, Berlin, pp 35-58

Yamashita S, Oshima A, Nikawa J, Hosaka K (1982) Regulation of the phosphatidylethanolamine methylation pathway in *Saccharomyces cerevisiae*. Eur J Biochem **128**: 589-595

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science **217**: 1214-1222

Yang W, Nadolska-Orczyk A, Wood KV, Hahn DT, Rich PJ, Wood AJ, Saneoka H, Premachandra GS, Bonham CC, Rhodes JC, Joly RJ, Samaras Y, Goldsbrough PB, Rhodes D (1995) Near-isogenic lines of maize differing for glycinebetaine. Plant Physiol **107**: 621-630