ENZYMES OF CHOLINE SYNTHESIS IN DICOT FAMILIES

A COMPARATIVE BIOCHEMICAL STUDY OF THE ENZYMES OF CHOLINE SYNTHESIS IN SEVERAL DICOTYLEDON FAMILIES.

By

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ABSTRACT

Choline is universal among plants as phosphatidylcholine and in many plants it serves as a precursor for the compatible osmolytes glycine betaine and choline-*O*-sulphate. In spinach, choline is synthesized by the sequential *N*-methylation of phosphoethanolamine (PEA) → phosphomethylethanolamine (PMEA) → phosphodimethylethanolamine (PDEA) → phosphocholine (PCho) and PCho is hydrolyzed to choline. The objective of this biochemical survey was to determine whether the activities of enzyme(s) converting PEA to PCho could be found in leaves of diverse plants and, if so, if common regulatory properties could be identified. Leaf tissue was harvested from 14 diverse dicot plants. Enzyme activities for the three sequential *N*-methylations were quantified in vitro and for representative assays using PEA as substrate, reaction products were identified by thin layer chromatography.

Extracts of all plants tested could metabolize PEA to PMEA, the rate of conversion varied from 0.04 to 25 nmol · min^{-1} · g^{-1} Fresh wt for soybean and cotton, respectively. In vitro PMEA → PDEA and PDEA → PCho rates varied between the plant species tested. Both steps were highest in sugar beet (22 and 24 nmol · min^{-1} · g^{-1} Fresh wt for PMEA → PDEA and PDEA → PCho, respectively) to below detection limits for soybean (<0.03 nmol · min^{-1} · g^{-1} Fresh wt).

Upon dark exposure, PEA → PMEA reaction rates were reduced from the light period levels but not in all cases was the activity reduced beyond the level

that could be attributed to a general loss in plant vigour. The reduction in *N*-methylation capacity was most pronounced in plants with the highest levels of PEA → PMEA activity, with reduction greatest in sugar beet > amaranth > spinach. Thus PEA → PMEA is catalyzed by a light-regulated enzyme in many but apparently not all dicot plants.

Datko and Mudd (1988a) have proposed that PEA → PMEA conversion is ubiquitous among plants and the enzyme responsible catalyzes a committing step for PCho synthesis. PEAMeT activity was found in leaf extracts of 10 additional dicot species. The level of in vitro activity and light regulation is not equivalent among all plants examined. If in vitro rates faithfully reflect the in vivo capacity to synthesize choline, why the variability among plants with respect to their capacity to synthesize a universal metabolite? Alternate routes of PCho/phosphatidyl choline synthesis may explain these differences (Datko and Mudd, 1988a,b; Hanson and Rhodes, 1983; Hitz *et al.*, 1981; Weretilnyk and Summers, 1992). Interestingly, the plants with the highest rates of PEA → PMEA activity, (cotton, sugar beet, amaranth, sunflower, spinach and statice) are all documented glycine betaine accumulating species. These plants may have higher rates of PEA methylation in order to meet requirements for osmolyte synthesis. Thus, it would be interesting to see if PEA metabolism to PCho is up-regulated in response to osmotic stress.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BADH	Betaine aldehyde dehydrogenase
BSA	Bovine serum albumin
CDP	Cytidine diphosphate
cpm	Counts per minute
dpm	Disintegrations per minute
DEA	Dimethylethanolamine
DTT	Dithiothreitol
EA	Ethanolamine
Na_2EDTA	Ethylenediaminetetraacetic acid disodium salt
Hepes	4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid
MDH	Malate dehydrogenase
MEA	Monomethylethanolamine
MMT	*S*-adenosyl L-methionine: L-methionine *S*-methyl transferase
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OAA	Oxaloacetate

LIST OF ABBREVIATIONS (CONT'D)

PCho	Phosphocholine
PDEA	Phosphodimethylethanolamine
PDEAMeT	Phosphodimethylethanolamine *N*-methyltransferase
PEA	Phosphoethanolamine
PEAMeT	Phosphoethanolamine *N*-methyltransferase
PMEA	Phosphomethylethanolamine
PMEAMeT	Phosphomethylethanolamine *N*-methyltransferase
Pi	Phosphate Ion
Ptd	Phosphatidyl- (prefix)
PVP	Polyvinylpyrrolidone
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase
SAM	*S*-Adenosyl-L-methionine
SE	Standard error
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
wt	Weight

LITERATURE REVIEW

Choline is extremely important in plants as choline has a dual role: it is present in plants as phosphatidyl choline (PtdCho), a lipid component of plants and it is required for glycine betaine synthesis in many higher plants (Hanson, 1993).

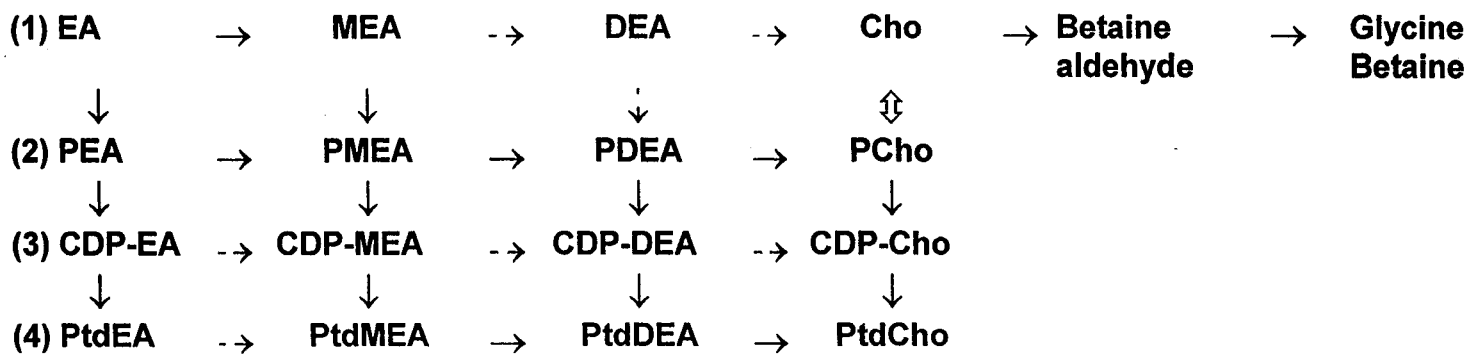
Choline Biosynthesis

There are several possible routes for choline synthesis among higher plants. A schematic representation is found in Figure 1 (Summers and Weretilnyk, 1993; Prud'homme and Moore, 1992a; Datko and Mudd, 1988a). Figure 1 presents the various possible routes in a metabolic grid highlighting sequential *N*-methylations directly of EA to Cho (free base route), PEA to PCho (phosphobase route) or PtdEA to PtdCho (phosphatidyl route), all which could conceivably give rise to choline (Rhodes and Hanson, 1993).

In leaves of spinach (*Spinacia oleracea* L.), phosphocholine (PCho) is the immediate precursor to choline. PCho synthesis involves three sequential *N*-methylations of phosphoethanolamine (PEA) $\rightarrow$ phosphomethylethanolamine (PMEA) $\rightarrow$ phosphodimethylethanolamine (PDEA) $\rightarrow$ PCho (Weretilnyk and Summers, 1992). Thus this pathway utilizes a phosphobase route.

Figure 1. Possible Pathways to Choline Synthesis

Metabolic grid showing possible routes of choline synthesis. Solid arrows indicate reactions for which in vitro measurements have been reported while dashed arrows indicate reactions for which no in vitro evidence is available. The abbreviations are: Cho, Choline; EA, ethanolamine; MEA, *N*-methylethanolamine; DEA, N,N-dimethylethanolamine. Phosphate esters are designated by P. Phosphatidyl derivatives designated by Ptd. Cytidine diphospho derivatives designated by CDP. Thus (1) shows the free base route to choline, (2) shows the phosphobase route to choline, (3) shows the cytidine diphospho intermediates, and (4) shows the phosphatidyl route to choline.


(1) EA
MEA
DEA
Cho
Betaine aldehyde
Glycine Betaine
(2) PEA
PMEA
PDEA
PCho
(3) CDP-EA
CDP-MEA
CDP-DEA
CDP-Cho
(4) PtdEA
PtdMEA
PtdDEA
PtdCho

Evidence that the phosphobase route for choline synthesis also predominated in sugar beet comes from the work of Hanson and Rhodes (1983). They sought to find the earliest methylation products of choline and betaine biosynthesis in leaf discs of salinized sugar beet (plants salinized to 150 - 200mM NaCl) using [^{14}C]EA, [^{14}C]PMEA, [^{14}C]formate or [^{14}C]PCho as precursors and then followed their incorporation into intermediates of the choline biosynthetic pathway. All intermediates formed were metabolites of the phosphobase route supporting the pathway of PEA $\rightarrow$ PMEA $\rightarrow$ PDEA $\rightarrow$ PCho to choline and then on to glycine betaine, with a minor use of the PCho towards PtdCho (Hanson and Rhodes, 1983). They suggested that a specific but unidentified choline phosphatase was likely to catalyze the hydrolysis of PCho to provide choline for glycine betaine.

Datko and Mudd (1988a,b) have done extensive work on the enzymes of PtdCho synthesis in *Lemna paucicostata*, soybean and carrot. These plants do not accumulate glycine betaine. *Lemna* uses a phosphobase route to sequentially *N*-methylate PEA to PCho for PtdCho synthesis (Mudd and Datko, 1986). Datko and Mudd (1988a) used tissue culture preparations of carrot (*Daucus carota* L.) and soybean (*Glycine max*) as well as soybean leaf discs to determine how PtdCho was synthesized in these species. Using labelled [^{3}H and ^{3}C] methionine, soybean and carrot tissue culture or soybean leaf discs performed an initial methylation of PEA to form PMEA. From this point on they differed in that further *N*-methylations for soybean were done with phosphatidyl

bases while carrot proceeded at both the phospho and phosphatidyl-base levels (Datko and Mudd, 1988a).

Choline synthesis has also been studied in castor bean endosperm by Prud'homme and Moore (1992a,b). These researchers found that the initial reaction in choline synthesis used the free base route, specifically EA → MEA. After this initial step, MEA was converted to PMEA and then choline synthesis was carried out following intermediates of the phosphobase route. In water-stressed barley, a glycine betaine accumulating monocot in the Gramineae, choline is synthesized by the phosphobase route and there is some use of phosphatidyl intermediates (Hitz *et al.*, 1981).

Lemna, soybean, carrot, barley, sugar beet and spinach, are all plants which *N*-methylate PEA → PMEA via the enzyme, *S*-adenosyl-L-methionine: phosphoethanolamine *N*-methyltransferase (PEAMeT). Datko and Mudd (1988a) proposed that the presence of this enzyme in diverse plants suggests that this methylation appears to be a common or conserved step in choline synthesis in spite of the divergence found in subsequent steps. Some evidence suggests that the enzyme that catalyzes this reaction, PEAMeT, has a role in regulating choline biosynthesis (Mudd and Datko 1989a). Cho applied exogenously to *Lemna*, carrot or soybean tissue, results in a reduction in the synthesis of Cho from EA (Mudd and Datko, 1989a,b). These experiments also identified a possible site of feedback inhibition for choline synthesis, which appears to be the first methylation

step, namely PEA → PMEA formation by PEAMeT. Mudd and Datko (1989a) speculated that PEAMeT could potentially regulate the flux through the choline synthesis pathway. Thus, in all of these studies, the enzyme which *N*-methylates PEA to PMEA, PEAMeT, is required for choline synthesis, possibly among all higher plants (Datko and Mudd, 1988a).

Phosphobase *N*-Methyltransferases in Spinach Leaves

The enzymes involved in the phosphobase pathway enroute to synthesis choline, PEAMeT, PMEAMeT (*S*-adenosyl-L-methionine: phosphomethylethanolamine *N*-methyltransferase, PMEA → PDEA) and PDEAMeT (*S*-adenosyl-L-methionine: phosphodimethylethanolamine *N*-methyltransferase, PDEA → PCho) have been identified and all three phosphobase *N*-methyltransferase activities are easily detected in crude leaf extract preparations of spinach (Weretilnyk and Summers, 1992; Summers and Weretilnyk, 1993; Weretilnyk *et al.*, 1995). PEAMeT activity in spinach leaves is highest at the end of the eight hour day and loses approximately half of its activity during the 16 hour night (Weretilnyk *et al.*, 1995). Upon resumption of the day cycle, PEAMeT activity increases steadily over the course of the light cycle. Crude leaf extracts prepared from spinach plants placed in the dark for 40 hours have in vitro PEAMeT activity levels that are low but above the detection limits of the

radioassay (Weretilnyk *et al* ., 1995). Thus the enzyme, PEAMeT, is light regulated in spinach leaves (Weretilnyk *et al.*, 1995). While PEAMeT activity shows light regulation, the activities of PMEAMeT and PDEAMeT do not undergo a continuous drop after exposure to a prolonged dark period. Thus, this supports the proposal that there are at least two enzymes present in the phosphobase route to synthesize choline (Weretilnyk and Summers, 1992). Summers and Weretilnyk (1993) also showed that the phosphobase pathway is upregulated under salt stress. In vitro enzyme assays of spinach leaf extracts showed an increase in all three phosphobase activities that were 2-3 fold higher than control unstressed spinach plants. In vivo radiotracer studies with [^{14}C]EA also showed that an elevated rate of Cho synthesis with salt stress is likely mediated by an increase in the activities of these three enzymes (Summers and Weretilnyk, 1993).

Glycine Betaine Biosynthesis

Glycine betaine is a quaternary ammonium compound that is accumulated in the plant in response to saline or dry environments (Wyn Jones and Storey, 1981: Rhodes and Hanson, 1993). Glycine betaine may act as a non-toxic osmotically active organic solute (osmolyte) that can be present in relatively high concentrations in the cytoplasm of the plant cell thus facilitating water uptake and retention by the plant (Wyn Jones and Storey, 1981; Rhodes and Hanson, 1993; Yancey *et al.*, 1982). Osmolyte compatibility results primarily from the absence of

perturbing effects on enzyme function and structure (Wyn Jones and Storey, 1981). Other compatible osmolytes include proline betaine, hydroxyproline betaine, β-alanine betaine and choline-*O*-sulphate (Hanson, 1992; Rhodes and Hanson, 1993).

In glycine betaine accumulating plants, in vivo tracer studies with radioactive or stable isotopes (Weretilnyk *et al.*, 1989) have confirmed that glycine betaine is synthesized in higher plants examined thus far by a two step oxidation of choline via the intermediate betaine aldehyde. Enzymes mediating both steps have been studied in the Chenopodiaceae, specifically, the chenopods sugar beet (Hanson and Wyse, 1982; Hanson and Rhodes, 1983) and spinach (Couglan and Wyn Jones, 1982). Both enzymatic reactions can be carried out by isolated chloroplasts of spinach (Hanson *et al.*, 1985). The first step is catalysed by a ferredoxin-dependent choline monooxygenase (CMO) (Brouquisse et al., 1989). CMO is a soluble enzyme located in the choroplast stroma. The reducing power in the form of reduced ferredoxin as an electron donor is photosynthetically generated and choline oxidation is strongly promoted by light both in vivo and in isolated chloroplasts (Hanson *et al.*, 1985).

The second enzyme in the glycine betaine pathway is catalyzed by a pyridine nucleotide-dependent betaine aldehyde dehydrogenase (BADH) that has a strong preference for NAD^+ (Weretilnyk and Hanson, 1989). In spinach leaves the majority (90%) of BADH activity is in the choroplast stroma, the remainder

apparently in a cytosolic isozyme (Weigel *et al.*, 1986). Since CMO appears to require photosynthetically reduced ferredoxin, betaine aldehyde is not likely to be enzymatically produced outside of chloroplasts (Hanson *et al.*, 1985). BADH has been purified to homogeneity from spinach leaves (Weretilnyk and Hanson, 1989) and the purified enzyme is a dimer with subunits of M_r of 60 000. The gene encoding BADH was first cloned from spinach (Weretilnyk and Hanson, 1990).

For glycine betaine accumulating plants, choline synthesis assumes a metabolic role in stress adaptation. Choline is directed toward glycine betaine synthesis and is presumed to be no longer available for phospholipid synthesis since glycine betaine appears to be a dead end product once formed (Hanson and Hitz, 1982). The research on glycine betaine formation and the enzymes involved in the two step oxidative conversion of choline to glycine betaine has now shifted to uncovering how plants furnish choline for the production of glycine betaine.

The importance of choline is seen in plant phospholipid metabolism and in the adaptation of plants to osmotic stress. Thus the focus of this research project was to perform a comparative biochemical study of the enzymes of choline biosynthesis in diverse dicot plants. Many of these plants were selected from a dicot family tree showing the phylogenetic relationships among plant orders (Weretilnyk *et al.*, 1989). The presence and levels of PEAMeT, PMEAMeT and PDEAMeT phosphobase *N*-methyltransferase activities were determined using

crude leaf extracts prepared from 14 plant species and a sensitive enzyme radioassay. The products of PEAMeT activity were identified by thin layer chromatography to ensure PMEA was the predominant product formed from PEA by extracts of all the plants studied. Furthermore, all plants were subjected to a continuous dark exposure treatment (exceeding 40 hrs) and the crude leaf extracts prepared from these plants were also assayed for phosphobase *N*-methyltransferase activities to determine if any exhibited light-regulated properties comparable to spinach.

This study will attempt to address the following questions: Is PEAMeT activity ubiquitous in plants as proposed by Datko and Mudd (1988a)? Do other plant species show any light regulation of PEAMeT, PMEAMeT and PDEAMeT enzymes? Finally, if present, are levels of enzyme activities comparable between plants or do some plants appear to have inherently higher capacities for choline synthesis?

MATERIALS AND METHODS

CHEMICALS AND RADIOCHEMICALS

All chemicals and enzymes were purchased from Sigma unless otherwise stated. All aqueous solutions were prepared using deionized water. The radioisotopes *S*-[methyl-^{3}H] adenosyl-L-methionine ([^{3}H]SAM, NET-155H), *S*-[methyl-^{14}C] adenosyl-L-methionine ([^{14}C]SAM, NEC-363) and L-[^{35}S]-methionine ([^{35}S]-methionine, NEG-009H) were purchased from NEN Life Science Products, Inc. The specific activity of [^{3}H]SAM ranged from 55 - 85 Ci•mmol^{-1} (0.55 mCi•mL^{-1}) and a SAM concentration ranging from 0.0065 to 0.0085 μmol•mL^{-1}. Upon arrival the stock [^{3}H]SAM was dispensed into 10 μL aliquots and stored at -20°C. [^{14}C]SAM had a specific activity ranging from 40 - 60 mCi•mmol^{-1} (0.02mCi•mL^{-1}) and was divided into 50 μL volumes and stored at -20°C. L-[^{35}S]-methionine (10 mCi•mL^{-1}) was also dispensed into 10 μL aliquots and stored at -80°C.

Unlabelled SAM, purchased from Boehringer Mannheim Canada (102407), was dissolved in 0.01 N H_2SO_4:ethanol (9:1,v/v) and the concentration determined using the molar extinction coefficient of 15 M^{-1}•cm^{-1} at 257 nm (Eloranta *et* al., 1976). Based upon the absorbance reading, the final SAM concentration was adjusted to 12 mM using 0.01 N H_2SO_4:ethanol (9:1,v/v), and then dispensed into 25 μL aliquots for storage at -20°C.

Dowex 50W(H^+) (X8, 200 mesh) resin was regenerated to a fully protonated form before use. First, 250 g of resin was mixed with 500 mL water, allowed to settle and the supernatant carefully decanted away from the settled resin. The resin was then resuspended with 500 mL 1.0 N HCl, mixed, allowed to settle and the supernatant drawn off. This washing procedure was repeated twenty times and then followed by at least twenty washings with water (or until the pH was between 5 - 7) to remove any excess acid. Dowex 1 (OH^-) (X8, 100-200 mesh) was completely regenerated to the OH^- form by washing the resin 25 times with 1.0 N NaOH. Dowex 50W (H^+) (X8, 100-200 mesh) was fully converted to the NH_4^+ form by washing 30 times with 1.0 N NH_4OH. Biorex 70 (Na^+) (100-200 mesh, Bio-Rad Laboratories) was fully converted to the H^+ form with 25 washes with 1.0 N HCl. In all cases, the acid or base treatment was followed with at least 20 washes with water.

Phosphobase Substrate Preparation

Commercially available PEA (Sigma, P-0503) was prepared by dissolving the PEA in 0.1N HCl to a final concentration of 100 mM followed by storage at -20°C. This stock solution was then diluted to 7.5 mM with water for phosphobase *N*-methyltransferase enzyme assays and stored at -20°C.

PMEA and PDEA substrates were synthesized from their phosphatidyl derivatives, PtdMEA and PtdDEA (Sigma, P-3274 and P-05399) using a modified

method from that reported by Datko and Mudd (1988a). In this procedure, 0.012g of PtdMEA and PtdDEA were dissolved in 1.0 mL 5% (v/v) Triton X-100 in each of two 13 mL polystyrene Falcon tubes and rotated end-over-end at room temperature for 48 hours. PMEA and PDEA substrates were then produced by phospholipase C (Grade 1 suspension, from *Bacillus cereus,* 4 enzyme units•μL^{-1}, Boehringer Mannheim Canada, 691950) digestion of PtdMEA or PtdDEA. For this step, 50 μL phospholipase C (200 enzyme units) was mixed with 75 μL 0.042 mM 3,3-dimethylglutarate buffer (pH 7.5, NaOH) and then desalted by centrifugation through a Sephadex G-25 (medium, Pharmacia) column equilibrated with the same buffer. Desalting by centrifugation was completed as described by Weretilnyk *et al.*, 1989. The desalted phospholipase C was then added to each phosphatidylbase tube after the 48 hour end-over-end rotation was complete. The tubes were incubated at 37°C in a water bath with slight agitation for 90 minutes. The water soluble PMEA and PDEA products were separated from the lipid soluble phosphatidyl compounds by adding 3.2 mL methanol and 1.6 mL chloroform, waiting two minutes, then adding 2.06 mL water and 4.8 mL chloroform. The mixture was vortexed to mix and then centrifuged at speed four for 1-2 min at room temperature in a clinical centrifuge fitted with a swing-out rotor. The contents of the tube should separate into an upper, aqueous phase and a lower lipid phase of approximately equal volume. If a clear boundary layer does not form, two to three drops of methanol are added and the tube is centrifuged as before. The top water soluble layer containing PMEA or PDEA was clear and

colourless while the bottom soluble layer was slightly cloudy and grey in colour. The top water soluble layer containing either PMEA or PDEA was transferred to a 13 x 100 mm test tube and concentrated by evaporation under nitrogen gas at 40°C (Meyer-N-Evap Analytical Evaporator, Organomation Inc.). The whitish residues were dissolved in 150 μL 0.1 N HCl and stored at -20°C.

The concentration of PMEA and PDEA was determined from the amount of inorganic phosphate (Pi) cleaved from the respective phosphobase. Pi concentration was determined following the method outlined by Martin and Tolbert (1983). PMEA and PDEA were hydrolysed by alkaline phosphatase (20 enzyme units•μL, Boehringer Mannheim Canada, 1097075) in the following assay medium: 5.0 μL of a 10 or 15-fold diluted phosphobase, 1.0 μL alkaline phosphatase and 225 μL buffer (50 mM $NaHCO_3$, pH 10.4, NaOH, 22°C, 1 mM $MgCl_2$ and 0.1 mM $ZnSO_4$) all combined in a final volume of 231 μL and then incubated at 37°C in a water bath for 20-24 hours. Controls containing 5.0 μL of the 10 or 15-fold diluted phosphobase and 226 μL water were placed at -20°C for the same time period. At the end of the incubation period, all tubes including those placed at -20°C were stopped with 250 μL 10% (w/v) trichloroacetic acid (TCA) and centrifuged at room temperature for 5 minutes at 14,000 rpm in an Eppendorf 5415C microcentrifuge. Next, 250 μL of the supernatant were transferred to a new tube containing 250 μL water, giving a total volume of 500 μL.

Pi standards of 0.5, 2.5, 5, 10, 15, 20 and 25 nmol Pi in a final volume of 500 μL were prepared by a dilution of a 1.0 mM KH_2PO_4 stock solution. An additional 500 μL of ammonium molybdate solution (0.25 g $(NH_4)_6Mo_7O_{24}•4H_2O$, 1.0 g sodium ascorbate, 3.0 mL concentrated H_2SO_4 made to 50 mL with water) was added to both Pi standards and the previously prepared 500 μL phosphobase reaction mixtures. The reactions were placed in a water bath for 90 minutes at 37°C and then the contents were immediately removed and their absorbance measured at 820 nm using a UVIKON 930 spectrophotometer. In the presence of Pi, the reaction mixture takes on a purple hue which is measured spectrophotometrically. The absorbance reading of the standards allows a Pi standard curve (absorbance at 820 nm versus nmoles Pi) to be drawn and used to determine the amount of Pi (nmoles) present in the phosphobase reaction tubes. Newly synthesized PMEA and PDEA concentrations ranged between 33 and 97 mM and each were diluted to 7.5 mM with water and stored at -20°C until used. To verify PMEA and PDEA product formation and that contamination did not occur, thin layer chromatography (see section entitled Thin Layer Chromatography) was performed on all newly prepared PMEA and PDEA stocks and the RF positions of the compounds resolved by TLC were compared to previously identified phosphobase compounds. As well, newly synthesized and diluted PMEA and PDEA solutions were tested as substrates in enzyme assays that were performed in parallel with assays using previously prepared phosphobase stocks. Comparable enzyme activity determinations for the older

and newly prepared substrates provided some assurance that the products were free of inhibitory contaminants and that the concentrations of the new substrates were not seriously in error.

PLANT MATERIAL

Seeds and Seeding

The seeds of fifteen dicot plant species representing diverse phylogenetic orders were chosen from a representative dicot family tree (Weretilnyk *et al.*, 1989). The seeds were obtained from various sources as outlined in Table I.

Each plant species required a different regime of controlled environmental conditions for growth and a summary of the growing conditions used for each plant can be found in Table II. As shown in Table II, all plants were grown in either coarse vermiculite or a 1:1:1 potting mixture of peat, vermiculite (Holiday Vermiculite, Vil Vermiculite Inc.) and perlite. For ease of discussion throughout the text, all plants will be referred to by their common names. Plants were grown in controlled environment chambers (Conviron E15) with a light intensity of 300 µmol• m^{-2} • s^{-1}. Temperature and daylight regimes (24°C , 8 h day; 20°C, 16 h night or 20°C, 16 h day; 20°C, 8h night) as well as potting conditions (350 mL plastic pots or 18 x 13.5 x 6 cm small plastic trays) for each plant species are found in Table II.

TABLE I. Plants Selected for Study.

Order[a]	Family	Species	Cultivar (if known)	Seed Source
Asterales	Asteraceae	*Helianthus annus*	Russian Mammoth	Tregunno Seeds, Hamilton, Ontario
		Lactuca sativa	Romaine	A.E. McKenzie Co. Ltd, Brandon, MB
			Esmeralda M.I pvp	Stokes Seeds Ltd., St. Catharines, Ontario
Caryophyllales	Chenopodiaceae	*Spinacia oleracea*	Savoy hybrid 612	Harris Moran Seeds, Rochester, New York
		Beta vulgaris	HM 8282	Alberta Sugar Co., Tabor, Alberta
	Amaranthaceae	*Amaranthus caudatus*	Love Lies Bleeding	Thompson and Morgan, Jackson, New Jersey
Capparales	Crucifereae	*Brassica napus*	OAC Summit	Crop Science Department, University of Guelph
Cucurbitales	Cucurbitaceae	*Cucurbita sativa*	Improved Long Green	Stokes Seeds Ltd., St. Catharines, Ontario
Fabales	Leguminosae	*Glycine max*	OAC Bayfield	Crop Science Department University of Guelph
		Pisum sativum	Thomas Laxton	Ontario Seed Company Ltd., Waterloo, Ontario
Malvales	Malvaceae	*Gossypium hirsutum*	Paymaster HS26	Horticultural Science, University of Florida at Gainesville
Plumbaginales	Plumbaginaceae	*Limonium perezii*		Thompson and Morgan, Jackson, New Jersey
		Limonium sinuatum		Thompson and Morgan, Jackson, New Jersey
Polemoniales	Convolvulaceae	*Convolvulus arvensis*		Valley Seed Service, Fresno California
Scrophulariales	Solanaceae	*Nicotiana tabacum*	Wisconsin 38	Horticultural Science, University of Florida at Gainesville
Umbellales	Umbelliferae	*Daucus carota*	Nantes Touchon	A.E. McKenzie Co. Ltd, Brandon, MB

[a] Orders listed alphabetically.

TABLE II. Planting Conditions Used in Growing Plants for Experimental Study.

Species[a]	Common Name	Growth Medium		Container		Environmental Conditions		Average Age at Harvest
		vermiculite	peat: perlite: vermiculite; 1:1:1	tray	pot	24° C 8 h day 20° C 16 h night	20° C 16 h day 20° C 8 h night	Days
Amaranthus caudatus	Amaranth		✔		✔		✔	52
Beta vulgaris	Sugar Beet	✔			✔		✔	36
Brassica napus	Canola		✔		✔		✔	32
Convolvulus arvensis	Bindweed		✔	✔			✔	42
Cucurbita sativa	Cucumber		✔		✔		✔	35
Daucus carota	Carrot		✔	✔			✔	33
Glycine max	Soybean		✔		✔		✔	46
Gossypium hirsutum	Cotton		✔		✔	✔		44
Helianthus annus	Sunflower		✔[b]		✔		✔	30
Lactuca sativa	Lettuce		✔	✔			✔	39
Limonium perezii	Statice		✔	✔			✔	124
Limonium sinuatum	Statice		✔	✔			✔	101
Nicotiana tobacum	Tobacco		✔	✔			✔	52
Pisum sativum	Pea	✔			✔		✔	25
Spinacia oleracea	Spinach	✔			✔	✔		44

[a] Species ordered alphabetically.

[b] fertilized with Osmocote[R].

Small holes at the base of the pots and trays provided ample drainage to prevent over watering and reduce algae growth.

Spinach seeds were planted approximately 1.0 cm deep in moist vermiculite. After seeding, water is withheld for three days then followed by daily watering for the next four days. Exactly one week from seeding, individual spinach seedlings were transplanted from small trays (18 x 13.5 x 6 cm) into individual 350 mL plastic pots where they remained until harvest, usually 4 to 7 weeks. Sunflower, soybean, canola, cotton and sugar beet seeds were sown directly in 350 mL plastic pots and seedlings eventually pruned to one plant per pot. All other seeds were planted in a 1:1:1, peat; perlite; vermiculite mixture in small trays covered with Saran Wrap (Dow) to prevent water loss. If too much condensation collected on the underside of the Saran Wrap, slits were cut into the plastic wrap to aerate and reduce algae growth. The small trays were placed within larger trays (55 x 28 x 6 cm) filled with half-strength Hoagland's solution (Hoagland and Arnon, 1950) to approximately 1.5 cm deep. Once the seedlings were large enough to survive transplantation, they were transferred to the appropriate growth medium (Table II).

Occasionally seeds germinated but seedlings failed to thrive. On these occasions the surface of the soil mix was drenched with a solution containing 10 % (w/v) Benelate fungicide. Also, prior to seeding, sunflower and soybean seeds were surface sterilized with dilute bleach (Chlorox Bleach diluted with water to a final conentration of 2.8% (w/v) sodium hypochlorite) for 5 to10 minutes. The

seeds were then rinsed thoroughly with water before seeding. Plants were watered daily with half strength Hoagland's solution (Hoagland and Arnon, 1950).

Controlled Environment Growth Conditions

The conditions of light duration and temperature used to grow the various plants are summarized in Table II. Spinach was grown under a short, 8 h day as it flowers under long days. By comparison, few other plants did well under the conditions favoured by spinach. Rather, with the exception of cotton, all other plants appeared much healthier grown under a longer (16 h), cooler (20°C) day.

Furthermore, it was noted that sunflower tended to develop chlorotic and necrotic lesions in the foliage regardless of day length. Since a nutrient deficiency was suspected, Osmocote (N: P: K^+, 9: 9: 18, Plant Products Co. Ltd.) granules were added to the potting mixture. Approximately one teaspoon of Osmocote was mixed well with the 1:1:1 soil mixture in each 350 mL plastic pot containing the newly transplanted sunflower seedlings.

Plants were harvested at various ages, a reflection of the length of time required to generate suffficient leaf material to harvest (Table II). Since light appears to regulate or influence the activity of at least one phosphobase *N*-methyltransferase in spinach (Weretilnyk *et al.*, 1989), unless otherwise stated, all plants were exposed to the same length of light period prior to harvest. Thus all

plants were harvested four hours after completion of the night cycle and resumption of the day period.

In experiments where a prolonged dark period was required, a subset of two plants was randomly selected and transferred to dark cabinets where temperature was maintained at 20°C and lights remained off for continuous periods in excess of 40 h. To ensure that the dark conditions were of sufficient duration to affect the level(s) of enzyme activity in spinach, any test species transferred to the dark was accompanied by two, randomly selected spinach plants. At the point of harvest, the spinach plants were harvested at the same time as any other species being tested.

To facilitate discussion in this thesis, all plants harvested 4 h into the day period will be considered as "control" plants as these conditions did not include a departure from their normal growing conditions. Plants exposed to prolonged dark periods (40 to 48 h) will be described as having undergone a dark "treatment".

Extract Preparation

For each extract preparation, leaves from a minimum of two individual plants were harvested at any one time. A single harvest of a plant of interest involved grinding leaves to produce six extract preparations: 1) control test plant; 2) control spinach; 3) 50:50 (w/w) mixture of control spinach and test plant;

4) dark-treated test plant; 5) dark-treated spinach; 6) 50:50 (w/w) mixture of dark-treated spinach and test plant.

All procedures were carried out at 4°C or on ice. The entire leaf consisting of the blade and not the midrib was coarsely chopped with a razor blade, ground in a mortar with sea sand (Fisher Scientific Limited) in a buffer (2.0 $mL \cdot g^{-1}$ Fresh wt) containing 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)-KOH, pH 7.8, 1.0 mM ethylenediaminetetraacetic acid disodium salt ($Na_2 \cdot EDTA$), 5.0 mM dithiothreitol (DTT, ICN Biochemcial), 5.0 mM sodium ascorbate, 10 mM sodium borate, and 20 mM sodium metabisulfite. The buffer was freshly prepared on the day of each harvest. The amount of leaf material ground ranged from 2 - 4 g of tissue randomly chosen from the approximately 8 g of chopped leaves. Some preparations such as those for cotton, statice and carrot included polyvinylpyrrolidone (PVP, 4% w/v) in the extraction buffer otherwise no enzyme activity was recovered from these plants. The homogenate was filtered through four layers of cheese cloth (Fisher Scientific Limited) then one layer of Calbiochem Miracloth (Rose Scientific). The resulting filtrate was transferred to a 15 mL Corex tube and centrifuged (Sorvall RC-5B Refrigerated Superspeed Centrifuge, SS-34 rotor) at 4°C and 10,000 g for 12 minutes. The pellet formed was discarded and the supernatant retained. At this point, the supernatant was either used directly to assay for enzyme activity or the crude extract was desalted by centrifugation through Sephadex G-25 columns (Weretilnyk *et al.*, 1989) prior to assay to remove low molecular weight

compounds. The Sephadex G-25 beads were equilibrated with 100 mM Hepes-KOH (pH 7.8), 1.0 mM $Na_2 \cdot$ EDTA, and 5.0 mM DTT. Therefore, two types of crude leaf extracts designated as 'undesalted' and 'desalted' were assayed for [^{3}H]SAM phosphobase *N*-methyltransferase activities. When possible, extracts were assayed for PEAMeT activity the same day they were prepared. Regardless of whether the assays were carried out on the day of extract preparation, all samples were flash frozen in liquid nitrogen and then stored at -80°C. All PMEAMeT and PDEAMeT activities reported were from assays of samples previously frozen and stored at -80°C. The frozen extract samples were quickly thawed by hand using gentle agitation. Only undesalted crude extracts were assayed for *S*-adenosyl L-methionine: L-methionine *S*-methyl transferase (MMT) activity. Only desalted crude extracts were employed in [^{14}C]PEAMeT assays.

Protein and Chlorophyll Determination

The concentration of protein in crude undesalted and desalted extracts was determined colormetrically by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as the standard. The assay was performed using the Bio-Rad MicroAssay Reagent (Bio-Rad Laboratories) following the protocol provided by the manufacturer.

Chlorophyll concentration was determined by removing 25 µL of the filtered brei (prior to centrifugation) and transferring the aliquot to 3.0 mL of

80% acetone and 20% water in a 13 x 100 mm foil wrapped test tube. The mixture was vortexed, then centrifuged at room temperature in an IEC clinical centrifuge for 4-5 min at step six. The absorbance of the supernatant was measured at 700 nm, 663 nm, and 645 nm using a UVIKON 930 spectrophotometer. The absorption coefficients of Arnon (1949) were used to determine the concentration of chlorophyll following the formula: $20.2\ (A_{645}-A_{700}) + 8.02\ (A_{663}-A_{700})$ = chlorophyll ($\mu g \cdot mL^{-1}$).

ENZYME ASSAYS

PHOSPHOBASE *N*- METHYLTRANSFERASES

Assay Conditions

Phosphobase *N*-methyltransferase enzyme assays were performed as described by Summers and Weretilnyk (1993). The phosphobase substrates used included PEA, PMEA and PDEA and methylation of these metabolites was assayed in tubes containing 100 µL of 150 mM Hepes-KOH, pH 7.8 and 1.5 mM $Na_2 \cdot$EDTA, 2.5 µL unlabelled 12 mM *S*-adenosyl-*L*-methionine (SAM), 1.0 µL labelled [^{3}H]SAM (0.55 µCi, NET-155H), 16.5 µL water, 5.0 µL of 7.5 mM phosphobase substrate (PEA, PMEA or PDEA) and 25 µL crude undesalted or desalted extract in a final volume of 150 µL. Controls were set up with water in

place of the phosphobase substrate. For extracts with low enzyme activity, 41.5 μL of undesalted or desalted crude extract was added to the assay tube and the separate water addition to the assay eliminated. The tubes were briefly spun in an Eppendorf 5415C microcentrifuge to mix the contents and then the tubes were incubated for 30 minutes at 30°C in a water bath. The reaction was stopped by dilution with 1.0 mL ice cold water, then an aliquot of 1.0 mL was removed from the stopped reaction mixture and applied to a 1.0 mL bed of Dowex 50W(H^+) resin in a disposable Evergreen column (Diamed, E/S 208-3384-060). The resin was washed with two washes of 0.5 mL water, the effluent discarded and the drained columns were transferred to clean 16 x 150 mm collection tubes. The phosphobases were eluted from the column with two washes of 5.0 mL of 0.1 N HCl and the eluate collected was vortexed to mix. A 1.0 mL aliquot of the eluate was then combined with 5.0 mL Beckman Ready Safe liquid scintillation cocktail (Beckman), vortexed then counted on the liquid scintillation counter (Beckman LS 1801). The remaining 9.0 mL was either discarded or stored at -20°C until sufficient product from comparable assays had accumulated to enable product identification by thin layer chromatography.

PEAMeT activity was also assayed in the presence of [^{14}C]SAM in lieu of [^{3}H]SAM. For these assays, reaction tubes contained 50 μL of 300 mM Hepes-KOH, pH 7.8 and 3.0 mM Na_2·EDTA, 2.2 μL 12 mM SAM, 10 μL labelled [^{14}C]SAM (200 nCi; NEC-363), 5.0 μL of 7.5 mM PEA substrate and 82.8 μL desalted extract in a final volume of 150 μL. The controls used water in

place of phosphobase substrate. The reactions were incubated for 60 min at 30°C in a water bath. The remainder of the procedure for assaying phosphobase *N*-methyltrnasferase activity was as described above for the [^{3}H]SAM based assay with the exception that all of the stopped reaction (1.15 mL) was applied to the Dowex 50W(H^+) column.

Calculation of phosphobase *N*-methyltransferase enzyme activity required several steps. First, counts per minute (cpm) obtained from a sample incubated with substrate was corrected for any apparent methylation activity independent of substrate (water control). Subtraction of control cpm from sample cpm yields a "net" cpm for substrate-dependent enzyme activity for one mL of column eluate. This value is multiplied by 10 (for 10 mL of 0.1 N HCl eluate) and corrected for the original assay volume of 1.15 mL to give the net cpm per assay. With a counting efficiency estimated to be 41.6% for [^{3}H]SAM under our counting conditions (Summers, unpublished), the cpm can be converted to dpm per assay. Since there are 2.22×10^6 dpm per μCi, dividing by dpm per μCi will give μCi per assay. Given each assay has 0.01833 μCi per nmol of SAM, dividing μCi per assay by μCi per nmol will give nmol per assay. This value, nmol per assay is then divided by the incubation period and the amount of extract used to give $nmol \cdot min^{-1} \cdot mL^{-1}$ extract. Taking into account that 1.0 g of tissue is ground in 2.0 mL buffer, multiplying by a factor of two will give activity on a gram fresh weight basis ($nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt). The same procedure is followed for calculating [^{14}C]SAM enzyme activity with the following considerations: the counting efficiency

of [^{14}C]SAM was assumed to be 100%, thus cpms were directly converted to dpms, the entire 1.15 mL stopped reaction mixture was added to the resin column and each assay had 6.67 nCi per nmol of SAM.

Assay Product Identification by Thin Layer Chromatography

Phosphobase *N*-methyltransferase assay products were identified using the 9.0 mL eluate of an assay chosen because it showed the highest radioactivity from among PEAMeT assays of comparable material. Each 9.0 mL eluate containing [^{3}H]- or [^{14}C]-labelled phosphobase reaction products from PEAMeT assays was stored at -20°C as described earlier. For analysis, the 9.0 mL eluate was thawed at room temperature and combined with 300 nmoles each of PEA, PMEA, PDEA and PCho to serve as carriers. The samples along with the phosphobase carriers were then concentrated by evaporation with nitrogen gas at 40°C (Meyer-N-Evap Analytical Evaporator, Organomation Inc.). Once dried, the samples were dissolved in 400 - 500 µL 0.1 N HCl, nitrogen gas evaporated and the resulting residue dissolved with 10 µL 0.1 N HCl. From this 10 µL volume, 1.0 µL was removed for scintillation counting in 5.0 mL liquid scintillation cocktail (Beckman Ready Safe) to quantify radioactivity. Of the remainder, 4.0 µL was spotted onto a Polygram silica G plate (20 x 20 cm, Macherey-Nagel Company), and the balance was stored at -20°C.

Prior to loading a sample, Polygram silica G plates were dried in a 40°C

oven for at least 24 hours. TLC tanks were lined with two sheets of 21 x 20 cm 3 MM chromatography papers (Whatman) to provide an equilibrated tank containing methanol, *n*-butanol, HCl and water (50:50:10:10, v/ v/ v/ v) in a 120 mL volume. The dried plate was placed inside the equilibrated tank the evening before the sample was applied. The plate was carefully set within grooved slots of the tank to keep it upright and prevent it from leaning on the tank wall. The next morning, the plate was removed from the tank and allowed to air dry for a minimum of two hours in a fume hood. At this point, the plate was ready for sample spotting. Exactly 1.0 cm from the bottom of the plate, a line was drawn by pencil extending the width of the plate and marked off at 1.0 cm intervals. A maximum of nine lanes, (1.0 cm wide), each separated by 1.0 cm, was spotted per plate. Each 4.0 µL sample was spotted as tiny beads across the 1.0 cm lane using a 10 µL Hamilton syringe (Fisher Scientific Limited model 701N). Approximately 1.0 µL of sample could be applied to the 1.0 cm lane at one time and then the sample was dried by passing a stream of warm air over the plate with a hair dryer between applications. Each phosphobase standard (300 nmoles) was also spotted onto individual lanes for comparison.

The TLC plate with phosphobase samples was placed in a paper-lined (3 MM chromatography papers; Whatman) tank with 110 mL solvent of methanol, *n*-butanol, HCl and water (50:50:5:5, v/ v/ v/ v) for six hours. After six hours, the plate was removed and air dried in a fume hood. At this point, any TLC plates carrying [^{14}C]phosphobases were exposed to X-ray film

(20.3 x 25.4 cm, X-OMAT AR, Eastman Kodak Co.) for a minimum of two weeks at -80°C. After the film was developed, the TLC plates were then sprayed with phosphomolybdate reagent (4.0 mL water, 0.5 g ammonium molybdate, 0.38 mL concentrated HCl, 2.5 mL 70% (v/v) perchloric acid and acetone to 50 mL) and allowed to dry. The phosphobases were visualized by exposure to short wavelength UV radiation for 1.5 h using the Mineralight UVS-54 lamp kept 5.0 cm above the plate. Purple spots were detected wherever standards were applied, including those added as carrier with radio-labelled samples. The relative mobility of the standards relative to radioactive zones on the plate enabled identification of spots for the [^{3}H] or[^{14}C]phosphobase products. Since it is not possible to use autoradiography for [^{3}H]-labelled products, TLC plates used to resolve and identify [^{3}H]phosphobases were not exposed to X-ray film. Rather, following removal from the TLC tanks, these plates were air dried then sprayed with the same phosphomolybdate reagent as described above and exposed to UV for development. The origin and any spots staining purple with the reagent were lightly outlined with a pencil, scraped from the plate and the powder transferred to a 10 mL scintillation vial containing 1.0 mL water, mixed and then 5.0 mL Beckman Ready Safe liquid scintillation cocktail was added. Vial contents were mixed by vortexing and the radioactivity quantified by counting on the liquid scintillation counter (Beckman LS 1801).

METHIONINE METHYLTRANSFERASE

Assay Conditions

The radioisotope used for *S*-adenosyl L-methionine: L-methionine *S*-methyl transferase (MMT) assays was L-[^{35}S]-methionine with a half life of 87.1 days. For the MMT assay, 10 μL of the stock [^{35}S] methionine was mixed with 25 μL 200 mM L-methionine, 115 μL water and 50 μL Dowex 50W (NH_4^+) slurry. The slurry was centrifuged (Eppendorf 5415 microcentrifuge) at room temperature for one minute at 14,000 rpm, the supernatant drawn off and aliquoted into 20-μL volumes for storage at -80°C.

The MMT assay used was modified from that reported by James *et al.* (1995). MMT activity was assayed in 1.5 mL microfuge tubes containing 2.0 μL of buffer (100 mM Hepes-KOH (pH 7.8), 1.0 mM $Na_2 \cdot$EDTA, and 5.0 mM DTT), 4.0 μL L-[^{35}S]-methionine (100 nCi, 1 mM), 4.2 μl 12 mM SAM and 41.8 μL extract in a final volume of 50 μL. Again, controls consisted of water replacing substrate, in this case SAM. The reactions were incubated for 60 minutes at 25°C in a water bath and then stopped by dilution with 895 μL ice cold water and 5.0 μL 50 mM L-methionine *S*-methylsulfonium iodide (SMM, Sigma, M-1881). The entire 950 μL stopped reaction was removed and applied to a 1.0 mL disposable column (Evergreen, Diamed, E/S 208-3384-060) containing Dowex 1 (OH^-) resin which was positioned to drain directly into a 1.0 mL column containing Biorex 70 (H^+) resin. Effluent from the Biorex 70 (H^+) resin drained into 16 x 150 mm collection

tubes. The columns, still in tandem, were washed by adding 5.0 mL water to the Dowex 1 (OH^-) matrix. Once drained of the water, the Dowex 1 (OH^-) containing columns were discarded and the material in columns containing Biorex 70 (H^+) were washed with two additional 5.0 mL aliquots of water and allowed to drain dry. The Biorex 70 (H^+) resin columns, once drained of water, were transferred to new 16 x 150 mm test tubes and the methylmethionine eluted from the Biorex 70 (H^+) resin with 5.0 mL 1.0 N HCl. The eluate collected was vortexed to mix and a 500 μL aliquot removed and added to 5.0 mL Beckman Ready Safe liquid scintillation cocktail. The cocktail and sample were vortexed and radioactivity counted on the Beckman LS 1801 liquid scintillation counter.

The counting efficiency of ^{35}S-methionine is assumed to be 100%. Since the half-life of ^{35}S-methionine is 87.1 days, MMT activity had to be corrected for decay of the radioisotope. The radiospecific activity of undiluted stock ^{35}S-methionine as of January 30, 1998 was 10 nCi · μL^{-1}. The amount of decay that had occurred by the day the assay was performed was calculated as the percent decayed using the following equation: % decayed = 100 -100/[antilog {0.301 (no. of days after January 30, 1998 ÷ 87.1 days)}]. Thus, the radiospecific activity used to calculate MMT activity was the correction factor multiplied by 10 nCi · μL^{-1}. The same procedure used to calculate [^{14}C]SAM:PEAMeT enzyme activity is followed for calculating [^{35}S]methionine methyltransferase activity with the following considerations: the entire 950 μL stopped reaction mixture was added to the resin column and each assay had 25 nCi · $nmol^{-1}$ methionine.

MALATE DEHYDROGENASE

Assay Conditions

The malate dehydrogenase spectrophotometric assay was modified from Summers *et al.* (1998). Malate dehydrogenase activity was assayed under the following assay conditions: 660 μL of 150 mM Hepes-KOH, pH 7.8 and 1.5 mM $Na_2 \cdot$ EDTA, 80 μL 2 mM NADH, 100 μL 2.5 mM OAA (oxaloacetate) as substrate, 150 μL water and 10 μL of diluted undesalted plant extract for a total volume of 1.0 mL. The undesalted plant extract was typically diluted 10-fold. The control contained no OAA and the water volume was increased to 250 μL. A blank reaction consisted of 990 μL water and the 10 μL diluted plant extract and was used to establish a zero absorbance reading on the spectrophotometer. The conversion of OAA to malate requires NADH and the spectrophotometer monitors a decrease in absorption of NADH to NAD^+ at 340 nm. The rate of decrease of NADH is divided by its molar extinction coefficient (ε) of 6.22 to give $\mu mol \cdot mL^{-1} \cdot min^{-1}$. MDH activity ($\mu mol \cdot mL^{-1} \cdot min^{-1}$) is then multiplied by the dilution of the 10 μL extract in 1 mL total assay volume as well by the dilution factor of the 10 μL extract. To report enzyme activity on a gram fresh weight basis ($nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt), multiplication by a factor of two for 1.0 g of tissue ground in 2.0 mL buffer is required.

RESULTS

Plant Growing Conditions

The planting medium and growth conditions routinely used for spinach proved unsuitable for many other plants species used in this study. As a consequence, alternative potting media and lighting regimes were tested in order to identify conditions that would lead to high germination rates, healthy seedling establishment and subsequent growth. Table III provides a summary of the average age of each plant at harvest, and the average time each plant spent in its light cycle and dark treatment before harvest. Of the plants shown in Table III, spinach was most frequently grown and harvested since it was harvested and ground each time a different species was tested (Materials and Methods). The date at which plants of different species were harvested was variable and ranged from approximately 24 days for pea to 124 days for statice. This reflected the diversity of growth rates seen among the plants. All plants were harvested in a vegetative state; none were flowering.

Light plays a critical role in the level of PEAMeT activity in spinach leaves (Weretilnyk *et al.* 1995). For standard PEAMeT activity determinations, all plants were harvested about four hours into their eight or sixteen hour light cycle. The mean hours spent in the light before harvest varied slightly for each plant and are recorded in Table III. It has been previously reported that PEAMeT activity declines to barely detectable levels in spinach plants placed in the dark for

TABLE III. Harvest Frequency for Each Plant Species.

Plant[a]	Trials	Average Age at Harvest[b]	Average Time (hrs) into Light Cycle	Average Time spent in Dark Treatment
Spinach	140	43.8 ± 1.5	5.1 ± 0.08	45.4 ± 0.18
Sugar beet	5	36.3 ± 2.3	5.0 ± 0.00	45.8 ± 0.14
Amaranth	10	52.0 ± 4.6	5.0 ± 0.00	45.5 ± 0.62
Statice	4	124.3 ± 4.2	5.2 ± 0.14	44.3 ± 0.27
Soybean	4	45.7 ± 13.2	4.8 ± 0.14	46.2 ± 0.76
Pea	6	24.8 ± 1.6	4.8 ± 0.14	45.0 ± 0.18
Cotton	5	43.5 ± 1.1	5.0 ± 0.24	45.2 ± 0.36
Carrot	8	33.3 ± 1.0	5.0 ± 0.00	44.7 ± 0.27
Canola	3	32.0 ± 2.4	5.7 ± 0.27	45.0 ± 0.47
Bindweed	5	42.5 ± 5.3	5.5 ± 0.24	46.3 ± 0.36
Tobacco	5	51.8 ± 8.5	5.3 ± 0.12	44.8 ± 0.42
Cucumber	6	35.0 ± 1.7	4.8 ± 0.12	45.8 ± 0.31
Sunflower	5	29.8 ± 6.2	5.2 ± 0.12	45.0 ± 0.35
Lettuce	8	38.5 ± 4.0	5.5 ± 0.20	46.2 ± 0.42

[a]data are means ± S.E. with a minimum replicate of n = 2 for cotton and maximum n = 53 for spinach
[b]days from seeding.

prolonged periods (Weretilnyk *et al.*,1995). Thus in order to dark-treat different plants, a period approaching or exceeding 45 hours of continuous dark was selected as a treatment to test the dark stability of PEAMeT activity in different plants. The dark treatments used are given in Table III and are reported as mean hours in the dark.

Phosphobase *N*-methylation in Spinach

Spinach has been studied in our laboratory and PEAMeT activity is readily detectable in leaf tissue (Summers and Weretilnyk, 1993; Weretilnyk and Summers, 1992; Weretilnyk et al., 1995). At least three ways can be used to report enzyme activity: on a gram fresh weight basis ($nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt), as specific activity on a protein basis ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein) or on a chlorophyll basis ($nmol \cdot min^{-1} \cdot mg^{-1}$ Chl). Measurements for PEAMeT activity for spinach leaf extracts assayed crude undesalted or desalted are given in Table IV. The measured enzyme activity, regardless of units of measurement, are significantly higher in desalted extracts versus undesalted extracts. For example, the crude undesalted spinach PEAMeT activity reported on a g Fresh wt basis has approximately 90% the activity of the same extract desalted. This suggests that enzyme measurements based upon crude undesalted extracts may underestimate the amount of activity and so may be considered conservative. Desalting was a routine step done for each plant extract. Table V, however, shows that desalting

TABLE IV. PEAMeT Activity Measurements Using Crude Leaf Extracts of Spinach Plants.

Extract[a]	Enzyme Activity		
	nmol • min^{-1} • g^{-1} Fresh wt	nmol • min^{-1} • mg^{-1} protein	nmol • min^{-1} • mg^{-1} Chl[b]
Undesalted	3.63 ± 0.17	0.29 ± 0.01	4.10 ± 0.17
Desalted	4.12 ± 0.21	0.50 ± 0.02	4.64 ± 0.21

[a] Data are means ± S.E for 69 repeats.
[b] Chlorophyll was measured using filtered brei (Materials and Methods).

Table V. PEAMeT Activity in Crude Leaf Extracts.

Plant	n	Enzyme Activity $nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt	
		Undesalted	Desalted
Bindweed	2	6.14 ± 0.42	2.68 ± 0.04
Cotton	2	25.40 ± 2.49	4.69 ± 2.98
Carrot	3	1.15 ± 0.08	0.65 ± 0.17

did not improve the apparent enzyme recovery of all plant species. For example, in bindweed, the extract was very viscous and desalting proved to be problematic. The consequence is shown in Table V. The crude undesalted extract had approximately two fold more activity than the crude desalted extract. The mucilaginous nature of the extract did not allow it to pass through the desalting beads easily, presumably reducing the recovery of enzyme activity in the resulting extract. Cotton and carrot also did not desalt well as seen in Table V. However, the recovery of the total volume of extract from the desalting procedure was not compromised. Since both of these extract preparations contained PVP (polyvinylpyrrolidone, 4% w/v), the effect that PVP has toward enzyme recovery by desalting is not known. Thus, while spinach PEAMeT appears to benefit from desalting as shown by having higher enzyme activity (Table IV), desalting is not always beneficial for the preparation of extracts from other plants. For the purpose of this thesis, activity measurements reported will not be based upon assays with desalted extracts.

While all three enzyme activity measurements can be used to report enzyme activity as was shown in Table IV for spinach, the values given in this thesis will be those based upon per g Fresh wt. Representation of enzyme activity on a g Fresh wt basis for crude, undesalted extracts was chosen instead of enzyme activity on a chlorophyll basis or a protein basis because the chlorophyll and protein levels showed variation between species (Table VI) and there was no reason to assume that variation in chlorophyll or protein levels would or should be

Table VI. Mean Protein and Chlorophyll Concentrations in Crude Leaf Extracts[a] of Plant Species Surveyed.

Plant[b]	protein $mg \cdot mL^{-1}$		chlorophyll $mg \cdot mL^{-1}$
	undesalted	desalted	undesalted
Spinach	6.16 ± 0.26	4.48 ± 0.15	0.41 ± 0.01
Sugar beet	8.32 ± 1.21	5.50 ± 0.14	0.66 ± 0.07
Amaranth	8.10 ± 0.52	6.38 ± 0.51	0.41 ± 0.03
Statice	6.85 ± 0.27	5.45 ± 0.14	0.37 ± 0.01
Soybean	5.10 ± 1.88	5.83 ± 0.43	0.34 ± 0.05
Pea	6.11 ± 0.36	4.82 ± 0.23	0.62 ± 0.08
Cotton	8.54 ± 0.56	6.66 ± 0.25	0.59 ± 0.03
Carrot	10.48 ± 0.51	8.67 ± 0.80	0.49 ± 0.03
Canola	7.50 ± 0.56	5.83 ± 0.45	0.54 ± 0.01
Bindweed	3.38 ± 0.65	1.65 ± 0.19	0.36 ± 0.03
Tobacco	8.08 ± 1.04	6.58 ± 0.82	0.59 ± 0.03
Cucumber	8.62 ± 0.76	6.79 ± 0.25	0.43 ± 0.03
Sunflower	9.38 ± 0.64	7.34 ± 0.24	0.52 ± 0.03
Lettuce	2.34 ± 0.65	1.82 ± 0.53	0.41 ± 0.01

[a] For all plants 0.5g of tissue was used in 1.0 mL buffer.
b Data are the means ± S.E. from a minimum of two repeat extractions for cotton to a maximum of 10 for spinach.

related to choline metabolism. For example, enzyme activity measurements based on protein concentration could underestimate the amount of enzyme activity present in crude extracts due to the unequal contribution of abundant enzymes such as Rubisco (ribulose 1,5-bisphosphate carboxylase) between the different plant species. Specifically, if two different extracts yield comparable enzyme activity per mL of extract, but one source of these extracts is prepared from a plant that has more protein such as Rubisco, then the protein determination used for specific activity of that sample will underestimate the phosphobase *N*-methyl transferase enzyme activity estimated from the radioassay. Consequently, for the purpose of this thesis, all data for [^{3}H] phosphobase *N*-methyl transferase enzyme activity, namely PEAMeT, PMEAMeT and PDEAMeT activities, are measured using crude, undesalted extracts and enzyme activity is reported based on a g Fresh wt basis ($nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt).

Although specific activity on a protein basis ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein) and on a chlorophyll basis ($nmol \cdot min^{-1} \cdot mg^{-1}$ Chl) are not provided for all plant species tested, protein and chlorophyll concentrations were determined and are given in Table VI. These values will permit conversion of enzyme activity from Fresh wt to one based upon protein or chlorophyll. One observation noted from Table VI was that protein values from crude, desalted extracts were less than their crude undesalted counterparts. The exception was seen for soybean, where crude, desalted extracts had higher protein concentrations. The difference in protein concentrations between crude undesalted and desalted extracts may be a result of

the desalting procedure which presumably removes low molecular weight compounds.

Plant Grinding Conditions and Controls Used

Since all three successive phosphobase *N*-methyl transferase activities (Table VII) can be detected in extracts of spinach leaves, spinach was chosen to serve as a "control" species against which the level of activity found in all other plants could be compared.

In grinding and assaying enzyme activity in some plants such as cotton, statice and carrot, little to no PEAMeT activity was found (Table VIII). The absence of PEAMeT activity in extracts of these plants could be explained by two reasons. The first and obvious reason is the absence of PEAMeT enzyme in the tissue. A second reason for an apparent reduction or absence of PEAMeT activity in vitro could be explained by the presence of inhibitory components in the plant such as phenolics released upon grinding the tissue and/or inactivation of enzymes by proteases or possible hydrolysis of phosphobase products by phosphatases during the assay. In either case, the result would lead to reduced estimates of enzyme activity for the intact plants. Determining the reason underlying the apparently low levels of PEAMeT activity was addressed using the control of grinding spinach together with the plant under study (Materials and Methods). If the contribution of total PEAMeT activity made by spinach in the combined extract is lower than

TABLE VII: Phosphobase *N*-Methyl Transferase Activities in Crude Undesalted Extracts of Spinach Leaves.

Substrate	n	Enzyme Activity	
		$nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt	$nmol \cdot min^{-1} \cdot mg^{-1}$ protein
PEA	54	3.69 ± 0.20	0.31 ± 0.02
PMEA	23	8.68 ± 0.84	0.75 ± 0.08
PDEA	23	6.63 ± 0.52	0.57 ± 0.05

TABLE VIII. Recovery of Spinach PEAMeT Activity Alone and in Mixed Grinds With Other Species.

Extract	Enzyme Activity nmol • min^{-1} • g^{-1} Fresh wt		$\frac{\text{Observed}}{\text{Predicted}}$ x 100%
	Observed	Predicted [a]	
Carrot	0.01		
Spinach	1.24		
Carrot and Spinach	0.22	0.625	35
Cotton	0.01		
Spinach	1.58		
Cotton and Spinach	0.52	0.795	65

[a] Based upon the expected contribution of each plant towards half of the observed enzyme activity if equal quantities of both plants are ground together and PEAMeT activities are completely in extracts of mixed grinds.

expected based upon an assay of spinach alone, then inhibitory components may be present in the tissue of the other plant that have reduced the activity of the spinach PEAMeT enzyme in the combined extract. For example, in Table VIII, one can see that the recovery of the combined extract of both cotton plus spinach and carrot plus spinach was very low prompting further investigation of the extracts in question. In order to improve the rates of enzyme activity for plants such as cotton and carrot, PVP (polyvinylpyrrolidone, 4% w/v) was added to the grinding buffer. Table IX shows PEAMeT activity in extracts prepared with or without PVP in the grinding buffer. All extracts for each plant species were prepared at the same time. Spinach PEAMeT enzyme activity is 1.4 fold higher without PVP in the extract than when PVP is present. Therefore, there is no apparent benefit to using PVP in the grinding buffer for spinach and its use may have an adverse affect on enzyme activity. In contrast, the inclusion of PVP in the grinding buffer increases activity in cotton, carrot and statice by at least seven fold or greater relative to its counterpart prepared without PVP, while more modest improvements are seen for cucumber and tobacco. In summary, the use of PVP is beneficial for tobacco and cucumber, but for cotton, statice and carrot, the use of PVP is essential. The addition of PVP also resolved the problem of the additivity in the combined extracts of spinach and the respective plant. Thus enzyme activity measurements reported in this thesis come from plant extracts where the recovery of the combined extract of spinach and the plant species tested was 100% $\pm$ 25% whether PVP was used or not.

TABLE IX. PVP Improves PEAMeT in Crude Undesalted Extracts of Some but Not All Plants.

Plant[a]	Enzyme Activity $nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt	
	- PVP	+ PVP
Spinach	4.62 ± 0.02	3.32 ± 0.00
Statice	0.01 ± 0.00	1.97 ± 0.01
Cotton	1.73 ± 0.01	39.21 ± 0.12
Carrot	0.14 ± 0.02	0.96 ± 0.01
Tobacco	0.23 ± 0.01	0.32 ± 0.00
Cucumber	0.16 ± 0.00	0.39 ± 0.00

[a] Data are single replicate measurements ± S.E.

PEAMeT Activity in Dicot Plants

After all the extraction and assay conditions were established, a bar graph of in vitro PEAMeT activity in control, crude undesalted leaf extracts of all plants surveyed was drawn (Figure 2). PEAMeT activtiy was detected in all plants, albeit at comparatively low levels for some (Figure 2). The highest PEAMeT activity was found in extracts of cotton at 25 ± 3 nmol·min^{-1}·g^{-1} Fresh wt and the lowest in those of soybean at 0.04 ± 0.01 nmol·min^{-1}·g^{-1} Fresh wt. The second highest PEAMeT activity was found in extracts of sugar beet at 16 ± 3 nmol·min^{-1}·g^{-1} Fresh wt, then followed by a range of activity values from 6 ± 1 nmol·min^{-1}·g^{-1} Fresh wt in amaranth to 0.11 ± 0.01 nmol·min^{-1}·g^{-1} Fresh wt in pea.

Thus in Figure 2 there are those plants that show PEAMeT activity higher than 4 ± 0.2 nmol·min^{-1}·g^{-1} Fresh wt or those with less than 2 ± 0.2 nmol·min^{-1}·g^{-1} Fresh wt enzyme activity. For example, tobacco had only 10% the activity of spinach. However, the PEAMeT activity measurements for pea and soybean were 0.11 ± 0.01 and 0.04 ± 0.01 nmol·min^{-1}·g^{-1} Fresh wt, respectively. Both of these measurements exceed what is considered the threshold of our detection limits for our radioassay at 0.03 nmol·min^{-1}·g^{-1} Fresh wt. To provide a more suitable comparison for PEAMeT activity among plant species showing less activity than spinach, the y-axis of Figure 2 has been redrawn in Figure 2A and only the data for plants with PEAMeT activity equivalent

FIGURE 2. PEAMeT Activity in Extracts Prepared from Leaves of Various Dicots.

PEAMeT activity ($nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt) of crude leaf extracts from plant species exposed to a minimum of four hours light prior to harvest. Complete description of extract preparation and assay conditions are outlined in Materials and Methods. Data are the means ± S.E. from a minimum of two repeat extractions for cotton to a maximum of five repeats for pea.

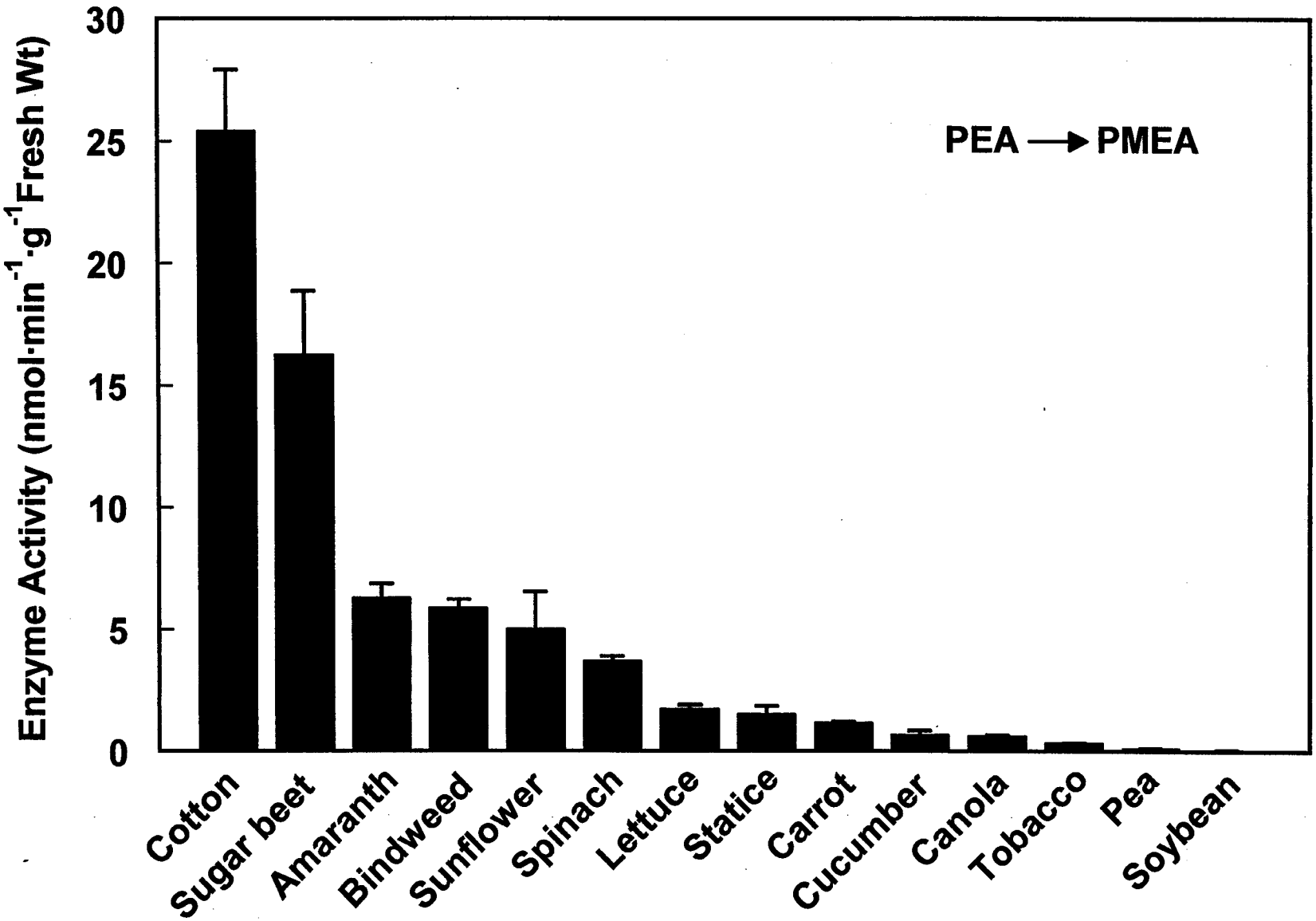
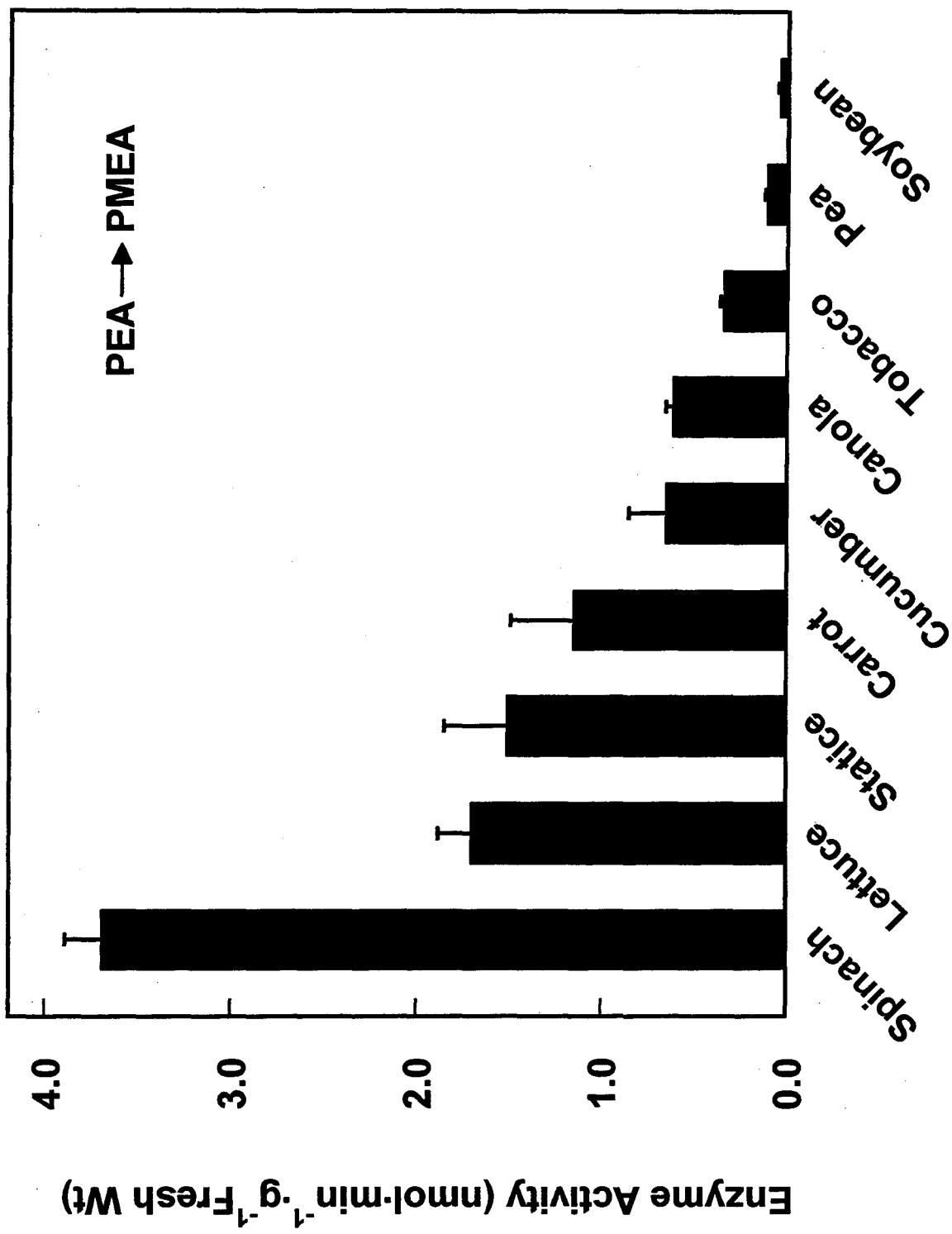

Enzyme Activity (nmol·min-1·g-1Fresh Wt)
30
25
20
15
10
5
0
PEA → PMEA
Cotton
Sugar beet
Amaranth
Bindweed
Sunflower
Spinach
Lettuce
Statice
Carrot
Cucumber
Canola
Tobacco
Pea
Soybean

FIGURE 2A. PEAMeT Activity in Extracts Prepared from Leaves of Various Dicots.

PEAMeT activity ($nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt) in crude undesalted leaf extracts of various plant species. Data are the same as in Figure 1, however the scale has been expanded to accommodate plants with activity equivalent to spinach and lower. Data are means ± S.E. with a minimum repeat of n = 3.


PEA → PMEA
Enzyme Activity (nmol·min-1·g-1Fresh Wt)
0.0
1.0
2.0
3.0
4.0
Spinach
Lettuce
Statice
Carrot
Cucumber
Canola
Tobacco
Pea
Soybean

to or less than that of spinach extract preparations are shown. Here it can be seen that both pea and soybean have PEAMeT activity levels above background.

As outlined in the Materials and Methods section, each extract was either assayed directly and referred to as undesalted extract or desalted prior to assay. PEAMeT activity determined for both crude undesalted and desalted extracts of all plants surveyed is reported in Table X. This table demonstrates that PEAMeT activity in crude undesalted and desalted samples assayed overlapped except for four (cotton, bindweed, carrot, soybean) of the 14 plant species. As already discussed, cotton, bindweed and carrot did not desalt well and so this result is not surprising. This table, along with Figure 2, shows that in vitro PEAMeT activity levels are variable between the plant species tested with a range of 600 fold from cotton with the highest level of activity to soybean with the lowest.

PEAMeT Activity Following a Dark Exposure Treatment

As discussed in the Materials and Methods, the "control" plants received at least four hours of light prior to harvest and some plants were placed in the dark for a period between 40 - 48 hours before harvest (dark-treated). The data collected for protein and chlorophyll concentrations of dark-treated plants are given in Table XI. For comparison, the corresponding protein and chlorophyll concentrations already reported in Table VI for plants harvested under control conditions are also provided. The range of chlorophyll values is from

TABLE X. PEAMeT Activity in Desalted and Undesalted Crude Leaf Extracts of Diverse Dicot Plants.

Plant[a]	n	Enzyme Activity nmol•min^{-1}•g^{-1} Fresh wt	
		Undesalted	Desalted
Cotton	2	25.40 ± 2.49	4.69 ± 2.98
Sugar beet	3	16.24 ± 2.63	11.55 ± 2.35
Amaranth	3	6.29 ± 0.58	6.41 ± 0.85
Bindweed[b]	2	6.14 ± 0.42	2.68 ± 0.04
Sunflower	4	5.01 ± 1.52	6.09 ± 1.65
Spinach	54	3.69 ± 0.20	4.28 ± 0.25
Lettuce	4	1.70 ± 0.18	1.99 ± 0.23
Statice	3	1.51 ± 0.34	1.34 ± 0.29
Carrot	3	1.15 ± 0.08	0.65 ± 0.17
Cucumber	4	0.65 ± 0.20	0.91 ± 0.26
Canola	3	0.61 ± 0.04	0.61 ± 0.08
Tobacco	4	0.34 ± 0.02	0.39 ± 0.04
Pea	5	0.11 ± 0.01	0.13 ± 0.03
Soybean	3	0.04 ± 0.01	0.15 ± 0.07

[a] Plants listed in descending order of PEAMeT activity for undesalted samples.
[b] Desalting was not performed for one repeat.

Table XI. Mean Protein and Chlorophyll Concentrations in Crude Leaf Extracts[a] of Control Plants and Dark-treated[b] Plants.

Plant[c]	Control			Dark-Treated		
	protein $mg \cdot mL^{-1}$		chlorophyll $mg \cdot mL^{-1}$	protein $mg \cdot mL^{-1}$		chlorophyll $mg \cdot mL^{-1}$
	undesalted	desalted	undesalted	undesalted	desalted	undesalted
Spinach	6.16 ± 0.26	4.48 ± 0.15	0.41 ± 0.01	5.48 ± 0.28	4.09 ± 0.17	0.35 ± 0.01
Sugar beet	8.32 ± 1.21	5.50 ± 0.14	0.66 ± 0.07	6.72 ± 0.58	5.64 ± 0.85	0.50 ± 0.03
Amaranth	8.10 ± 0.52	6.38 ± 0.51	0.41 ± 0.03	6.84 ± 0.35	5.19 ± 0.15	0.29 ± 0.02
Statice	6.85 ± 0.27	5.45 ± 0.14	0.37 ± 0.01	6.59 ± 0.10	5.45 ± 0.06	0.35 ± 0.02
Soybean	5.10 ± 1.88	5.83 ± 0.43	0.34 ± 0.05	7.50 ± 0.42	6.37 ± 0.52	0.34 ± 0.04
Pea	6.11 ± 0.36	4.82 ± 0.23	0.62 ± 0.08	5.05 ± 0.72	4.18 ± 0.34	0.66 ± 0.06
Cotton	8.54 ± 0.56	6.66 ± 0.25	0.59 ± 0.03	8.51 ± 0.68	6.34 ± 0.42	0.43 ± 0.00
Carrot	10.48 ± 0.51	8.67 ± 0.80	0.49 ± 0.03	9.30 ± 1.12	7.90 ± 0.60	0.44 ± 0.01
Canola	7.50 ± 0.56	5.83 ± 0.45	0.54 ± 0.01	4.83 ± 0.29	3.91 ± 0.26	0.40 ± 0.01
Bindweed	3.38 ± 0.65	1.65 ± 0.19	0.36 ± 0.03	3.26 ± 0.04	1.85 ± 0.13	0.35 ± 0.04
Tobacco	8.08 ± 1.04	6.58 ± 0.82	0.59 ± 0.03	5.48 ± 0.52	3.68 ± 1.06	0.41 ± 0.05
Cucumber	8.62 ± 0.76	6.79 ± 0.25	0.43 ± 0.03	6.73 ± 0.30	5.64 ± 0.10	0.36 ± 0.01
Sunflower	9.38 ± 0.64	7.34 ± 0.24	0.52 ± 0.03	8.47 ± 0.69	7.31 ± 0.61	0.41 ± 0.02
Lettuce	2.34 ± 0.65	1.82 ± 0.53	0.41 ± 0.01	2.88 ± 0.22	2.02 ± 0.26	0.31 ± 0.03

[a] For all plants 0.5g of tissue was used in 1.0 mL buffer.
[b] Time of dark treatment exceeding 40 hrs.
[c] Data are the means ± S.E. from a minimum of two repeat extractions for cotton to a maximum of ten for spinach.

approximately 0.29 to 0.66 mg Chl $\cdot mL^{-1}$. Fewer than half of the plants in Table XI have less protein (mg protein $\cdot mL^{-1}$) after a dark treatment relative to their controls. There are only four plants that show no change in chlorophyll concentrations following >40 h dark exposure while all other plants have decreased chlorophyll levels after a dark treatment.

As for control plants, dark-treated plants were harvested at the same time and were ground in a buffer with or without PVP. The results of PEAMeT activity assayed in crude extracts prepared with and without PVP following a dark treatment are found in Table XII. The results for control plants have already been shown in Table IX but are repeated here to enable direct comparisons. Table XII shows that the inclusion of PVP in the grinding buffer increases PEAMeT activity significantly in extracts of all dark-treated plants listed. Both cotton and statice PEAMeT activities are 11 and 16 fold higher, respectively, with PVP. Despite the beneficial effect of PVP, in all dark-treated plants, the PEAMeT activity decreased after a dark treatment whether PVP was used or not.

Table XIII gives PEAMeT activity measurements after a dark exposure treatment for all of the plants surveyed. The data for control plants already reported in Table X are repeated in Table XIII for ease of comparison. As for plants exposed to lights before harvest, crude desalted extracts of the dark-treated plants frequently showed no difference from measurements with crude undesalted extracts with the exceptions being those prepared from cotton, bindweed (which does not desalt well as previously discussed) and spinach. In comparing the

TABLE XII. Effect of Grinding With PVP on PEAMeT Activity in Crude Undesalted Extracts of Control and Dark-treated Plants.

	Enzyme Activity nmol • min^{-1} • g^{-1} Fresh wt			
	Control		Dark-Treated	
Plant[a]	- PVP	+ PVP	- PVP	+ PVP
Spinach	4.62 ± 0.02	3.32 ± 0.00	0.00 ± 0.00	0.12 ± 0.01
Statice	0.01 ± 0.00	1.97 ± 0.01	0.04 ± 0.00	0.64 ± 0.01
Cotton	1.73 ± 0.01	39.21 ± 0.12	0.88 ± 0.02	10.07 ± 0.09
Carrot	0.14 ± 0.02	0.96 ± 0.01	0.00 ± 0.00	0.11 ± 0.01
Tobacco	0.23 ± 0.01	0.32 ± 0.00	0.05 ± 0.01	0.13 ± 0.00
Cucumber	0.16 ± 0.00	0.39 ± 0.00	0.15 ± 0.01	0.24 ± 0.01

[a] Data are single repeat measurements ± S.E.

TABLE XIII. PEAMeT Activity in Crude Leaf Extracts of Control and Dark-treated[a] Plants.

Plant[b]	n	Enzyme Activity nmol • min^{-1} • g^{-1} Fresh wt			
		Control		Dark-treated	
		Undesalted	Desalted	Undesalted	Desalted
Cotton	2	25.40 ± 2.49	4.69 ± 2.98	4.22 ± 0.35	1.16 ± 0.73
Sugar beet	3	16.24 ± 2.63	11.55 ± 2.35	0.26 ± 0.07	0.30 ± 0.05
Amaranth	3	6.29 ± 0.58	6.41 ± 0.85	0.41 ± 0.22	0.75 ± 0.23
Bindweed[c]	2	6.14 ± 0.42	2.68 ± 0.04	2.63 ± 0.08	2.17 ± 0.08
Sunflower	4	5.01 ± 1.52	6.09 ± 1.65	1.74 ± 0.43	2.63 ± 0.51
Spinach	54	3.69 ± 0.20	4.28 ± 0.25	0.24 ± 0.02	0.32 ± 0.02
Lettuce	4	1.70 ± 0.18	1.99 ± 0.23	0.81 ± 0.20	1.07 ± 0.24
Statice	3	1.51 ± 0.34	1.34 ± 0.29	0.83 ± 0.26	1.08 ± 0.32
Carrot	3	1.15 ± 0.08	0.65 ± 0.17	0.09 ± 0.01	0.09 ± 0.01
Cucumber	4	0.65 ± 0.20	0.91 ± 0.26	0.37 ± 0.12	0.64 ± 0.16
Canola	3	0.61 ± 0.04	0.61 ± 0.08	0.10 ± 0.05	0.06 ± 0.03
Tobacco	4	0.34 ± 0.02	0.39 ± 0.04	0.16 ± 0.05	0.20 ± 0.07
Pea	5	0.11 ± 0.01	0.13 ± 0.03	<0.03 ± 0.01	<0.03 ± 0.01
Soybean	3	0.04 ± 0.01	0.15 ± 0.07	<0.03 ± 0.01	<0.03 ± 0.01

[a] Time of dark treatment exceeding 40 hrs
[b] Plants were ranked in descending order of PEAMeT activity
[c] Desalting was not determined for one repeat.

activities from control plants versus dark-treated plants, PEAMeT activity decreases after a dark exposure (40 - 48 hrs) for all of the plants, but the extent of this decrease is not the same. Figure 3 shows the PEAMeT activity remaining in prepared undesalted crude leaf extracts after a dark treatment as a percent of PEAMeT activity detected in control plants. Sugar beet had two percent of the PEAMeT activity found in control, light exposed plants remaining after a dark period, whereas the activity remaining in amaranth, carrot and spinach was between 6 - 8%. Decreased PEAMeT activity following dark-treatment among other plants ranged from as low as 15% in canola to 42% in bindweed. In tobacco, cucumber and statice the amount of activity was approximately 50% with the best recovery being approximately 65% for statice (Figure 3).

PEAMeT, PMEAMeT and PDEAMeT Enzymes in Spinach

Characteristic PEAMeT, PMEAMeT and PDEAMeT activities for crude extract preparations of spinach leaves left undesalted and desalted are given in Table XIV. Plants from which these extracts were prepared were exposed to a minimum 4 h light period prior to harvest. Phosphobase *N*-methyltransferase enzyme activity is reported as $nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt and $nmol \cdot min^{-1} \cdot mg^{-1}$ protein. As already shown in Table X for spinach PEAMeT activity and again here in Table XIV along with the subsequent two methylation activities, activity measurements from desalted extracts are not significantly different from those of

FIGURE 3. Percent PEAMeT Activity Remaining in Crude Undesalted Leaf Extracts of Various Plants After a Dark Treatment.

Bars depict the ratio of PEAMeT activity from dark-treated plants over the PEAMeT activity from control, hence light exposed plants, expressed as a percentage. See Materials and Methods for dark treatment conditions, extract preparation and assay conditions. Data are means ± S.E. from a minimum of two repeat extractions for cotton to a maximum of five for pea.

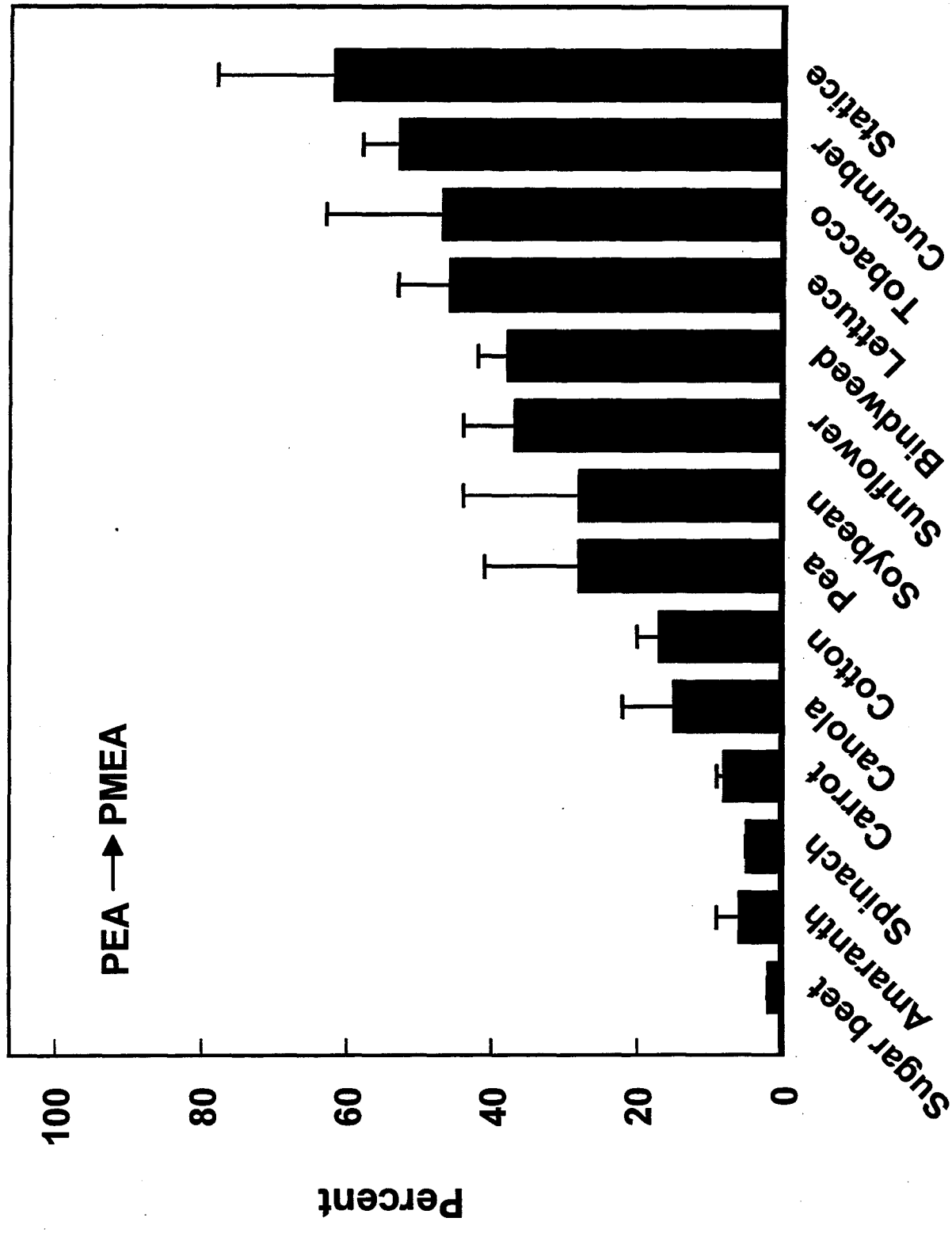

PEA → PMEA
Percent
0
20
40
60
80
100
Sugar beet
Amaranth
Spinach
Carrot
Canola
Cotton
Pea
Soybean
Sunflower
Bindweed
Lettuce
Tobacco
Cucumber
Statice

TABLE XIV: Phosphobase *N*-Methyl Transferase Activity in Leaf Extracts of Control Spinach Leaves.

Substrate	n	Treatment	Enzyme Activity	
			$nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt	$nmol \cdot min^{-1} \cdot mg^{-1}$ Protein
PEA	54	undesalted	3.69 ± 0.20	0.31 ± 0.02
		desalted	4.28 ± 0.25	0.51 ± 0.03
PMEA	23	undesalted	8.68 ± 0.84	0.75 ± 0.08
		desalted	9.69 ± 0.80	1.10 ± 0.08
PDEA	23	undesalted	6.63 ± 0.52	0.57 ± 0.05
		desalted	7.34 ± 0.52	0.84 ± 0.06

undesalted extracts. The order ranking the levels of phosphobase *N*-methyltransferase enzyme activity did not change, with PEAMeT activity lowest, followed by PDEAMeT activity and highest, PMEAMeT activity. Interestingly, PMEAMeT activity was 2.4 fold higher than PEAMeT activity in a undesalted or desalted extract preparation and PDEAMeT activity was 1.8 fold higher than PEAMeT activity.

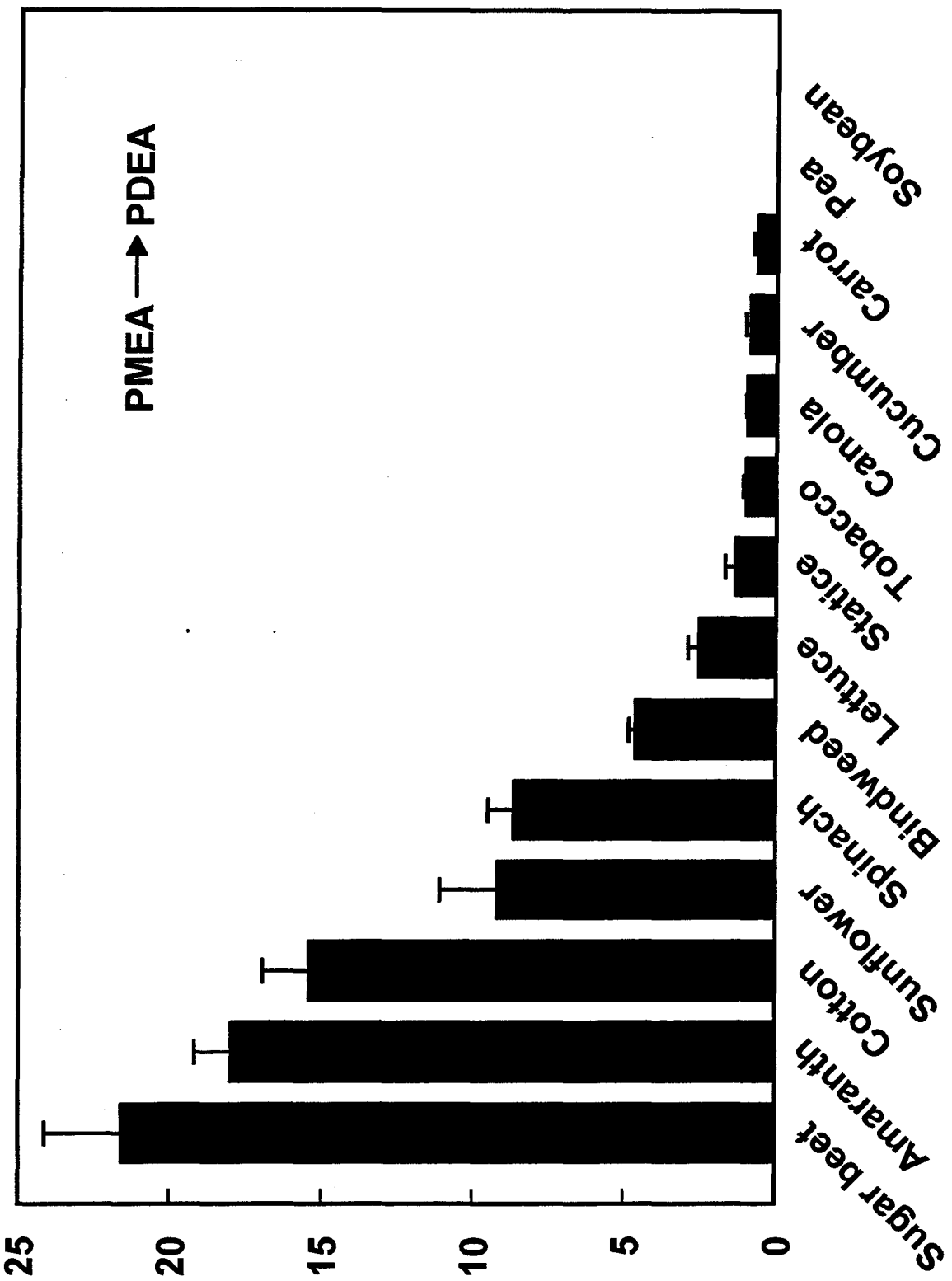
PMEAMeT and PDEAMeT activities were assayed in all of the plants surveyed. Since the spinach enzyme(s) could be detected in crude undesalted extracts of spinach with apparent recoveries of 90% for both activities (Table XIV), undesalted extracts prepared for the other plants were assumed to provide suitable material for a comparative biochemical study. For each species, PMEAMeT and PDEAMeT activities were measured under identical in vitro assay conditions (Materials and Methods).

PMEAMeT (PMEA → PDEA) Activity in Diverse Dicot Plants

PMEAMeT activity was highest in sugar beet at 22 ± 3 $nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt, followed by amaranth and then cotton. A comparison of PMEAMeT activity in Figure 4 showed that in general, there were several plants with relatively high levels of PMEAMeT activity such as sugar beet, amaranth, cotton, sunflower, spinach and bindweed while plants such as lettuce, statice, tobacco, canola,

FIGURE 4. PMEAMeT Activity in Extracts Prepared from Leaves of Various Dicots.

PMEAMeT activity ($nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt) of crude leaf extracts from plant species exposed to a minimum of four hours light prior to harvest. Complete description of extract preparation and assay conditions are outlined in Materials and Methods. Data are means ± S.E. with a minimum repeat of n = 2 for cotton to a maximum of n = 4 for sunflower.


PMEA → PDEA
Enzyme Activity (nmol·min-1·g-1Fresh Wt)
0
5
10
15
20
25
Sugar beet
Amaranth
Cotton
Sunflower
Spinach
Bindweed
Lettuce
Statice
Tobacco
Canola
Cucumber
Carrot
Pea
Soybean

cucumber and carrot had lower activities, < 3 nmol · min^{-1} · g^{-1} Fresh wt. In contrast, both pea and soybean had no detectable activity (<0.03 nmol · min^{-1} · g^{-1} Fresh wt).

Figure 4 shows that bindweed is only at 50% of the PMEAMeT activity of spinach PMEAMeT. The numerical values of in vitro PMEAMeT activity shown graphically in Figure 4 are found in Table XV along with those of desalted crude extract preparations. The PMEAMeT activity measurements using undesalted and desalted extracts give the same estimates for 11 of the 14 plants but higher enzyme activity estimates were found for undesalted extracts of amaranth and bindweed while tobacco PMEAMeT activity measurements from desalted extracts were higher.

PDEAMeT (PDEA → PCho) Activity in Diverse Dicot Plants

PDEAMeT enzyme activity depicted in Figure 5 shows sugar beet with the highest activity at 24 ± 4 nmol · min^{-1} · g^{-1} Fresh wt followed by cotton and then amaranth. Notably there is a two fold difference in activity between sugar beet and the next highest measurement, that of cotton PDEAMeT, followed by a gradual decrease in activity amongst the remaining plants. The lowest PDEAMeT activity was found for pea at 0.05 ± nmol · min^{-1} · g^{-1} Fresh wt while no activity was detected in soybean (<0.03 nmol · min^{-1} · g^{-1} Fresh wt). Undesalted and desalted crude extract preparations were used to measure in vitro PDEAMeT activity and are reported in Table XVI. The enzyme activity measured in crude undesalted extracts

TABLE XV. PMEAMeT Activity in Desalted and Undesalted Crude Extracts of Diverse Dicot Plants.

Plant[a]	n	Enzyme Activity nmol•min^{-1}•g^{-1} Fresh wt	
		Undesalted	Desalted
Sugar beet	3	21.60 ± 2.54	17.78 ± 2.76
Amaranth	3	18.00 ± 1.19	15.23 ± 0.51
Cotton	2	15.43 ± 1.52	10.54 ± 3.45
Sunflower	4	9.20 ± 1.90	9.46 ± 1.49
Spinach	23	8.68 ± 0.84	9.69 ± 0.80
Bindweed	1	4.39 ± 0.00	2.33 ± 0.00
Lettuce	3	2.55 ± 0.42	2.27 ± 0.42
Statice	3	1.38 ± 0.32	1.45 ± 0.34
Tobacco	3	1.03 ± 0.06	1.30 ± 0.16
Canola	3	1.00 ± 0.02	0.94 ± 0.32
Cucumber	3	0.90 ± 0.13	0.61 ± 0.17
Carrot	3	0.67 ± 0.11	0.54 ± 0.22
Pea	3	<0.03 ± 0.01	<0.03 ± 0.01
Soybean	3	<0.03 ± 0.01	<0.03 ± 0.01

[a] Plants listed in descending order of PMEAMeT activity for undesalted samples.

FIGURE 5. PDEAMeT Activity in Extracts Prepared from Leaves of Various Dicots.

PDEAMeT activity ($nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt) of crude leaf extracts from plant species exposed to a minimum of four hours light prior to harvest. Complete description of extract preparation and assay conditions are outlined in Materials and Methods. Data are means ± S.E. with a minimum repeat of n = 2 for cotton to a maximum of n = 4 for sunflower.

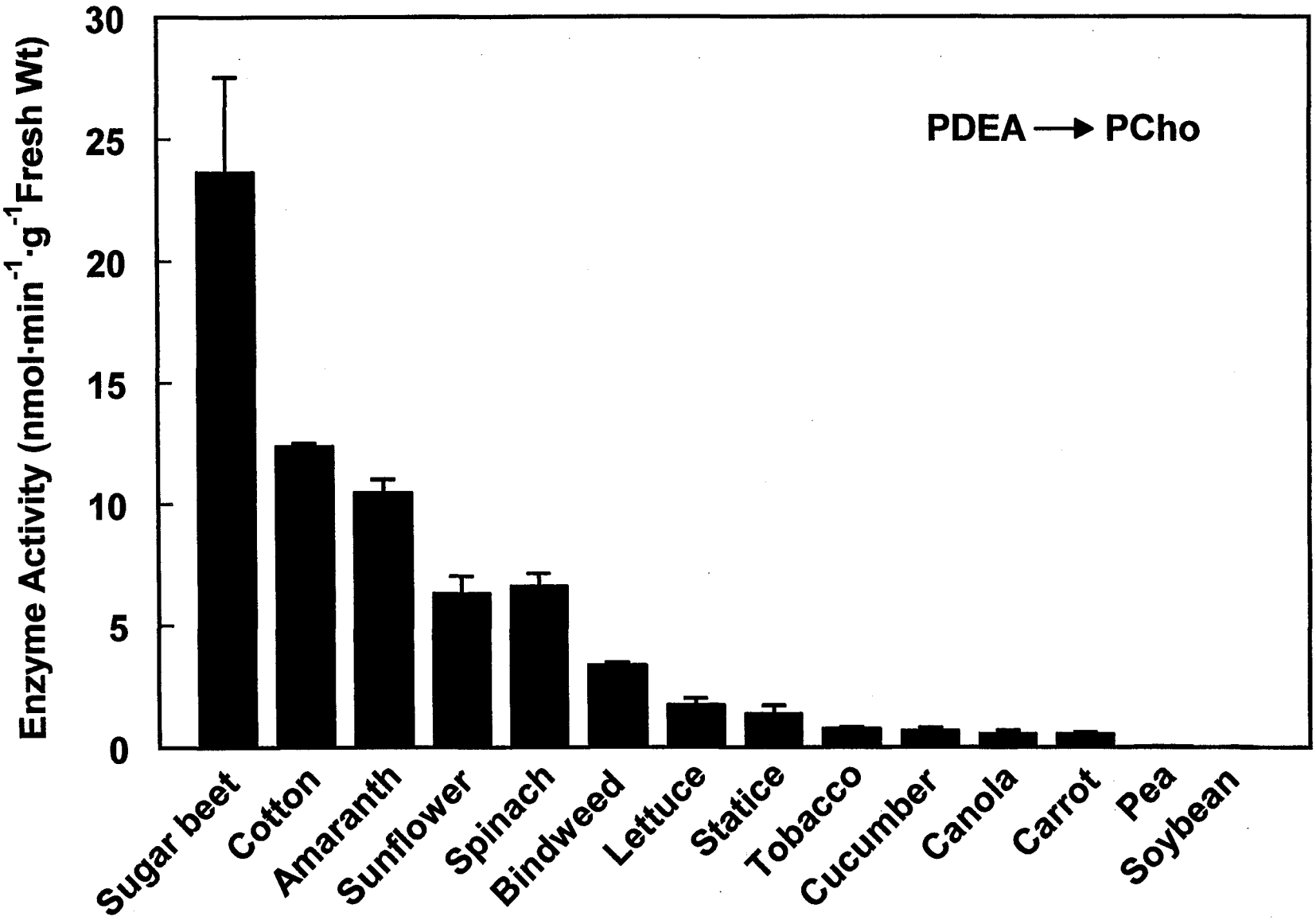

Enzyme Activity (nmol·min-1·g-1Fresh Wt)
30
25
20
15
10
5
0
PDEA → PCho
Sugar beet
Cotton
Amaranth
Sunflower
Spinach
Bindweed
Lettuce
Statice
Tobacco
Cucumber
Canola
Carrot
Pea
Soybean

TABLE XVI. PDEAMeT Activity in Desalted and Undesalted Crude Extracts of Diverse Dicot Plants.

Plant[a]	n	Enzyme Activity nmol•min^{-1}•g^{-1} Fresh wt	
		Undesalted	Desalted
Sugar beet	3	23.67 ± 3.88	19.61 ± 2.02
Cotton	2	12.39 ± 0.11	4.58 ± 1.16
Amaranth	3	10.47 ± 0.55	10.64 ± 0.73
Sunflower	4	6.32 ± 0.71	4.97 ± 0.64
Spinach	23	6.63 ± 0.52	7.34 ± 0.52
Bindweed	1	3.36 ± 0.00	1.03 ± 0.00
Lettuce	3	1.72 ± 0.27	1.14 ± 0.25
Statice	3	1.36 ± 0.34	1.15 ± 0.21
Tobacco	3	0.79 ± 0.04	0.75 ± 0.05
Cucumber	3	0.68 ± 0.12	0.43 ± 0.11
Canola	3	0.56 ± 0.14	0.65 ± 0.15
Carrot	3	0.55 ± 0.07	0.46 ± 0.18
Pea	3	0.05 ± 0.00	<0.03 ± 0.02
Soybean	3	<0.03 ± 0.01	<0.03 ± 0.08

[a] Plants listed in descending order of PDEAMeT activity for undesalted samples.

has already been shown graphically in Figure 5 and gives the same PDEAMeT activity estimates as from desalted extracts for nine of the 14 plants.

In summarizing the data shown in Figures 2, 4 and 5, a pattern emerges which shows that plants have varying levels of PEAMeT, PMEAMeT and PDEAMeT activities. In general, plants that rank high with respect to the level of PEAMeT activity also rank highly with respect to PMEAMeT and PDEAMeT activities.

Phosphobase *N*-methyltransferase Activity After a Dark Treatment

The activity of the three phosphobase *N*-methyltransferases in extracts of spinach leaves after exposure of the leaves to a prolonged period of darkness is shown in Table XVII. For comparative purposes, the enzyme activities of control spinach plants (shown in Table XV) are repeated in this table. Table XVII also shows PEAMeT, PMEAMeT and PDEAMeT activities after a dark treatment (40-48 hr). The activities for all three phosphobase *N*-methyltransferase enzyme activities decrease following dark exposure. However, the drop in PEAMeT activity after a dark exposure is greatest, leaving only 6% of the light PEAMeT activity remaining relative to the activity of control, light exposed plants. The changes seen in PMEAMeT and PDEAMeT were not as great and activities following dark treatment were on the same order of magnitude with 65 -70% activity remaining for both.

TABLE XVII. Phosphobase *N*-Methyltransferase Activity in Crude Extracts of Spinach Leaves.

Substrate	n	Treatment	Enzyme Activity in Control Plants		Enzyme Activity in Dark-treated Plants	
			nmol • min^{-1} • g^{-1} Fresh wt	nmol • min^{-1} • mg^{-1} protein	nmol • min^{-1} • g^{-1} Fresh wt	nmol • min^{-1} • mg^{-1} protein
PEA	54	undesalted	3.69 ± 0.20	0.31 ± 0.02	0.24 ± 0.02	0.02 ± 0.00
		desalted	4.28 ± 0.25	0.51 ± 0.03	0.32 ± 0.02	0.04 ± 0.00
PMEA	23	undesalted	8.68 ± 0.84	0.75 ± 0.08	5.64 ± 0.59	0.53 ± 0.05
		desalted	9.69 ± 0.80	1.10 ± 0.08	6.93 ± 0.54	0.85 ± 0.05
PDEA	23	undesalted	6.63 ± 0.52	0.57 ± 0.05	4.36 ± 0.38	0.39 ± 0.03
		desalted	7.34 ± 0.52	0.84 ± 0.06	4.95 ± 0.36	0.62 ± 0.04

PMEAMeT Activity Following a Dark Exposure Treatment

PMEAMeT activity was assayed in vitro using crude undesalted and desalted leaf extracts prepared from all plants surveyed following a dark exposure treatment and results are found in Table XVIII. Data for activity of plants grown under control light conditions are repeated from Table XV. As for PEAMeT, PMEAMeT activity decreases after a dark treatment and this decrease is shown regardless of whether the samples are desalted or not. Figure 6 shows the percent PMEAMeT activity remaining after a prolonged dark period (40 - 48 hr) relative to the level of activity in control plants for crude undesalted extracts. The greatest reduction in PMEAMeT activity upon dark exposure was found for carrot at 20% remaining following exposure of plants to continuous dark. The reduction for most other plants (e.g. amaranth to tobacco) was less with the amount of PMEAMeT activity remaining in the range of approximately 40 to 80%.

PDEAMeT Activity Following a Dark Exposure Treatment

The final phosphobase *N*-methyltransferase to review is PDEAMeT. PDEAMeT activity in undesalted and desalted crude leaf extracts after a dark exposure period is seen in Table XIX along with the activity measurements for control plants shown previously (Table XVI). PDEAMeT activities for pea and soybean were either low and variable or below the limits of detection and so a statement concerning light responsive behaviour for either enzyme is not well

TABLE XVIII. PMEAMeT Activity in Crude Leaf Extracts of Control and Dark-treated[a] Plants.

Plant[b]	n	Enzyme Activity nmol • min^{-1} • g^{-1} Fresh wt			
		Control		Dark-treated	
		Undesalted	Desalted	Undesalted	Desalted
Amaranth	3	18.00 ± 1.19	15.23 ± 0.51	7.12 ± 0.50	6.29 ± 0.25
Cotton	2	15.43 ± 1.52	10.54 ± 3.45	8.51 ± 1.56	5.03 ± 1.38
Sunflower	4	9.20 ± 1.90	9.46 ± 1.49	4.10 ± 1.31	4.08 ± 0.67
Spinach	23	8.68 ± 0.84	9.69 ± 0.80	5.64 ± 0.59	6.93 ± 0.54
Bindweed	1	4.39 ± 0.00	2.33 ± 0.00	1.76 ± 0.00	0.96 ± 0.00
Lettuce	3	2.55 ± 0.42	2.27 ± 0.42	1.22 ± 0.29	1.14 ± 0.28
Statice	3	1.38 ± 0.32	1.45 ± 0.34	0.86 ± 0.21	1.10 ± 0.27
Tobacco	3	1.03 ± 0.06	1.30 ± 0.16	0.76 ± 0.05	1.17 ± 0.10
Canola	3	1.00 ± 0.02	0.94 ± 0.32	0.26 ± 0.06	0.35 ± 0.10
Cucumber	3	0.90 ± 0.13	0.61 ± 0.17	0.59 ± 0.07	0.42 ± 0.10
Carrot	3	0.67 ± 0.11	0.54 ± 0.22	0.15 ± 0.02	0.10 ± 0.03
Pea	3	<0.03 ± 0.01	<0.03 ± 0.01	<0.03	<0.03 ± 0.01
Soybean	3	<0.03 ± 0.01	<0.03 ± 0.01	<0.03	0.10 ± 0.07

[a] Time of dark treatment exceeding 40 hrs.
[b] Plants were ranked in descending order of PMEAMeT activity for undesalted samples.

FIGURE 6. Percent PMEAMeT Activity Remaining in Crude Undesalted Leaf Extracts of Various Plants After a Dark Treatment.

Bars depict the ratio of PMEAMeT activity from dark-treated plants over the PMEAMeT activity from control, hence light exposed plants, expressed as a percentage. See Materials and Methods for dark treatment conditions, extract preparation and assay conditions. Data are means ± S.E. with a minimum repeat of n = 2.

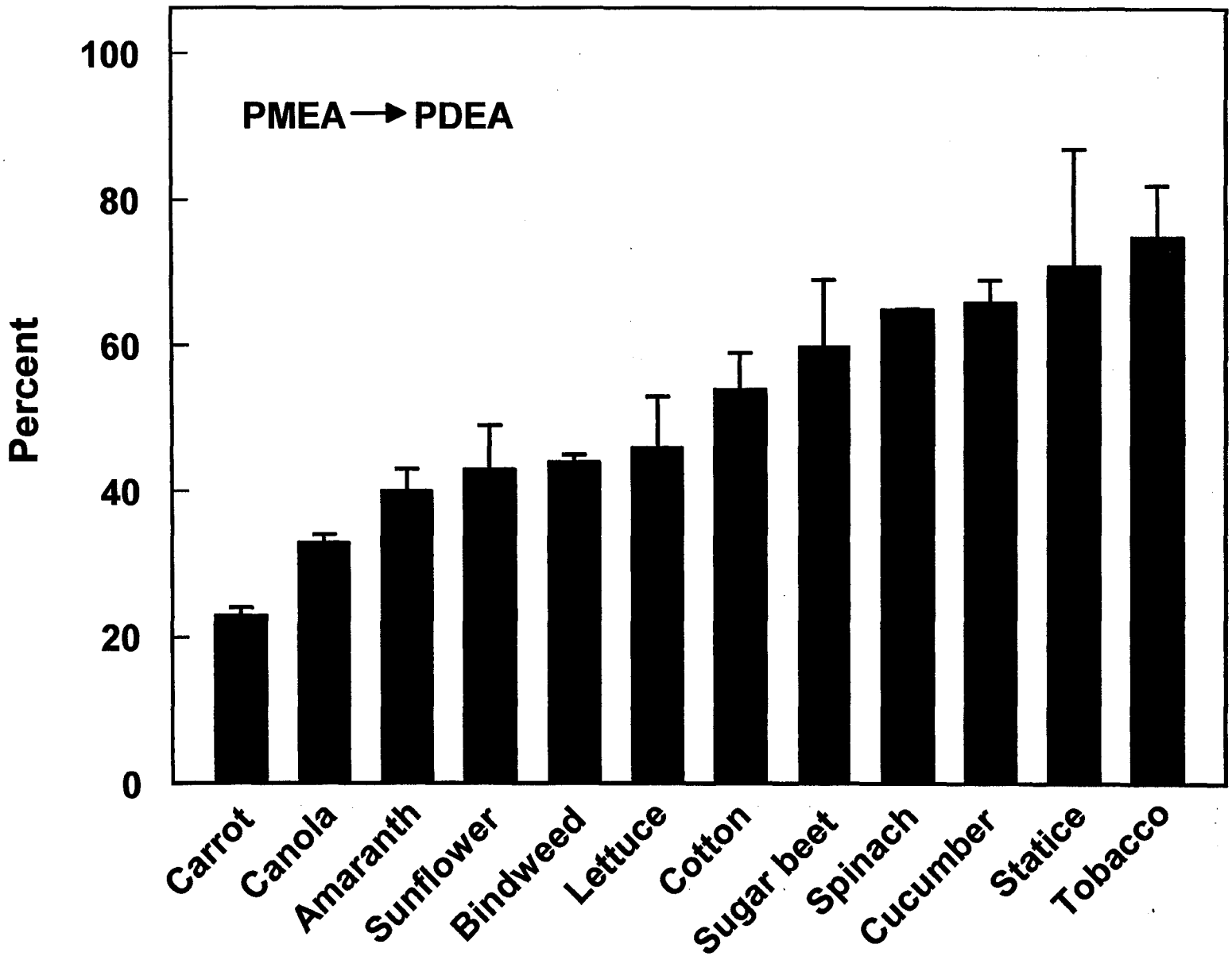

PMEA → PDEA
Percent
100
80
60
40
20
0
Carrot
Canola
Amaranth
Sunflower
Bindweed
Lettuce
Cotton
Sugar beet
Spinach
Cucumber
Statice
Tobacco

TABLE XIX. PDEAMeT Activity in Crude Leaf Extracts of Control and Dark-treated[a] Plants.

Plant[b]	n	Enzyme Activity nmol•min^{-1}•g^{-1} Fresh wt			
		Control		Dark-treated	
		Undesalted	Desalted	Undesalted	Desalted
Cotton	2	12.39 ± 0.11	4.58 ± 1.16	7.48 ± 0.89	3.67 ± 0.87
Amaranth	3	10.47 ± 0.55	10.64 ± 0.73	5.28 ± 0.86	4.86 ± 0.56
Sunflower	4	6.32 ± 0.71	4.97 ± 0.64	2.87 ± 0.68	2.45 ± 0.42
Spinach	23	6.63 ± 0.52	7.34 ± 0.52	4.36 ± 0.38	4.95 ± 0.36
Bindweed	1	3.36 ± 0.00	1.03 ± 0.00	1.11 ± 0.00	0.67 ± 0.00
Lettuce	3	1.72 ± 0.27	1.14 ± 0.25	0.89 ± 0.15	1.02 ± 0.25
Statice	3	1.36 ± 0.34	1.15 ± 0.21	0.95 ± 0.21	0.95 ± 0.21
Tobacco	3	0.79 ± 0.04	0.75 ± 0.05	0.66 ± 0.04	0.62 ± 0.05
Cucumber	3	0.68 ± 0.12	0.43 ± 0.11	0.56 ± 0.02	0.34 ± 0.01
Canola	3	0.56 ± 0.14	0.65 ± 0.15	0.19 ± 0.04	0.33 ± 0.04
Carrot	3	0.55 ± 0.07	0.46 ± 0.18	0.10 ± 0.02	0.10 ± 0.03
Pea	3	0.05 ± 0.00	<0.03 ± 0.02	<0.03	<0.03

[a] Time of dark treatment exceeding 40 hrs.
[b] Plants were ranked in descending order of PDEAMeT activity for undesalted samples.

defended. However, PDEAMeT activity in all other plants decreases after a dark treatment but the extent of the decrease is variable. The percent PDEAMeT activity remaining after a prolonged dark period (40 - 48 hr) was measured using crude undesalted extracts and is shown in Figure 7. Carrot had the lowest percent (18%) of PDEAMeT activity remaining after a dark treatment relative to the level of enzyme activity in control plants. All plants from canola on had over 38% PDEAMeT activity remaining and cucumber, statice and tobacco displayed little PDEAMeT light regulation. These plants were only positioned relative to one another in this figure based upon their positions in Figure 6.

During a preliminary extract preparation and radioassay of *Limonium perezii* for PEAMeT activity, it was noticed that *L. perezii* appeared to have a PEAMeT activity which was the least light responsive compared to other plants tested at that point in the survey. This prompted an investigation into the PEAMeT activity of another *Limonium* species, namely, *Limonium sinuatum*. Table XX shows all phosphobase activities and the percent PEAMeT, PMEAMeT and PDEAMeT activity remaining after a dark exposure for the two *Limonium* species. For each enzyme, activity measurements did not differ and thus data from only one species, that of *L. perezii*, was chosen arbitrarily for the survey. The percent PMEAMeT and PDEAMeT activity remaining after a dark exposure was similarly high to that of PEAMeT seen in Figure 2 and here in Table XX. Again, comparing the

FIGURE 7. Percent PDEAMeT Activity Remaining in Crude Undesalted Leaf Extracts of Various Plants After a Dark Treatment.

Bars depict the ratio of PDEAMeT activity from dark-treated plants over the PDEAMeT activity from control, hence light exposed plants, expressed as a percentage. See Materials and Methods for dark treatment conditions, extract preparation and assay conditions. Data are means ± S.E. with a minimum repeat of n = 2.

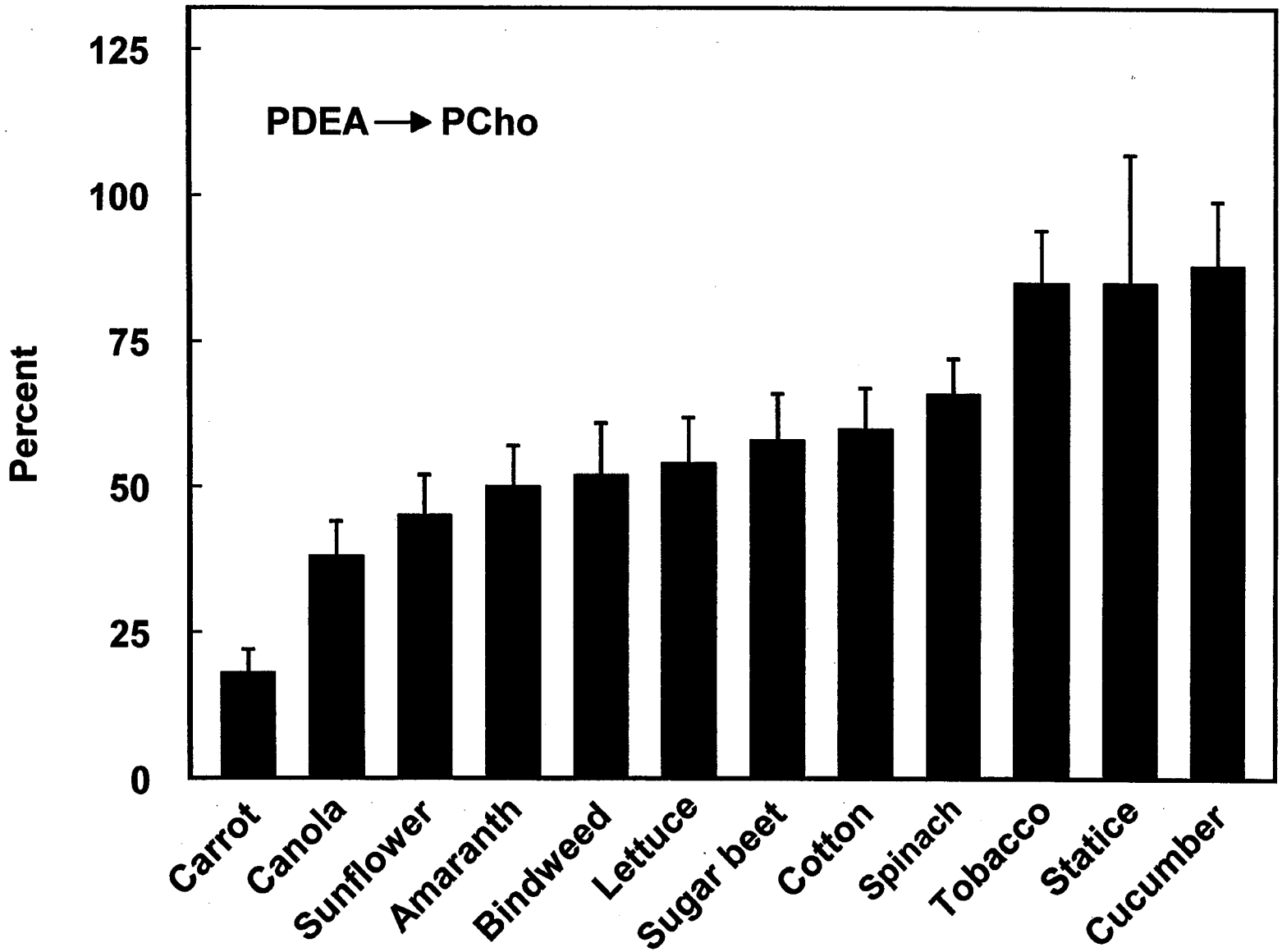

PDEA → PCho
Percent
125
100
75
50
25
0
Carrot
Canola
Sunflower
Amaranth
Bindweed
Lettuce
Sugar beet
Cotton
Spinach
Tobacco
Statice
Cucumber

TABLE XX. Phosphobase *N*-methyltransferase activity in Two *Limonium* Species.

Plant[a]	Treatment	Enzyme Activity nmol•min^{-1}•g^{-1} Fresh Wt			D/C PEAMeT ratio (%)	D/C PMEAMeT ratio (%)	D/C PDEAMeT ratio (%)
	Control or Dark	PEAMeT	PMEAMeT	PDEAMeT			
L. *perezii*	C	1.51 ± 0.34	1.38 ± 0.32	1.36 ± 0.34	62.0 ± 16.0	71.0 ± 16.0	85.0 ± 22.0
L. *sinuatum*	C	1.22 ± 0.05	1.35 ± 0.01	1.36 ± 0.22	76.0 ± 10.0	60.0 ± 3.0	94.0 ± 28.0
L. *perezii*	D	0.83 ± 0.26	0.86 ± 0.21	0.95 ± 0.21			
L. *sinuatum*	D	0.90 ± 0.10	0.95 ± 0.15	1.11 ± 0.13			

[a] Data are means ± S.E. for three repeat extractions.

dark/control ratios for all enzymes tested, there is no difference in *Limonium* species in percent activity remaining after a dark treatment.

Thin Layer Chromatography of Phosphobases Produced from PEAMeT Assay

During the assay, PEAMeT transfers a radioactive methyl group from SAM to PEA thus producing radiolabelled PMEA. Such product formation has been reported for spinach (Summers and Weretilnyk, 1993), soybean and carrot (Datko and Mudd, 1988b) and sugar beet (Hanson and Rhodes, 1983) but has not been verified for other plant species tested. To identify the assay products of the plants used in this study, thin layer chromatography was used. This was also done as a precaution since other substrates present in crude extracts such as DNA, lignins and pectins could also be methylated by SAM and possibly contribute to our rate estimates. Thus routinely, the 9.0 mL of 0.1 N HCl Dowex 50W (H^+) column eluate containing radiolabelled phosphobase *N*-methyltransferase reaction products (see Materials and Methods) originating from crude undesalted or desalted extracts with high PEAMeT activity was selected to identify the product(s) formed. Each column eluate was concentrated by nitrogen gas and spotted onto a TLC plate (see Materials and Methods). The inclusion of non-radioactive phosphobase standards (PEA, PMEA, PDEA and PCho) with reaction products spotted onto the TLC plate allowed for easy identification of products as purple

spots when the plate was sprayed with a molybdate reagent. Once the position of each phosphobase standard was identified, spots were scraped from the TLC plate and the radioactivity in the spot quantified. Table XXI shows that using only PEA as substrate, [^{3}H]PMEA, [^{3}H]PDEA and [^{3}H]PCho are formed during the 30 minute incubation period of the PEAMeT assay. Indeed, for the extracts from plants listed in Table XXI, the predominant product was [^{3}H]PMEA (84-94%), while [^{3}H]PDEA and [^{3}H]PCho were frequently formed, albeit at lower levels, (6-15%) and (<2 %) respectively.

TLC was also used to detect [^{14}C]phosphobase products formed during the PEAMeT assay from plants that did not have high PEAMeT activity. Identical procedures to that of [^{3}H]phosphobases were carried out as described in the Materials and Methods except that spots from the silica plate were not scraped but the entire plate was subjected to autoradiography at -80°C. As shown in Figure 8, the predominant ^{14}C-radiolabelled product was [^{14}C]PMEA and was found in all plants, even faintly for soybean and pea.

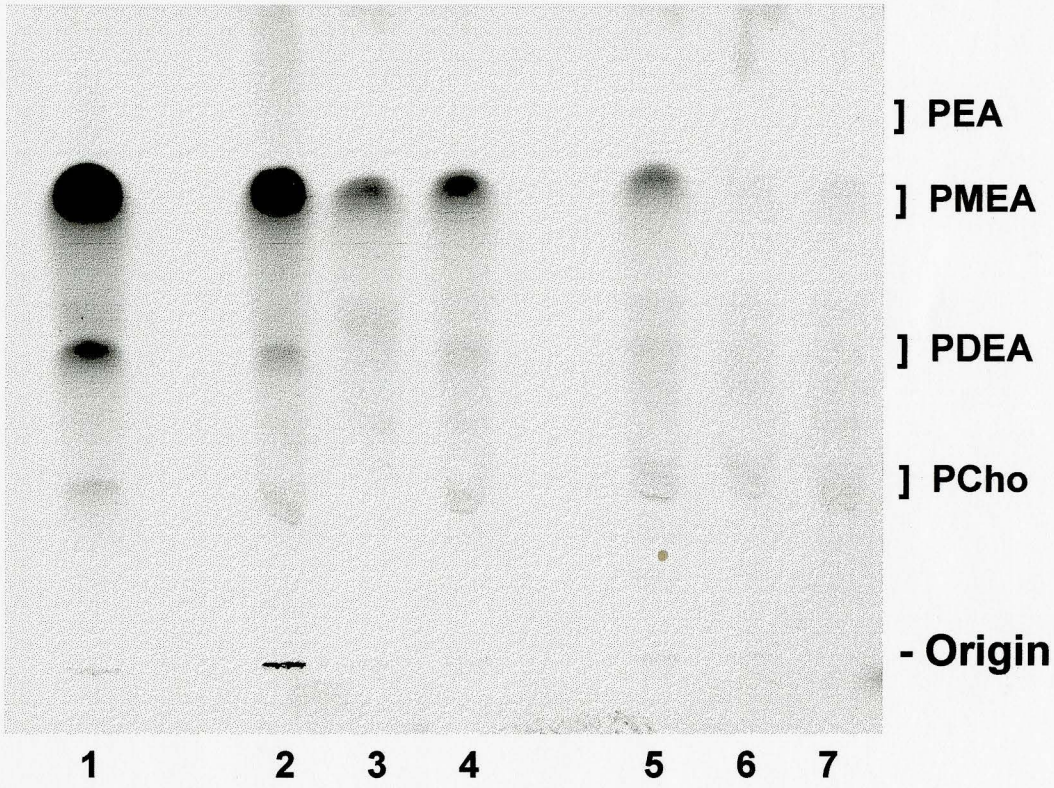
Table XXI. Use of Thin Layer Chromatography to Detect Phosphobase Products Produced in PEAMeT Assay.

Plant	Radioactivity (cpm)[a]		%[^{3}H]Radiolabelled phosphobase Product[b]		
	Applied	Recovered	PMEA	PDEA	PCho
Spinach	10496	3879	89	10	1
Cotton	34200	17266	88	10	2
Sugar beet	8472	3429	94	6	0
Amaranth	10756	3512	85	13	2
Bindweed	11256	5334	84	15	1
Sunflower	14544	6931	90	9	1
Lettuce	2776	1394	91	9	0
Statice	6524	4251	89	10	1

[a] cpm scraped from spot on silica plate.
[b] Expressed as % of recovered.

Figure 8. Autoradiograph of TLC Silica Plate Showing PMEA as the Predominant Product of the PEAMeT Assays Performed With Extracts of Plants With Low Levels of Enzyme Activity.

TLC conversion of [^{14}C]SAM to [^{14}C]phosphobases by leaf extracts from the leaves of plants with PEAMeT activity less than 2 nmol $\cdot$ min^{-1} $\cdot$ g^{-1} Fresh wt and spinach done as a control from (lane 1-7) spinach, canola, carrot, cucumber, tobacco, soybean and pea. PEA methyltransferase was assayed with [^{14}C]SAM as described in Materials and Methods. The products from PEA methyltransferase reactions were concentrated and then separated on silica gel G plates as described in Materials and Methods. The figure shows an autoradiograph of the TLC plate. The location of the origin and the running positions of authentic PEA, PMEA, PDEA and PCho are indicated.


] PEA
] PMEA
] PDEA
] PCho
- Origin
1
2
3
4
5
6
7

DISCUSSION

Extract and Assay Conditions

In this study, many different plant species were grown and then ground to prepare an extract suitable to measure phosphobase *N*-methyltransferase activities. Since there were no pre-tested protocols available to grow many of the plants or prepare the extracts, many of these conditions had to be empirically determined. At the onset we did not know if using crude undesalted or desalted extracts would matter, if PVP would benefit or inhibit enzyme activity, or even how to best report rates of enzyme activity. As a consequence, extracts of each plant were prepared and ground under a variety of conditions, and the extracts used to quantify enzyme activity. Once extracts were prepared, suitable dilutions had to be performed and then aliquots of the dilutions tested by enzyme assay in order to ensure that rates of activity in the assay were linear over the incubation period. Thus considerable attempts by trial and error were done to provide plants of healthy appearance and extracts whose enzyme activities could be reproducibly and reliably measured. By completing experiments on each plant a minimum of twice (cotton) or at least three times (all other species), variability between extract preparations could be evaluated. All the data reported in this thesis includes means with a measure of variation provided by standard errors. For example, Figure 2 shows the variability around the mean PEAMeT enzyme reaction rates

and that different levels of enzyme activities were observed between the various species tested.

For the purpose of this comparative biochemical study, Fresh wt was deemed to be the most suitable unit for enzyme activity measurements. The variability of protein and chlorophyll levels between plants led to less meaningful comparisons than one based upon Fresh wt since Fresh wt reflected a constant mass of tissue ground independent of how green it was or how much protein it might have had. For leaf tissue, water to dry mass contents are relatively uniform (95% water content) (Taiz and Zeiger,1991) while chlorophyll and protein contents can be more variable (Table VI).

Grinding spinach leaves with leaves of other plants proved to be a very useful control. For example, had this not been carried out, the apparently negligible activity first obtained for cotton would have been accepted as a reproducible and reliable estimate of PEAMeT activity instead of a serious underestimate (compare cotton PEAMeT activity for Table VIII and Table X).

Phosphobase *N*-methyltransferase Activities

This is not the first report of phosphobase *N*-methyltransferase activities as they have been detected in crude extracts prepared from spinach leaves (Summers and Weretilnyk, 1993; Weretilnyk and Summers, 1992; Weretilnyk et al., 1995). Summers and Weretilnyk (1993) reported phosphobase

N-methyltransferase enzyme activities of 0.52 nmol·min^{-1}·mg^{-1} protein for PEAMeT, 1.18 nmol·min^{-1}·mg^{-1} protein for PMEAMeT and 0.81 nmol·min^{-1}·mg^{-1} protein for PDEAMeT, respectively. The values reported in this thesis are based upon a greater number of replicates and are 0.31 ± 0.02 nmol·min^{-1}·mg^{-1} protein for PEAMeT (4 ± 0.2 nmol·min^{-1}·g^{-1} Fresh wt), 0.75 ± 0.08 nmol·min^{-1}·mg^{-1} protein for PMEAMeT (9 ± 0.8 nmol·min^{-1}·g^{-1} Fresh wt) and 0.57 ± 0.05nmol·min^{-1}·mg^{-1} protein for PDEAMeT (7 ± 0.5 nmol·min^{-1}·g^{-1} Fresh wt), (Table VII). These values reported in this thesis are lower but compare favourably with the published estimates. Estimates of activity of these enzymes in soybean and carrot have also been published previously by Mudd and Datko (1989b). They found PEAMeT, PMEAMeT and PDEAMeT activity in carrot tissue culture to be 0.06 nmol·mg^{-1}protein·min^{-1}, 0.04 nmol·mg^{-1}protein·min^{-1} and 0.06 nmol·mg^{-1}protein·min^{-1}, respectively. Their published values are reported on a protein basis and by using the protein concentration value for carrot of 10.48 mg protein·mL^{-1} from Table VI, we can convert enzyme activity reported in this thesis on a g Fresh wt basis to a protein basis. Consequently, from this study, PEAMeT activity was determined to be 0.06 nmol·mg^{-1}protein·min^{-1}, PMEAMeT activity to be 0.03 nmol·mg^{-1}protein·min^{-1} and PDEAMeT activity to be 0.03 nmol·mg^{-1}protein·min^{-1} for carrot. These enzyme activity measurements for carrot leaf tissue compare well with those of Mudd and Datko (1989b) for tissue culture samples. In a similar manner, soybean PEAMeT activity reported in this thesis on a g Fresh wt basis was converted to 0.004 nmol·mg^{-1}protein·min^{-1}

(using 5.10 mg protein $\cdot mL^{-1}$ from Table VI). PEAMeT enzyme activity was determined for soybean tissue culture by Mudd and Datko (1989b) and was reported to be 0.0014 nmol $\cdot mg^{-1}$protein $\cdot min^{-1}$. The PEAMeT activity measurement for soybean in this thesis is three fold higher than that reported by Mudd and Datko (1989b). Thus enzyme activities for leaf tissue reported in this thesis are comparable if not higher (as was for soybean) than those published by Mudd and Datko (1989b) using extracts from tissue cultures. It apppears that another legume, pea, has levels of enzyme activity which are similar to soybean. It is somewhat reassuring that the low levels of phosphobase *N*-methyltransferase activity we report agree with estimates already published by Mudd and Datko (1989b) who also included verification of reaction products.

Detection of PEAMeT Activity in All Plants Surveyed

One of the most important findings in this thesis is the presence of PEAMeT enzyme activity in leaf extracts of all plants surveyed and that the level of activity appears to vary significantly between them (Figure 2). The apparent ubiquity of this enzyme supports the hypothesis proposed by Datko and Mudd (1988a) that the conversion of PEA to PMEA is a common committing step for choline synthesis in higher plants. Although PEAMeT enzyme activity is variable from a high of 25 $\pm$ 2 nmol $\cdot min^{-1} \cdot g^{-1}$ Fresh wt in cotton to a low of 0.04 $\pm$ 0.01 nmol $\cdot min^{-1} \cdot g^{-1}$ Fresh wt in soybean, it is detectable and activity

measurements are reproducible. This report expands the survey of PEAMeT enzyme activity measurements to species that have not been previously tested including pea, lettuce, canola, cucumber, amaranth, bindweed, cotton, sugar beet, sunflower and *Limonium* species.

Figure 2 depicts the levels of PEAMeT activity found in extracts of leaves of the dicot plants under study in this thesis. A natural question which arises is whether in vitro enzyme activity is a true reflection of in vivo enzyme rates. If in vitro enzyme activity is not a true reflection of in vivo rates, then this suggests that the enzyme is not functioning at maximal capacity. For example, limitations such as substrate availability in a plant cell or the possibility of compartmentation of the enzyme away from its substrate, may serve to limit the activity of an enzyme in a plant cell. However, there is some evidence to support in vitro activity as providing a reasonable if not true reflection of in vivo capacity. By looking at salinized plants, specifically spinach, Summers and Weretilnyk (1993) found that the phosphobase route and not the free base route is the predominant pathway for Cho synthesis in spinach. Using in vitro enzyme activity measurements from extracts of spinach leaves from plants salinized step-wise to 100mM, 200mM or 300mM, they showed that the increase in in vitro activity was accompanied by increases in the flux rate through the phosphobase pathway by in vivo radiolabelling of phosphobase intermediates of Cho synthesis of all three enzymes in the phosphobase pathway for Cho synthesis. Thus this pathway in spinach is upregulated, presumably to meet the demand for more glycine betaine to adjust to an osmotically challenging

environment. If the metabolic control to upregulate the choline biosynthetic pathway exists in spinach, then perhaps all plants have a variable capacity to synthesize a compound they may all need and use. Datko and Mudd's (1988a) proposal that the PEA to PMEA step is required for all higher plants en route to the synthesis of choline, suggests that the variable PEAMeT activity rates shown in Figure 2 may reflect underlying differences in the capacity for different plants to synthesize choline. None of the plants in this study were intentionally osmotically stressed, and so presumably, not actively accumulating glycine betaine to any extent beyond those needed for growth under non-stressed conditions. This would suggest that plants such as canola, tobacco, carrot, pea, soybean and cucumber cannot synthesize as much choline as plants with higher levels of PEAMeT such as spinach, cotton, sugar beet, amaranth, bindweed, lettuce and statice.

After the initial methylation of PEA to PMEA, the plants have different pathways available in order to synthesize choline (Summers and Weretilnyk, 1993; Rhodes and Hanson, 1993). For example, evidence from Datko and Mudd (1988a) shows that carrot, after the initial methylation of PEA, carries out all subsequent methylations at both the phosphobase and phosphatidylbase levels. In fact, the data indicating that carrot PEAMeT activity was two fold higher than either PMEAMeT or PDEAMeT activity was similar to what Mudd and Datko (1989b) found. In carrot tissue culture, PEAMeT activity was roughly 1.5 fold higher than PMEAMeT activity, while the level of PEAMeT activity was not different from PDEAMeT activity and their in vivo radiolabelling experiments showed that

the plants do not utlize the PMEA → PDEA step as well as the PMEA → PtdMEA → PtdDEA route. Thus the reduced levels of PMEAMeT and PDEAMeT in carrot could signify leaves used here are also using the phosphatidylbase route to synthesize choline. Likewise, the enzyme activity measurements reported in this study for soybean are consistent with the findings of Datko and Mudd (1988b). In this thesis, soybean enzyme activity measurements for PMEAMeT and PDEAMeT enzymes were below the detection limits of our radioassay. In soybean tissue culture, Datko and Mudd (1988b) found no activity of the second and third phosphobase *N*-methyltransferase enzymes to produce PDEA and PCho enroute to choline. Instead, they demonstrated that after the committing step of PEA to PMEA, soybean utilizes a phosphatidyl base route to form PtdCho and very likely explains the absence of enzyme rates found in this thesis for PMEAMeT and PDEAMeT. Pea leaf methyltransferase activity reported here also had no detectable PMEAMeT activity and PDEAMeT activity was found to be very low but above our limits of detection at 0.05 $nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt. Since the PDEA → PCho assay products were not identified, it is possible that these low rates indicate that another substrate present in the crude undesalted extracts was methylated in vitro. In this regard, activity was not detected using desalted extracts of pea (Table XIX).

Gylcine Betaine Accumulation in Dicot Plants

Figure 9 has been redrawn from Figure 2, this time indicating which plants are glycine betaine accumulators and which have not been found to accumulate this osmolyte. A striking observation from Figure 9 is that the glycine betaine accumulators "cluster" at one end of the graph - the high PEAMeT activity end. Cotton, sugar beet, amaranth, bindweed, sunflower and spinach are known to accumulate glycine betaine. Several *Limonium* species also accumulate glycine betaine as do the species used in this study (Hanson *et al.*, 1991). None of the other plants are known to accumulate glycine betaine (Weretilnyk et al., 1989, Rhodes and Hanson, 1993)

A review by Rhodes and Hanson (1993) identifies families and genera that accumulate glycine betaine to levels that could be osmotically significant, if gylcine betaine is confined mainly to cytoplasmic compartments of the plant cell. Data reported on a Fresh wt basis is converted to a dry wt basis by assuming 10% of the Fresh wt is dry matter. In accumulating genera, species have glycine betaine levels of approximately 5-100 $\mu mol \cdot g^{-1}$ dry wt under non-stressed conditions or 40-400 $\mu mol \cdot g^{-1}$ dry wt under natural or experimental saline or dry conditions. In non-accumulating genera, glycine betaine levels are less than 1 $\mu mol \cdot g^{-1}$ dry wt . Table XXII provides some reported values of glycine betaine levels within the leaves of dicot plants represented in this study. If all of these plants *N*-methylate PEA to PCho, the high amount of betaine in spinach, sugar beet, amaranth, sunflower and bindweed would require a high flux of PMEA

Figure 9. PEAMeT Activity in Crude Leaf Extracts of Various Glycine Betaine Accumulating and Non-Accumulating Dicots.

In vitro PEAMeT activity ($nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt) in crude undesalted leaf extracts of plant species tested. Glycine betaine accumulating (solid bar) or non-accumulating species (hatched bar) are identified. Complete description of harvesting and assay conditions are outlined in Materials and Methods. Data are the means ± S.E. from a minimum of two repeat extractions for cotton to a maximum of five repeats for pea.

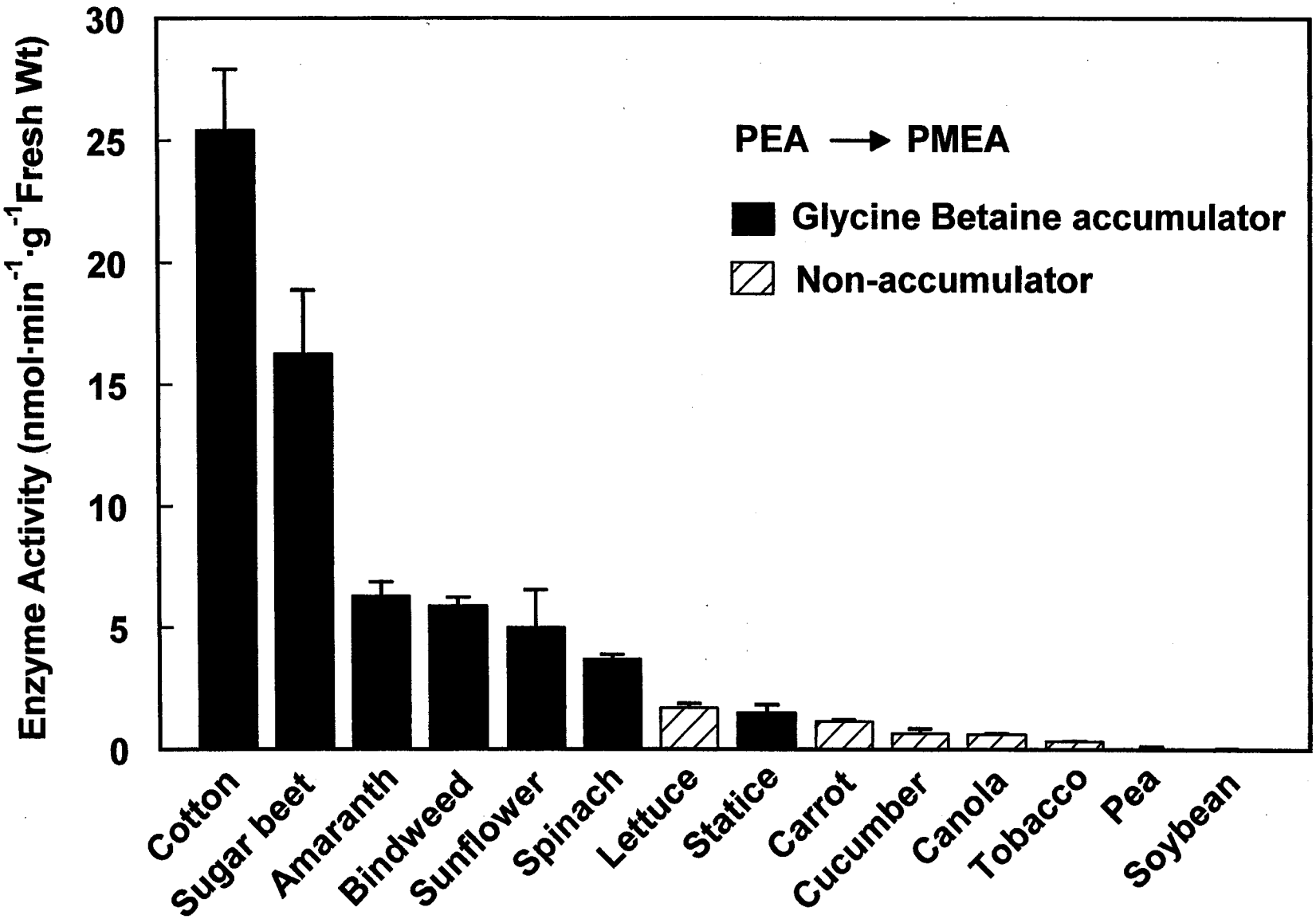

Enzyme Activity (nmol·min-1·g-1Fresh Wt)
30
25
20
15
10
5
0
PEA → PMEA
Glycine Betaine accumulator
Non-accumulator
Cotton
Sugar beet
Amaranth
Bindweed
Sunflower
Spinach
Lettuce
Statice
Carrot
Cucumber
Canola
Tobacco
Pea
Soybean

Table XXII. Summary of Glycine Betaine Accumulation in Various Dicot Plant Leaves.

Plant	Glycine Betaine	Glycine Betaine $\mu mol \cdot g^{-1}$ dry wt		Reference
	Accumulator (Y/N)	Control	Salinized (150-300mM)	
Spinach	Y	120.0	371	Weretilnyk *et al.*, 1989
Sugar beet	Y	108.0	263	Hanson and Wyse, 1982
Amaranth	Y	85.0	164	Weretilnyk *et al.*, 1989
Statice[a]	Y	8.0	120	Hanson *et al.*, 1991
Soybean	N			
Pea	N	1.9		Selvaraj *et al.*, 1995
Cotton[b]	Y	210.0	900	Gorham, 1996
Carrot	N			
Canola	N	4.9		Selvaraj *et al.*, 1995
Bindweed	Y	57.0	116	Weretilnyk *et al.*, 1989
Tobacco	N	0.32 2.8	N/A	Weretilnyk *et al.*, 1989; Selvaraj *et al.*, 1995
Cucumber	?			
Sunflower	Y	63.0	108	Weretilnyk *et al.*, 1989
Lettuce	N	0.13	N/A	Weretilnyk *et al.*, 1989

[a] The salinized treatment for statice was done at 400-500mM.
[b] Measurements reported from glycine betaine values from expressed cell sap.

through the choline synthesis pathway. It is tempting to speculate that leaves of plants with higher in vitro PEAMeT activity shown in Figure 9 reflect a greater need for choline as a consequence of glycine betaine synthesis. Thus, the high rates of PEAMeT enzyme activity seen in Figure 9 for some, but not all plants, may be necessary to accomodate this increased flux for osmolyte accumulation.

The capacity to synthesize glycine betaine may be an archetypal angiosperm characteristic expressed strongly in some plants and weakly in others (Weretilnyk *et al.*, 1989). According to a review by Hanson and Rhodes (1993), no glycine betaine accumulators are known in the families Portulacaceae, Magnoliaceae and Cruciferae of which canola is a member. Also, based upon the low glycine betaine levels, the authors did not consider *Lactuca* and *Nicotiana* to be accumulators.

The PEAMeT activity level at approximately 2 ± 0.2 nmol $\cdot$ min^{-1} $\cdot$ g^{-1} Fresh wt in romaine lettuce is an interesting finding given that it is not known to accumulate glycine betaine (Weretilnyk *et al.*, 1989). Different cultivars within the Gramineae are known to vary widely with respect to glycine betaine accumulation (Ishitani *et al.* 1993) and the same could hold true for lettuce. To investigate if the PEAMeT activity present in lettuce was specific to the romaine cultivar or not, leaves of Esmeralda M.I. pvp. (boston type), another cultivar of *Lactuca sativa* was also ground and assayed for PEAMeT activity. Comparable results for both cultivars and leaves from dark treated plants are shown in Table XXIII showing the presence of comparable levels of PEAMeT activity in the two cultivars tested.

TABLE XXIII. PEAMeT Activity of Two Varieties of *Lactuca sativa.*

Type	Cultivar	Enzyme Activity nmol•min^{-1}•g^{-1} Fresh wt	
		Control	Dark-treated
Romaine		1.48 ± 0.06	0.80 ± 0.02
Boston	Esmeralda M.I. pvp	1.46 ± 0.08	0.79 ± 0.00

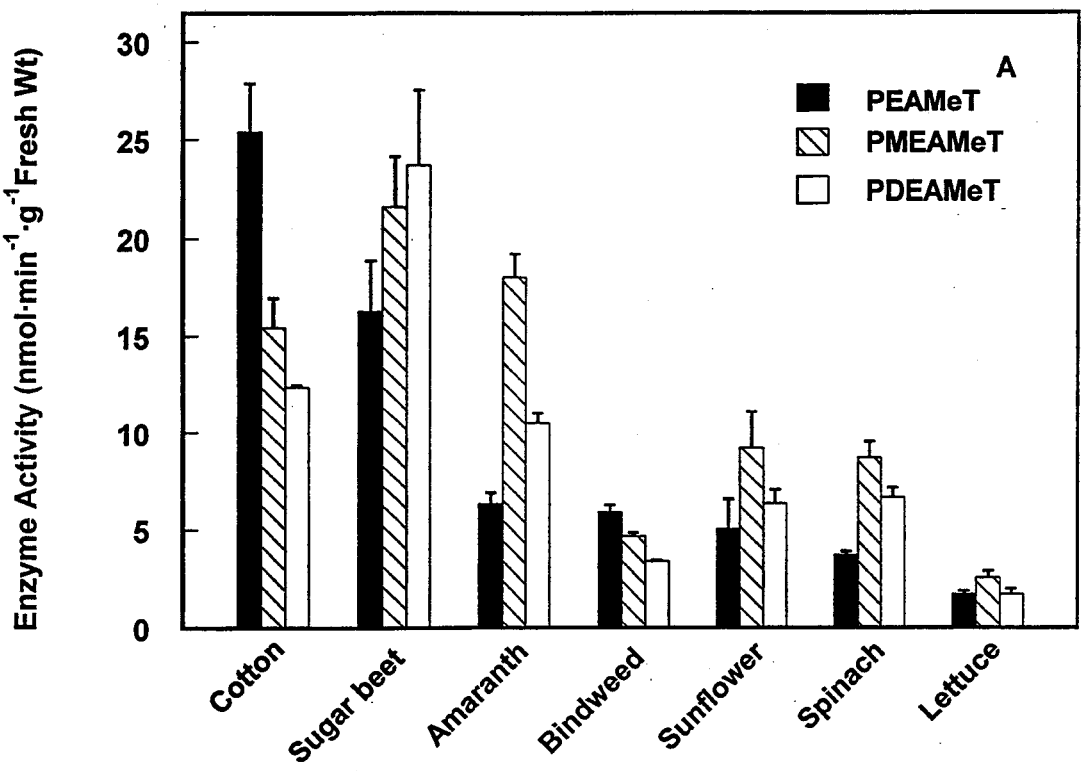
Weretilnyk *et al.*, (1989) found very little glycine betaine synthesis and no accumulation in cos lettuce leaf tissue compared to spinach and other glycine betaine accumulators. If romaine has a level of PEAMeT activity roughly half that of the glycine betaine accumulator spinach, this is presumably the level of choline synthesis required for growth and not osmolyte synthesis. However, the possibility that choline-*O*-sulphate is produced by this plant cannot be discounted since this determination was not performed.

PEAMeT, PMEAMeT and PDEAMeT Activity in Dicot Plants Surveyed

A composite of all three phosphobase *N*-methyltransferase activities from the various dicot plants is shown in Figure 10 Panels A and B. This figure summarizes the potential contribution of each methylation activity towards PCho synthesis for the different plant species surveyed and provides between-species comparison. As shown in Figure 10, the plants differ with respect to the relative rates of the three methylation activities. That is, for cotton and bindweed PEA → PMEA activity was highest relative to the two subsequent reactions while for amaranth, sunflower and spinach, PMEA → PDEA activity predominates (Figure 10A). Indeed, cotton had approximately two fold more PEAMeT activity than either PMEAMeT or PDEAMeT while for amaranth the converse relationship appears to hold with PMEAMeT and PDEAMeT activities, both were approximately two fold higher than PEAMeT activity.

Figure 10, Panels A and B. PEAMeT, PMEAMeT, and PDEAMeT Activity in Crude Undesalted Leaf Extracts from Various Dicot Plants.

Composite of PEAMeT (PEA → PMEA - filled bar), PMEAMeT (PMEA → PDEA - hatched bar), and PDEAMeT (PDEA → PCho - open bar) in vitro enzyme activity for each plant species tested. Description of extract preparation and assay conditions are outlined in Materials and Methods. Plants are arranged from highest PEAMeT activity to lowest. Note the difference in the y-axis scale between panels A and B. Data are means ± S.E. for a minimum of two repeats (cotton) to a maximum of five repeats (pea).


A
PEAMeT
PMEAMeT
PDEAMeT
Enzyme Activity (nmol·min-1·g-1Fresh Wt)
30
25
20
15
10
5
0
Cotton
Sugar beet
Amaranth
Bindweed
Sunflower
Spinach
Lettuce

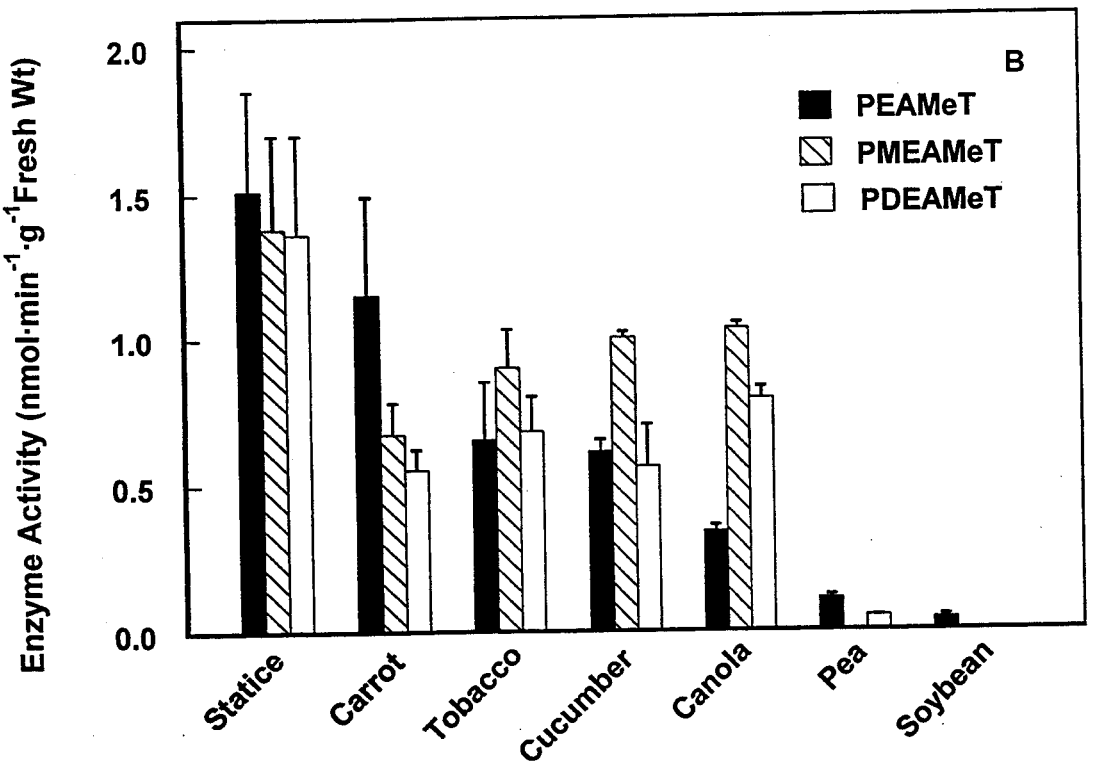

B
PEAMeT
PMEAMeT
PDEAMeT
Enzyme Activity (nmol·min-1·g-1Fresh Wt)
2.0
1.5
1.0
0.5
0.0
Statice
Carrot
Tobacco
Cucumber
Canola
Pea
Soybean

Figure 10B shows enzyme activity using a scale that more appropriately displays measurements for plants with lower activities. Again there are differences among the plants with respect to relative levels of activity for the three enzymes. Canola showed higher PMEAMeT and PDEAMeT activity relative to PEAMeT activity, a situation analogous to that seen in spinach in Figure 10A. The activity of the phosphobase *N*-methyltransferase enzyme PEAMeT was highest in carrot, pea and soybean. No apparent difference in the levels of activity for all three reactions was found for statice or tobacco (Figure 10B).

Control Phosphobase *N*-methyltransferase Activities Following Dark Treatment

Placing plants in continuous dark for a period approaching or exceeding 45 hours led to decreases in PEAMeT activity following dark exposure (Table XIII), a feature previously reported for spinach (Weretilnyk *et al.*, 1995). However, the level of PEAMeT activity remaining following a dark treatment varied among the plant species studied. This suggests that the rate of decline in PEAMeT activity is not the same among different plants. Sugar beet had only two percent of the light period PEAMeT activity remaining after a long dark exposure while amaranth, carrot and spinach activities dropped to 6-8%. Interestingly, sugar beet, amaranth and spinach are all in the Order Caryophyllales and show somewhat comparable reductions in PEAMeT activity

upon exposure to prolonged dark periods. Some plants, such as sunflower and bindweed had relatively high control rates of PEAMeT activity (5 - 6 $nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt) but did not show as great a reduction in PEAMeT activity after a dark treatment as the plants from the Order Caryophyllales. The percent PEAMeT activity remaining after a dark exposure for sunflower and lettuce, both in the Order Asterales and bindweed in the Order Polemoniales was approximately 40%. For the remaining plants, residual PEAMeT activity following dark exposure ranged from approximately 20 - 50% with the highest levels found for statice in excess of 70%. Thus PEAMeT activity in many plants is responsive to light conditions as spinach is reported to be, but the magnitude of the response varies. Whether the same mechanism(s) operate(s) to reduce activity in all plants is unknown.

Like PEAMeT activity, PMEAMeT and PDEAMeT activities in extracts of spinach leaves decreased after exposure to a prolonged period of darkness (Table XVII). As previously published (Weretilnyk *et al*, 1995), PEAMeT activity dropped to only six percent of the light cycle activity level and 71 and 68% of PMEAMeT and PDEAMeT activity, respectively, remained after prolonged exposure to darkness in spinach.

The percent PMEAMeT activity remaining after a dark treatment in Figure 5 indicates cucumber, statice and tobacco were the least light responsive. While sugar beet, amaranth and spinach displayed the highest degree of light regulation of the PEAMeT enzyme, all three plants had greater than 40% PMEAMeT activity

remaining after a dark treatment. This would indicate that there is at least more than one phosphobase *N*-methyltransferase enzyme present as has been proposed for spinach (Summers and Weretilnyk, 1993; Weretilnyk and Summers, 1992) and carrot (Datko and Mudd, 1988a). A similar scenario is found with PDEAMeT activity after a dark treatment. That is, carrot and canola had over 40% PDEAMeT activity remaining after a prolonged dark period and cucumber, statice and tobacco were the least light responsive with over 75% PDEAMeT activity remaining.

MMT and MDH Assays

A concern was raised about whether or not plants exhibiting low phosphobase *N*-methyltransferase activity, including, extracts from plants subjected to a 40 - 48 hr dark period (Table VIII), were still metabolically active. It is conceivable that PEAMeT activity could not be found in some plant species because the extracts were prepared from unhealthy or dying plants. Also, SAM hydrolase activity in various plants may reduce the SAM available during assays, resulting in underestimates of enzyme activity. There could also be a dark-activated phosphatase hydrolysing phosphobase substrates or products. These concerns were addressed using a variety of controls. The utility of the mixed grinds with spinach has been discussed previously. However, two additional enzymes were assayed in plant extracts namely, methionine

methyltransferase, (MMT) and malate dehydrogenase, (MDH). The presence or absence of these enzymes provided independent assessments for the metabolic "competence" of the plants tested. The presence of MMT activity, for example, ensured that the extract did not harbour components that acted specifically or with greater efficacy on SAM-utilizing methyltransferases. MDH activity, on the other hand, is a more generally used housekeeping enzyme (Summers *et al.*, 1998), and perhaps a more widely used and accepted measure of plant viability and extract quality.

The results of MMT assays done on crude leaf extracts of both control and dark treated plants are given in Table XXIV. The highest activity was found in the extract of cotton control plants at 1 ± 0 nmol $\cdot$ min^{-1} $\cdot$ g^{-1} Fresh wt. All plants grown under control conditions had higher MMT activity than spinach which proved to be the lowest at 0.01 nmol $\cdot$ min^{-1} $\cdot$ g^{-1} Fresh wt. In some extracts such as canola, sunflower and lettuce, less activity was seen in leaf extracts of dark-treated plants but these are still higher rates than that of spinach MMT activity in leaf tissue of similarly treated plants. Soybean and pea plants grown under control conditions had extracts with little phosphobase *N*-methyltransferase activities (Figures 2, 4 and 5), however, MMT activity was readily detected at 76 and 45 times higher levels, respectively, than MMT activity of spinach. In fact, soybean at 0.8 ± 0 nmol $\cdot$ min^{-1} $\cdot$ g^{-1} Fresh wt was second only to sunflower in MMT activity. James et al., (1995) purified MMT 620-fold to apparent homogeneity from leaves

TABLE XXIV. *S*-Adenosyl L-Methionine: L-Methionine *S*-Methyl Transferase (MMT) Activity in Crude Extracts of Diverse Dicot Species.

Plant[a]	Enzyme Activity nmol•min^{-1}•g^{-1} Fresh wt		Ratio (D/C %)
	Control	Dark Treatment	
Spinach	0.01 ± 0.01	0.20 ± 0.03	200
Sugar beet	0.58 ± 0.02	0.46 ± 0.00	79
Amaranth	0.20 ± 0.00	0.21 ± 0.01	105
Statice	0.35 ± 0.01	0.23 ± 0.02	66
Soybean	0.76 ± 0.00	0.66 ± 0.00	87
Pea	0.45 ± 0.00	0.20 ± 0.00	44
Cotton	1.09 ± 0.01	0.48 ± 0.03	44
Carrot	0.07 ± 0.00	0.05 ± 0.00	71
Canola	0.85 ± 0.02	0.63 ± 0.01	74
Bindweed	0.24 ± 0.01	0.17 ± 0.01	71
Tobacco	0.26 ± 0.02	0.19 ± 0.01	73
Cucumber	0.21 ± 0.01	0.32 ± 0.04	152
Sunflower	0.94 ± 0.14	0.72 ± 0.02	76
Lettuce	0.60 ± 0.03	0.51 ± 0.05	85

[a]Data are means of duplicate measurements ± S.E.

of the dicot *Wallastonia biflora*. MMT is the first enzyme in the biosynthetic pathway from methionine to form DMSP, another osmolyte. James *et al.*, (1995) assayed for MMT activity from a crude extract preparation of *Wallastonia biflora* leaves and found the activity to be 0.74 $nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt. This value is comparable to those reported in Table XIV.

Even in plants such as amaranth and sugar beet where prolonged dark periods led to a substantial reduction in PEAMeT activity (Figure 3), for these plants no comparable reduction in MMT activity was seen after 44 h or longer in the dark. What seemed striking and anomalous was that the activity of MMT was two fold higher in the dark than the light for spinach. This observation was studied in greater detail by Candace Webb and the results of her assays using extracts prepared for this study are shown in Table XXV. The extracts of spinach control plants had 3.6 fold higher MMT activity when desalted than the same extracts left crude. Thus Table XXIV suggests that MMT activity in spinach extracts is inhibited by a low molecular weight compound or enzyme that is removed by centrifugation of the extract through Sephadex G25. Furthermore, it would appear that the inhibitory component is either reduced, absent or not inhibitory in extracts of dark treated tissue. As well, Table XXV suggests that the MMT activities for the crude extracts reported in Table XXIV can only be considered conservative estimates and that actual rates in control plants may be higher if MMT activity in these extracts would also benefit from desalting. While such assays on desalted extracts were not carried out, it is noteworthy that PEAMeT shows little to no

Table XXV. MMT Activity in Crude Leaf Extracts of Spinach Plants Grown Under Control Conditions or Exposed to a Prolonged Dark Period[a].

Treatment	Enzyme Activity $nmol \cdot min^{-1} \cdot g^{-1}$ Fresh wt		Ratio D/C (%)
	Control	Dark-treated	
Undesalted	0.03 ± 0.01	0.09 ± 0.02	300
Desalted	0.11 ± 0.01	0.11 ± 0.01	100

[a] Time of dark treatment exceeding 40 hrs.

inhibition in crude extracts (Table VIII) and that MMT activity levels in 10 of the 14 plants tested had greater than 70% the level of MMT activity in the dark. Thus the reduction of PEAMeT activity in many of these plants in response to prolonged dark exposure (Figure 2) to approximately 40% and lower cannot be completely attributed to generic methyl-transferase inhibition either artifactually generated in the assay or produced in the plants.

MDH, responsible for converting malate to OAA, is ubiquitous in plants (Moller and Lin, 1986) and it was detected in all crude extracts prepared from the leaves of plants surveyed. MDH activity rates (measured by Candace Webb) are shown in Table XXVI. The highest MDH activity, 46.4 $\mu mol \cdot min^{-1} \cdot g^{-1}$ Fresh wt, was found in the cucumber control plant and the lowest was the dark treated carrot at 7.0 $\mu mol \cdot min^{-1} \cdot g^{-1}$ Fresh wt. Though the rates of MDH activity were variable between the plant sources tested, even plants with low PEAMeT activity relative to spinach have comparable or even higher rates of MDH activity to spinach and in no case does MDH activity drop below 60% in the dark. If this roughly 40% decline from control levels can be taken to indicate a reduced metabolic competence of the plants, then a reduction in phosphobase *N*-methyltransferase activity by 40% or less, depending on the plant, could be attributed to a general decline in the health and vigor of the plants and not entirely upon any light-responsive regulatory properties these transferases might have. A case in point is tobacco where PEAMeT activity was reduced to approximately 50% after a dark exposure treatment (Figure 3). Table XXVI indicates that the dark treated tobacco

Table XXVI. MDH Activity in Crude Leaf Extracts of Plants Grown Under Control Conditions or Exposed to a Prolonged Dark Period[a].

Plant[b]	Enzyme Activity $\mu mol \cdot min^{-1} \cdot g^{-1}$ Fresh wt		Ratio (D/C %)
	Control	Dark Treatment	
Spinach	19.4 ± 0.3	21.0 ± 0.3	104
Sugar beet	23.1 ± 1.5	21.1 ± 0.4	91
Amaranth	41.1 ± 2.9	41.5 ± 0.4	101
Statice	25.9 ± 1.1	19.2 ± 0.3	74
Soybean	29.6 ± 0.1	44.1 ± 0.6	149
Pea	29.5 ± 0.1	26.4 ± 0.3	89
Cotton	45.5 ± 0.8	31.8 ± 0.1	70
Carrot	7.5 ± 0.2	6.2 ± 0.0	82
Canola	19.9 ± 0.0	16.0 ± 0.1	80
Bindweed	18.9 ± 0.0	13.3 ± 0.1	70
Tobacco	38.9 ± 0.6	25.8 ± 0.2	66
Cucumber	46.4 ± 0.6	29.6 ± 0.0	64
Sunflower	10.6 ± 0.1	11.5 ± 0.0	108
Lettuce	15.0 ± 0.2	16.1 ± 0.2	107

[a] Time of dark treatment exceeding 40 hrs.
[b]Data are means of duplicate measurements ± S.E.

plants had 66% the MDH activity of control plants. Consequently, it would be difficult to conclude that tobacco is light regulated to the same extent as is suggested by Figure 3. Likewise, a more pronounced reduction in PEAMeT as opposed to MDH activity would suggest a more specific dark-mediated affect on enzyme activity. For example, in the case of sugar beet where PEAMeT activity is reduced to 2% the control level, MDH activity remained statistically unchanged in control versus dark.

One should not overlook that PMEAMeT and PDEAMeT activity remained at relatively high levels with respect to PEAMeT activity in the glycine betaine accumulating and non-accumulating plants exposed to a prolonged dark treatment (Tables XIX and XX). The PMEAMeT and PDEAMeT enzyme activities of statice, tobacco and cucumber were almost as high as the control rates after a dark treatment (Tables XIX and XX). These tables also show that PMEAMeT and PDEAMeT methyltransferases are present and detectable in extracts of dark-treated plants, including extracts of plants with little to no PEAMeT activity (eg. sugar beet, amaranth and spinach).

PMEA is Predominant Product Formed From PEAMeT Assay

The radiolabelled enzyme assay products were identified by thin layer chromatography as previously described (Smith, 1995). Smith (1995) discussed the inherent problems of using crude preparations containing more than one

phosphobase *N*-methyltransferase in establishing whether PEAMeT could catalyze more than just the first *N*-methyl transfer. He reported that a partially purified spinach leaf extract preparation (enriched between 22- and 38-fold for PEAMeT activity), converted PEA → PMEA → PDEA → PCho with only PEA as the substrate in the assay after 30 minutes in the ratio of 77:17:6 (PMEA:PDEA:PCho). Although the extract preparations used for this thesis are not purified beyond desalting and thus still considered to be crude extract preparations, phosphobase reaction products for spinach, cotton, sugar beet, amaranth, bindweed, sunflower, lettuce and statice plants (Table XXI), all showed similar ratios of PMEA: PDEA: PCho production at an approximately 89:10:1 ratio. The remaining plants under investigation including those with low PEAMeT activity, such as pea, soybean, carrot, canola, tobacco and cucumber all showed PMEA formation as the predominant radiolabelled product from PEA.

Summary

This study gives phosphobase *N*-methyltransferase enzyme activity measurements for PEAMeT, PMEAMeT and PDEAMeT, all enzymes in the choline biosynthetic pathway using extracts prepared from phylogenetically diverse dicot species. Supporting evidence that PEAMeT enzyme is ubiquitous to plants was found during this survey in the dicot plants tested. Plants found to have higher levels of PEAMeT activity (cotton, bindweed, sugar beet, amaranth, spinach

and sunflower) in crude leaf extracts were also glycine betaine accumulators, whereas, plants that had lower levels of PEAMeT activity (canola, tobacco, cucumber, soybean, carrot and pea) in crude leaf extracts were non-accumulators. This finding suggests that a low flux of PEA → PMEA leads to choline synthesis required only for phospholipid metabolism and little else. Salt stressing the dicot plants studied in this survey would be useful to see if PEAMeT activity is upregulated during times of osmotic stress.

The results of the levels of PMEAMeT and PDEAMeT activities remaining in control plants after exposure of plants to a dark period of approximately 45 hours show different levels of activity remaining and perhaps suggest different regulation of these phosphobase enzymes. For plants such as statice and tobacco, there appears to be little light regulation of these methyltransferases. Yet, the light regulation of PEAMeT is large (92 - 98% reduction) for spinach, amaranth and sugar beet plants. Thus the mechanism for light regulation among plants is not known and more research is needed to explain why the plants differ in this regard.

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