PURIFICATION OF ETHANOLAMINE KINASE FROM SPINACH

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF ETHANOLAMINE KINASE FROM SPINACH

by

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ABSTRACT

Ethanolamine kinase (EC 2.7.1.82) catalyses the reaction of ethanolamine and Mg^{2+}-ATP to produce phosphoethanolamine and Mg^{2+}-ADP. For spinach (*Spinacia oleracea*) the activity of ethanolamine kinase is increased in leaf extracts of salinized plants. A comparison of ethanolamine kinase activity between extracts from control and salinized plants after native polyacrylamide gel electrophoresis shows that ethanolamine kinase activity migrates to the same position on a gel. This observation suggests that salinization does not induce the activity of a novel ethanolamine kinase isozyme.

Ethanolamine kinase has been purified 6,537 fold to apparent homogeneity from spinach leaves by ammonium sulphate fractionation and sequential fractionation by both open-bed and HPLC chromatography, using ion-exchange and hydrophobic interaction matrices. The enzyme has an estimated molecular weight of 80,000 D by gel filtration chromatography and a subunit size of 38,000 D by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Ethanolamine kinase has a broad pH optimum between pH 7 and 9 and the optimal ratio of Mg^{2+}:ATP for the reaction is 1:1 at 5 mM. The apparent K_m value for the substrate ethanolamine is 16 μM and the V_{max} is 438 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} protein. Monomethylethanolamine and dimethylethanolamine serve as substrates for ethanolamine kinase but not trimethylethanolamine (*ie* choline). Enzyme activity is slightly stimulated by NaCl

and KCl and inhibited to varying degrees by phosphate, ammonium, phosphoethanolamine and phosphodimethylethanolamine. Not surprisingly enzyme activity is also inhibited by ADP and to varying degrees by the divalent cations Mn^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+} and Ni^{2+}.

This work is the first purification and biochemical characterization of ethanolamine kinase in spinach and is the first step towards understanding the contribution ethanolamine kinase makes towards the synthesis of choline.

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INTRODUCTION

Any environmental factor that influences cellular water content can be a threat to life since 85 to 95% of cellular volume in metabolically active cells is made up of water (Somero, 1992). Even though water stress can be a problem for animals, plants are more sensitive to environmental changes in water availability since most plants lack the capacity to escape poor environmental conditions. Plants are therefore often exposed to varying degrees of water (osmotic) stress brought about by salinity, desiccation or freezing at some stage in their ontogeny (Morgan, 1984). Plants have had to evolve in response to water stress, with most of the evolutionary responses presumably occurring when plants moved to dry land from an aquatic environment. An important aspect of plant adaptation to water deficits (Morgan, 1984) and salinity stress (Wyn Jones *et al*, 1977) is metabolic adaptation by the accumulation of certain cytoplasmic molecules to combat the resulting osmotic stress. By better understanding how osmotic stress-tolerant plants manipulate their biochemical pathways to counteract environmentally imposed osmotic stress, genetic engineering of these pathways into species that are less tolerant of osmotic stress may become possible.

Osmotic Stress Resistance in Plants

Plant Water Relations

There are only a few strategies that plants use to deal with plant water deficits. Plants adapt to water stress by employing developmental and metabolic changes in response to total water potential (reviews: Turner, 1979; Jones *et al*, 1981). Water potential is the chemical potential of water of a plant cell (Salisbury and Ross, 1985). Water potential is made up of the sums of the solute (osmotic), turgor (pressure) and matric potentials (Salisbury and Ross, 1985). Solute potential reflects the difference in concentration of solutes between the inside and the outside of the cell or between the plant and the soil. Turgor is maintained by the solute potential and plays a role in cell expansion, growth and many biochemical, physiological and morphological processes (Jones *et al*, 1981), gas exchange capacity in leaves, and the regulation of certain metabolic cellular functions such as growth by cell elongation (Hanson and Hitz, 1982). Matric potential is the overall reduction in water potential caused by the interaction of the cell wall with water in the surrounding soil particles, which reduces the tendency of water molecules to react chemically or to evaporate (Salisbury and Ross, 1985).

Under conditions of salinity or drought stress, the water potential of the plant drops. This is due to an excess concentration of salts in the soil solution which tends to cause water to diffuse out of the plant cells (Salisbury and Ross, 1985).

Osmotic Stress Escape

Plants that escape conditions of osmotic stress differ from other plants only in

the way they survive during dry periods. Drought escape is seen in desert ephemerals which have no special physiological, biochemical or morphological mechanisms to cope with water deficits (Mulroy and Rundel, 1977).

Osmotic stress is escaped either by rapid phenological development or developmental plasticity. Those plants with rapid phenological development complete their life cycle before serious water deficits develop and these plants withstand dry seasons as fleshy rhizomes insulated from dry soil or as seeds (Jones *et al*, 1981). Desert annuals often rely on developmental plasticity and have flowers with a minimum of vegetative structure and are indeterminate in their growth habitat. In conditions of limiting rainfall few flowers and seeds are formed. In plentiful rain there is an abundance of vegetative growth, flowers and seeds (Mulroy and Rundel, 1977).

Osmotic Stress Avoidance

Plants that are said to avoid conditions of osmotic stress do so by ensuring that tissue dehydration does not occur by maintaining a high tissue water potential. This is achieved by the careful regulation of water uptake and water loss.

Water uptake is enhanced by increased hydraulic conductance. The hydraulic resistances in the plant are kept low enough for water to be freely available to major metabolic centres within the shoot (Jones *et al*, 1981). A change in rooting patterns serves to increase the volume of soil tapped to increase the amount of water tapped. This is accomplished by growing deeper roots, by increasing the root density or by changing the water flow properties through the roots. Support for this adaptive

mechanism comes from evidence that drought resistance is known to result in a greater proportion of total plant mass as roots (Begg and Turner, 1976; Fischer and Turner, 1978).

Water loss is reduced by manipulating the epidermal conductance of the plant. This is achieved by regulating the opening and closing of the stomates (Nobel, 1976). This is a strategy that is useful for succulents; an advantage to long-term survival, but closing the stomates also decreases the amount of carbon assimilated decreasing net productivity (Fischer and Turner, 1978; Turner, 1979) and thus would not be a useful strategy to have in crop plants (Hsiao *et al*, 1976). By closing the stomates to prevent water loss, another undesirable effect is that the temperature increases in plant cells (Gates, 1968). Some plants counteract this increase in tissue temperature by rolling their leaves or letting their leaves hang limp (Begg and Turner, 1976).

A decrease in absorbed radiation also serves to reduce the amount of water lost by the plant. This can be achieved by the control of leaf movements and changes in the reflectance characteristics of the leaves. In the plant *Stylosanthes humilis*, leaves are aligned parallel to incident radiation as plant water deficit increases (Begg and Torsell, 1974). Radiation load can also be decreased by the production of hairs (Begg and Turner, 1976; Ehleringer *et al*, 1976), surface wax (Chatterton *et al*, 1975) or salt on the leaves, all of which increase the reflectance of light from the leaves (Jones *et al*, 1981).

Another way in which plants can reduce the amount of water lost through transpiration is to change the total evaporative surface area of the leaves. An example of this strategy would be a plant that bears large, thin leaves in the wet winter season

and replaces these winter leaves with small, thicker leaves in the drier summer season (Taiz and Zeiger, 1991). Another example is seen in some crop plants which allow large, photosynthetically less active older leaves to die off (Constable and Hearn, 1978).

Osmotic Stress Adaptation

Plants need to retain cellular turgor and volume properties. The most notable symptom of low turgor pressure is lack of growth by cell elongation (Hsiao, 1973). Not only is cellular growth affected by a decrease in turgor pressure but many enzymatic reactions both associated with and detached from the plasma membrane are turgor-dependent (Hsiao, 1973). At decreased water potential, there is a maintenance of turgor by solute accumulation (Wenkert *et al*, 1978) or by an increase in cell wall elasticity (Ordin *et al*, 1956). High cell wall elasticity enhances the ability of tissue to maintain increased turgor pressure as compared to tissue with decreased cell wall elasticity (Weatherley, 1970). Adaptation at the metabolic level consists of solute accumulation (Hanson and Hitz, 1982). Solute accumulation serves to reduce the solute potential of plant cells which reduces water loss and increases water uptake (Wyn Jones and Gorham, 1983).

Solute Potential Reduction and the Accumulation of Solutes

A relatively energetically inexpensive way for a plant to decrease its solute potential is to take up salts such as K^+, Na^+ or Cl^- from the environment (soil) (Wyn Jones *et al*, 1979; Salisbury and Ross, 1985). The problem with this strategy is

that high levels of cytoplasmic salts cause great disruptions in the proper functioning of cellular processes (Yancey *et al*, 1982). The key properties of cells that are strongly disrupted by elevated salt concentrations are: rates of enzymatic catalysis such as K_m (Bowlus and Somero, 1979) and V_{max} (Borowitzka and Brown, 1974; Stewart and Lee, 1974); binding of ligands by enzymes; protein subunit assembly; protein compartmentation; protein solubility; transmembrane potentials; and interactions between phospholipid bilayers and peripheral membrane proteins (Yancey *et al*, 1982; Somero, 1992). Disruptions occur even when salt levels are a few tenths molar greater than normal (Yancey *et al*, 1982). Even with these possible cellular disruptions some organisms such as halophilic archaebacteria successfully accumulate salts as a means of lowering solute potential (Lanyi, 1974).

Salt Accumulation in the Archaebacteria

In halophilic archaebacteria such as *Halobacterium* spp., Na^+ and K^+ can be accumulated to levels greater than 7 M (Brown and Simpson, 1972; Lanyi, 1974). Most proteins would become nonfunctional at this salt level (reviewed by Flowers *et al*, 1977). In order to function with high salt concentrations within their cells, these archaebacteria have altered protein structures so that they absolutely require high salt concentrations to function (Lanyi, 1974). For example, archaebacterial ribosomes have identical sedimentation coefficient values to eubacteria ribosomes but are unstable at salt concentrations lower than 3.5 M and maximal activity is achieved at 4.0 M salt (Visentin *et al*, 1972).

Archaebacterial proteins contain an unusually high level of acidic amino acid

residues and low levels of basic residues (Lanyi, 1974). This results in the proteins bearing a high net negative charge which requires a higher concentration of surrounding cytoplasmic cationic counterions such as K^+ or Na^+ for the protein to fold into the native, compact structure needed for activity (Lanyi, 1974). This activation is completed by a relatively low (few tenths molar) salt concentration which is a reflection of specific and strong ionic binding of the accumulated cations to the carboxylate groups on the protein (Lanyi, 1974; Somero, 1992). In fact, archaebacterial proteins may require salt levels of at least 1 M concentration for peak enzymatic activities (Lanyi, 1974).

The accumulation of salts from the environment is an energetically inexpensive and successful strategy for dealing with low water potential. These organisms gain from a metabolic cost reduction but are absolutely dependent on a high salt environment and are unable to survive in the face of widely fluctuating salinities (Yancey *et al*, 1982; Somero, 1992).

Solute Accumulation in Eubacteria and Eukaryotes

Eubacteria and eukaryotes have taken a different evolutionary route in adapting to osmotic stress. These organisms accumulate low molecular weight organic molecules to extremely high concentrations in the cytoplasm of the cell thereby lowering the solute potential of the cell without perturbing protein function (Somero, 1992). The accumulation of nontoxic molecules, first described by Brown and Simpson (1972) as "compatible solutes" or osmolytes, appears to be an evolutionarily simpler means for coping with high intracellular osmolarity (Yancey *et al*, 1982).

For an organism to employ a compatible solute accumulation strategy, what is required is the acquisition of mechanisms to create the osmolyte and to regulate osmolyte concentrations to reflect changes in total water potential. It is thought that only a few enzymes are responsible for osmolyte accumulation and that these enzymes are regulated by small changes in salt concentration (Schoffeniels, 1976; Kauss *et al*, 1979). Other metabolic changes that would need to be considered are the creation of mechanisms to remove salts from the cytoplasm to the external environment or to the vacuole (Yancey *et al*, 1982), the creation of or the modification of pathways for osmolyte synthesis, and changes in K_m values of enzymes using one or more of the organic osmolytes as substrates. Though these changes seem to be complex, regulation requires a limited number of protéins, thus the majority of cellular proteins are not altered, these changes being minor compared with the massive changes in protein sequences of halobacteria (Yancey *et al*, 1982). The osmolyte accumulation strategy enables eubacteria and eukaryotes to adapt to widely fluctuating environmental conditions, an ability the halophilic archaebacteria lack (Yancey *et al*, 1982; Somero, 1992).

Classes of Osmolytes

There seems to be a selective basis for using only a few classes of osmolytes (Somero, 1992). There are only five classes of organic solutes that are the major contributors to the intracellular osmolyte pool in the most osmotically concentrated cells. These classes of osmolytes are: 1, sugars and polyhydric alcohols such as glycerol, mannitol, sucrose and sorbitol; 2, amino acids and amino acid derivatives

such as glycine, alanine, proline, taurine and ß-alanine; 3, methylamines such as betaine, choline-*O*-sulphate, trimethylamino-*N*-oxide and sarcosine; 4, urea; and 5, ß-dimethylsulfoniopropionate. There is a wide distribution of compatible solutes accumulated amongst diverse organisms, perhaps indicating the convergent evolution of strategies (Yancey *et al*, 1982; Rhodes, 1987; Somero, 1992).

This discussion will focus on the methylamine, *N,N,N*-trimethylglycine betaine (betaine). Betaine is among the most potent of all the osmolytes in protecting enzymes from inhibitory effects of increased salt concentrations (Pollard and Wyn Jones, 1979; Warr *et al*, 1988) and in promoting bacterial growth under high levels of salt (Le Rudulier *et al*, 1984).

Betaine

Phylogenetic Distribution of Betaine

Fast atom bombardment mass spectrometry to detect betaine in monocots (Rhodes *et al*, 1987) and dicots (Weretilnyk *et al*, 1989) of related and divergent plants shows that betaine accumulation occurs throughout the plant kingdom. Families which contain members who accumulate betaine as confirmed by MS or ^{2}H NMR analyses of leaf tissues (review: Rhodes and Hanson, 1993) include the Chenopodiaceae (Rhodes *et al*, 1987; Weretilnyk *et al*, 1989; McCue and Hanson, 1992), Amaranthaceae, Asteraceae, Solanaceae, Leguminoseae (Weretilnyk *et al*, 1989), Gramineae (Rhodes *et al*, 1987; Arakawa *et al*, 1990), Compositae, Convolvulaceae (Rhodes *et al*, 1987; Weretilnyk *et al*, 1989) and the Plumbaginaceae (Hanson *et al*, 1991). Within these families accumulation may occur in all genera or

only in some. Comparative biochemical and immunological evidence suggests that the capacity to synthesize betaine is an archetypal angiosperm characteristic, expressed strongly by some living plants and weakly by others (Weretilnyk *et al*, 1989; Rhodes and Hanson, 1993).

Plants without betaine accumulation show low but still detectable quantities of betaine (Weretilnyk *et al*, 1989). Using antibodies to betaine aldehyde dehydrogenase (one of the betaine biosynthetic enzymes) in Western blot hybridization and immunotitrations confirm immuno-relatedness within divergent and conserved species, pointing towards a single origin for betaine synthesis in dicots (Weretilnyk *et al*, 1989).

Betaine as a Cytoplasmic Osmoticum

There is histochemical, biochemical and physiological evidence that betaine is an inert and nontoxic cytoplasmic osmoticum (Wyn Jones and Storey, 1981; Robinson and Jones, 1986). Betaine facilitates the uptake and retention of water in plants under osmotic stress (Wyn Jones *et al*, 1977) which helps to maintain osmotic equilibrium between the cytoplasm and the vacuole of the plant cell (Wyn Jones *et al*, 1979; Hanson and Wyse, 1982). The vacuole solute potential is lowered by Na^+ and Cl^- accumulation (Wyn Jones *et al*, 1977; Wyn Jones *et al*, 1979; Yancey *et al*, 1982) whereas the cytoplasm accumulates betaine (Gorham and Wyn Jones, 1983). The localization of betaine in the cytoplasm reduces the amount needed to be synthesized. The metabolic cost is therefore reduced, which saves valuable fixed carbon and nitrogen reserves (Le Rudulier *et al*, 1984).

Not only do osmolytes protect against drought stress, but these compounds are also thought to protect plant enzymes against heat inactivation (Paleg *et al*, 1981), to stabilize folded protein structures, to promote subunit interactions (Incharoensakdi *et al*, 1986) and prevent inhibition of enzyme activity caused by inorganic salts (Pollard and Wyn Jones, 1979) and urea. These are commonly observed manifestations of the protective effects of the osmolytes *in vitro* (Le Rudulier *et al*, 1984; Rhodes, 1987). Solutes also appear to interact with and stabilize membranes (or membrane-bound proteins) (Wyn Jones and Storey, 1981) and are implicated in *in vivo* cryoprotection (Bokarev and Ivanova, 1971).

Betaine Biosynthesis

In higher plants, betaine is formed from choline (Cho) by a two step oxidation process. First, Cho is converted to betaine aldehyde by Cho monooxygenase (Brouquisse *et al*, 1989), then betaine aldehyde is converted to betaine by betaine aldehyde dehydrogenase (BADH) (Weigel *et al*, 1986). BADH has been purified and cloned from spinach (Weretilnyk and Hanson, 1990) and sugarbeet (McCue and Hanson, 1992). It has been sequenced and antibodies have been produced against it in spinach (Arakawa *et al*, 1987; Weretilnyk and Hanson, 1990). Cho monooxygenase (Brouquisse *et al*, 1989), has yet to be isolated and characterized but work is under way (Burnet *et al*, 1993).

In chenopods, Cho monooxygenase and BADH are both localized in the chloroplast in spinach (Hanson *et al*, 1985) and in sugarbeet (Weigel *et al*, 1988), in contrast to mammals where Cho oxidation to betaine is mitochondrial (Glenn and

Vanko, 1959). Mammals are able to catabolize betaine unlike plants which do not appear to catabolize betaine (Hanson and Hitz, 1982). In principle, this lack of catabolism renders betaine a highly effective osmotic solute, since large increases in betaine pool size can be achieved quite quickly (Rhodes, 1987).

In order to learn how Cho is partitioned between betaine production and phospholipid biosynthesis, more must be learned about Cho biosynthesis in betaine accumulating and betaine non-accumulating plants.

Pathway(s) of Choline Biosynthesis

Cho is an important osmoregulatory compound serving in the synthesis of osmolytes such as betaine (Wyn Jones *et al*, 1979) and choline-*O*-sulphate (Hanson *et al*, 1991). Cho is also an essential component of plant membrane phospholipids (Mudd, 1980). Despite Cho's important role in plant lipid metabolism, little is known about the enzymes involved in its biosynthesis except from a few radiotracer experiments (Hitz *et al*, 1981; Hanson and Rhodes, 1983; Mudd and Datko, 1986) and crude enzyme studies (Moore, 1976; Datko and Mudd, 1988b).

Possible Choline Biosynthetic Routes

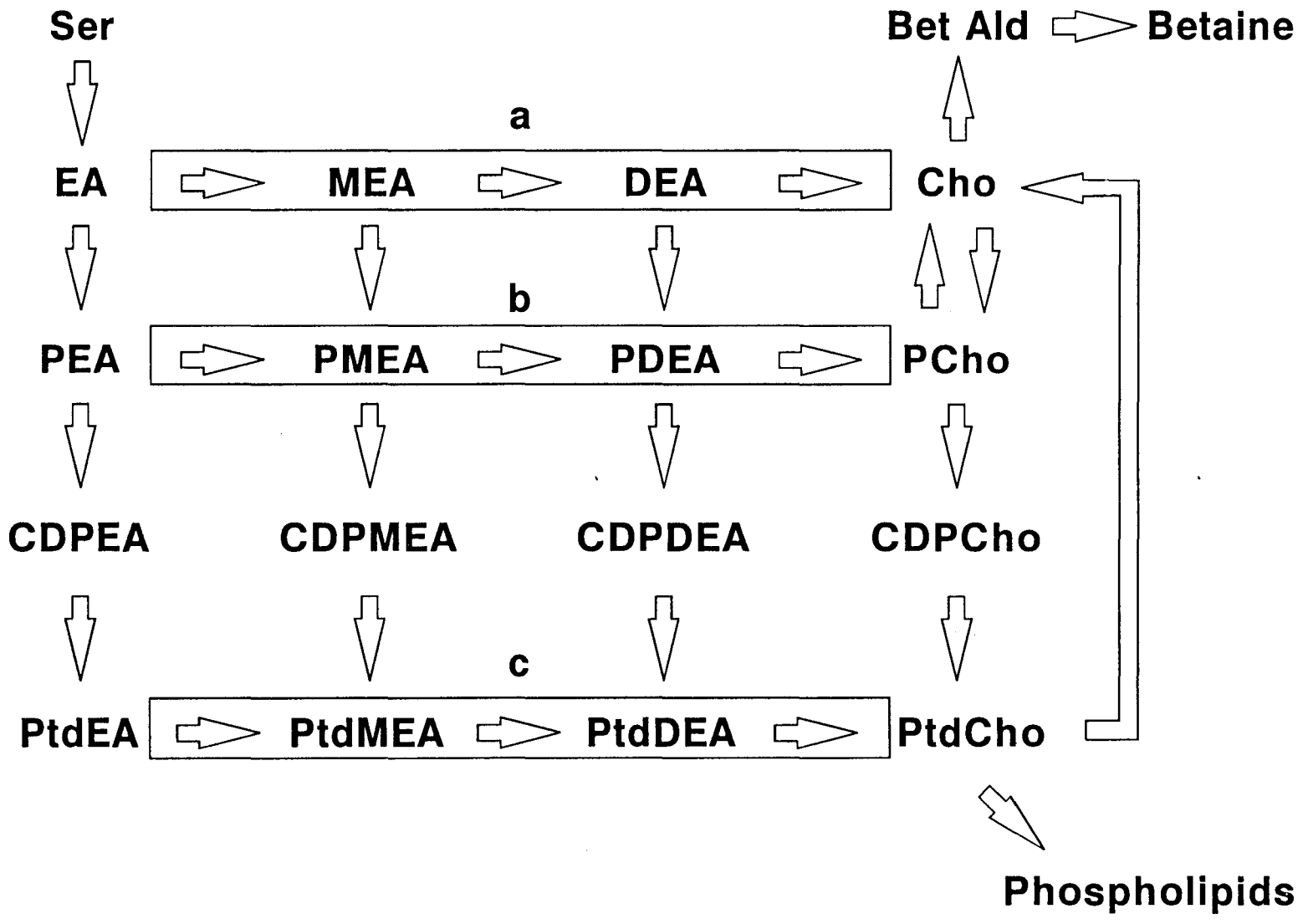
The possible routes of Cho synthesis from serine as deduced by *in vivo* radiotracer experiments using several different plants are given in Figure 1.

Ethanolamine Synthesis

Not much is known about the pathway leading to ethanolamine (EA) formation

Figure 1 *The Alternate Pathways of Choline Biosynthesis*

The many possible routes of Cho biosynthesis in plants. There are three interconnected series of *N*-methylation reactions; the freebase (box a), the phosphobase (box b) and the phosphatidylbase (box c) routes. Abbreviations: Ser, serine; EA, ethanolamine; MEA, monomethylethanolamine; DEA, dimethylethanolamine; Cho, choline; PEA, phosphoethanolamine; PMEA, phosphomonomethylethanolamine; PDEA, phosphodimethylethanolamine; PCho, phosphocholine; CDPEA, cytidine diphosphate phosphoethanolamine; CDPMEA, cytidine diphosphate phosphomonomethylethanolamine; CDPDEA, cytidine diphosphate phosphodimethylethanolamine; CDPCho, cytidine diphosphate phosphocholine; PtdEA, phosphatidylethanolamine; PtdMEA, phosphatidylmonomethylethanolamine; PtdDEA, phosphatidyldimethylethanolamine; PtdCho, phosphatidylcholine; Bet Ald, betaine aldehyde; Betaine, glycine betaine.


Ser
Bet Ald
Betaine
a
EA
MEA
DEA
Cho
b
PEA
PMEA
PDEA
PCho
CDPEA
CDPMEA
CDPDEA
CDPCho
c
PtdEA
PtdMEA
PtdDEA
PtdCho
Phospholipids

in plants. There is radiotracer evidence for EA being derived from serine (Hanson and Scott, 1980; Coughlan and Wyn Jones, 1982). Serine is derived from the decarboxylation of glycine produced in the mitochondrial photorespiratory nitrogen cycle (Keys *et al*, 1978). How EA is formed from serine is not clear, but in *Lemna paucicostata* the decarboxylation of serine itself is favoured but the route serine --> phosphatidylserine --> phosphatidylethanolamine (PtdEA) --> EA could not be ruled out (Mudd and Datko, 1989c) especially since phosphatidylserine decarboxylase activity has been detected in other plants (review: Moore, 1982). Thus the origins of EA are still uncertain. Another aspect of betaine biosynthesis which is not clear is why betaine is formed by the seemingly more complicated route through serine, EA and Cho and not by the direct *N*-methylation of glycine (Rhodes, 1987).

Choline Synthesis from Ethanolamine

As Figure 1 suggests, in higher plants there are several biosynthetic routes for the production of Cho, with different routes predominating in different species (and perhaps even tissues). Essentially, these routes involve three parallel, interconnected series of *N*-methylated intermediates of EA (freebases), phosphoethanolamine (PEA) (phosphobases) or PtdEA (phosphatidylbases) (Fig. 1) (see: Weretilnyk and Summers, 1992; Rhodes and Hanson, 1993).

In plants which do not accumulate betaine there appears to be a great divergence in the pathways of *N*-methylation used to form Cho. In *Lemna paucicostata* (Mudd and Datko, 1986), the *N*-methylations follow the phosphobase route. In suspension cultures of soybean (*Glycine max*) and carrot

(*Daucus carota*) the phosphatidylbase route for the *N*-methylations of EA predominates with carrot also using the phosphobase route (Datko and Mudd, 1988a). In castor bean endosperm it is EA that is *N*-methylated to form MEA and the subsequent *N*-methylations occur through either the freebase or phosphobase routes to form Cho (Prud'homme and Moore, 1992).

For betaine accumulating plant species studied to date, the *N*-methylations of EA to form Cho seem to follow the phosphobase route and/or the phosphatidyl base route. In the monocot barley, both the phosphobase and phosphatidylbase routes are used (Hitz *et al*, 1981; Giddings and Hanson, 1982). For the chenopod sugarbeet, Hanson and Rhodes (1983) report PEA being *N*-methylated to form phosphocholine (PCho) with a specific phosphatase hydrolysing PCho to form Cho.

For the betaine accumulating chenopod spinach, there is a discrepancy in the literature as to how EA is converted to Cho. Marshall and Kates (1974) proposed that the *N*-methylations occur at the phosphatidylbase route while Coughlan and Wyn Jones (1982) maintain that the *N*-methylations occur at the freebase level. In the study of Marshall and Kates (1974), the phosphobase and freebase routes were not investigated. There is no direct evidence to support the *N*-methylation of EA and there is recent evidence that in both salinized and non-salinized spinach, EA is converted to Cho through the phosphobase route (Weretilnyk and Summers, 1992; Summers and Weretilnyk, 1993) similar to that reported for sugarbeet (Hanson and Rhodes, 1983). Spinach is closely related to sugarbeet so it seems likely that these two species would utilize a similar pathway to form Cho, especially since divergent plants (barley, carrot and soybean) show the same pathway as sugarbeet of

N-methylating PEA (Weretilnyk and Summers, 1992). Thus the *N*-methylation of EA reported for spinach (Coughlan and Wyn Jones, 1982) is likely in error and phosphobase *N*-methylation is the major route of Cho synthesis in betaine accumulating plants of the Chenopodiaceae (Weretilnyk and Summers, 1992; Summers and Weretilnyk, 1993) (Fig. 2).

Regulation of the Choline Biosynthesis Pathway and Betaine Biosynthesis

Metabolically Active Choline Pool

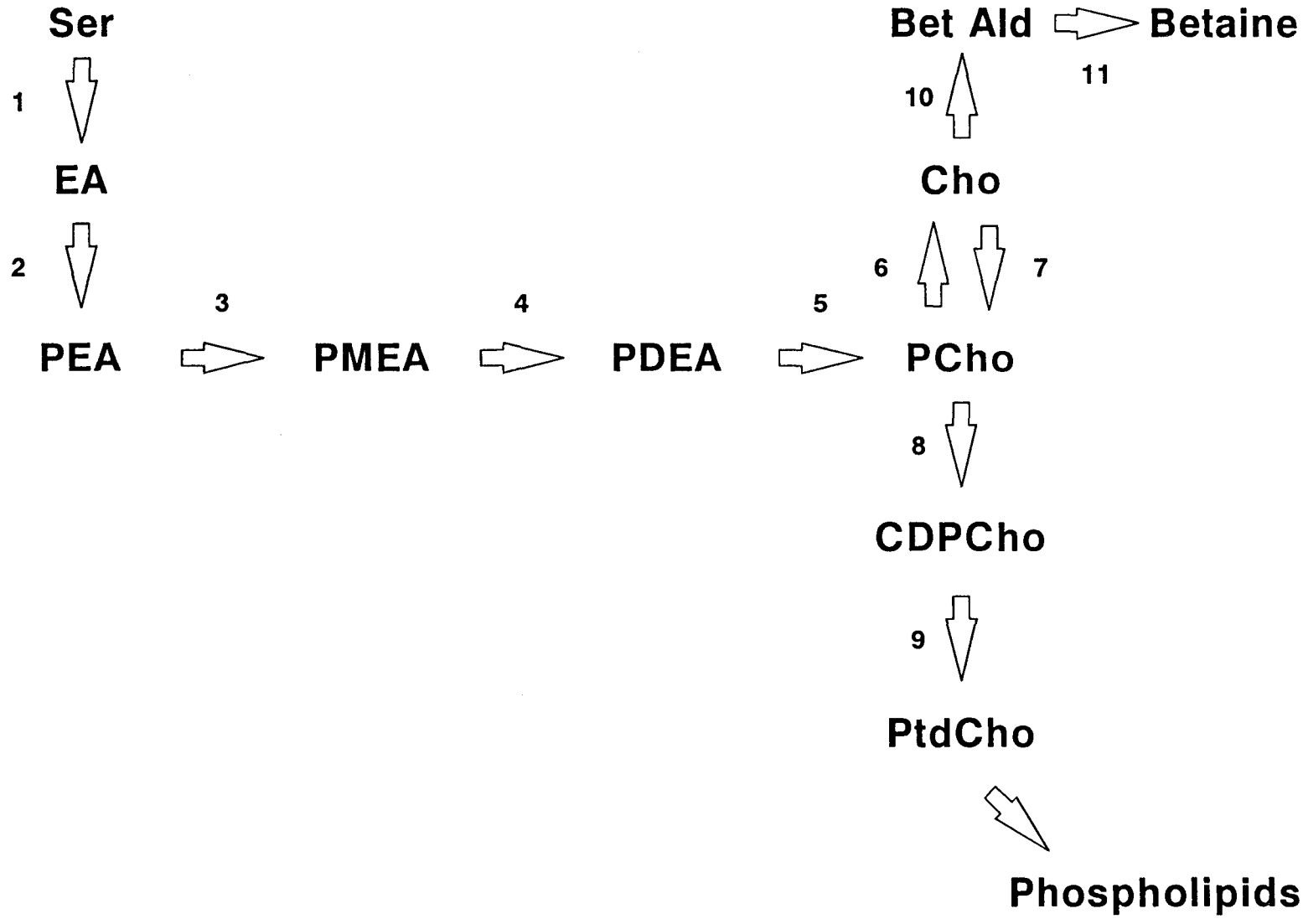
Cho (PCho) pool sizes are high in extracellular plant saps (Maizel *et al*, 1956; Martin and Tolbert, 1983). In animals and microorganisms extracellular Cho is transported across the membrane as one of the major sources of Cho for phospholipid biosynthesis (Kent, 1990). In cultures of *Lemna paucicostata*, EA and Cho can be taken up by cells and immediately phosphorylated and used to form phospholipids (Datko and Mudd, 1986; Mudd and Datko, 1989a, 1989b). Even though *Lemna* can take up Cho, the PCho pool size for phospholipid biosynthesis remains small (Mudd and Datko, 1986). It is thought that there are two pools of Cho in plant tissues; a pool that is not readily accessible to phospholipid and betaine biosynthesis and another that is synthesized in the Cho biosynthesis pathway which provides the required Cho for phospholipid biosynthesis, and betaine under conditions of saline stress (Hanson and Rhodes, 1983; Mudd and Datko, 1986; Prud'homme and Moore, 1992).

Choline Pool Size Specific for Betaine Synthesis

Not only is the amount of Cho which can be converted into betaine or

Figure 2 *Proposed Route of Choline Biosynthesis in Spinach*

The route of choline biosynthesis in spinach is through the phosphobase pathway (Fig. 1, box b) adapted from Weretilnyk and Summers (1992), Rhodes and Hanson (1993) and Summers and Weretilnyk (1993). The enzymes involved in this pathway are: 1, serine decarboxylase; 2, ethanolamine kinase (EAK); 3, phosphoethanolamine *N*-methyltransferase (PEAMeT); 4, phosphomonomethylethanolamine *N*-methyltransferase (PMEAMeT); 5, phosphodimethylethanolamine *N*-methyltransferase (PDEAMeT); 6, phosphocholine phosphatase; 7, choline kinase (ChoK); 8, phosphocholine cytidylyltransferase; 9, phosphocholine phosphotransferase; 10, choline monooxygenase (Cho monooxygenase); 11, betaine aldehyde dehydrogenase (BADH).


Ser
Bet Ald
Betaine
1
10
11
EA
Cho
2
6
7
3
4
5
PEA
PMEA
PDEA
PCho
8
CDPCho
9
PtdCho
Phospholipids

phospholipids quite small but the amount of Cho that can be converted specifically into betaine is also regulated. The Cho oxidative enzymes are localized in the chloroplast (Hanson *et al*, 1985) thus the amount of Cho made available for betaine synthesis is thought to be regulated by this compartmentalization (Hitz *et al*, 1981; Hanson and Rhodes, 1983; Weretilnyk and Summers, 1992). Hanson *et al* (1985) estimated that the Cho pool located in chloroplasts comprises 1.4% of the total Cho pool of the cell. In response to osmotic stress, regulation of betaine synthesis may occur through changing the pool size of Cho in the chloroplast or by increasing the flux through the Cho oxidation pathway through regulation of the activities of Cho monooxygenase and BADH (Hanson *et al*, 1985; Weigel *et al*, 1986).

Betaine Synthesis Enzymes

A gradually imposed increase in osmotic stress results in a steady decrease in the solute potential of the plant and an increase in betaine production and accumulation (Hanson and Hitz, 1982; Weigel *et al*, 1986). This accumulation is directly proportional to the severity of the osmotic stress (Coughlan and Wyn Jones, 1980; Hanson and Wyse, 1982). The increase in the rate of betaine production does not affect the pool sizes of Cho for spinach (Coughlan and Wyn Jones, 1982) but the activities of Cho monooxygenase (Brouquisse *et al*, 1989) and BADH (Weigel *et al*, 1986) rise incrementally in response to increasing salinity levels.

Through use of antibodies to BADH, it has been shown that increased BADH activity in spinach (Weretilnyk and Hanson, 1989), and sugarbeet (McCue and Hanson, 1992) is accompanied by an increase in BADH protein and translatable

mRNA levels (Weretilnyk and Hanson, 1989, 1990). This suggests that betaine accumulation in leaves of stressed plants is regulated, at least in part, by the changes in the expression of genes for the biosynthetic enzymes. Evidence from the analysis of genetic crosses, suggests that there are only a small number of nuclear-encoded genes controlling betaine accumulation (Hanson and Grumet, 1985) and that these genes are highly pleiotropic in their action, simultaneously influencing growth, total solute potential and betaine accumulation level (Grumet *et al*, 1987).

Choline Synthesis Enzymes

In betaine non-accumulators, phospholipid biosynthesis is the only major fate of Cho moieties, whereas in betaine accumulators, Cho is used to synthesize both phospholipids and betaine. Two possible scenarios for how Cho is partitioned between the two possible routes are that the route for production of Cho destined for betaine synthesis could be different than that used to produce Cho for phospholipid biosynthesis or the pathways are shared and the enzymes in the pathway itself are regulated to meet the demands for both end products (Weretilnyk and Summers, 1992).

Very little is known about flux rate through the Cho biosynthesis pathway under saline conditions. Radiotracer studies with barley (Hitz *et al*, 1981) and spinach (Coughlan and Wyn Jones, 1982) show that the higher rate of betaine synthesis in osmotically stressed leaves also involves an increase in the rate of Cho synthesis (Hitz *et al*, 1981, Coughlan and Wyn Jones, 1982; Hanson and Rhodes, 1983). Also, the activities of EA kinase (EAK) and PEA *N*-methyltransferase

(PEAMeT), phosphomonomethylethanolamine (PMEA) *N*-methyltransferase (PMEAMeT) and phosphodimethylethanolamine (PDEA) *N*-methyltransferase (PDEAMeT) increase in response to salinity stress (Summers and Weretilnyk, 1993). The increases in activity observed are modest and similar to the increases for the Cho oxidative enzymes (Weigel *et al*, 1988; Brouquisse *et al*, 1989).

The modest enzyme increases involved in Cho synthesis and Cho oxidation in response to increased salinity may be coincidental or a behaviour reflecting a coordinating mechanism to regulate the rate and/or the amount of Cho made by plants and the amount of Cho made available for betaine synthesis (Weretilnyk and Summers, 1992). It is not known whether the increase in activity shown in the Cho biosynthesis enzymes is an activation of existing enzymes or *de novo* synthesis as is the case with BADH (Weretilnyk and Summers, 1992). Before one can consider genetically engineering the capacity for betaine accumulation into osmotic stress sensitive plants, one must learn more about the pathways involved in Cho and betaine synthesis and their regulation.

Genetic Engineering of Betaine Accumulation in Non-Accumulating Plant Species

Betaine accumulation is widespread among plants but many important crop species such as tomato, tobacco (Weretilnyk *et al*, 1989), potato and rice (McCue and Hanson, 1990) do not accumulate betaine and are sensitive to osmotic stress (Weretilnyk *et al*, 1989). These plants are in the same families with species that do accumulate betaine, which makes it likely that these betaine non-accumulators could be candidates for genetically engineering the capacity for betaine accumulation.

Genetic engineering of the capacity for betaine accumulation is not likely to be a simple task even in closely related species. For example, in tomato and tobacco a two-step Cho oxidation pathway exists, which is likely to be the same as that in their betaine accumulating relatives, and yet these plants are unable to accumulate betaine under conditions of osmotic stress (Weretilnyk *et al*, 1989). This means that Cho production may be the limiting factor for betaine synthesis.

Thus a better understanding of how Cho biosynthesis is regulated and how Cho is partitioned between betaine and phospholipid production is essential before proceeding with interspecies gene transfer techniques.

Regulation of Choline Biosynthesis Pathway

Exogenous Cho added to *Lemna paucicostata* (Mudd and Datko, 1989a) or soybean and carrot cultures (Mudd and Datko, 1989b) or PCho to sugar beet leaf discs (Hanson and Rhodes, 1983) decreased the rate of synthesis of Cho from EA. In *Lemna paucicostata*, added EA decreases the level of the synthesis of EA derivatives (Mudd and Datko, 1989c). Also PEA accumulation decreases by the same level that EA synthesis is decreased (Mudd and Datko, 1989a). These observations all suggest that Cho biosynthesis could be regulated near the beginning of the pathway in Figure 2, and these authors highlighted the *N*-methylations of PEA to produce Cho.

There is no variation in the Cho biosynthesis route from EA to PEA or PEA to PMEA between many plant species studied to date (see Weretilnyk and Summers, 1992; Rhodes and Hanson, 1993; Summers and Weretilnyk, 1993) with the sole exception of castor bean endosperm which methylates EA to MEA (Prud'homme and

Moore, 1992). Thus the reaction of PEA to PMEA is thought to be a common committing step of EA derivatives for the formation of Cho (Mudd and Datko, 1986; Datko and Mudd, 1988a; Mudd and Datko, 1989a) and experiments designed to identify feedback inhibition, point to the *N*-methylation steps in the phosphobase route, specifically the *N*-methylation of PEA by PEAMeT (Mudd and Datko, 1989a, 1989b).

When the *N*-methyltransferase enzyme(s) are feedback inhibited by exogenous Cho, PEA synthesis is also down-regulated sufficiently to compensate for the lower amounts of PEA used (Mudd and Datko, 1989a). Thus it appears that PEA *N*-methylation is a highly regulated process. EAK activity may also be regulated either in conjunction increased PEA levels produced by a decrease in flux through PEAMeT though increased PEA does not accumulate in sufficient amounts to negate the regulatory effect of decreased activity of PEAMeT (Mudd and Datko, 1989a). This makes both EAK and PEAMeT enzymes worth characterizing in plants.

Ethanolamine Kinase

Historically, studies on EAK have focused on whether EAK and Cho kinase (ChoK) are distinct enzymes or whether they are the same enzyme catalyzing the two separate reactions (reactions 2 and 7 respectively, Fig. 2). In general, EAK and ChoK are separate enzymes in microorganisms such as *Plasmodium falciparum* (a protozoan vector for malaria) (Ancelin and Vial, 1986), a rumen protozoan *Entodinium caudatum* (Broad and Dawson, 1974), dermatophytes *Microsporum gypseum* and *Epidermophyton folccosum* (Kasinathan *et al*, 1983) and yeast

(Wittenburg and Kornberg, 1953). There are separate enzymes for both EAK and ChoK activities in insects such as the fat-body from *Phormia regina* larvae (Shelley and Hodgson, 1971), *Culex pipiens fatigans* larvae (the vector for Bancroftian filariasis) (Ramabraham and Subrahmamyan, 1981), cockroach (*Periplaneta americana*) (Kumar and Hodgson, 1970) and plants such as soybean (*Glycine max*) (Warfe and Harwood, 1979a, 1979b; Mellor *et al*, 1986), rapeseed (*Brassica campestris*) (Ramasarma and Wetter, 1957) and spinach (*Spinacia oleracea*) (Tanaka *et al*, 1966; Macher and Mudd, 1976).

In higher animals, there is evidence that the two enzymes are separate in rat brain (Upreti *et al*, 1976; Kunishita *et al*, 1987; Uchida and Yamashita, 1990), rabbit brain (Haubrich, 1973), rodent Ehrlich ascites carcinoma cell extracts (Sung and Johnstone, 1967), rooster liver (*Gallus gallus*) (Paddon *et al*, 1982), and human liver (Draus *et al*, 1990). However, it has been shown that there is one enzyme with both EAK and ChoK activities in rat kidney (Ishidate *et al*, 1985a; Ishidate and Nakazawa, 1992), rat brain (Uchida and Yamashita, 1992), mammary tissue of cows (Infante and Kinsella, 1976), and primate lung (African Green monkey) (Ulane *et al*, 1978). In rat liver there are conflicting reports on the nature of EAK and ChoK. Some researchers report that ChoK and EAK are separate enzymes in rat liver (Weinhold and Rethy, 1972, 1974; Upreti *et al*, 1976; Brophy *et al*, 1977) while others report a single enzyme with dual activities (Brophy and Vance, 1976; Porter and Kent, 1990, 1992). These contradictory reports may reflect the presence of many different EAK and ChoK enzymes or enzymes with both EAK and ChoK activity present in various organisms or between tissues within one organism. In rats, there are multiple

molecular forms of an enzyme with both EAK and ChoK activity in kidney, lung, liver and intestinal cytosols (Ishidate *et al*, 1985b) but most of these are immunochemically identical (Ishidate *et al*, 1984; Ishidate *et al*, 1985b).

Ethanolamine Kinase Purification and Biochemical Properties From Various Organisms

Despite the numerous reports on EAK in the literature, little information is available on the biochemical characterization of EAK in the various organisms studied. EAK has been purified (to various degrees) from only a few different tissues of these species and in animals the characteristics of the enzyme vary considerably. From the characterization of EAK for all organisms to date there is little evidence to suggest that all EAK enzymes are phylogenetically related and since there are no clones or antibodies for EAK available, one cannot estimate how similar EAK is in the various animals and plants or even between tissues of certain animal species.

Molecular Weight

In studies of EAK from mammals, the native size of the enzyme varies from 36,000 (Weinhold and Rethy, 1974) to 87,600 D (Uchida and Yamashita, 1992). In the microorganism *Culex pipiens fatigans*, EAK is 44,000 D (Ramabrahmam and Subrahmanyam, 1981). In the two plant studies there is also great discrepancy in the size of EAK with a report for soybean of 37,000 D (Warfe and Harwood, 1979a) and in spinach 110,000 D.

Assay Conditions for Maximal Activity

In the organisms studied to date, the pH range for maximal EAK activity has been basic ranging from pH 7 to 10. For example, rat liver EAK has a broad pH optimum from 7 to 8.5 (Weinhold and Rethy, 1974), in human liver a sharp pH optimum of 8.5 was found (Draus *et al*, 1990) and in rat Ehrlich ascites tumour cells a pH optimum of 8.5 to 10 was found (Sung and Johnstone, 1967). In all of the studies, only a few buffer systems were tested. In many studies only narrow pH ranges were tested, not going higher than pH 9. It is difficult to say whether the variation in pH optima is an artifact of experimental design or true variation between EAK properties between different species.

Out of all the organisms studied to date, when the Mg^{2+} and ATP requirements for both EAK and ChoK were investigated it was found that the ratio of Mg^{2+} to ATP was either 1:1 (Ancelin and Vial, 1986; Ramasarma and Wetter, 1957) or 2:1 (Ishidate *et al*, 1985a; Infante and Kinsella, 1976; Weinhold and Rethy, 1974). For EAK and ChoK enzymes exhibiting maximal activity at 2:1 Mg^{2+} to ATP, excess Mg^{2+} was required. In one case excess Mg^{2+} was required at a ratio of 3:2 in human liver EAK (Draus *et al*, 1990).

Kinetic Parameters

The kinetic parameters for EAK are quite varied. The K_m values of EAK for EA vary from 8 μM in germinating soybean (Warfe and Harwood, 1979a) to 0.25 mM in human liver (Draus *et al*, 1990) and in rat kidney (Ishidate *et al*, 1985a), and to 7.7 mM in rat liver (Brophy *et al*, 1977).

Purification of Ethanolamine Kinase From Higher Plants

Soybean

EAK has only been partially purified from two plant species, soybean and spinach. In germinating soybean seeds, Warfe and Harwood (1979a) obtained two partially purified EAK preparations having 300 and 800 fold purifications, respectively. They found that EAK has a polypeptide size of 17-19,000 D and can form a dimer of 37,000 D. The pH optimum for the enzyme is approximately 8.5 and the enzyme has a K_m of 8 μM for EA. EAK was extremely sensitive to sulphydryl reagents and used Mg^{2+}-ATP as a substrate at a ratio of 1 to 1 (3 mM). Mn^{2+} and Ca^{2+} could substitute for Mg^{2+} but lowered the overall activity 0.59 and 0.70 fold, respectively when included in the assay system. EDTA was completely inhibitory. EAK activity was unaffected by added Cho. Furthermore, the EAK preparation had no ChoK activity. The authors claimed that EAK was purified to homogeneity, but no polyacrylamide gel was provided as proof.

Spinach

In spinach, Macher and Mudd (1976) partially purified EAK from spinach leaf tissue 60 fold. This enzyme had a native molecular weight of 110,000 D (no denatured polypeptide size estimate was given) and a K_m for EA of 42 μM. EAK activity was maximal from approximately pH 8.5 up to 9.5 with no sharp optimum peak. The K_m for Mg^{2+}-ATP was 63 μM and 1.36 mM Mg^{2+}-ATP was inhibitory. The Mg^{2+} to ATP ratio was 2:1 suggesting that excess Mg^{2+} was required for maximal EAK activity. Ca^{2+} was a potent inhibitor of EAK activity. MEA, DEA

and ADP were inhibitors of PEA formation. EAK migrated to a different position in native polyacrylamide gels than ChoK, EAK occupying a position in the gel between two zones of ChoK activity. However, the partially pure preparation of EAK still appeared to retain some ChoK activity (as determined by assaying for ChoK). Even though Macher and Mudd (1976) biochemically characterized the enzyme, their results are likely to have been influenced by possible contaminating enzymes in their 60 fold purified extract.

The fact that the biochemical properties of EAK from the two species are so different suggests that a purification of EAK to homogeneity would be an important first step to establishing unambiguous physical and catalytic properties for this enzyme.

Research Strategy

Spinach is a moderately salt tolerant chenopod that accumulates betaine under conditions of osmotic stress. When subjected to saline conditions of up to 300 mM NaCl (Weigel *et al*, 1986), plant growth is not greatly affected (Coughlan and Wyn Jones, 1980). Though the chenopod sugarbeet can withstand 500 mM NaCl (McCue and Hanson, 1992), spinach is easier to grow under controlled environmental conditions and it contains fewer phenolics which would hamper the recovery of active enzymes (Coombs, 1982).

In this thesis, the purification of EAK from non-salinized spinach tissue is outlined, along with the biochemical characterization of this enzyme. It is known that EAK activity increases in plants grown in saline conditions (Weretilnyk and Summers,

1992; Summers and Weretilnyk, 1993). It is not definitively known whether EAK and ChoK are separate enzymes in spinach or whether one or more (iso)enzymes is responsible for increased EAK activity in salinized tissue. There is evidence that in spinach the same Cho biosynthesis pathway is used by salinized and non-salinized plants (Weretilnyk and Summers, 1992; Summers and Weretilnyk, 1993) (Fig. 2).

The preparation of purified spinach EAK will enable future peptide sequencing, antibody production and further molecular analyses to help elucidate the role, if any, for EAK in the regulation of Cho biosynthesis in response to salt stress.

MATERIALS AND METHODS

Chemicals and Materials

DTT and ATP were purchased from ICN. Sephadex G-25, BSA (Sigma Fraction V bovine serum albumin), Dowex 50-H^+, DEAE-Sepharose CL-6B, phenyl-Sepharose CL-4B, ω-aminohexyl-agarose and gel filtration standards were obtained from Sigma. The MonoQ (5/5) and phenyl-Superose (5/5) HPLC columns were purchased from Pharmacia. The Waters Protein Pak Glass 300SW HPLC column and the Waters UltraLoop were purchased from Millipore. Formula 989 liquid scintillation counter (LSC) cocktail was obtained from New England Nuclear (NEN). The Protein Assay Dye Reagent Concentrate, SDS-PAGE molecular weight standards and gel dryer cellophane were purchased from BioRad. Phosphoenol pyruvate (PEP) (crystallized monosodium salt) and NADH (disodium salt grade II) were purchased from Boehringer Mannheim Canada (BMC). The Diaflo YM10 membranes and the Centricon 10 microconcentrators were purchased from Amicon. All other biochemicals were purchased from Sigma unless otherwise specified.

Enzymes

Pyruvate kinase (EC 2.7.1.40) isolated from rabbit muscle (2000 $IU \cdot mL^{-1}$, 25°C) and lactate dehydrogenase (EC 1.1.1.27) isolated from hog muscle (5500 $IU \cdot mL^{-1}$, 25°C) were purchased from BMC.

Radioisotopes

All radioisotopes were purchased from NEN. Radioactive EA was purchased in two batches, the first [1,2-^{14}C]EA (A) was 4.8 mCi • mmol^{-1} and 0.2 mCi • mL^{-1} and the second [1,2-^{14}C]EA (B) was 3.0 mCi • mmol^{-1} and 0.2 mCi • mL^{-1}. Radioactive Cho, [methyl-^{14}C]Cho, was 53.0 mCi • mmol^{-1} and 0.2 mCi • mL^{-1}.

Preparation of Materials

Regenerated Dowex 50-H^+

The Dowex 50-H^+ resin required regeneration before use. The H^+ form was produced by making a slurry of 200 to 300 g of resin with 500 mL water in a 1 L beaker. The resin was allowed to settle and the supernatant was aspirated off of the resin. To the resin, 500 mL of 1 N HCl was added. The resin was briefly and gently mixed with the acid and the resin allowed to settle. The supernatant was aspirated from the resin and 1 N HCl was added again. At least 20 changes of 1 N HCl were required to ensure that the resin had been completely regenerated to contain only H^+ side arms. The H^+ regenerated resin was then washed in a similar manner with water until the pH of the supernatant was 6 to 7.

Regenerated Dowex 50-NH_4^+

About 100 mL of regenerated Dowex-H^+ was placed into a 500 mL beaker and after the resin settled the water was aspirated off. The resin was then briefly and gently mixed with 200 mL of 1 M NH_4Cl and the resin allowed to settle. The supernatant was aspirated off and more 1 M NH_4Cl was added. At least 20 changes

of 1 M NH_4Cl were required to ensure that the resin had H^+ side arms replaced by NH_4^+ ions. The NH_4^+ regenerated resin was then washed in a similar manner with water until the pH of the supernatant was 6 to 7.

Preparation of Phosphomethylethanolamine and Phosphodimethylethanolamine

PMEA and PDEA were prepared by Dr. Peter Summers by phospholipase C (Sigma, Type XI from *B. cereus*) treatment of PtdMEA or PtdDEA as outlined by Datko and Mudd (1988b).

Plant Materials and Growth Conditions

Spinach (*Spinacia oleracea* L.) seeds, cv Savoy Hybrid 612 (Harris Moran Seeds, Rochester, New York), were sown 1 cm deep in moist, medium vermiculite. The pots were not watered for 3 days and then watered for 4 days, after which single seedlings were transplanted to individual pots (350 mL capacity) of medium vermiculite. The spinach plants were grown under an 8 h, 24°C day (approximately 300 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity) and a 16 h, 20°C night, and were watered daily with half-strength Hoagland's solution (Hoagland and Arnon, 1939). Plants were exposed to 4 h of light before harvest.

The step-wise salinization of the spinach was performed as described by Weigel *et al* (1986). Salinization commenced approximately two weeks after transplant. The plants were initially watered with 50 mM NaCl in half-strength Hoagland's solution (400 mL per day per plant). The salt concentration was then raised by 50 mM every three days until a desired salt concentration of 200 mM was

reached. Plants were maintained at the final NaCl level for at least seven days prior to harvest.

Crude Extract Preparation

To obtain crude extract, plant leaves (with major veins removed) were coarsely chopped with a razor blade and then either ground in a chilled mortar and pestle or homogenized in a Waring blender using 2 mL buffer (100 mM Tris-HCl pH 7.8 (4°C), 2 mM Na_2EDTA, 5 mM DTT, 1 mM $MgCl_2$ and 10% (v/v) glycerol) per gram fresh weight of tissue. The brei was filtered through four layers of cheesecloth and one layer of Miracloth (Calbiochem) that was suspended over a beaker kept on ice. An aliquot of this filtrate was used to determine chlorophyll concentration and the remainder was centrifuged at 10,000 • *g* for 10 min at 4°C. The supernatant was desalted by centrifugation through Sephadex G-25 and the desalted extract used for the measurement of enzyme activity and protein concentration.

Chlorophyll Determination

To determine the concentration of chlorophyll, 25 μL of plant filtrate was combined with 3 mL 80% (v/v) acetone and then vortexed. The solution was then centrifuged for 5 min using a clinical centrifuge. The absorbance of the supernatant was then measured at 700 nm, 663 nm and 645 nm using a UVIKON 930 (Kontron Instruments) spectrophotometer. The concentration of the chlorophyll (in μg chlorophyll • mL^{-1}) was calculated using the formula (Arnon, 1949):

$$[\text{Chlorophyll}] = 20.2(A_{645} - A_{700}) + 8.02(A_{663} - A_{700})$$

Protein Determination

Protein concentration was determined colourimetrically by the method of Bradford (1976) using BSA as a standard. BSA standards were serially diluted to give 125, 62.5, 31.25 and 15.625 $g \cdot L^{-1}$ concentrations. Each tube contained 100 μL of standard or appropriately diluted sample, 800 μL of water and 200 μL of Protein Assay Dye Reagent Concentrate. The resultant mixture was vortexed gently. The absorbance of each sample was measured against a blank (containing only water and dye) at 595 nm using a UVIKON 930 spectrophotometer.

Enzyme Assays

Ethanolamine Kinase Assay

EAK activity was determined using the method of Mudd and Datko (1989a). Each 200 μL assay contained 100 mM Tris-HCl pH 8.5 (22°C), 1.8 mM $MgCl_2$, 0.9 mM ATP, 0.2 mM KCl and 52.13 μM [1,2-^{14}C]EA (A) or 83.34 μM [1,2-^{14}C]EA (B) and enzyme sample. The mixture was incubated for 30 min at 30°C. The reaction was stopped by the addition of 1 mL of ice-cold water, and then the mixture was vortexed and put on ice. A 1 mL aliquot was then removed and applied to a 1 mL column of regenerated Dowex-H^+. The product, ^{14}C-labelled PEA, was eluted with 9 mL of 0.1 N HCl leaving the unreacted substrate, [1,2-^{14}C]EA, bound to the Dowex matrix. The eluate was vortexed and a 1 mL aliquot was withdrawn and added to 10 mL Formula 989. Production of [1,2-^{14}C]PEA was quantified using a Beckman LS 1801 scintillation counter (100% counting efficiency). EAK activity was calculated as nmol PEA produced $\cdot$ $min^{-1} \cdot mg^{-1}$ protein or mIU $\cdot$ mg^{-1} protein where

1 mIU is equivalent to 1 nmol product formed $\cdot$ min^{-1}.

Spectrophotometric Assay for Ethanolamine Kinase

An alternate assay method for determining the activity of EAK was to monitor the formation of ADP spectrophotometrically using the method of Uchida and Yamashita (1992). EAK was coupled with the pyruvate kinase-lactate dehydrogenase (PK-LDH) enzyme system:

EA + ATP ----------> PEA + ADP 1) EAK

ADP + PEP ----------> pyruvate + ATP 2) PK

pyruvate + NADH ----------> lactate + NAD 3) LDH

In this assay, the amount of ADP produced by EAK is proportional to the depletion of NADH which can be quantified by following the decrease in absorbance at 340 nm. Each 1 mL assay contained 100 mM Tris-HCl pH 8.5 (22°C), 5 mM $MgCl_2$, 0.5 mg $\cdot$ mL^{-1} BSA, 0.2 mM KCl, 5 mM ATP, 1 mM PEP, 0.4 mM NADH, 2 mM substrate (EA, MEA, DEA, Cho, serine or tyrosine), 7 IU PK, 32 IU LDH and sample (extract containing EAK). First, a mixture of Tris, $MgCl_2$, KCl, BSA and water was brought to 30°C. All of the remaining components except for the sample were then added and the absorbance of this assay mixture at 340 nm was measured until a baseline was achieved. The reaction was started with the addition of sample and the decrease in absorbance was followed in a 1 cm cuvette using a UVIKON 930 spectrophotometer at room temperature. A control lacking substrate was also carried out. EAK activity (with t in minutes) was calculated by the following equation:

$$\text{Activity } (\mu\text{mol PEA} \cdot \text{min}^{-1}) = [A_{340}(\text{sample}) - A_{340}(\text{control})]/6.22t$$

Choline Kinase Assay

ChoK activity was assayed by the method of Mudd and Datko (1989a). The 200 μL reaction mixture contained 100 mM Tris-HCl pH 8.5 (22°C), 8 mM $MgCl_2$, 8 mM ATP, 0.6 mM Cho, 4.75 μM [methyl-^{14}C]Cho and enzyme sample. After 20 min at 30°C the reaction was stopped with 1 mL ice-cold water, the mixture vortexed and the tube placed on ice. An aliquot of 1 mL was removed and applied to a 1 mL column of regenerated Dowex-NH_4^+. The radiolabelled product, [methyl-^{14}C]PCho, was eluted with 4 mL of water while the unreacted substrate, [methyl-^{14}C]Cho, remained bound to the Dowex resin. The eluate was vortexed and a 1 mL aliquot was withdrawn and added to 10 mL Formula 989 and [methyl-^{14}C]PCho was quantified using a Beckman LS 1801 scintillation counter (100% counting efficiency). ChoK activity was calculated as nmol PCho formed $\cdot$ min^{-1} $\cdot$ mg^{-1} protein or mIU $\cdot$ mg^{-1} protein.

Phosphobase Phosphatase Assay

The presence of PCho phosphatase, PEA phosphatase and other nonspecific (with respect to substrate) phosphatases was determined in acidic conditions (Tanaka *et al*, 1966). For acidic conditions, each 350 μL assay contained 100 mM sodium acetate-HCl pH 5.0 (22°C), 5 mM PCho or PEA and enzyme sample. PEA phosphatase activity was also tested in alkaline conditions with each 350 μL assay containing 100 mM Tris-HCl pH 8.5 (22°C), 5 mM PCho or PEA and enzyme sample. Each reaction was incubated at 30°C for 30 min and was terminated by the addition of 100 μL 7.5% (w/v) TCA. Controls for the phosphobase phosphatase

reactions (both acidic and basic conditions) had the TCA added before the addition of enzyme sample. After adding TCA, the solutions were vortexed and then centrifuged using a clinical centrifuge.

An aliquot of the clarified supernatant was withdrawn and the P_i concentration was measured colourimetrically by the method of Martin and Tolbert (1983). Phosphate standards were prepared from KH_2PO_4 to make 500 μL volumes containing 0, 0.5, 2.5, 5, 10, 15, 20 or 25 nmol of phosphate. To each 500 μL of sample or standard, an additional 500 μL of freshly made ammonium molybdate solution (4 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 114 mM L-ascorbic acid) was added. Once the ammonium molybdate solution was added, each tube was vortexed, sealed with parafilm and incubated for 90 min at 37°C. After incubation, the absorbance of the reaction mixture was measured at 820 nm using a UVIKON 930 spectrophotometer. Phosphatase activity was calculated in nmoles P_i produced $\cdot$ min^{-1} $\cdot$ mg^{-1} protein or mIU $\cdot$ mg^{-1} protein.

Polyacrylamide Gradient Gel Electrophoresis (PAGE) Analyses

Native Polyacrylamide Gel Electrophoresis Analyses and Enzyme Activity Measurements

The proteins in extracts containing EAK activity were separated in native state by gradient PAGE using a 1.5 mm thick 6-9% (w/v) gel using the buffer system of Davis (1964). In each sample well a minimal volume (5 to 10 μL) of dye solution (0.1% (w/v) bromophenol blue and 20% (v/v) glycerol) was loaded. The sample dye was overlaid with protein sample and the protein sample was overlaid with electrolyte

buffer. The gel was run at a constant voltage of 200 V at 4°C until the dye ran off the gel (approximately 14 h). Following electrophoresis, lanes of the gel were sliced into segments and EAK and ChoK activities were measured *in situ* using a modification of the method of Ishidate *et al* (1985b).

To test for EAK activity, a lane of a gel containing resolved proteins was cut into 40, 3 mm segments and each segment was transferred to a microfuge tube containing 100 μL incubation buffer (200 mM Tris-HCl pH 8.5 (22°C), 3.6 mM $MgCl_2$ and 0.4 mM KCl) and 2.75 μL water. Gel segments were submerged by sharply tapping the closed microfuge tube against the bench top once or twice (the gel pieces slid to the bottom of the tube easily). The submerged gel pieces were kept at 4°C overnight. To assay, the microfuge tubes were brought up to 30°C in a water bath (approximately 15 to 20 minutes) and then 2.25 μL of reaction mix (2 μL 90 mM ATP and 0.25 μL [1,2-^{14}C]-EA (A)) was added and the reaction was allowed to proceed for 60 min at 30°C. Stopping the reaction, processing the sample and counting was identical to the radioisotope EAK assay described previously.

To test for ChoK activity a lane of a gel containing resolved proteins was also sliced into 40, 3 mm segments. Each segment was submerged in 100 μL incubation buffer (200 mM Tris-HCl pH 8.5 (22°C) and 16 mM $MgCl_2$) with 1.55 μL water and kept at 4°C overnight. After preincubating the tubes at 30°C as described above, 8.45 μL of reaction mix (3.2 μL 500 mM ATP, 4.5 μL 24 mM Cho and 0.75 μL [methyl-^{14}C]Cho) was added to each tube and the reaction was allowed to proceed at 30°C for 60 min. The remainder of the assay was identical to the radioisotope ChoK assay described previously.

Sodium Dodecyl Sulphate (Denaturing Conditions) Polyacrylamide Gel Electrophoresis Analyses

Proteins in samples were solubilized in an equal volume of sample buffer containing 60 mM Tris-HCl pH 6.8 (22°C), 10% (v/v) glycerol, 1% (w/v) SDS, 1% (w/v) DTT and 0.002% (w/v) bromophenol blue (Merrick, 1983). The SDS-treated enzyme mixture was heated at 90°C for 3 min. The denatured proteins were then quickly loaded onto a 1.5 mm thick 7-15% (w/v) polyacrylamide gradient gel containing SDS (Chua, 1980). The buffer system of Neville (1971) was used. SDS-PAGE molecular weight standards were diluted 1:100 (v/v) in sample buffer, denatured (90°C, 3 min) and loaded (10 μL/lane). The gel was calibrated with rabbit muscle phosphorylase *b* (97.4 kD), bovine serum albumin (66.2 kD), hen egg white ovalbumin (45.0 kD), bovine carbonic anhydrase (29.0 kD), soybean trypsin inhibitor (21.5 kD) and hen egg white lysozyme (14.4 kD). Sample and standards were overlaid with electrolyte buffer and electrophoresis was carried out at a constant current of 15 mA/1.5 mm thick gel at 15°C until the dye ran off the bottom of the gel (approximately 5 h).

Silver Staining and Drying of Polyacrylamide Gels

After electrophoresis the proteins in the polyacrylamide gels were fixed in 50% (v/v) methanol overnight and then stained with silver according to the method of Wray *et al* (1981). Once stained for protein, the gels were placed in a gel drying solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid and 3% (v/v) glycerol) for 2 h and then dried between two sheets of gel dryer cellophane in front of a fan

(Wallevik and Jensenius, 1982).

Enzyme Purification

Unless otherwise stated, all purification procedures were performed at 4°C. For all purification steps, [1,2-^{14}C]EA (A) was used except for the phenyl-Superose step in Purification II (see Results) determinations where [1,2-^{14}C]EA (B) was used. For the HPLC steps a Waters 625 LC System (Millipore) was used.

$(NH_4)_2SO_4$ Precipitation

Crude enzyme preparations were subjected to $(NH_4)_2SO_4$ precipitation. To adjust the concentration of $(NH_4)_2SO_4$ in the enzyme sample, tables provided by Wood (1976) were used. In a preliminary experiment, it was determined that greater than 66% of the EAK activity but only 10% of the total protein present in crude extract was found to precipitate between 2.2 to 3.2 M $(NH_4)_2SO_4$. Thus, the crude enzyme sample was first raised to 2.2 M $(NH_4)_2SO_4$ by adding finely ground $(NH_4)_2SO_4$ slowly, while stirring. After the $(NH_4)_2SO_4$ was completely dissolved, the sample was equilibrated for 30 min with stirring. The sample was then centrifuged for 10 min at $10,000 \cdot g$ and the pellet was discarded (after resuspending the pellet and checking for EAK activity). The supernatant was then raised to 3.2 M $(NH_4)_2SO_4$ and centrifuged as before. This time the supernatant was discarded (after checking for EAK activity) and the pellet was resuspended in a minimal amount of Buffer A (100 mM Tris-HCl pH 7.8 (4°C), 2 mM Na_2EDTA, 5mM DTT, 1 mM $MgCl_2$ and 10% (v/v) glycerol) and dialysed overnight against Buffer A (3 changes of 10x the volume of the

resuspended enzyme sample volume).

Diethylaminoethyl-Sepharose CL-6B (Anion Exchange Chromatography)

An 80 mL diethylaminoethyl-Sepharose (DEAE-Sepharose) CL-6B column was equilibrated with Buffer A and loaded with the dialysed $(NH_4)_2SO_4$ fraction at a flow rate of 0.5 $mL \cdot min^{-1}$. The non-adsorbed proteins were eluted with 2 L Buffer A and adsorbed proteins were eluted by an 800 mL linear gradient of 0 to 0.3 M NaCl in Buffer A at a flow rate of 1 $mL \cdot min^{-1}$. To ensure maximal recovery of adsorbed EAK, the gradient was followed by 100 mL Buffer A containing 1 M NaCl. Fractions of 8 mL were collected and assayed for EAK activity and those with low protein levels and high activity were pooled and made 1 M with respect to $(NH_4)_2SO_4$ using tables from Wood (1976).

Phenyl-Sepharose CL-4B (Hydrophobic Interaction Chromatography)

A phenyl-Sepharose CL-4B column (80 mL) was equilibrated with 1 M $(NH_4)_2SO_4$ in Buffer A and loaded with the DEAE-Sepharose purified sample at a flow rate of 0.5 $mL \cdot min^{-1}$. Non-adsorbed proteins were eluted with 600 mL of 0.6 M $(NH_4)_2SO_4$ in Buffer A and then adsorbed proteins were eluted with an 800 mL linear gradient of 0.6 to 0 M $(NH_4)_2SO_4$ in Buffer A at a flow rate of 1 $mL \cdot min^{-1}$. To ensure maximal recovery of adsorbed EAK, the gradient was followed by 500 mL Buffer A. Fractions of 8 mL were collected and assayed for EAK activity. Aliquots of fractions were desalted by centrifugation through Sephadex G-25 prior to assay. Fractions with high enzymatic activity and low protein concentration were pooled,

concentrated approximately 15 fold and then dialysed using 3 x 50 mL changes of Buffer A using a stirred ultrafiltration cell (Amicon) with a Diaflo YM10 membrane.

ω-Aminohexyl-Agarose (Hydrophobic/Ionic Interaction Chromatography)

A 25 mL hydrophobic ω-aminohexyl-agarose column was run using ionic conditions. The dialysed phenyl-Sepharose purified sample was loaded at a flow rate of 0.5 $mL \cdot min^{-1}$. Non-adsorbed proteins were eluted with 600 mL Buffer A and adsorbed proteins were eluted with an 800 mL linear gradient of 0 to 1 M NaCl in Buffer A at a flow rate of 1 $mL \cdot min^{-1}$. To ensure maximal recovery of adsorbed EAK, the gradient was followed by 100 mL of Buffer A containing 1 M NaCl. Fractions of 7 mL were collected and assayed for EAK activity and those fractions with high activity and low protein content were pooled, concentrated approximately 25 fold and dialysed with 3 x 50 mL changes of Buffer A in an ultrafiltration cell (YM10 membrane).

MonoQ (5/5) HPLC (Anion Exchange Chromatography)

A 1 mL MonoQ (5/5) column was equilibrated in Buffer A. The ω-aminohexyl-agarose purified sample was loaded through a 25 mL UltraLoop and non-adsorbed proteins were eluted at a flow rate of 0.5 $mL \cdot min^{-1}$ with 30 mL Buffer A. Adsorbed proteins were eluted using a 15 mL linear gradient of 0 to 0.3 M NaCl in Buffer A at a flow rate of 0.5 $mL \cdot min^{-1}$. Fractions of 0.5 mL were collected and analyzed by SDS-PAGE and those fractions with low protein complexity and high EAK activity were pooled and concentrated (to a minimal volume; approximately

150 μL) by centrifugation using a Centricon 10 microconcentrator. The concentrated sample was made 1 M with respect to $(NH_4)_2SO_4$ with the addition of 3.8 M $(NH_4)_2SO_4$ (containing 2 mM Na_2EDTA and 50 mM Tris-HCl pH 7.8 (4°C)) using the tables by Wood (1976) and applied to the phenyl-Superose hydrophobic interaction column.

Phenyl-Superose (5/5) HPLC (Hydrophobic Interaction Chromatography)

A 1 mL phenyl-Superose (5/5) column was equilibrated in Buffer A containing 1 M $(NH_4)_2SO_4$. The enzyme sample was injected in a series of 50 μL injections while the column was being washed with buffer A containing 1 M $(NH_4)_2SO_4$ at a flow rate of 0.15 mL $\cdot$ min^{-1} (with a total wash volume of 25 mL) until non-adsorbed proteins were removed from the column. Adsorbed proteins were then eluted with a 15 mL gradient of 1 to 0 M $(NH_4)_2SO_4$ in Buffer A and fractions of 150 μL were collected. The fractions with high EAK activity and low protein complexity (as determined by SDS-PAGE) were pooled, concentrated (to a minimal volume; approximately 150 μL) and dialysed (5 x 2 mL changes of Buffer A) using a Centricon 10 microconcentrator.

Biochemical Characterization of Ethanolamine Kinase

Molecular Weight Determination for Ethanolamine Kinase Using Protein Pak Glass 300SW HPLC (Gel Filtration)

An estimate of the molecular weight for EAK in native state was obtained using a 13.3 mL (7.5 mm x 30 cm) Protein Pak 300SW HPLC column. The column

was run at 22°C with a horizontal orientation and equilibrated in Buffer B (50 mM Tris-HCl pH 7.5 (22°C), 1 mM $MgCl_2$, 100 mM KCl and 1 mM DTT). The column was first calibrated with gel filtration molecular weight markers purchased as a kit from Sigma. The standards were sweet potato β-amylase (200 kD), yeast alcohol dehydrogenase (150 kD), bovine serum albumin (66 kD), bovine erythrocyte carbonic anhydrase (29 kD) and horse heart cytochrome *c* (12.4 kD). The standards were individually dissolved in Buffer B containing 5% (v/v) glycerol at the following concentrations: albumin, 10 $mg \cdot mL^{-1}$; alcohol dehydrogenase, 5 $mg \cdot mL^{-1}$; β-amylase, 4 $mg \cdot mL^{-1}$; carbonic anhydrase, 3 $mg \cdot mL^{-1}$ and cytochrome *c*, 2 $mg \cdot mL^{-1}$. For chromatography, 200 μL of each standard protein was combined and the resulting mixture filtered through a 0.45 μm membrane. Each 50 μL injection of standards contained 240 μg total protein. Column fractions were collected as 150 μL fractions. Standards were followed by a 50 μL injection of sample purified by the phenyl-Superose column and the eluate was also collected as 150 μL fractions. The void volume was determined by the injection of 50 μL of 1 $mg \cdot mL^{-1}$ Blue dextran (2000 kD). Fractions of the enzyme sample with high EAK activity and low protein complexity (as determined by SDS-PAGE) were pooled and stored at -80°C.

Kinetic Experiments

Unless otherwise specified, kinetic analyses used the ω-Aminohexyl-agarose purified sample of Purification III (see Results).

pH Optimum

To determine the pH at which EAK activity is at an optimum, various buffers of overlapping pH were used in the enzyme assays. The buffers are listed in Table I and were used at a concentration of 100 mM in the assay mixture which also contained 1.8 mM $MgCl_2$, 0.9 mM ATP, 0.2 mM KCl and 83.34 μM [1,2-^{14}C]EA (B). The reaction was carried out at 30°C for 30 min.

Linearity of Ethanolamine Kinase Assay With Respect to Time

Linearity of the EAK assay as a function of time was determined using the following assay conditions; 100 mM Tris-HCl pH 8.5 (22°C), 1.8 mM $MgCl_2$, 0.9 mM ATP, 0.2 mM KCl and 83.34 μM [1.2-^{14}C]EA (B). The reaction was carried out at 30°C and EAK activity was measured at 5 min intervals.

KCl Requirements

To determine whether varying the concentration of KCl in the assay mixture had an effect on EAK activity, an assay mixture containing 100 mM Tris-HCl pH 8.5 (22°C), 1.8 mM $MgCl_2$, 0.9 mM ATP and 83.34 μM [1,2-^{14}C]EA (B) was used with varying KCl concentrations of 8, 20, 40, 100, 200, 300, 500 or 1000 μM. Assays were carried out at 30°C for 30 min.

Mg^{2+} and ATP Optimum

The effects of Mg^{2+} and ATP concentrations on EAK activity were investigated simultaneously. The assay conditions used were 100 mM Tris-HCl

Table I *Buffers and pH Ranges for pH Optimum Experiment*

Chemical names for the buffers and their abbreviations are as follows: 2-(N-morpholino)ethanesulphonic acid (Mes); [*bis*-(2-hydroxyethyl)-amino]*tris*-(hydroxymethyl)methane (Bis-Tris); 1,3-*bis*[*tris*(hydroxymethyl)methylamino] propane (Bis-Tris propane); piperazine-N,N'-*bis*(2-ethanesulfonic acid) (Pipes); 3-(N-morpholino)propanesulfonic acid (Mops); N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes); N-[*tris*-(hydroxymethyl)methyl]glycine (Tricine); 2-(cyclohexylaminoethanesulfonic acid) (Ches); 3-(cyclohexylamino)propanesulfonic acid (Caps).

pH (22°C)	Buffer	pH adjusted with	pK_a
3.0	glycine	HCl	2.35 (25°C)
	sodium citrate	citric acid	3.13 (25°C)
4.0	sodium citrate	citric acid	
	sodium acetate	acetic acid	4.76 (25°C)
5.0	sodium citrate	citric acid	4.76 (25°C)
	sodium acetate	acetic acid	
5.5	sodium citrate	citric acid	
	sodium acetate	acetic acid	
	Mes	NaOH	6.15 (20°C)
6.0	sodium citrate	citric acid	6.40 (25°C)
	Mes	NaOH	
	Bis-Tris	HCl	6.50 (25°C)
6.5	Mes	NaOH	
	Bis-Tris	HCl	
	Bis-Tris propane	HCl	6.80 (20°C)
	Pipes	NaOH	
	Mops	NaOH	7.20 (20°C)
7.0	Bis-Tris	HCl	
	Bis-Tris propane	HCl	
	Pipes	NaOH	
	Mops	NaOH	
	Tris	HCl	8.30 (20°C)
	Hepes	NaOH	7.55 (20°C)
7.5	Bis-Tris propane	HCl	
	Pipes	NaOH	
	Mops	NaOH	
	Tris	HCl	
	Hepes	NaOH	
	Tricine	NaOH	8.15 (20°C)

8.0	Bis-Tris propane	HCl	
	Tris	HCl	
	Hepes	NaOH	
	Tricine	HCl	
8.5	Bis-Tris propane	HCl	
	Tris	HCl	
	Tricine	HCl	
9.0	Bis-Tris propane	HCl	
	Tris	HCl	
	glycine	NaOH	9.78 (25°C)
	Ches	NaOH	9.50 (25°C)
9.5	Bis-Tris propane	HCl	
	glycine	NaOH	
	Ches	NaOH	
10.0	glycine	NaOH	
	Ches	NaOH	
	Caps	NaOH	10.40 (20°C)
11.0	Caps	NaOH	

pH 8.5 (22°C), 0.2 mM KCl, 83.34 μM [1,2^{14}C]EA (B) and $MgCl_2$ concentrations of 0.5, 1.0, 1.5, 2.0, 5.0 or 10.0 mM. For each different $MgCl_2$ concentration, ATP concentrations of 0.5, 1.0, 1.5, 2.0, 5.0 and 10.0 mM were used. The reactions were carried out at 30°C for 30 min.

Ethanolamine Concentration and Maximal Ethanolamine Kinase Activity

To determine the K_m and V_{max} for EAK using EA as a substrate, optimized assay conditions (100 mM Tris-HCl pH 8.5 (22°C), 5 mM $MgCl_2$, 5 mM ATP and 0.2 mM KCl) were used with varying EA concentrations (2.5, 5, 7.5, 10, 15, 20, 30, 50, 125, 250, 1000 or 5000 μM EA). The reactions were carried out at 30°C for 30 min. For concentrations of EA from 2.5 to 250 μM, only [1,2-^{14}C] EA (B) was used to supply the EA for the reaction. For the assay with 1000 μM EA, 0.5 mM [1,2-^{14}C]EA (B) was supplemented with 0.5 mM non-radiolabelled EA. The 5000 μM assay contained 0.667 mM [1,2-^{14}C]EA (B) and 4.333 mM non-radiolabelled EA.

K_m and V_{max} were estimated by two non-linear, curve-fitting programs. Both the enzyme kinetics program Cosy v. 4.0 (1989) by Marc Eberhard (Basel, Switzerland) and Fig.P v. 6.0 (1991) by Biosoft (Ferguson, MO) used the least-squares Marquardt fit (Marquardt, 1963). The data was also converted to linear form and K_m and V_{max} were estimated using the double-reciprocal plot (1/v vs 1/s), the Eadie-Hofstee plot (v vs v/s) and the half-reciprocal plot (s/v vs s) where "s" represents EA concentration and "v" EAK activity.

Direct Linear Plot

To determine the K_m and V_{max} for EAK using a nonparametric analysis, each s and v value were converted into a straight line intercepting the x-axis at (-s,0) and the y-axis at (0,v). For each line an equation was determined in the form $y=mx + b$. By subtracting two equations, the point of intersection of the two lines is achieved which yields a point (K_m', V_{max}'). Each of the three replicates is treated individually and intersections of lines with the same s value were not included. This method yielded a series of values for K_m or V_{max} and the median value is the one that best approximates the true K_m or V_{max} (Cornish-Bowden and Eisenthal, 1974).

Other Kinetic Studies

Unless otherwise specified, all assays examining the effect of metabolites or ions on EAK activity used the optimized radioisotope assay containing 100 mM Tris-HCl pH 8.5 (22°C), 5 mM $MgCl_2$, 5 mM ATP, 0.2 mM KCl and 30 μM [1,2-^{14}C]EA (B). The reactions were carried out for 30 min at 30°C. All supplements to this assay are given in the appropriate Table or Figure.

RESULTS

Part I: Salinization, Ethanolamine Kinase Activity and a Flow Chart of Ethanolamine Kinase Purifications

Salinization and Ethanolamine Kinase Activity

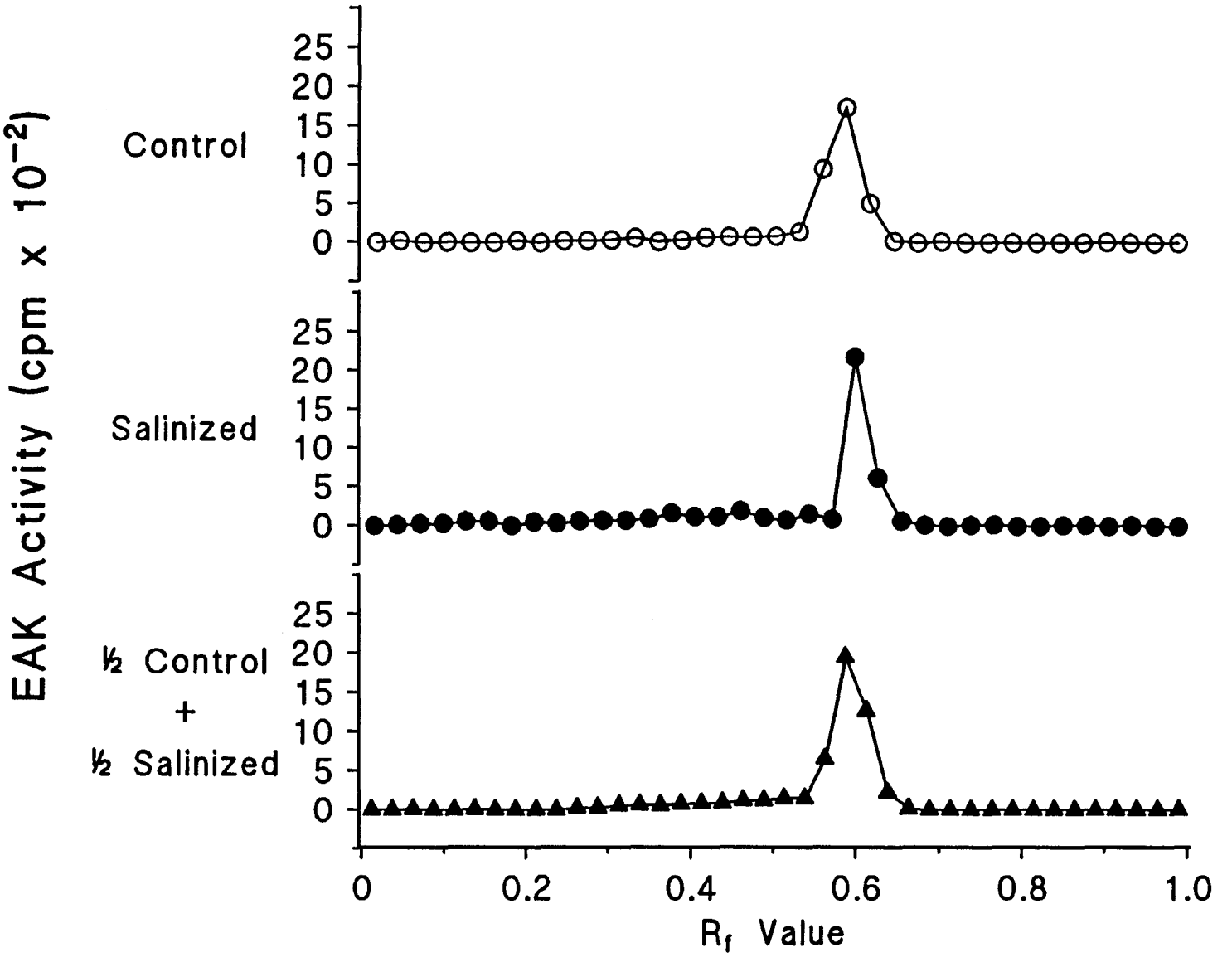
In spinach, the activity of EAK is elevated in plants that are salinized. This increase in activity is modest, only 1.5 fold when plants are salinized to 200 mM NaCl (Weretilnyk and Summers, 1992). To explore the possibility that a novel EAK isozyme is induced in response to the increase in salinity, the migration patterns of EAK in extracts from control plants (grown without added salt) and plants salinized to 200 mM NaCl were compared by Native-PAGE. Figure 3 shows that a single peak of EAK activity is found when proteins extracted from control plants were resolved by Native-PAGE. Similarly, one peak is also seen when extract from salinized plants was subjected to Native-PAGE. EAK activity in either extract migrated to the same position in the gel, at approximately 0.6 R_f units. To ensure that the peaks truly coincided, a further extract was run containing a mixture of equal amounts of extract from control and salinized plants. For this mixed sample, only one peak of EAK activity is detected.

Ethanolamine Kinase Purification Scheme

EAK was purified from spinach tissue grown under non-saline conditions.

Figure 3 *Migration Pattern of Ethanolamine Kinase in Native Polyacrylamide Gels*

Extracts from control plants (○), plants salinized to 200 mM NaCl (●) and a mixture of control and salinized extracts (▲) were separated electrophoretically by Native-PAGE. Gels were sliced into segments and the segments were used to assay for EAK activity. The resulting enzyme activity expressed as cpm x 10^{-2} per slice is plotted against relative position on the gel. Protein loaded for each sample was 200 μg. EAK activity of the initial extracts was 0.42, 0.91, and 0.60 mIU $\cdot$ mg^{-1} protein for the control, salinized and the mixture of control and salinized samples, respectively.


EAK Activity (cpm x 10^{-2})
Control
Salinized
½ Control
+
½ Salinized
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1.0
R_f Value

Due to the large quantity of tissue required for the purification of EAK, crude extract was prepared from five different harvests (shown in the top portion of Fig. 4). Each crude sample was processed to the $(NH_4)_2SO_4$ step and then frozen using liquid N_2 and then stored at -80°C until all five harvests were completed. Detailed data for the crude samples and $(NH4)_2SO_4$ fractionations are presented in Appendix A. A summary of the crude and $(NH_4)_2SO_4$ steps is presented in Table II.

As can be seen from the flow chart in Figure 4, the five $(NH_4)_2SO_4$ preparations were pooled before application to a DEAE-Sepharose anion-exchange column. EAK that adsorbed to the column was eluted and further processed (as described in the Methods and Materials section) and will, henceforth be referred to as "Purification I". Some EAK activity did not adsorb to the DEAE-Sepharose matrix presumably due to overloading, although the amount of protein applied to the column did not exceed the theoretical binding capacity of the matrix. This non-adsorbed fraction was collected as "Flow-through (A)" (Fig. 4). Flow-through (A) was then reapplied to a cleaned and regenerated DEAE-Sepharose matrix and the EAK activity that adsorbed to this column was eluted and further processed to comprise "Purification II". Some EAK activity still did not adsorb to the DEAE-Sepharose matrix and was collected as "Flow-through (B)". Flow-through (B) was reapplied to a cleaned and regenerated DEAE-Sepharose matrix. The EAK activity that adsorbed to the DEAE-Sepharose matrix this time was eluted and further processed as "Purification III" (Fig. 4).

Figure 4 *Flow Chart of Ethanolamine Kinase Purification*

Crude (A), (B), (C), (D) and (E) represent the five different harvests of spinach tissue. These preparations were individually processed to give $(NH_4)_2SO_4$ precipitated fractions with EAK activity (A), (B), (C), (D) and (E) respectively. The five $(NH_4)_2SO_4$ samples were pooled to form an "$(NH_4)_2SO_4$-pooled" (A.S. pooled) fraction which was subsequently processed as three purifications I, II and III. Details of the purification steps are described in the text.

Abbreviations used in the figure: DEAE-Sepharose CL-6B anion exchange chromatography (DEAE), phenyl-Sepharose CL-4B hydrophobic interaction chromatography (P/Seph), ω-aminohexyl-agarose hydrophobic/ionic interaction chromatography (A/Hex), MonoQ (5/5) HPLC anion exchange chromatography (M/Q), phenyl-Superose (5/5) HPLC hydrophobic interaction chromatography (P/Sup), Protein Pak Glass 300SW HPLC gel filtration (Gel/Filt) and eluate containing non-adsorbed EAK activity (Flow-through).

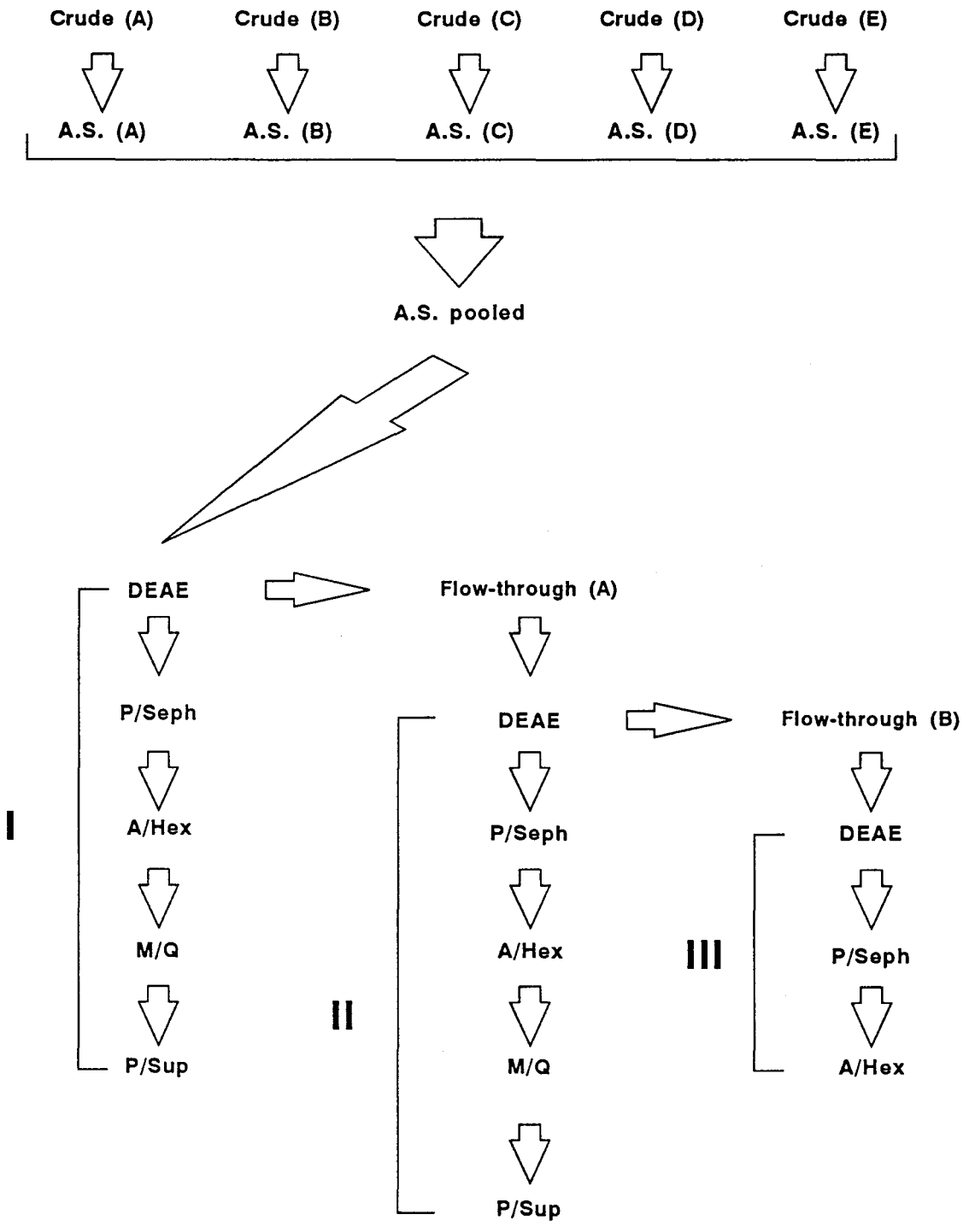

Crude (A)
Crude (B)
Crude (C)
Crude (D)
Crude (E)
A.S. (A)
A.S. (B)
A.S. (C)
A.S. (D)
A.S. (E)
A.S. pooled
DEAE
P/Seph
A/Hex
M/Q
P/Sup
I
Flow-through (A)
DEAE
P/Seph
A/Hex
M/Q
P/Sup
II
Flow-through (B)
DEAE
P/Seph
A/Hex
III

Table II *Purification Steps to Form the $(NH4)_2SO_4$-pooled Sample*

Five crude samples were individually fractionated using $(NH4)_2SO_4$. The extent of purification achieved by this step and recovery estimates of EAK activity for these individual $(NH_4)_2SO_4$ fractions were calculated using their respective initial crude estimates of activity. In order to obtain a representative value for a crude sample that would approximate the actual activity had all five extracts been pooled prior to further purification, an arithmetic mean of the five crude samples was calculated for specific activity and protein concentration. The volume, total protein and total activity values in Table II for the average crude sample are sums of all five of the crude preparations. The average value for the specific activity of the crude preparation was 0.055 $mIU \cdot mg^{-1}$ protein with a total of 3511 mIU of EAK activity. These values were used as the basis of comparison for the remaining steps in the purification of EAK.

Step	[Protein]	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	mg·mL^{-1}	*mL*	*mg*	*mIU*	*mIU·mg^{-1} protein*	*fold*	*%*
Crude (A)	4.90	2170	10633	681	0.064	1	100
Crude (B)	3.24	2400	7776	1404	0.053	1	100
Crude (C)	4.38	2510	10994	330	0.030	1	100
Crude (D)	4.41	4650	20456	916	0.045	1	100
Crude (E)	3.49	3950	13786	1172	0.085	1	100
Crude average	4.08	15680	53928	3511	0.055	1	100
A.S. (A)	6.93	136	942	1404	1.49	23	206
A.S. (B)	7.10	160	1136	909	0.80	15	221
A.S. (C)	6.76	220	1487	595	0.40	13	180
A.S. (D)	11.95	221	2641	1722	0.65	14	188
A.S. (E)	13.59	160	2174	1848	0.85	10	158
A.S. pooled	8.48	870	7378	6569	0.89	16	188

Part II: Purification of Ethanolamine Kinase to Apparent Homogeneity

Ethanolamine Kinase: Purification I

Purification of EAK from crude leaf extract began with the addition of solid $(NH_4)_2SO_4$. To precipitate EAK activity in the crude extract, 2.2 M $(NH_4)_2SO_4$ was required. This concentration of $(NH_4)_2SO_4$ eliminated more than 85% of protein contaminants from the sample, produced a 16 fold increase in EAK specific activity from that of the initial crude leaf extract, and gave a recovery of EAK activity greater than that predicted from the crude sample (Table III). Reasons for the higher than predicted recovery will be discussed later.

The dialysed 2.2 to 3.2 M $(NH_4)_2SO_4$ fraction was then applied to a DEAE-Sepharose column. Figure 5 shows a typical elution profile for this column. Although most of the adsorbed protein eluted at the same NaCl concentration as EAK activity, only 10% of the proteins from the $(NH_4)_2SO_4$ precipitated sample bound to the matrix, so the specific activity was doubled after this step (Table III). Only 32% of total EAK activity was recovered after this step. This low recovery can be attributed to not all of the EAK activity adsorbing to the first DEAE-Sepharose column and this "missing" recovery was recouped by the other two purifications (II and III) as discussed later.

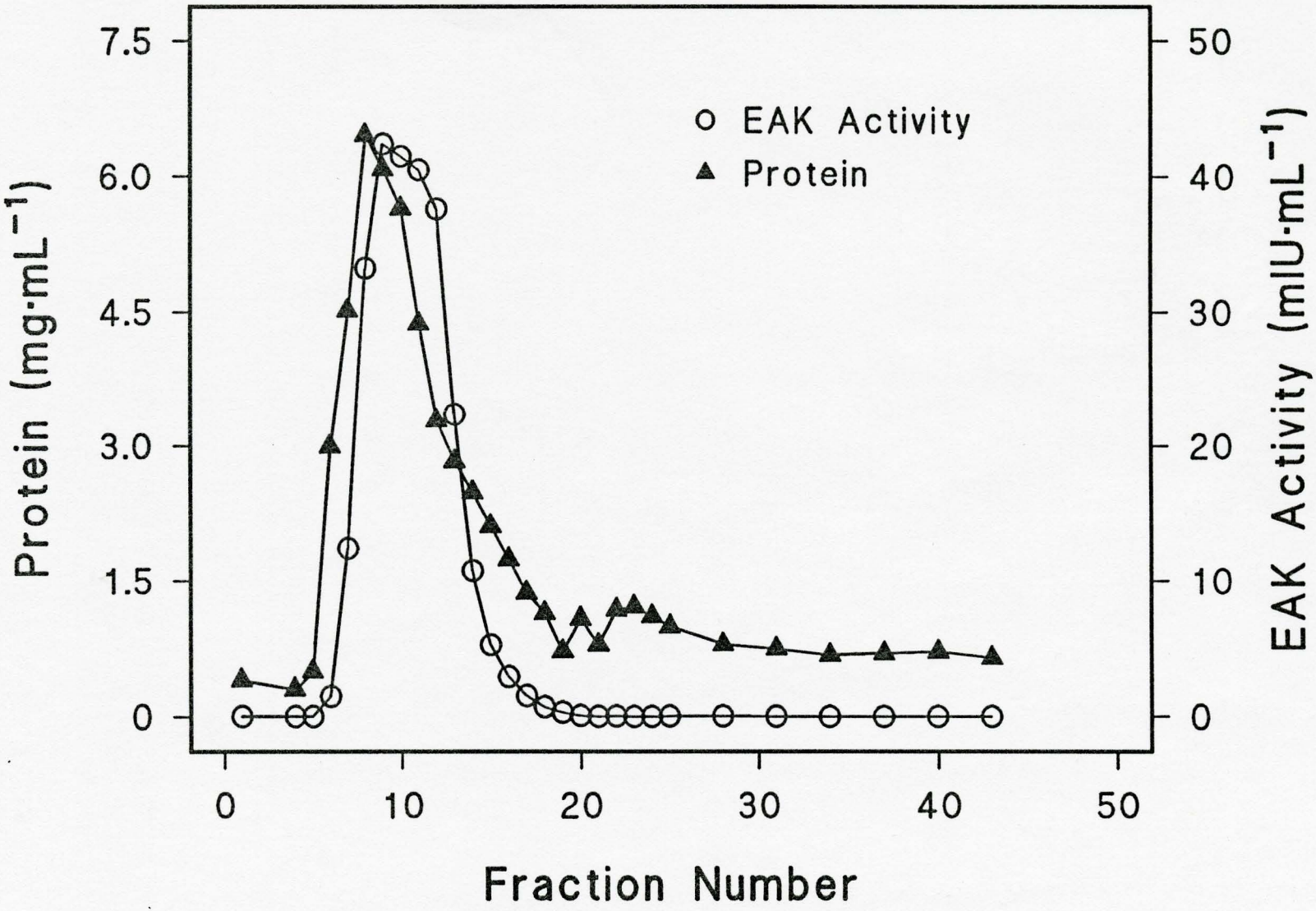
Fractions from the ion exchange step containing high EAK activity were pooled and applied to a phenyl-Sepharose column. The phenyl-Sepharose step increased the degree of purification 17 fold over that of the DEAE-Sepharose step to a cumulative 216 fold purification of EAK specific activity from that of the initial crude extract (Table III). Most of the protein (90%) either did not adsorb to the

Table III *Ethanolamine Kinase Purification I*

Step	[Protein]	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	mg · mL^{-1}	*mL*	*mg*	*mIU*	*mIU · mg^{-1} protein*	*fold*	*%*
Crude average	4.08	15680	53928	3511	0.055	1	100
A.S. pooled	8.48	870	7378	6569	0.89	16	188
DEAE	2.79	236	658	1127	1.71	31	32
P/Seph	4.53	14	63	760	11.99	216	22
A/Hex	3.39	4	14	443	32.70	590	13
M/Q	6.80	0.34	2.3	446	192.87	3481	13
P/Sup	0.018	0.112	0.002	0.73	362.17	6537	0.02

Figure 5 *Diethylaminoethyl-Sepharose Elution Profile*

A representative elution profile for the DEAE-Sepharose purification step (Table III). Adsorbed proteins were eluted according to the procedure in the Materials and Methods section. EAK activity shown is cpm x 10^{-2} obtained from 95 μL of sample assayed from each fraction. Note that the EAK activities estimated in fractions 6 to 17 are likely to be underestimates of true EAK activity (see text page 78 for discussion). Protein was estimated colourimetrically using Bradford's reagent.


EAK Activity
Protein
Protein (mg·mL⁻¹)
EAK Activity (mIU·mL⁻¹)
Fraction Number
7.5
6.0
4.5
3.0
1.5
0
50
40
30
20
10
0
0
10
20
30
40
50

column matrix or eluted at a higher $(NH_4)_2SO_4$ concentration than the EAK activity (Fig. 6). There was a 10% loss of total activity between this and the previous DEAE-Sepharose purification step. This loss may be due, in part, to excluding samples with EAK activity but low specific activity from the final pooled and concentrated sample.

The sample purified by phenyl-Sepharose was then applied to an ω-aminohexyl-agarose column. The elution profile for this column (Fig. 7) shows that the majority of adsorbed protein (78%) was completely eluted before the fractions containing EAK activity. Consequently, this step increased the fold purification by a factor of 2.5 to a final 590 fold from the initial crude EAK specific activity (Table III). A 10% loss of activity occurred between this and the previous step.

EAK-containing fractions from the ω-aminohexyl-agarose column were pooled, concentrated and dialysed and then loaded onto a MonoQ HPLC column. Of the total protein loaded onto the MonoQ column, 85% was either not adsorbed by the matrix or, if adsorbed, the protein eluted after the peak of EAK activity (Fig. 8). The recovery of EAK activity from the MonoQ step was 100% of that recovered from the previous ω-aminohexyl-agarose step and there was an increase in the fold purification by 6 to a total of 3481 fold from that of the initial specific activity in the crude extract (Table III).

The MonoQ purified sample was then applied to a phenyl-Superose HPLC column. Figure 9 shows that the phenyl-Superose column was an exceedingly useful column to separate EAK from protein contaminants. This is true despite the poor (0.2%) recovery of EAK activity (Table III). The modest 2 fold increase in specific

Figure 6 *Phenyl-Sepharose Elution Profile*

A representative elution profile for the phenyl-Sepharose purification step. Conditions for elution are as described in the Materials and Methods section. EAK activity given is cpm x 10^{-2} obtained from 95 μL of sample assayed from each fraction. Note that the EAK activities estimated in fractions 75 to 97 are likely to be underestimates of true EAK activity (see text page 78 for discussion). Protein was estimated by UV absorbance at 280 nm.

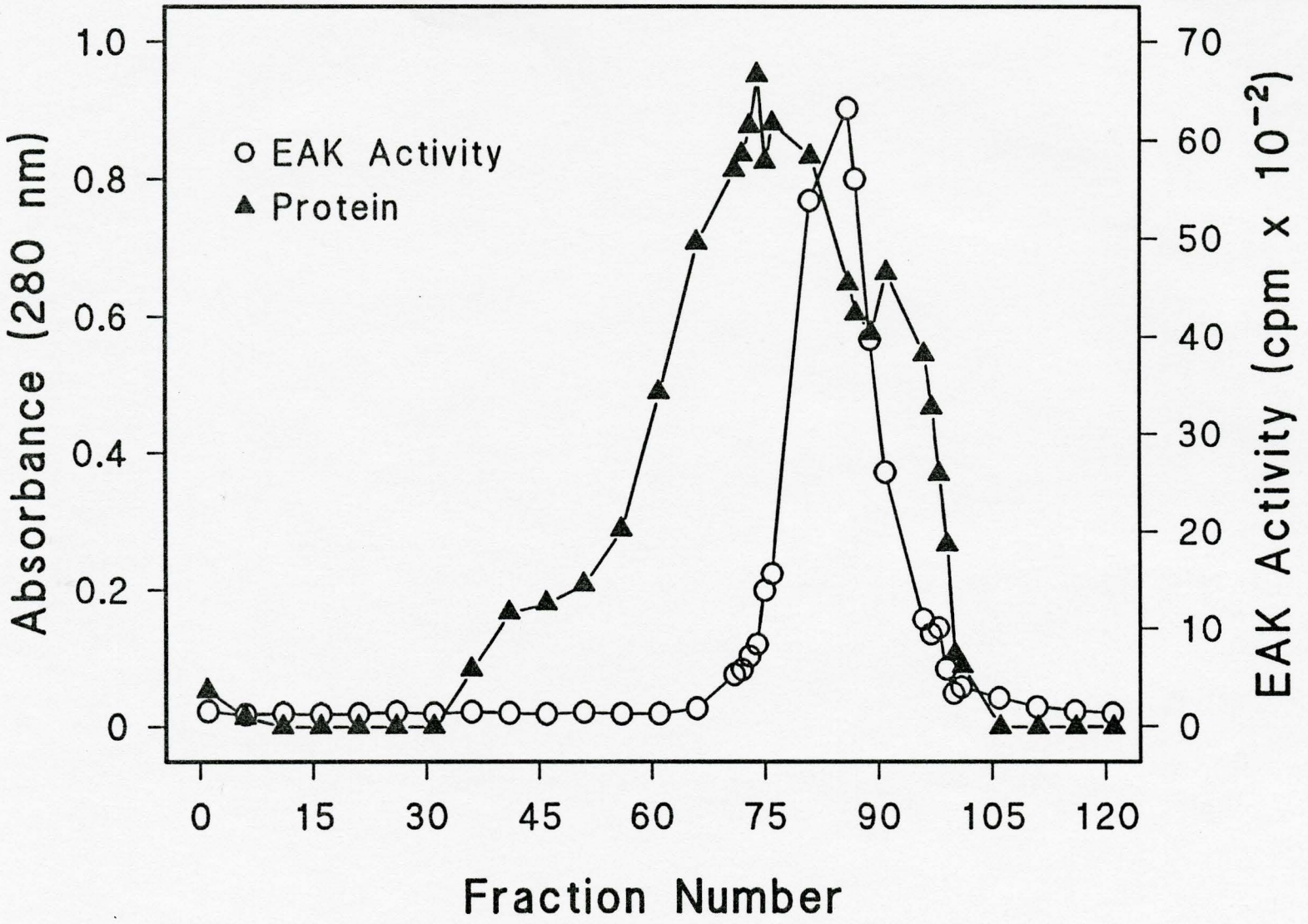

Absorbance (280 nm)
1.0
0.8
0.6
0.4
0.2
0
EAK Activity
Protein
70
60
50
40
30
20
10
0
EAK Activity (cpm x 10^{-2})
0
15
30
45
60
75
90
105
120
Fraction Number

Figure 7 *ω-Aminohexyl-Agarose Elution Profile*

A representative elution profile for the ω-aminohexyl-agarose column. Elution conditions are as described in the Materials and Methods section. EAK activity is given as cpm x 10^{-2} obtained from 95 μL of sample assayed from each fraction. Note that the EAK activities estimated in fractions 13 to 20 are likely to be underestimates of true EAK activity (see text page 78 for discussion). Protein was estimated by UV absorbance at 280 nm.

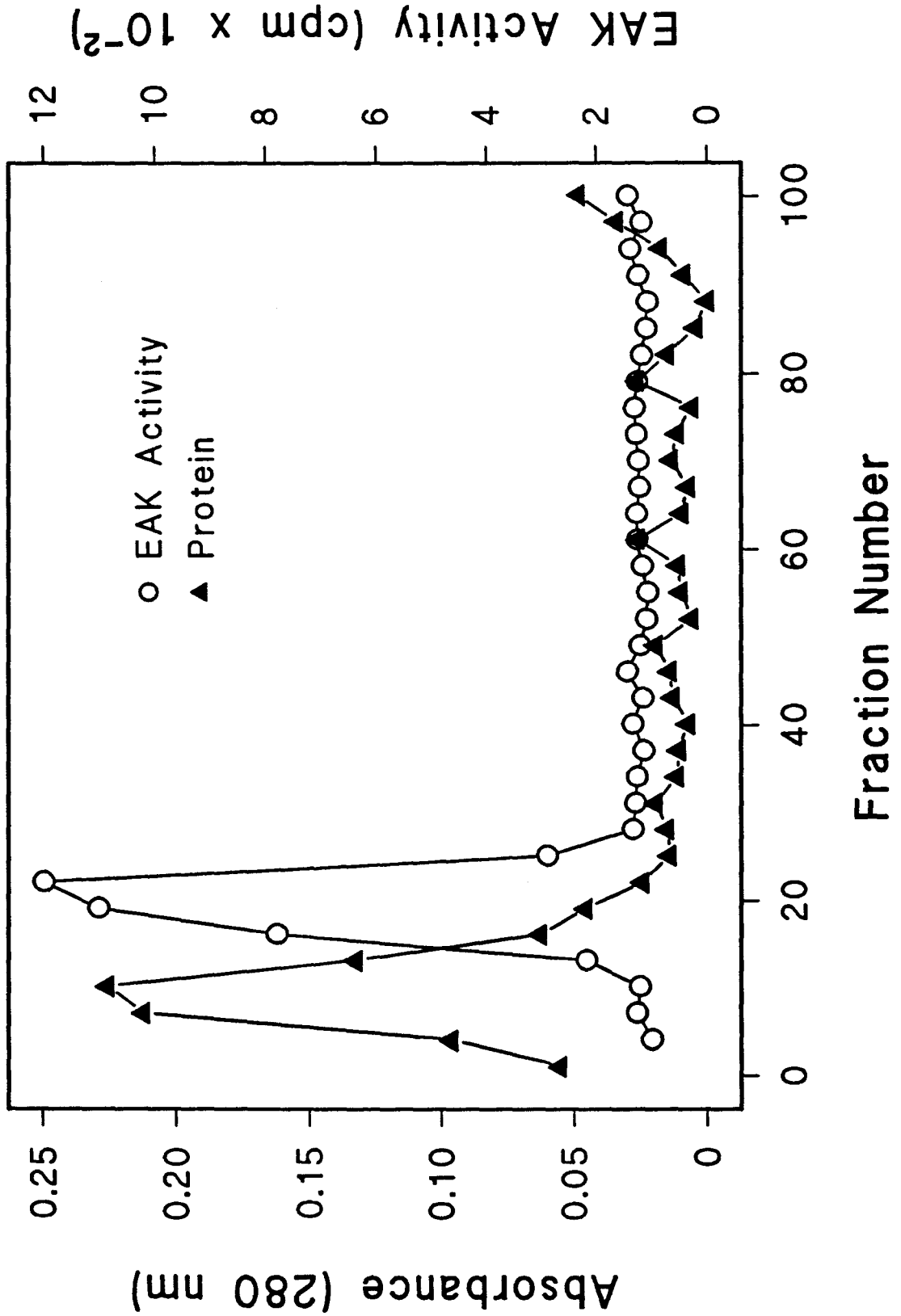

Absorbance (280 nm)
0.25
0.20
0.15
0.10
0.05
0
EAK Activity (cpm x 10^-2)
12
10
8
6
4
2
0
o EAK Activity
▲ Protein
0
20
40
60
80
100
Fraction Number

Figure 8 *MonoQ Elution Profile*

This is a representative elution profile of MonoQ purification step. Elution is as outlined in the Materials and Methods section. EAK activity is cpm x 10^{-3} obtained from 95 μL of sample from each fraction. Note that the EAK activities in fractions 22 to 41 are likely to be underestimates of true EAK activity while the EAK activity in fraction 26 is likely a better estimate of the true EAK activity (see text page 78 for discussion). Protein was estimated using UV absorbance at 280 nm.

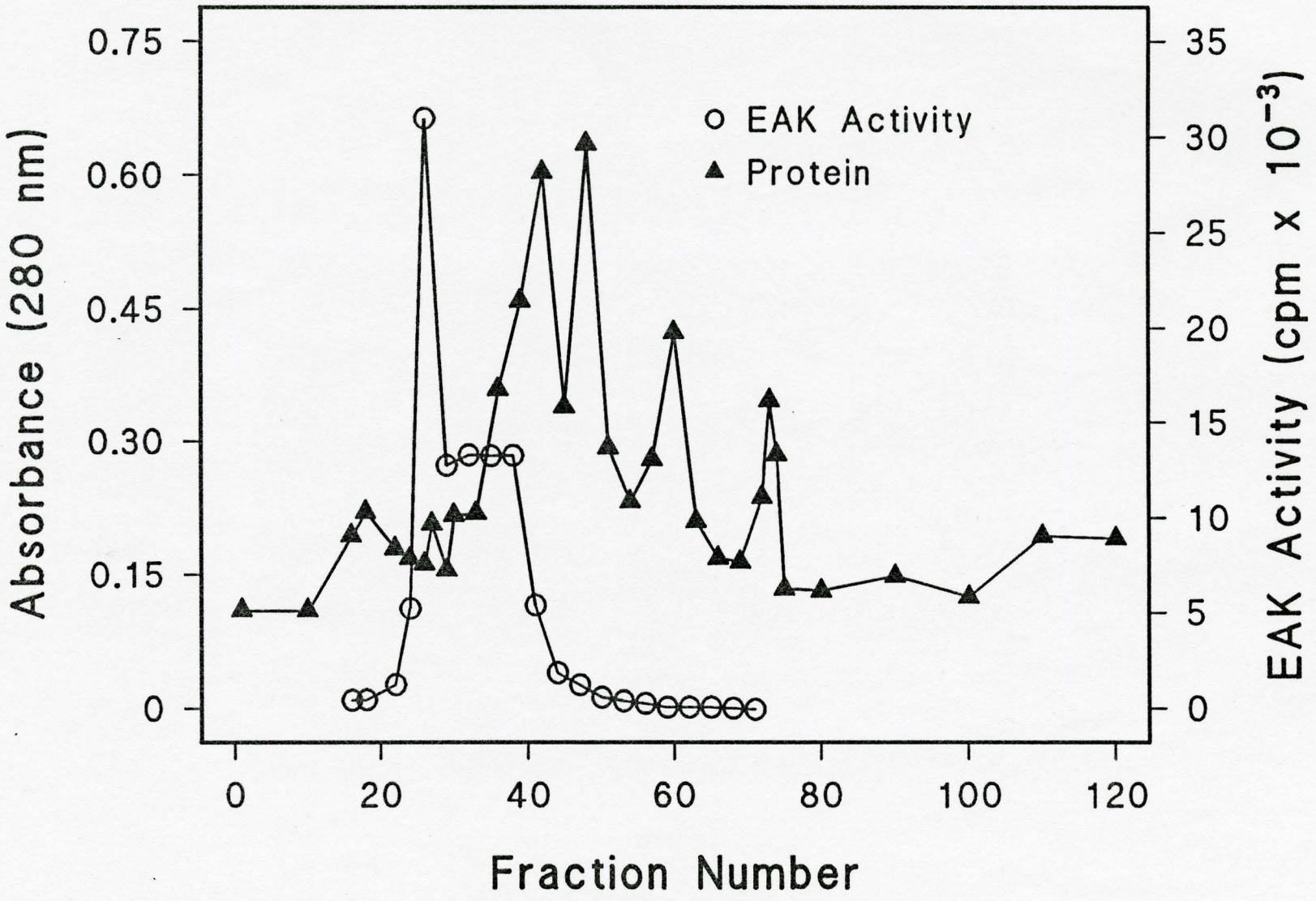
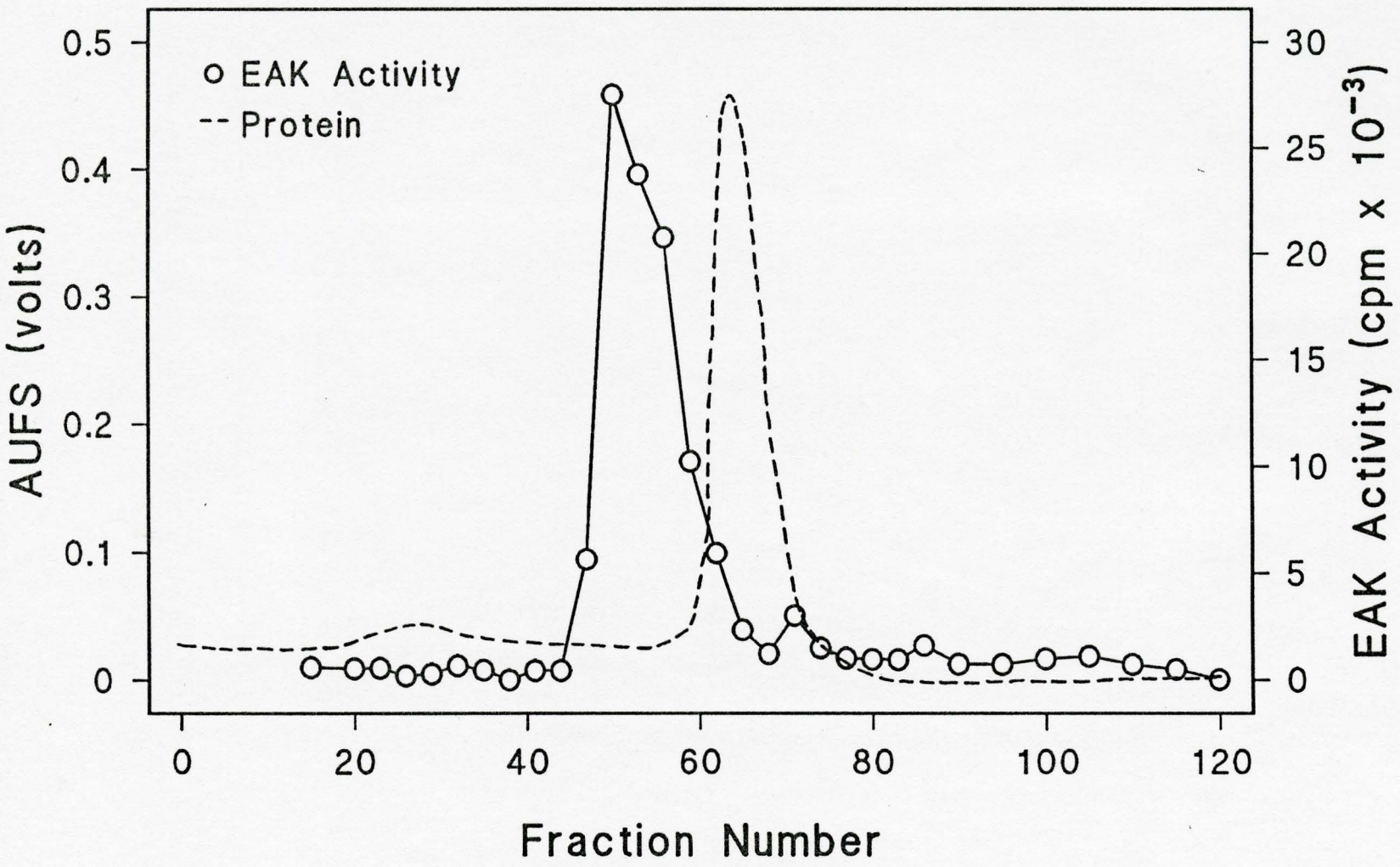

Absorbance (280 nm)
0.75
0.60
0.45
0.30
0.15
0
EAK Activity
Protein
35
30
25
20
15
10
5
0
EAK Activity (cpm x 10^{-3})
0
20
40
60
80
100
120
Fraction Number

Figure 9 *Phenyl-Superose Elution Profile*

This is a representative elution profile for the phenyl-Superose column. Elution conditions are as outlined in the Materials and Methods section. EAK activity is cpm x 10^{-3} obtained from 95 μL of sample assayed from each fraction. Note that the EAK activities in fractions 47 to 65 are likely to be underestimates of true EAK activity (see text page 78 for discussion). Protein was estimated by UV absorbance at 280 nm.


o EAK Activity
-- Protein
AUFS (volts)
0.5
0.4
0.3
0.2
0.1
0
30
25
20
15
10
5
0
EAK Activity (cpm x 10^{-3})
0
20
40
60
80
100
120
Fraction Number

activity achieved by this step yields a 6537 fold increase over the specific activity for the crude leaf extract.

Extracts from all of the steps of Purification I (Table III) were analyzed by SDS-PAGE (Fig. 10). The successive purification steps had the effect of reducing the number of protein contaminants present. For the phenyl-Superose purification step (Fig. 10, lane 8) only one polypeptide of approximately 38,000 D can be seen. As mentioned earlier, this sample represents approximately a 6,500 fold increase in specific EAK activity over that of the initial crude extract (Table III).

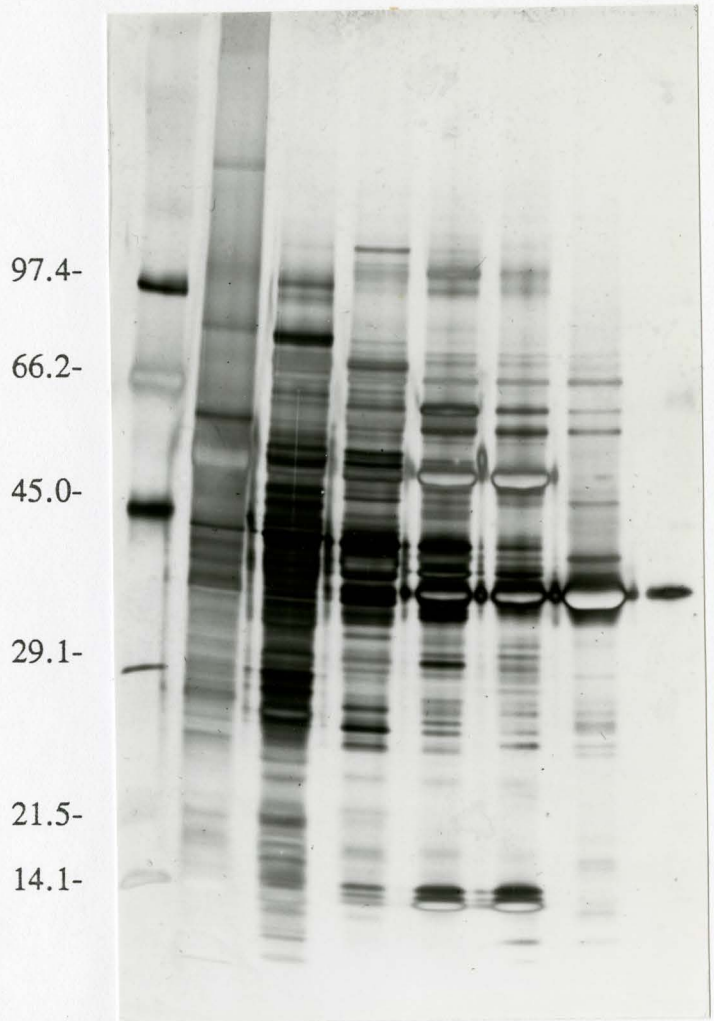

(Mr)
1
2
3
4
5
6
7
8
97.4-
66.2-
45.0-
29.1-
21.5-
14.1-

Figure 10 *Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of Polypeptides Present in Samples From Each Step of Purification I*

Protein samples loaded include: 0.2 μg of each molecular weight protein standard with $M_r \times 10^{-3}$ (lane 1); 5 μg Crude (C) purified sample (lane 2); 5 μg $(NH_4)_2SO_4$-pooled fraction (lane 3); 5 μg DEAE-Sepharose purified sample (lane 4); 5 μg phenyl-Sepharose purified sample (lane 5); 5 μg ω-aminohexyl-agarose purified sample (lane 6); 5 μg MonoQ purified sample (lane 7) and 0.02 μg of phenyl-Superose purified sample (lane 8). The proteins in the gel were stained with silver reagent.

Part III: Preparation of Ethanolamine Kinase Used for Kinetic Analyses

Ethanolamine Kinase: Purification II

Comparison of Table III with the summary shown in Table IV indicates that Purification II had a higher fold purification and recovery of EAK activity than Purification I (Table III) from the DEAE steps to the MonoQ steps. However, it proved extremely difficult to completely remove contaminating proteins even using the phenyl-Superose column and a homogeneous preparation was not achieved. EAK in this sample appears to be the major protein band, based on SDS-PAGE analysis and silver staining (data not shown), however there was insufficient sample to use for further studies.

Ethanolamine Kinase: Purification III

In Purification I (Table III) only 32% of the total EAK activity was recovered after the DEAE purification step. Taking the combined recoveries of all three DEAE-Sepharose column separations (32% (Table III), 69% (Table IV) and 50% (Table V), respectively) there was a greater than 100% combined recovery of total EAK activity from the $(NH_4)_2SO_4$ step to the DEAE-Sepharose step.

The ω-aminohexyl-agarose step in Purification III had a 4078 fold greater specific activity than crude extract (Table V). This represents a greater increase in activity than the 590 fold purification from initial crude specific activity that was achieved by the comparable ω-aminohexyl-agarose step of Purification I (Table III). The SDS-PAGE analysis of the pooled and concentrated sample from the ω-aminohexyl-agarose step of Purification III (Table V) shows that there are still some

Table IV *Ethanolamine Kinase Purification II*

Step	[Protein]	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	mg · mL^{-1}	*mL*	*mg*	*mIU*	*mIU · mg^{-1} protein*	*fold*	*%*
Crude average	4.08	15680	53928	3511	0.055	1	100
A.S. pooled	8.48	870	7378	6569	0.89	16	188
Flow-through (A)	3.12	1186	3700	6727	1.82	33	192
DEAE	15.62	25	391	2421	6.20	112	69
P/Seph	2.41	10	24	1637	67.92	1226	47
A/Hex	1.56	1.5	2.3	472	201.55	3638	13
M/Q	4.93	0.11	0.52	167	321.97	5811	5
P/Sup-1	0.17	0.08	0.014	2.01	148.33	2677	0.06
P/Sup-2	0.47	0.13	0.061	9.85	161.49	2915	0.28
P/Sup-3	0.54	0.13	0.067	2.50	37.13	670	0.07
P/Sup-4	1.26	0.09	0.113	6.93	61.40	1108	0.20
P/Sup-5	0.30	0.08	0.022	3.10	142.19	2567	0.09

Table V *Ethanolamine Kinase Purification III*

Step	[Protein]	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	$mg \cdot mL^{-1}$	*mL*	*mg*	*mIU*	*$mIU \cdot mg^{-1}$ protein*	*fold*	*%*
Crude average	4.08	15680	53928	3511	0.06	1	100
A.S. pooled	8.48	870	7378	6569	0.89	16	188
Flow-through (B)	2.78	910	2530	3651	1.44	26	104
DEAE	3.84	57	219	1737	7.93	143	50
P/Seph	1.02	24	24	769	31.41	567	22
A/Hex	0.20	3	0.5	122	225.79	4078	4

protein contaminants co-purifying with EAK (Fig. 11, lane 7). Despite containing other proteins, this sample had a high specific activity (225.79 mIU $\cdot$ mg^{-1} protein) and a total of 122 mIU of EAK activity (Table V). The high specific activity and the availability of a reasonable supply of enzyme made the ω-aminohexyl-agarose sample from Purification III a candidate for use in kinetic studies. However, this sample would only be suitable for kinetic studies if it was free of potentially interfering enzymes such as phosphobase phosphatases and if the residual ChoK activity was comparable to the ChoK of the ω-aminohexyl-agarose purified sample of Purification I. The use of this less pure EAK sample for kinetic studies was considered since there was not enough material available from either of the more highly purified preparations from Purifications I and II.

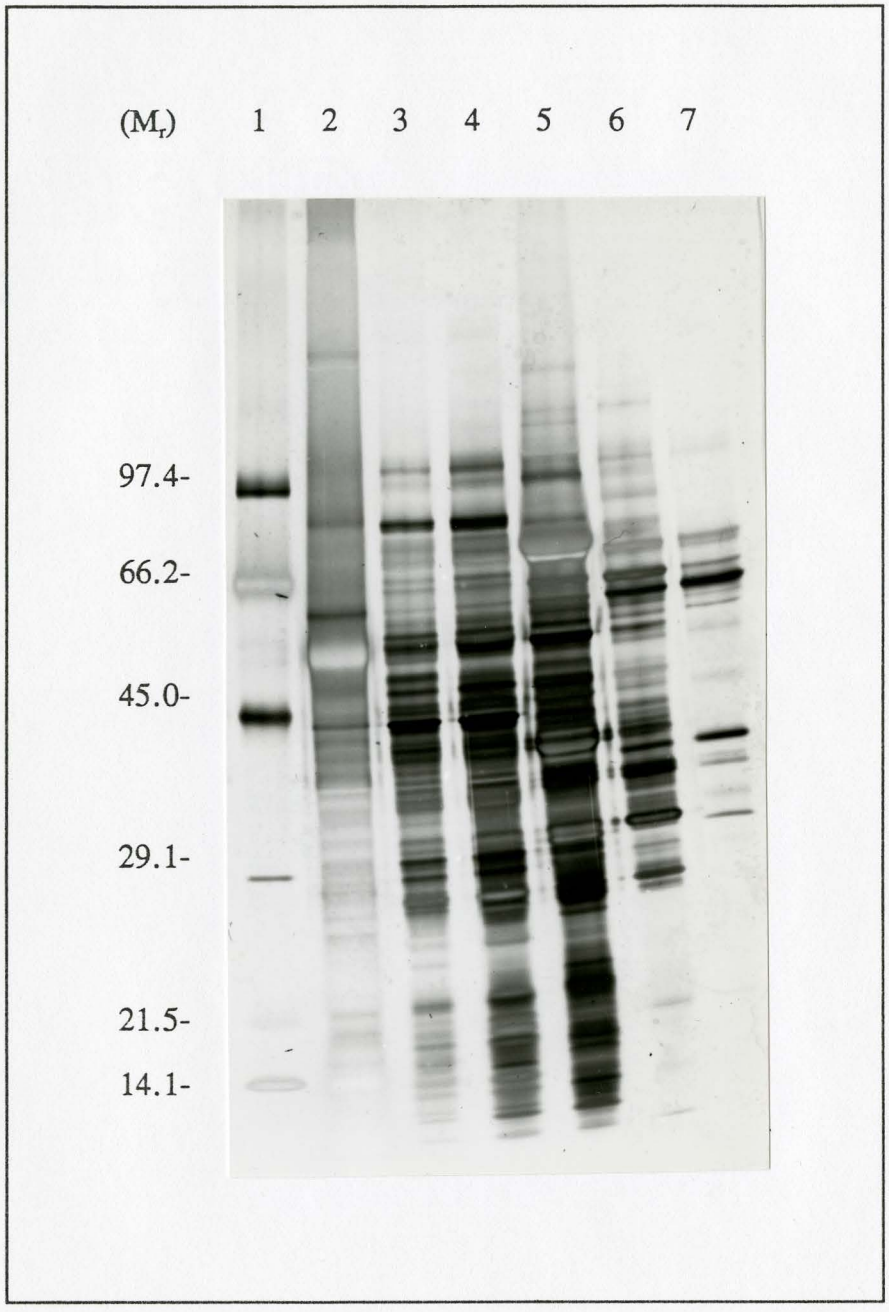
Suitability of ω-Aminohexyl-Agarose Sample From Purification III for Kinetic Analyses

Phosphobase Phosphatase Activity

The presence of phosphobase phosphatase enzyme(s) in an EAK containing sample could lead to PEA being converted back to EA, providing an underestimate of EAK activity. Furthermore, the P_i generated by a PEA phosphatase could inhibit EAK giving anomalous estimates of activity. Other phosphobase pathway intermediates used in kinetic studies could potentially be converted to freebases as well. This makes an accurate and constant determination of reaction components very difficult, especially since it is not known if one or several different phosphobase phosphatases are present in a sample and whether the

Figure 11 *Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of Polypeptides Present in Samples From Each Step of Purification III*

Protein samples loaded include: 0.2 μg of each molecular weight protein standard with $M_r \times 10^{-3}$ (lane 1); 5 μg Crude (C) purified sample (lane 2); 5 μg $(NH_4)_2SO_4$-pooled fraction (lane 3); 5 μg Flow-through (B) purified sample (lane 4); 5 μg DEAE-Sepharose purified sample (lane 5); 2 μg of phenyl-Sepharose purified sample (lane 6) and 1 μg of ω-aminohexyl-agarose purified sample (lane 7). Lane 7 contained the sample used for kinetic analyses. The proteins in the gel were stained with silver reagent.


(Mr)
1
2
3
4
5
6
7
97.4-
66.2-
45.0-
29.1-
21.5-
14.1-

phosphatase(s) present have broad or narrow substrate specificities.

The possible interference of phosphobase phosphatase activity was tested in various samples from crude extract to the ω-aminohexyl-agarose sample from Purification III using PEA or PCho as substrates (Table VI). For PEA phosphatase, acid and alkaline conditions were tested using conditions for PCho phosphatase since a published enzyme assay for this enzyme is not available. PCho (crude) and PEA (flow-through fractions from DEAE III column) samples were hydrolysed by phosphatase enzyme(s) under acid and alkaline conditions. However, no hydrolysis of PEA or PCho was detected in the ω-aminohexyl-agarose III sample at either pH.

Relationship of Ethanolamine Kinase and Choline Kinase Activities in Purified Spinach Leaf Extract Samples

In pooled fractions of an ω-aminohexyl-agarose purified sample, EAK and ChoK activities migrate to different positions in a gel when subjected to Native-PAGE. Figure 12 shows that there are at least two different ChoK activity peaks associated with an ω-aminohexyl-agarose purified sample obtained from a purification obtained previous to the current study. There also appears to be a minor ChoK activity peak that coincides with the EAK activity peak. The presence of EAK and multiple forms of ChoK still present in the ω-aminohexyl-agarose purified sample used for the analysis in Figure 12 suggests that further purifications are required to completely separate EAK from contaminating ChoK enzymes.

In Purification I, it appears as though EAK has been purified away from ChoK, since the EAK to ChoK ratio increased throughout the purification

Table VI *Phosphobase Phosphatase Activity in Purified Spinach Extracts*

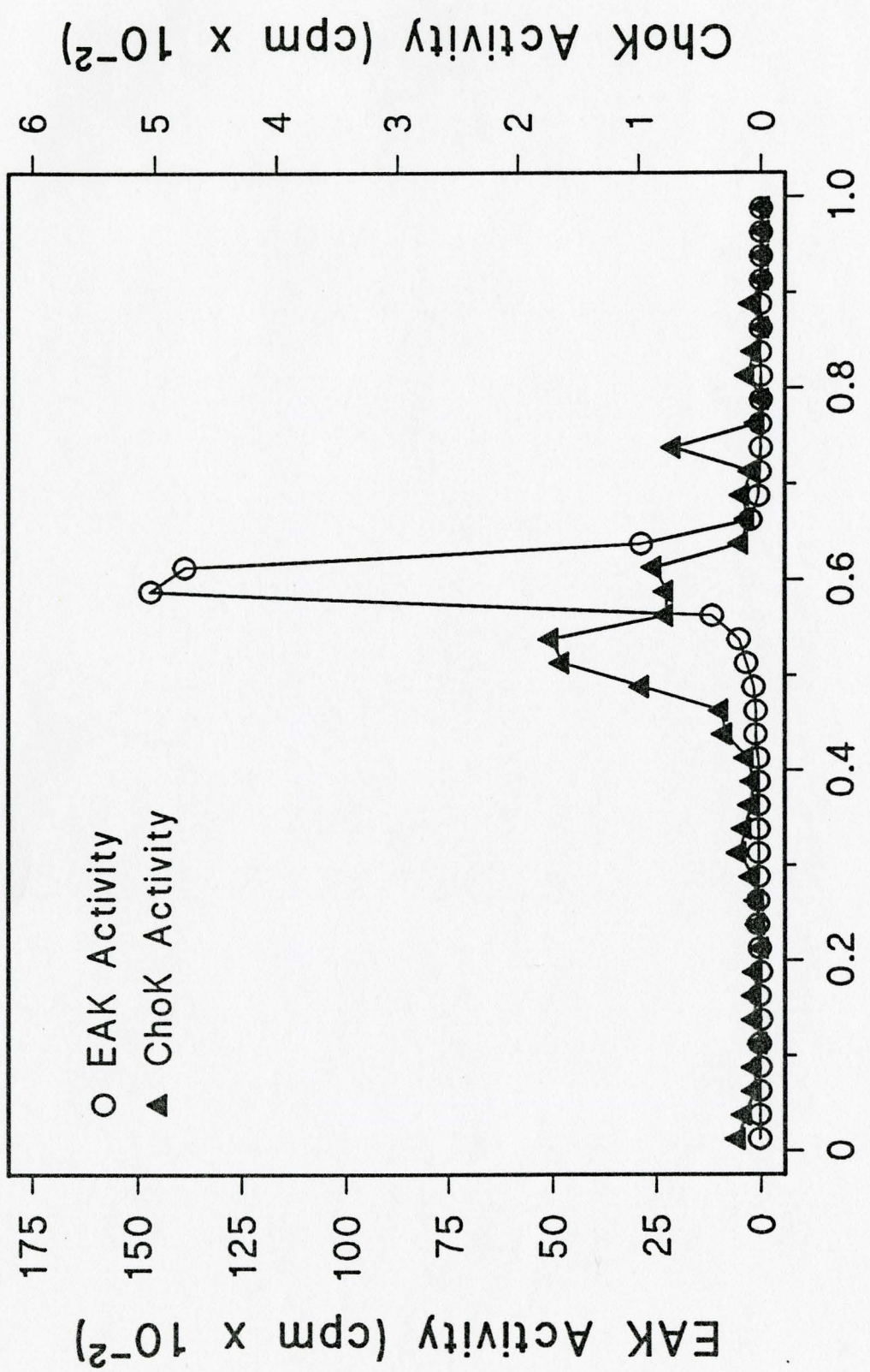
Phosphatase activity was measured in crude and DEAE III flow-through column samples and in samples from two steps of Purification III. EAK activity and EAK to phosphatase ratio of activities are also given.

Sample (n^a)	Substrate (pH)	Phosphatase Activity[b]	EAK Activity	EAK to Phosphatase
		mIU·mg^{-1} protein	*mIU·mg^{-1} protein*	*ratio*
Crude (2)	PCho (5.0)	2.30 ± 0.04	0.27	0.12
DEAE III (4)	PCho (5.0)	0.08 ± 0.01	3.36	40.73
A/Hex III (2)	PCho (5.0)	ND[c]	225.79	---
Flow-through (C)[d] (2)	PEA (5.0)	0.056 ± 0.004	0.55	9.82
Flow-through (C)[d] (2)	PEA (8.5)	0.013 ± 0.002	0.55	42.31
A/Hex III (2)	PEA (5.0)	ND	225.79	---
A/Hex III (2)	PEA (8.5)	ND	225.79	---

[a]number of replicates [b]mean ± S.E. [c]not detected
[d]flow-through from DEAE column

Figure 12 *Migration Patterns of Ethanolamine Kinase and Choline Kinase in Native Polyacrylamide Gels*

A Native-PAGE analysis of an ω-aminohexyl-agarose purified sample obtained from a purification of EAK performed prior to the current study. Gels were sliced into segments and the segments were used to assay for EAK activity (○) and ChoK activity (▲). Protein loaded per lane was 198 μg of the sample with a specific activity of 6.43 mIU · mg^{-1} protein. Enzyme activity is reported as cpm x 10^{-2} obtained per gel slice assayed.


EAK Activity (cpm x 10⁻²)
ChoK Activity (cpm x 10⁻²)
O EAK Activity
▲ ChoK Activity
175
150
125
100
75
50
25
0
6
5
4
3
2
1
0
0
0.2
0.4
0.6
0.8
1.0
Rf Value

(Table VII). However, some residual ChoK activity is still detected in the purified sample (0.12 mIU $\cdot$ mg^{-1} protein). This residual ChoK specific activity remained relatively constant from the DEAE-Sepharose column fractionation onwards at a ratio for EAK to ChoK of 0.12.

In Purification II the EAK to ChoK ratio increased until the ω-aminohexyl-agarose step where the ratio started to level off (Table VII). In Purification III the EAK to ChoK ratio remained fairly constant, with both activities increasing to the same degree throughout the purification. In both cases, this suggests that a ChoK enzyme is being co-purified along with EAK. This result is not unanticipated since neither preparation yielded a single homogeneous band.

Sample Used in Kinetic Analyses

The ω-aminohexyl-agarose sample from Purification III appears to be free of PCho and PEA phosphatase activities, eliminating possible production of free phosphate or the conversion of newly formed phosphobases back to the free base form in an assay. Though there appears to be ChoK activity associated with the ω-aminohexyl-agarose sample from Purification III, EAK specific activity is approximately 5 times higher than ChoK specific activity. The low ChoK activity should not interfere with EAK assays but may raise problems in interpretation if K_m values for the various free base substrates (for example, Cho) were to be carried out. Thus with these considerations in mind, the ω-aminohexyl-agarose sample from Purification III was used for the kinetic analyses unless otherwise specified.

Table VII *Ethanolamine Kinase to Choline Kinase Ratios*

The EAK and ChoK activities were measured in the steps for all three purifications (see Figure 4) with the EAK to ChoK ratio shown.

Sample	EAK Specific Activity	ChoK Specific Activity	EAK:ChoK
	mIU · mg^{-1} protein	*mIU · mg^{-1} protein*	*ratio*
Purification I			
Crude average	0.06	0.15	0.4
A.S. pooled	0.89	0.35	2.5
DEAE	1.71	0.13	13
P/Seph	11.99	0.18	68
A/Hex	32.7	0.28	117
M/Q	193	0.12	1556
Purification II			
Crude average	0.06	0.15	0.4
A.S. pooled	0.89	0.35	2.5
Flow-through A	1.82	0.57	3.2
DEAE	6.2	0.85	7.3
P/Seph	67.9	1.55	44
A/Hex	202	7.6	27
Purification III			
Crude average	0.06	0.15	0.4
A.S. pooled	0.89	0.35	2.5
Flow-through B	1.44	0.48	3.0
DEAE	7.93	1.61	4.9
P/Seph	31.4	3.7	8
A/Hex	226	45	5

Part IV: Biochemical Characterization of Ethanolamine Kinase

Molecular Weight Estimation for Ethanolamine Kinase

The molecular weight of EAK was estimated by gel filtration chromatography and by SDS-PAGE. In the first approach, the volume of buffer required to elute EAK from a gel filtration column is determined. When this elution volume (V_e) is compared with the void volume (V_o) of the column a "rate" of passage through the column is obtained. When this elution rate is compared to the elution rates of standard proteins of known molecular weight, it can be estimated that EAK has a native size of 80,000 D (Fig. 13). When an ω-aminohexyl-agarose purified sample is further processed by gel filtration, and the resultant fractions which contain EAK activity are analyzed by SDS-PAGE, a single major polypeptide at a relative molecular weight of 38,000 is found by silver staining (Fig. 14).

Development of Conditions for an Optimized Ethanolamine Kinase Assay

pH Optimum

The effect of buffers with overlapping pH distributions were used to determine the pH optimum for EAK activity. Figure 15 shows that EAK has a broad pH optimum from pH 7 to about pH 9. All buffers within this pH range with the exception of tricine seem to be compatible. In the range of buffers used above pH 9 glycine drastically decreases EAK specific activity relative to the activity observed using the other buffers (Bis-Tris propane, Ches and Caps). The two buffers which gave maximal EAK specific activity are Tris and Bis-Tris propane. To ensure that a more pure EAK sample would display the same pH optimum, the experiment was

Figure 13 *Estimation of the Relative Molecular Weight of Ethanolamine Kinase by HPLC Gel Filtration*

The least-squares line shown through the standard proteins (●), which omits the data point for BSA, predicts a M_r 80,000 for EAK (○). The regression fit was $r^2=0.9927$. Anomalous migration of the BSA standard has been noted by others (Weretilnyk and Hanson, 1989).

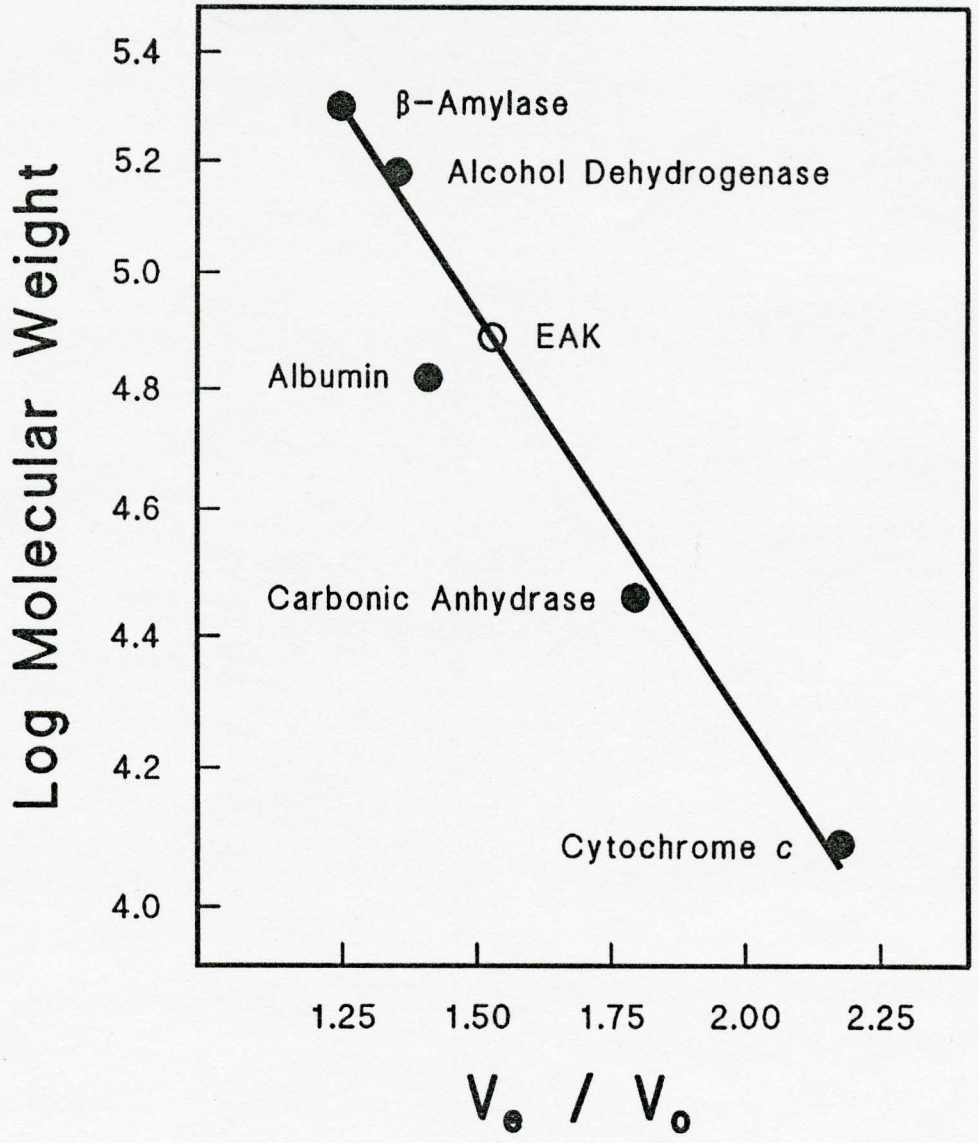

Log Molecular Weight
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
β-Amylase
Alcohol Dehydrogenase
EAK
Albumin
Carbonic Anhydrase
Cytochrome c
1.25
1.50
1.75
2.00
2.25
Ve / Vo

Figure 14 *Molecular Weight Estimation of Ethanolamine Kinase by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis*

Sample and standards were analyzed by SDS-PAGE, and a least-squares fit of standards (●) yielded a fit of $r^2=0.9930$ and predicts a molecular weight of 38,000 for EAK (○). Protein loaded was 5 μg.

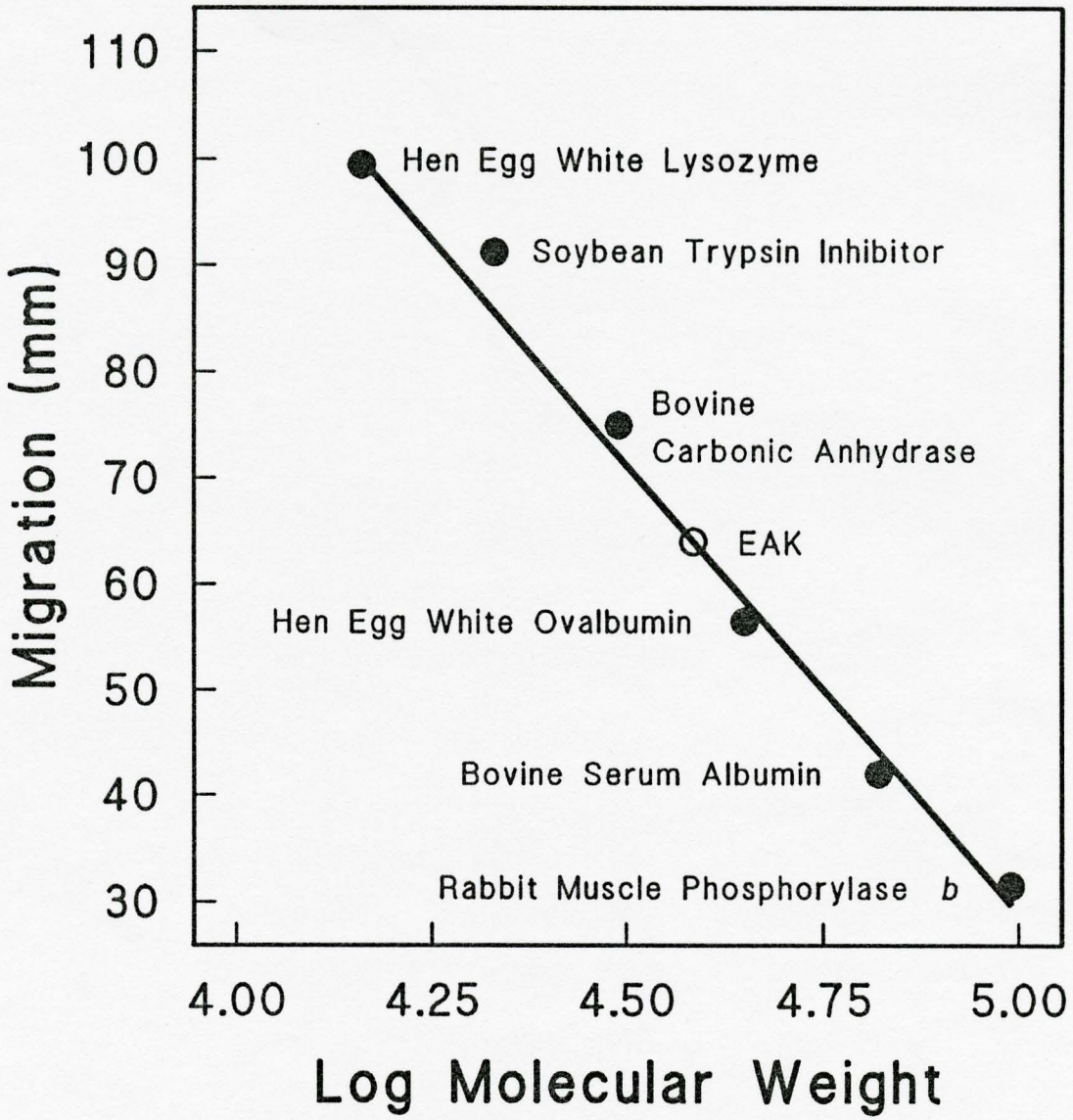
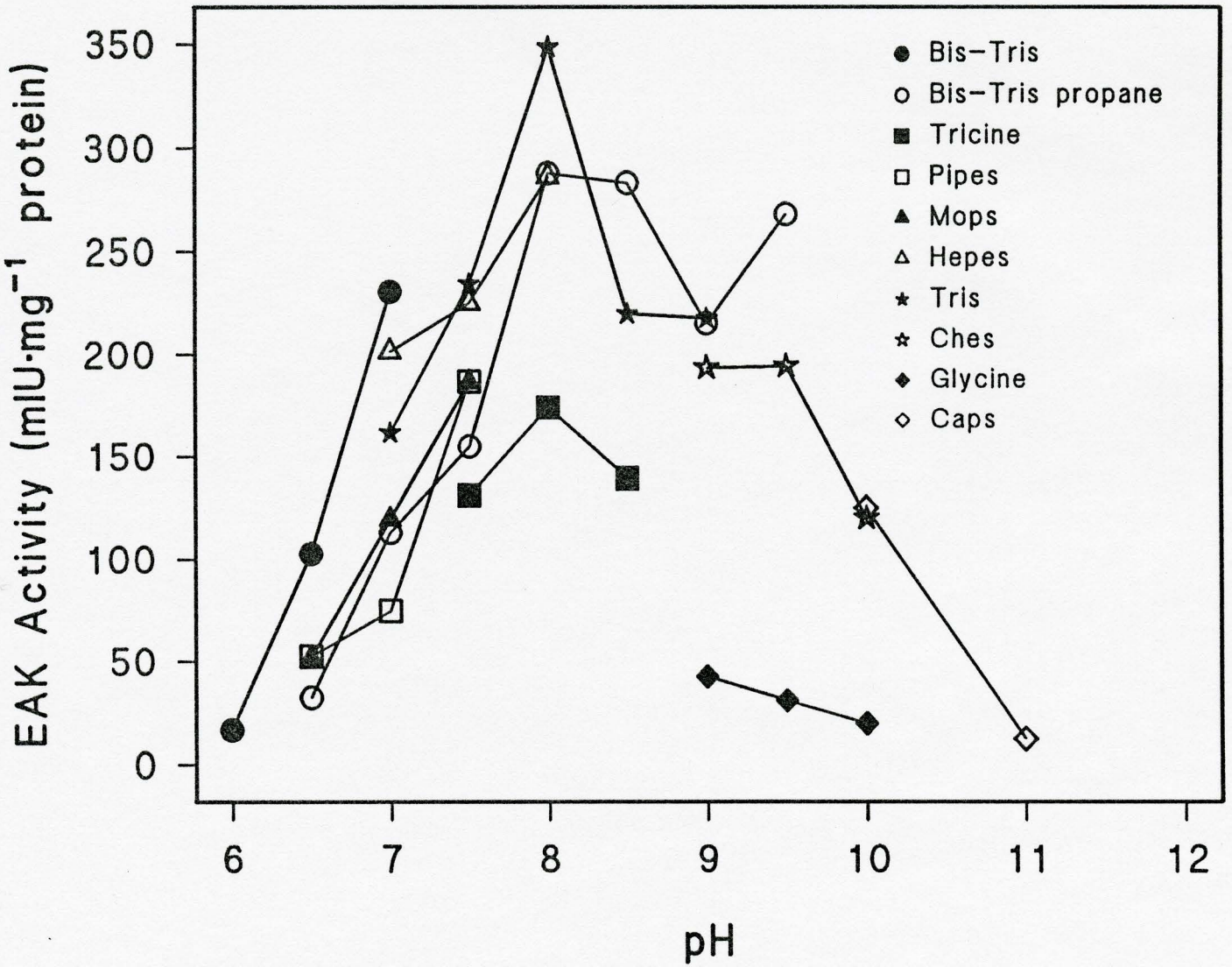

110
100
90
80
70
60
50
40
30
Migration (mm)
Hen Egg White Lysozyme
Soybean Trypsin Inhibitor
Bovine
Carbonic Anhydrase
EAK
Hen Egg White Ovalbumin
Bovine Serum Albumin
Rabbit Muscle Phosphorylase b
4.00
4.25
4.50
4.75
5.00
Log Molecular Weight

Figure 15 *Ethanolamine Kinase Activity as a Function of pH*

EAK activity measurements for buffers below pH 6 were excluded since very low to below background levels of enzyme activity were obtained. Values are single trials in a single experiment.


EAK Activity (mIU·mg⁻¹ protein)
350
300
250
200
150
100
50
0
6
7
8
9
10
11
12
pH
Bis–Tris
Bis–Tris propane
Tricine
Pipes
Mops
Hepes
Tris
Ches
Glycine
Caps

repeated using the phenyl-Superose sample from Purification II. In this case EAK activity peaks at pH 8 and remains high until pH 9.5 for both Tris and Bis-Tris propane showing that a more pure sample displays the same pH optimum (data not shown). Thus, pH 8.5 with Tris-HCl buffer was kept as the buffer of choice.

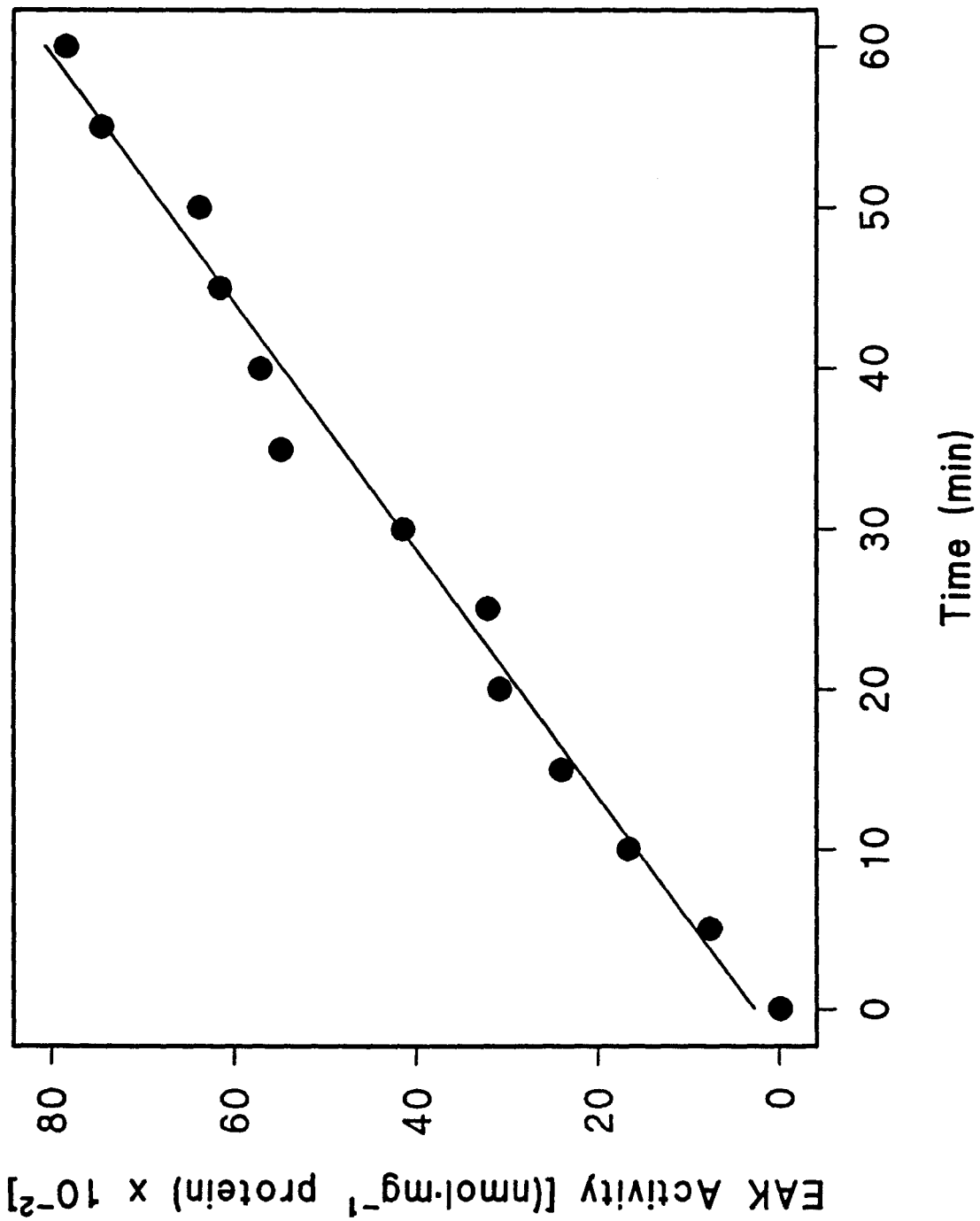
Linearity of Ethanolamine Kinase Reaction for Both Assay Length and Protein Concentration

At all stages in the purification of EAK, enzyme activity was not linear with increasing protein concentration (data not shown). Dilution of enzyme sample was required in order to ensure that EAK activity was being measured accurately. Due to the large numbers of assays required to produce an elution profile, accurate dilutions were not done. Only a qualitative, relative amount of EAK activity in the fractions was required (Fig. 5,6,7,8 and 9). In all other analyses (except for gel slice experiments) EAK activity was determined using a sample diluted to give activity in a linear range when plotted as a function of protein concentration.

Before any kinetic studies were performed, the linearity of EAK activity with respect to assay time required verification. The original assay protocol (Mudd and Datko, 1989a) called for an assay duration of 30 minutes so a time period from 0 to 60 minutes was tested. Figure 16 shows that the reaction is linear for the entire time period tested (with up to 0.38 μg of protein in the ω-aminohexyl-agarose III enzyme sample per 200 μL assay), thus 30 minutes was retained for the EAK assay.

Figure 16 *Ethanolamine Kinase Activity With Varying Length of Assay Time*

A least-squares regression of r^2=09873 was obtained for assay time with respect to EAK activity. Values are single trials in a single experiment. Sample used for the study was 0.38 g of the ω-aminohexyl-agarose sample from purification III per 200 μL assay.


EAK Activity [(nmol·mg⁻¹ protein) x 10⁻²]
0
20
40
60
80
0
10
20
30
40
50
60
Time (min)

KCl Concentration and Ethanolamine Kinase Activity

KCl has been suggested as being required for maximal EAK activity in soybean seeds (Warfe and Harwood, 1979a) and Mudd and Datko (1989a) included 200 μM KCl in the assay for plant EAK. In order to optimize EAK activity, the effect of KCl concentration on EAK activity was tested (Table VIII). Varying the concentration of KCl in the reaction from 8 μM to 1000 μM had no apparent effect on EAK activity. The original 200 μM KCl recommended by Mudd and Datko (1989a) was kept in the optimized EAK assay conditions.

Mg^{2+} and ATP Concentrations and Ethanolamine Kinase Activity

The assay conditions recommended by Macher and Mudd (1976) for spinach EAK contained 1.8 mM $MgCl_2$ and 0.9 mM ATP, yielding a 2:1 ratio Mg^{2+} to ATP. In the present study when the amounts of Mg^{2+} and ATP are varied, the greatest amount of EAK activity is obtained when $MgCl_2$ and ATP levels are both 5.0 mM (Fig. 17). In fact, for the various concentrations of Mg^{2+} and ATP tested, the highest rate of enzyme activity is achieved when the Mg^{2+} to ATP ratio is 1:1 (Fig. 17).

To determine if 5.0 mM $MgCl_2$ is the optimum Mg^{2+} level, the experiment was repeated with a more pure sample (Purification II, phenyl-Superose pooled sample 2) and a constant 1:1 ratio of Mg^{2+} to ATP. Since approximately equivalent EAK activity is achieved for all concentrations of Mg^{2+} tested from 2 to 10 mM, 5 mM was used in all subsequent assays (Table IX).

The experiment involving the measurement of EAK activity with changing ATP concentration and constant Mg^{2+} concentration was repeated with a fairly crude

Table VIII *Ethanolamine Kinase With Varying Concentrations of KCl*

The results given are for a single trial in a single experiment.

KCl Concentration	EAK Activity
μM	*mIU·mg^{-1} protein*
8	174
20	172
40	225
100	169
200	226
300	185
500	238
1000	189

Figure 17 *Ethanolamine Kinase Activity and Mg^{2+} to ATP Ratio*

For each concentration of $MgCl_2$, ATP was added at concentrations of 0.5 to 10 mM. Data is displayed in terms of Mg^{2+} to ATP ratio, and each value is the result of a single trial in a single experiment.

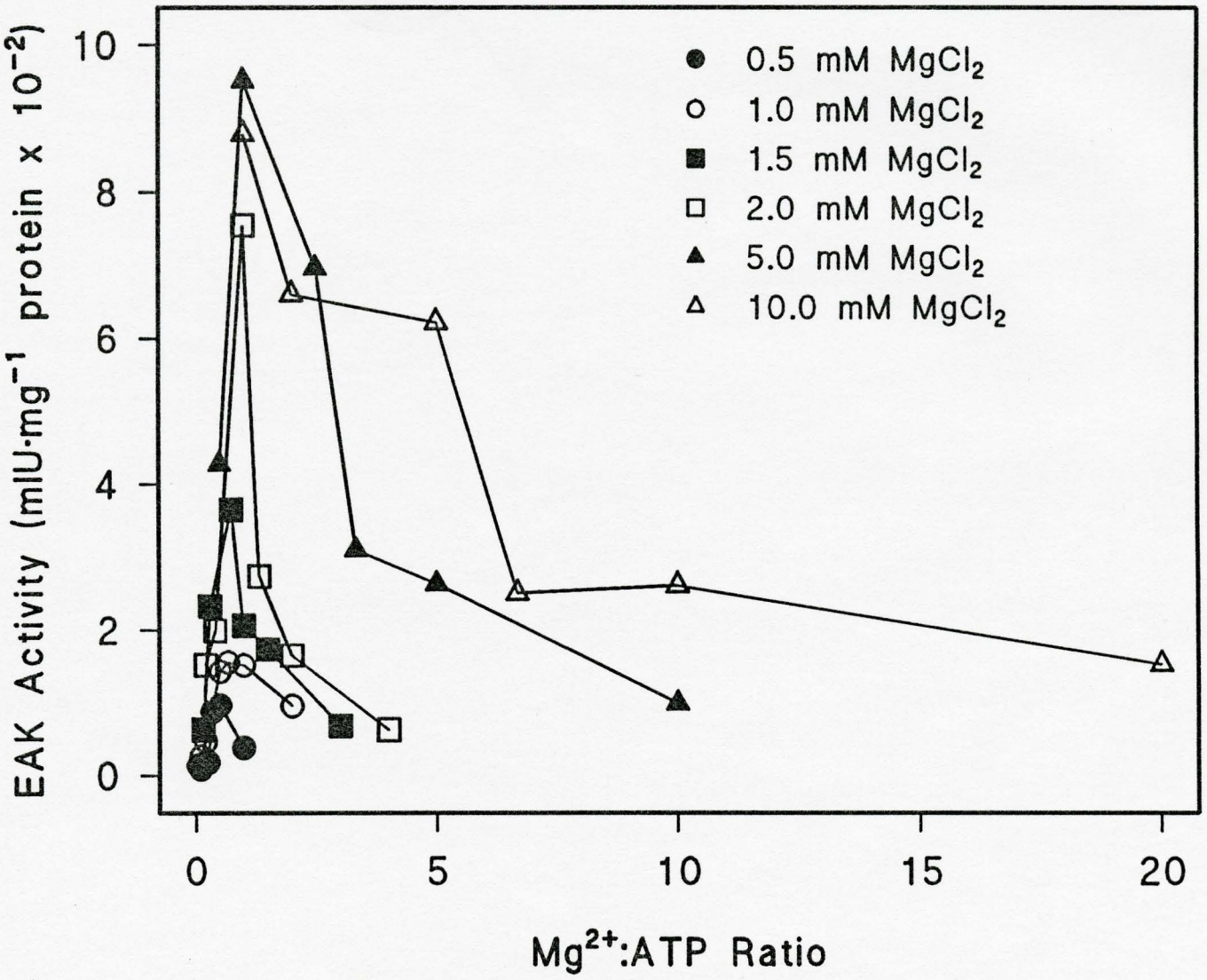

EAK Activity (mIU·mg^{-1} protein x 10^{-2})
10
8
6
4
2
0
0
5
10
15
20
Mg^{2+}:ATP Ratio
0.5 mM MgCl$_2$
1.0 mM MgCl$_2$
1.5 mM MgCl$_2$
2.0 mM MgCl$_2$
5.0 mM MgCl$_2$
10.0 mM MgCl$_2$

Table IX *Ethanolamine Kinase Activity as a Function of Varying Mg^{2+} Concentration*

The concentration of $MgCl_2$ was varied but a constant Mg^{2+} to ATP ratio of 1:1 was maintained. Values are of a single trial in a single experiment.

$MgCl_2$ Concentration	EAK Activity
mM	*mIU·mg^{-1} protein*
2	28
3	38
4	48
5	49
6	47
7	44
8	47
9	55
10	44

sample (Flow-through from DEAE-Sepharose III column). With a constant 5 mM of $MgCl_2$, the highest EAK activity is seen with 5 mM ATP (Table X). Thus for all kinetic studies, $MgCl_2$ and ATP concentrations were both changed to 5 mM. A better estimate of the requirements that EAK has for Mg^{2+} and ATP could be obtained by determining the apparent K_m values for Mg^{2+} and ATP, but unfortunately there was insufficient sample for this analysis.

Optimized Ethanolamine Kinase Assay Conditions

In summary, the optimized assay conditions for EAK are 100 mM Tris-HCl pH 8.5 (22°C), 200 μM KCl, 5 mM $MgCl_2$ and 5 mM ATP at 30°C for 30 min.

Kinetics of Ethanolamine Kinase

Once the optimal assay conditions for EAK were determined, the effects of varying substrate (EA) concentration on enzyme activity were investigated (Fig. 18). The best method of determining K_m and V_{max} is to fit the data directly to the Michaelis-Menten equation using a non-linear least-squares procedure, this calculation requiring the use of a computer to accurately extrapolate beyond experimental values (Cornish-Bowden, 1979). After a fit to an equation has been achieved, residuals should be plotted and analyzed to ensure that the equation actually fits the data (Cornish-Bowden, 1981). Residuals are the differences between the observed rates (v) and the corresponding calculated rates (v') from the best-fit equation. This difference (v-v') should tend to increase in absolute magnitude as calculated (v') increases, but the relative differences [(v-v')/v'] should be scattered in a parallel band

Table X *Ethanolamine Kinase Activity as a Function of $MgCl_2$ Concentration*

For these assays ATP was held constant at 5 mM. The sample used was the flow-through from the DEAE-Sepharose III step. Values are of a single trial in a single experiment.

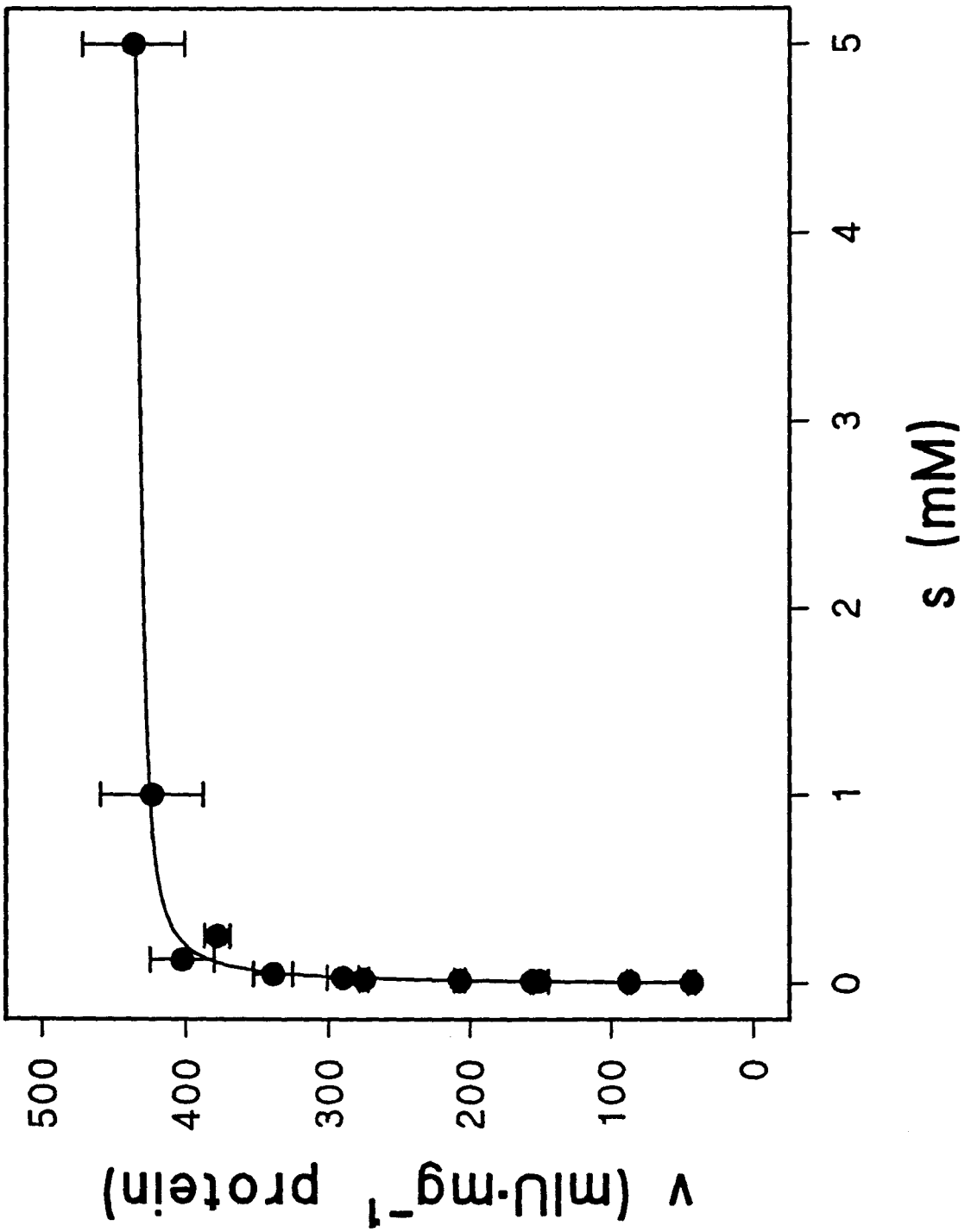
$MgCl_2$ Concentration	EAK Activity
mM	*$mIU \cdot mg^{-1}$ protein*
0.5	0.45
1.0	0.58
1.5	0.60
2.0	0.68
5.0	1.28
10.0	0.72

Figure 18 *Fit of Ethanolamine Kinase Activity With Respect to Ethanolamine Concentration to the Michaelis-Menten Equation*

EAK activity (v) with varied concentrations of EA (s). The curve plotted is the non-linear curve-fit as calculated by Fig.P (Biosoft). The Michaelis-Menton Equation is generally written as:

$$v = \frac{V_{max}}{1 + K_m/s} = \frac{V_{max} \cdot s}{K_m + s}$$

This equation describes a rectangular hyperbola in v, s space passing through the origin with asymptotes $v=V_{max}$ and $s=-K_m$. The best-fit curve gives an equation for the rectangular hyperbola of $y=(28.48 \pm 3.22)x + 430 \pm 15$. The error bars indicate the S.E. of the mean ($n=3$) for a single experiment.


v (mU·mg⁻¹ protein)
500
400
300
200
100
0
0
1
2
3
4
5
s (mM)

about zero (Cornish-Bowden, 1979). Figure 18 shows a curve-fit as calculated by the Fig.P program for EAK activity with varying EA concentration, this curve-fit passingthe residuals test. The curve-fit in Figure 18 gives a K_m of 15.1 μM and a V_{max} of 430 mIU $\cdot$ mg^{-1} protein, while another curve fit analysis (Marquardt, 1963) gives a K_m of 16.0 μM and a V_{max} of 463 mIU $\cdot$ mg^{-1} (curve-fit not shown).

Transformation of the data from Figure 18 into linear forms provides an alternate means of calculating K_m and V_{max} (Figures 19, 20 and 21). Along with these analyses, K_m and V_{max} can also be estimated using the nonparametric direct linear plot. Table XI shows a summary of the K_m and V_{max} estimates derived using all linear and non-linear methods. The most accurate method, due to its nonparametric nature (Cornish-Bowden and Eisenthal, 1974), should be the direct linear plot giving a K_m of 15.5 μM and V_{max} of 483 mIU $\cdot$ mg^{-1} protein. Values obtained by this method are well in agreement with both of the non-linear fits. The method of choice to represent the data from Figure 18 in linear form is the half-reciprocal plot (s/v vs s) plot which predicts a K_m of 15.9 μM and a V_{max} of 438 mIU $\cdot$ mg^{-1} protein. These are very close to those estimates provided by the other derivations. The close agreement seen in values obtained by the various tests suggests that the data in Figure 18 are close to being parametric in nature. Greater deviation is seen in the Eadie-Hofstee plot (v vs v/s), although the K_m of 17.1 μM and V_{max} of 437 mIU $\cdot$ mg^{-1} protein are still within range of error of the Fig.P curve-fit results. The method that stands out as being distinctly different and likely in error is the double reciprocal plot (1/v vs 1/s), in that it gives a K_m which is calculated to be twice that estimated by the other methods and a V_{max} that is also anomalously high.

Figure 19 *Double Reciprocal Plot of Ethanolamine Kinase Activity With Respect to Ethanolamine Concentration*

A graphical representation of a linearized regression fit for the data in Figure 18. The x-axis is the reciprocal of EAK activity (1/v) and the y-axis is the reciprocal of EA concentration (1/s). The double reciprocal plot has the equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{s} + \frac{1}{V_{max}}$$

The least-squares line had an equation of $y=5.080x10^{-5}x + 0.001707$ and a fit of $r^2=0.9809$.

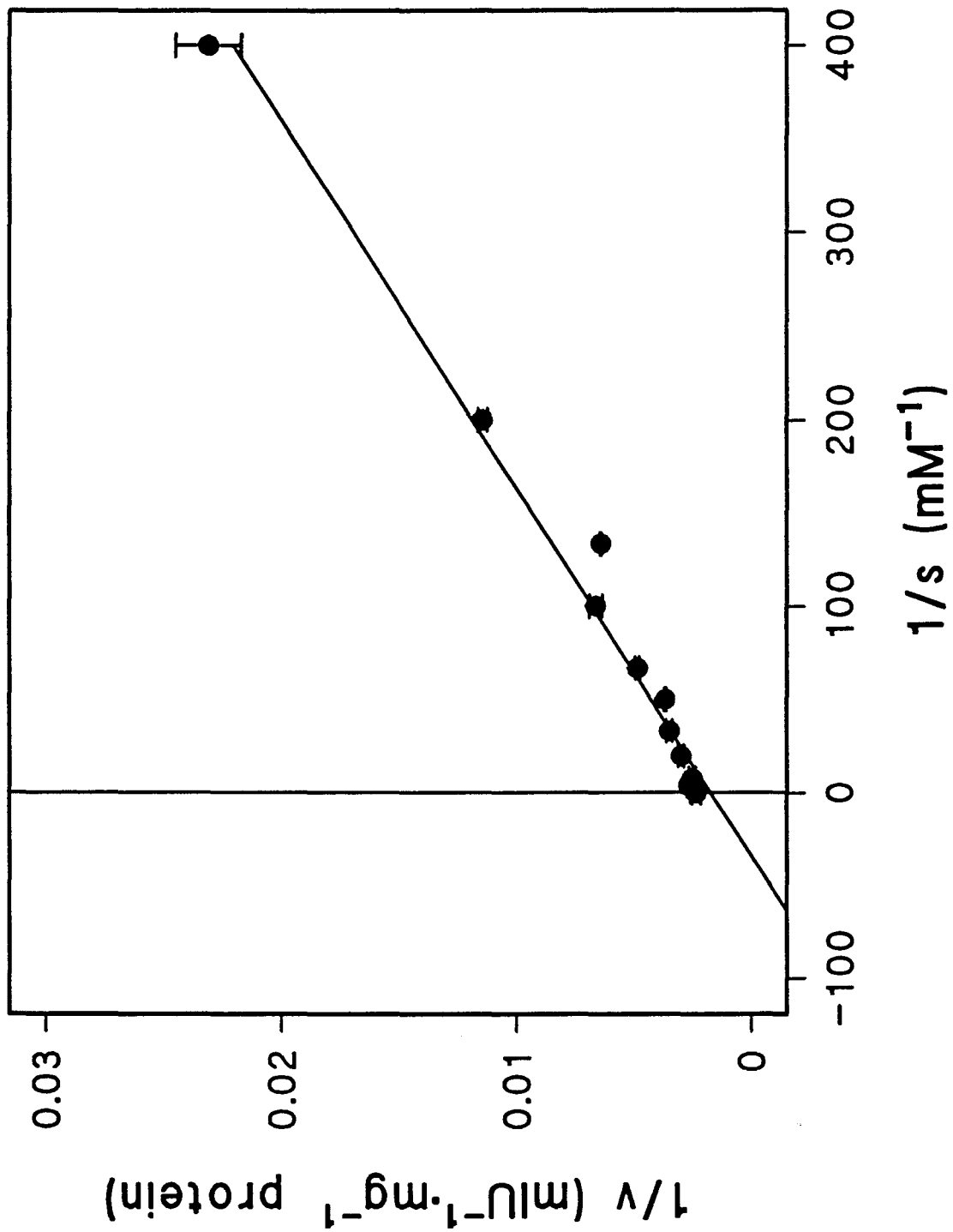

0.03
0.02
0.01
0
1/v (mlU⁻¹·mg⁻¹ protein)
−100
0
100
200
300
400
1/s (mM⁻¹)

Figure 20 *Eadie-Hofstee Plot of Ethanolamine Kinase Activity With Respect to Ethanolamine Concentration*

A linear transformation of the data in Figure 18 is shown, with the x-axis representing EAK activity divided by EA concentration (v/s) and the y-axis representing EAK activity (v). The Eadie-Hofstee Plot has the equation:

$$v = \frac{-K_m v}{s} + V$$

The least-squares fit of the linear regression was $y=-0.017086x + 437.1592$ with $r^2=0.8703$.

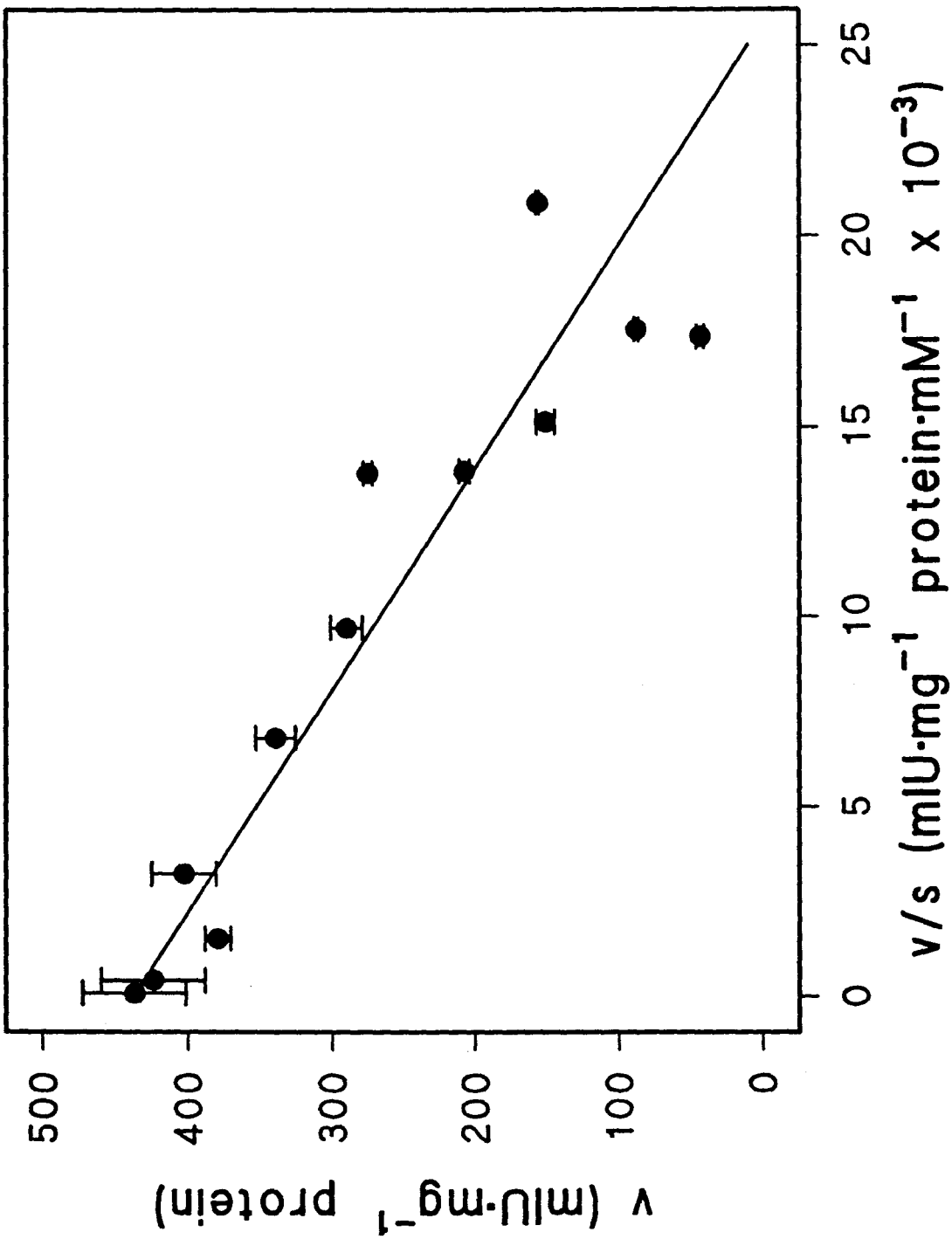

v (mU·mg⁻¹ protein)
500
400
300
200
100
0
0
5
10
15
20
25
v/s (mlU·mg⁻¹ protein·mM⁻¹ x 10⁻³)

Figure 21 *Half Reciprocal Plot of Ethanolamine Kinase Activity With Respect to EA Concentration*

This form of a linear regression of the data from Figure 18 has EA concentration divided by EAK activity (s/v) on the y-axis and EA concentration (s) on the x-axis. The half reciprocal plot has the equation:

$$\frac{s}{v} = \frac{s}{V_{max}} + \frac{K_m}{V_{max}}$$

The least-squares analysis of the linear regression was $y=0.002281x + 4.454x10^{-5}$ with $r^2=0.99996$.

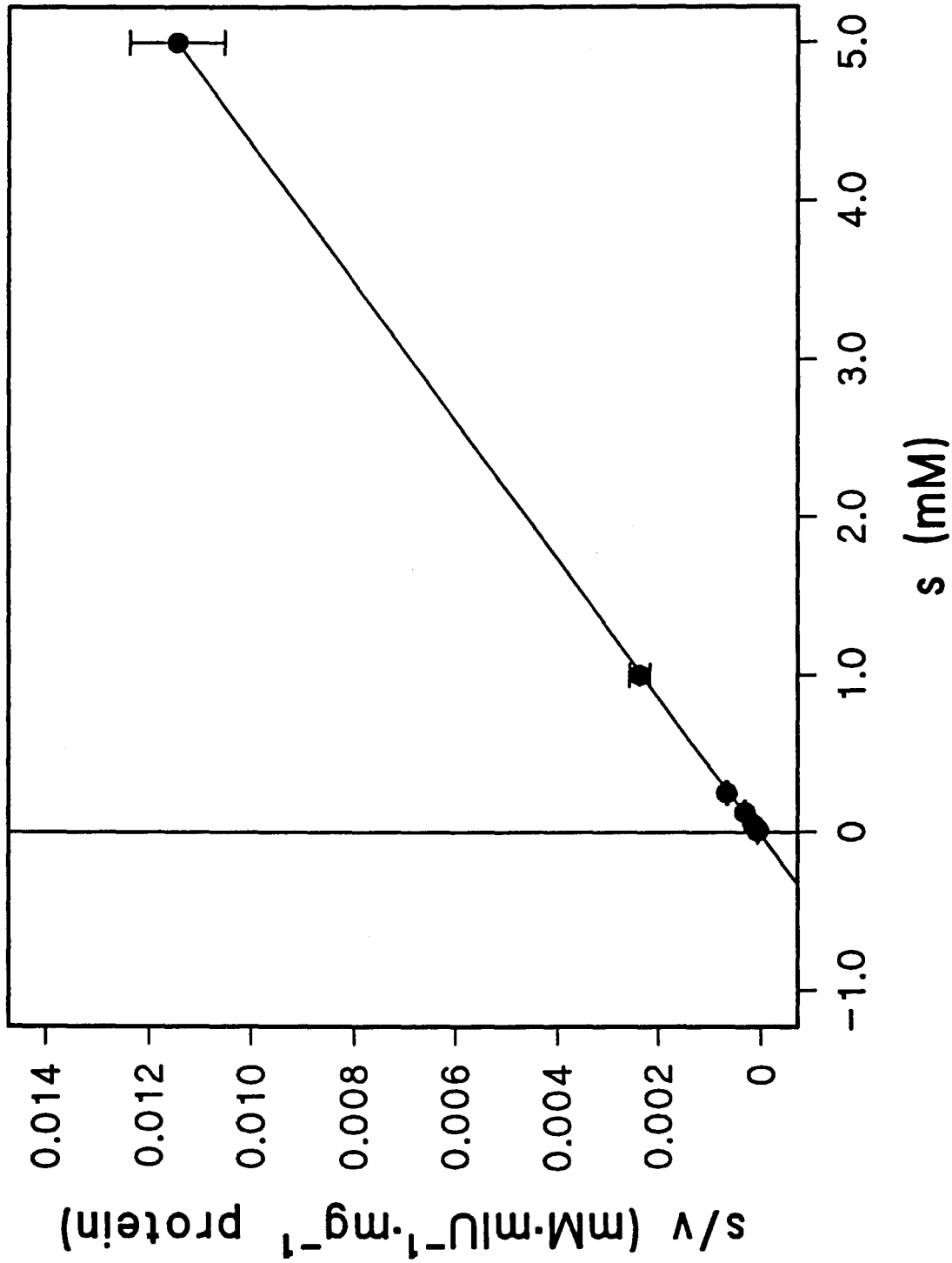

s/v (mM·mU⁻¹·mg⁻¹ protein)
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0
−1.0
0
1.0
2.0
3.0
4.0
5.0
s (mM)

Table XI *Summary of Kinetic Values for Ethanolamine Kinase Activity With Respect to Ethanolamine Concentration Calculated Using Different Methods*

Various methods were used to estimate the K_m for EA and the V_{max} for EAK under optimized assay conditions. Standard errors are only available for the curve-fit using Fig.P (Biosoft).

Method	K_m	V_{max}
	μM	$mIU \cdot mg^{-1}$ *protein*
double reciprocal plot	29.8	587
Eadie-Hofstee plot	17.1	437
half reciprocal plot	15.9	438
direct linear plot	15.5	438
curve-fit (Cosy, Eberhard)	16.0	464
curve-fit (Fig.P, BioSoft)	15.1 ± 1.7^a	430 ± 15

[a]mean $\pm$ S.E.

Effect of Various Choline and Betaine Precursors on Ethanolamine Kinase Activity

Various compounds in the Cho biosynthesis grid (Fig. 1) were tested to see if they had an effect on EAK activity. Optimal assay conditions were used (containing 6 nmol of radiolabelled EA) along with the addition of 60 or 600 nmol of each compound listed in Table XII. Non-radioactive EA added to the assay reduces the EAK activity quite significantly (12% of control activity at 60 nmol and no detectable activity in the 600 nmol EA sample). This reduction in activity is simply the decrease in detectable radioactive PEA being formed due to the dilution of radiolabelled EA available in the assay mixture. For the other free bases, reduced EAK activity is obtained with MEA (38% of control activity at 60 nmol and 4% of control activity at 600 nmol) and DEA (69% of control activity at 60 nmol and 26% of control activity at 600 nmol) but there is no change in EAK activity when Cho is added. Among the phosphobases, the only statistically significant reductions in EAK activity are with PEA and PDEA (32% and 71% of control activity, respectively). All three phosphobases were virtually phosphate-free with PEA contributing 0.621 mM of phosphate per assay and PMEA and PDEA each contributing 0.003 mM of phosphate per assay (Dr. Peter S. Summers, personal communication). Thus, the effects that PEA and PDEA have on reducing EAK activity are due to the phosphobases themselves and not to the presence of free phosphate in the assay.

Since the data of Table XII represents a competition experiment, a spectrophotometric assay was carried out to determine if the reduction in EAK activity by MEA and DEA can be attributed to these compounds serving as substrates

Table XII *Effect of Choline, Betaine and Their Biosynthetic Pathway Intermediates on Ethanolamine Kinase Activity*

EAK activity as affected by the presence of 60 or 600 nmol of various compounds. Data presented are the mean ± S.E. (n=3) for a single experiment.

Compound	Amount Added	EAK Activity	Percent Control[a]
	nmol	*mIU · mg^{-1} protein*	*%*
EA	60	57 ± 4	12
	600	0 ± 1	0
MEA	60	184 ± 7	38
	600	18.2 ± 0.4	4
DEA	60	341 ± 23	69
	600	129 ± 11	26
Cho	600	464 ± 39	95
Serine	600	468 ± 30	95
PEA	600	156 ± 39	32
PMEA	600	495 ± 34	101
PDEA	600	349 ± 6	71
PCho	600	495 ± 20	101
BetAld	600	487 ± 23	99
Betaine	600	448 ± 47	91

[a]EAK Activity of Control = 490 ± 8 mIU · mg^{-1} protein

as opposed to inhibitors. Table XIII suggests that MEA and DEA are slightly better substrates for EAK than EA. EAK affinity for MEA and DEA would best be assessed by estimates of K_m and V_{max} but there was insufficient sample to attempt these analyses. Although there is more sample available that is less pure, the spectrophotometric assay is less sensitive and subject to more interference than the radioisotope assay so that only a highly purified EAK preparation would be suitable to determine these substrate affinities. Cho as a substrate yields a low, but still detectable EAK activity (Table VII) which was not unanticipated since ChoK activity was detected in the enzyme sample using a more sensitive radioactive assay. Serine and tyrosine are not suitable substrates.

Effects of Compounds on Ethanolamine Kinase Activity

While the purification scheme for EAK was being elucidated, the effects of various ions commonly used in buffer systems for EAK activity were investigated. In Table XIV, the addition of 10 mM of each salt does not appear to have an effect on EAK activity except in the case of LiCl which shows a slight stimulation. When the salt concentration is raised to 100 and 200 mM the effects of the various ions on EAK activity are more apparent. Salts that give a consistent increase in activity include NaCl and KCl (at levels 10^3 fold higher than in the optimized assay) while NH_4Cl, $(NH_4)_2SO_4$, and KH_2PO_4 all produce a decrease in EAK specific activity. In the case of LiCl, EAK activity goes up with 10 and 100 mM LiCl and then down with 200 mM LiCl (Table XIV).

The effect of various divalent cations on EAK activity was analyzed. As

Table XIII *Possible Alternative Substrates for Ethanolamine Kinase*

Possible substrates for EAK were tested using the spectrophotometric assay for EAK as described in the Materials and Methods section.

Substrate	Repeats	EAK Activity[a]
	#	$mIU \cdot mg^{-1}$ *protein*
EA	5	21.5 ± 0.9
MEA	4	38 ± 3
DEA	2	36 ± 5
Cho	2	5.0 ± 0.2
Serine	1	ND[b]
Tyrosine	1	ND

[a]mean ± S.E. [b]not detected

Table XIV *Effects of Various Ions on Ethanolamine Kinase Activity*

Data presented are the mean ± S.E. (n=3) for a single experiment for each condition.

Compound	Final Assay Concentration	EAK Activity	Percent Control[a]
	mM	*mIU·mg^{-1} protein*	*%*
NH_4Cl	10	609 ± 3	105
	100	574 ± 21	99
	200	333 ± 37	57
$(NH_4)_2SO_4$	10	575 ± 110	99
	100	339 ± 16	58
	200	179 ± 10	31
KH_2PO_4	10	621 ± 38	107
	100	407 ± 6	70
	200	80 ± 20	14
NaCl	10	575 ± 35	99
	100	757 ± 54	130
	200	657 ± 53	113
KCl	10	519 ± 31	89
	100	599 ± 71	103
	200	715 ± 80	123
LiCl	10	715 ± 53	123
	100	791 ± 82	136
	200	441 ± 16	76

[a]EAK Activity of Control = 581 ± 20 mIU·mg^{-1} protein

shown by Table XV, Mn^{2+} reduced the activity of EAK to almost background at 5 mM. Note that this concentration of Mn^{2+} is equivalent to that of Mg^{2+} (5 mM). Reduction in EAK activity with Ca^{2+} is almost as dramatic (a 90% loss of activity at 5 mM). Co^{2+} and Ba^{2+} decrease EAK activity to about half while Ni^{2+} had less of an effect on EAK activity (a loss of only 30% of activity at 5 mM).

ADP also inhibits EAK activity (Table XVI). At 5 mM ADP (equivalent to the ATP level), EAK activity is halved. At 50 mM, 10 fold the ATP level used in the optimal assay (5mM), EAK activity is almost reduced to background levels. Thus ADP appears to be an effective inhibitor of EAK activity. The true effectiveness of ADP inhibition could only be estimated by a K_i determination, but there was insufficient sample for this analysis.

Table XV *Effect of Divalent Cations on Ethanolamine Kinase Activity*

EA Kinase was assayed in the presence of 5 mM Mg^{2+} with an additional 1 fold, 2 fold or 10 fold concentration of each ion tested. Data presented are the mean ± S.E. (n=2) for a single experiment for each condition.

Compound	Final Assay Concentration	EAK Activity	Percent Control[a]
	mM	*mIU·mg^{-1} protein*	%
$MnCl_2 \cdot 4H_2O$	5	35 ± 17	6
	10	18 ± 2	3
	50	7.7 ± 0.1	1
$CaCl_2 \cdot 2H_2O$	5	55 ± 8	10
	10	40 ± 1	7
	50	20 ± 15	3
$CoCl_2 \cdot 6H_2O$	5	256 ± 5	44
	10	129 ± 23	22
	50	54 ± 25	9
$BaCl_2$	5	272 ± 34	47
	10	238 ± 11	41
	50	96 ± 1	17
$NiCl_2 \cdot 6H_2O$	5	410 ± 44	71
	10	366 ± 57	63
	50	11 ± 1	2

[a]control = 581 ± 20 mIU·mg^{-1} protein

Table XVI *Effect of ADP Concentration on Ethanolamine Kinase Activity*

The concentrations used were 1 fold, 2 fold and 10 fold that of ATP (5 mM) in the assay. Data presented are the mean ± S.E. (n=3) of a single experiment.

Final Concentration of ADP	EAK Activity	Percent Control[a]
mM	*mIU·mg^{-1} protein*	*%*
5	192 ± 7	49
10	126 ± 2	32
50	27 ± 1	7

[a]control = 396 ± 8 mIU·mg^{-1} protein

DISCUSSION

Salinization and Ethanolamine Kinase Activity

In salinized spinach plants EAK activity is increased about 1.5 fold (Weretilnyk and Summers, 1992). Figure 3 shows that EAK plant extract from plants salinized to 200 mM has a specific activity that is 2.2 fold greater than that of the control plant extract, which is comparable to the results of Weretilnyk and Summers (1992). The migration patterns of both of these extracts using Native-PAGE is compared in Figure 3 and shows that EAK activity is located at the same position of 0.6 R_f units. This suggests that there is only one EAK enzyme present in both salinized and control plants and that the increased activity is not likely due to the up-regulation of a second, electrophoretically distinct isozyme.

Resolving enzyme activity in gels does not conclusively show that there is truly only one EAK enzyme present in spinach plants. However, if there are multiple forms of EAK present, then they are electrophoretically indistinguishable. In a few other investigations various isozymes of EAK and ChoK have been shown to be readily separable. Different isozymes containing both EAK and ChoK activity in rat kidney, liver, lung and intestinal cytosols were differentially separated by Ishidate *et al* (1985b). Two forms of ChoK in spinach were separated by Macher and Mudd (1976) and in this thesis (Fig. 12). The presence of only one form of EAK in spinach makes it likely that EAK activity is somehow up-regulated by increased salinity

levels. Purification and biochemical characterization of EAK is necessary in order to obtain more information about EAK in spinach.

Purification of EAK from non-salinized tissue is desirable because 80% more non-salinized spinach leaves can be produced in the same amount of growth cabinet space as salinized spinach. Also the watering of plants using normal Hoagland's solution is less time consuming since during salinization care must be taken to insure that no solution touches the plant leaves. Large amounts of spinach leaf tissue is required since EAK activity measurements suggest that this enzyme is not an abundant protein in spinach plants. This can be seen from the estimates of total protein present in the crude samples compared to the limited amount of homogenous EAK produced (only 0.02% of total protein was recovered as EAK) (Table III). The estimate for the abundance of choline oxidation enzymes places the upper limit for these enzymes at 0.01% of total soluble protein (Hanson *et al*, 1986) which is likely to be similar for EAK. The minimum amount of purified protein required for amino acid sequencing is 5-10 pmol (Hunkapiller and Hood, 1983) and for raising antibodies up to 10 mg may be required (Klymkowsky and Dutcher, 1984). Obtaining even these minute quantities becomes a major technical challenge (Hanson *et al*, 1986) when isolating an enzyme as limited in supply as EAK. The task is even greater if biochemical characterization is required, since much more purified enzyme is required. In this thesis, 7 kg of spinach leaves (see Appendix) were required for my purification scheme and there was still insufficient homogeneous EAK for detailed biochemical analyses. If there is only one isozyme of EAK (as suggested by Native-PAGE), then a more practical source of EAK would be from unsalinized plants, thus unsalinized

spinach plants were used for the purification of EAK.

Ethanolamine Kinase Purification

EAK is found in the supernatant of crude leaf extracts centrifuged to 10,000 • *g*, consistent with a soluble enzyme, and has been purified to apparent homogeneity from spinach leaves (Figure 10). In this study, all of the EAK purification steps listed in Table III eliminated many contaminating proteins, but a key purification step was the phenyl-Superose HPLC column. This hydrophobic chromatography step produces a single polypeptide band as shown by SDS-PAGE (Fig. 10, lane 8). The phenyl-Superose step resulted in the separation of EAK away from a polypeptide pigmented with a yellow colouration which co-purified with EAK in all previous steps (Fig. 10, lanes 1-7). The purified EAK has a specific activity of 362 mIU • mg^{-1} protein which is a 6,537 fold increase from the specific activity of crude extract (0.055 mIU • mg^{-1} protein). This is significantly greater than the previously published attempt from spinach leaves by Macher and Mudd (1976). Macher and Mudd (1976) did a preliminary investigation into the biochemical characteristics of EAK from store bought spinach and obtained a specific activity of 14.80 mIU • mg^{-1} protein which was 59.2 fold over the specific activity of their crude extract.

Warfe and Harwood (1979a) purified EAK from germinating soybean to what the authors claimed was apparent homogeneity. Their final specific activity was 10,730 mIU • mg^{-1} protein with a fold purification of 864 over that of crude extract. The specific EAK activity reported from soybean is 30 fold higher than that obtained

for pure EAK in spinach leaves (Table III) but the purification is 8 fold lower. This could be due to a greater abundance of EAK enzyme in germinating soybean seeds and a lower abundance of EAK with higher activity in the mature spinach leaves or possibly that the purified soybean EAK was not truly homogeneous. Warfe and Harwood (1979a) claim that the enzyme was purified to apparent homogeneity but did not include a gel in their publication so the homogeneity of their preparation cannot be confirmed. They also stained with Coomassie brilliant blue which stains proteins with a 100 fold lower sensitivity than the silver staining technique employed in the current study (Switzer *et al*, 1979). This means that impurities present in the soybean extract may not have shown in their analysis, suggesting that the differences found in activity and fold purification values could be due to purity differences, not necessarily a species or tissue type difference between EAK from spinach leaves and germinating soybean seeds.

Ethanolamine Kinase and Choline Kinase

This study adds to the existing evidence that EAK is a distinct enzyme from ChoK in plants. It appears that there are at least two distinct ChoK enzymes (Fig. 12) and only one EAK enzyme in spinach (Fig. 3, Fig. 12). The results shown in Figure 12 agree with the results obtained by Macher and Mudd (1976) who found ChoK activity in their partially pure EAK sample that could be electrophoretically separated from the activity of EAK. In the study by Macher and Mudd (1976), there appeared to be a minor peak of ChoK activity coinciding with the EAK peak which is more pronounced in this study (Fig. 12). This ChoK peak was not reported as being

significant by the authors but in light of the results of the current study, this may suggest that EAK would have some very low capacity to phosphorylate choline in spinach leaves. This is supported by the low residual amount of ChoK activity present in the homogenous preparation of EAK (Fig. 10) but the EAK:ChoK ratio is over 1,500:1 (Table VII).

Protein Concentration and its Effects on Linearity of Ethanolamine Kinase Reaction and Enzyme Stability

Throughout the purification of EAK, enzyme activity was not linear with protein concentration. This was corrected for by diluting the EAK-containing extract with buffer during the assay. This non-linear property of EAK activity is not unusual. Infante and Kinsella (1976) determined that in lactating bovine mammary gland, EAK and ChoK had distinct active sites possibly on the same protein since only EAK activity showed a non-linear enzyme activity with respect to increasing protein concentration. This is in contrast with the study by Macher and Mudd (1976) with EAK in spinach leaves where product formation increased linearly with added protein. This may be due in part to the very low protein concentration of the partially pure EAK extract that they obtained (0.04 $mg \cdot mL^{-1}$). The EAK present in this sample might not have reached the critical concentration where further dilution would be required for linearity in an assay.

For the EAK from spinach in this study, one possible explanation for non-linearity is that there is a build up of the reaction products Mg^{2+}-ADP (Table XVI) and PEA (Table XII) which have been shown to inhibit enzyme activity

in vitro. Dilution of the enzyme sample to be assayed would decrease the concentrations of these inhibitory compounds. As samples became more highly purified, the dilution factor continually needed to be increased, supporting this explanation (data not shown).

EAK purified from spinach leaves in this study was stable to repeated freeze thaw cycles provided the total protein concentration was kept above approximately 1 $mg \cdot mL^{-1}$ (data not shown). The EAK from spinach showed low activity when dilute but EAK activity was recovered by concentrating the sample to greater than 1 $mg \cdot mL^{-1}$ protein, showing that the activity losses were not irreversible. Many researchers have reported that EAK becomes unstable when highly purified, but the effect of sample concentrations were not considered. Macher and Mudd (1976) found that spinach leaf EAK was stable in crude preparations (protein concentration of 5.24 $mg \cdot mL^{-1}$) but was unstable when the sample was pure but less concentrated (protein concentration of 0.04 $mg \cdot mL^{-1}$). The reported instability may therefore have been due to dilution effects. Similar losses of activity were reported for rat liver EAK (Weinhold and Rethy, 1974), and in *Culex pipiens fatigans* EAK (Ramabrahmam and Subrahmanyam, 1981) but in these cases the protein concentrations were not given so a comparison cannot be made. It may be that for these reported cases of EAK instability, the activity may also have been lost by dilution effects on EAK activity.

In the purification of ChoK and enzymes with both ChoK and EAK activities, BSA is commonly added to prevent inactivation. Such is the case of ChoK purified from rat liver where BSA was added to the enzyme extract at a concentration of

2 mg • mL^{-1} for stabilization (Brophy *et al*, 1977). For the purification of ChoK from various animal and plant species, BSA was often added to the initial purification steps as a precaution against protease activity, such as for the purification of ChoK from soybean (Warfe and Harwood, 1979b) but in many cases it was used to counteract dilution effects. In rat liver, ChoK purified sample became inactivated during dialysis unless BSA was added (Brophy and Vance, 1976). In rat kidney Ishidate and Nakazawa (1992) keep protein concentration at about 20 mg • mL^{-1} before dialysis of the purified sample. Concentration (dilution) may therefore affect stability more than purity.

Biochemical Characterization of Ethanolamine Kinase

Since there was insufficient pure EAK to use for both sequencing as well as for all of the biochemical analyses, the less pure ω-aminohexyl-agarose sample from Purification III (Table V) was used for the biochemical analyses. This sample, though not completely pure (Fig. 11, lane 7), was useful since it had no detectable phosphobase phosphatase activity (Table VI), although it did have some ChoK activity (45 mIU • mg^{-1} protein, 0.2 fold of EAK activity) (Table VII).

Phosphobase Phosphatase

The determination of phosphobase phosphatase activity in EAK containing extracts was crucial before further biochemical analyses were performed. That is, since EAK produces a phosphobase (PEA), any phosphatase capable of hydrolysing this product could give rise to anomalously low estimates of EAK activity. Also,

EAK seems to be inhibited by phosphate, PEA and PDEA (Table XII, Table XIV), and MEA and DEA also serve as substrates (Table XIII). This means that any phosphobase phosphatase activity in a sample would have profound effects on the measurements of enzyme kinetics for EAK.

Choline Kinase

The ChoK activity (7.6 mIU $\cdot$ mg^{-1} protein) (Table VII) is possibly due to the presence of a ChoK enzyme in the ω-aminohexyl-agarose sample from Purification III. Native-PAGE could be used to separate EAK from ChoK, but this technique would require at least 100 μg of sample to be able to detect the ChoK activity and there was not enough sample remaining after all of the biochemical analyses to address this possibility.

In the future, all of the ChoK enzyme in the sample will have to be eliminated before the K_m values for free base substrates are determined. Also, if ChoK is not removed from the sample, the issue of whether ChoK could phosphorylate EA should be investigated before making an estimate of the K_m values for Mg^{2+} and ATP. In this study it appears that ChoK was not used as a substrate by EAK. This is shown by the ratio of EAK activity to ChoK activity in the ω-aminohexyl-agarose sample from Purification III being 5:1 (Table VII) and the ratio of EAK activity with EA as a substrate to EAK activity with Cho as a substrate being 4.3:1. Thus all of the Cho used as a substrate could be accounted for by the ChoK activity present in the sample.

Molecular Weight Estimation for Ethanolamine Kinase

The native molecular weight of EAK in spinach plants appears to be 80,000 D (Fig. 13). By denaturing SDS-PAGE, a single polypeptide of 38,000 D is found (Fig. 14). This is consistent with EAK being a dimer. In the previous spinach EAK study (Macher and Mudd, 1976), a native M_r of 110,000 was estimated by gel filtration on Sephadex G-200. This method of molecular weight determination, using an open-bed chromatography column, is not as accurate as the method employed in this study which uses an analytical HPLC chromatographic column. The difference in molecular weight between the two studies is likely to be a reflection of the technologies used. Macher and Mudd (1976) did not determine subunit molecular weight.

In germinating soybean (Warfe and Harwood, 1979a), gel filtration using Bio-Gel P-100 was used to estimate a native M_r of 37,000 for EAK, with a subunit weight of 17,000 D. SDS-PAGE revealed that the subunit weight was actually 19,000 D and the peak with the higher molecular weight off of the gel filtration column contained three bands, 18,000, 35,000 and 45,000 D. The interpretation given by the authors for these results is that the enzyme can associate-dissociate to give monomeric and dimeric forms, with the 17,000-19,000 D EAK associating to give the 35,000 D higher molecular weight form. The 35,000 D form of soybean EAK is similar in size to the 38,000 molecular weight for spinach leaf EAK polypeptide shown in Figure 14. These differences in polypeptide and native M_r for EAK in the two studies indicates that there are different EAK enzymes in the two different tissues (leaves and germinating seeds) or between the two different plants.

Ramabrahmam and Subrahmanyam (1981) determined the Native M_r of EAK from *Culex pipiens fatigans* to be 44,000 D by gel filtration (Sephadex G-200). Ishidate *et al* (1984) found that EAK in rat kidney had a molecular weight of 42,000 D by SDS-PAGE forming a dimer of 75-80,000 D. Weinhold and Rethy (1974) purified EAK and ChoK from rat liver and found that a peak with only EAK activity had a Native M_r of 36,000 and a peak with both EAK and ChoK activities had a Native M_r of 166,000 (by Sephadex G-200). Uchida and Yamashita (1992) give molecular weight for rat brain EAK of 44,000 D by SDS-PAGE and a Native M_r of 87,600 by Superose-12 chromatography. In human liver, EAK was found to have a molecular weight of 42,000 D by SDS-PAGE analysis (Draus *et al*, 1990).

Ishidate *et al* (1985b) found that there was a diversity in molecular forms of ChoK in rat tissues and that the differences shown by Native-PAGE are partially due to the difference in their molecular size. Thus, the size of EAK is not necessarily constant between species. Spinach EAK is closest in agreement with the study by Ishidate *et al* (1984) giving a Native M_r of 75-80,000 for rat kidney EAK with subunits of 40-45,000 D.

pH Optimum

Figure 15 shows that EAK from spinach has a broad basic pH optimum from pH 7 to 9. This pH optimum agrees with the pH dependence curves shown in many EAK purifications. The only buffers which seem to inhibit EAK activity are glycine and tricine (Fig. 15), and phosphate buffers which were avoided due to the inhibitory effects of PO_4^{-3} ions on spinach EAK activity (Table XIV). In spinach leaves Macher

and Mudd (1976) found that EAK activity was maximal at the highest range of all the buffers used (HEPES, Tris-HCl and Tris-glycine from pH 7.5 to 9.5) with Tris-glycine providing the greatest activity. The glycine containing buffer which yielded the highest EAK activity in Macher and Mudd's (1976) study were found to be strongly inhibitory in the current study.

Glycine containing buffers yield the highest EAK activity in many animal species. In lactating bovine mammary gland EAK had a pH optimum of 8 (Infante and Kinsella, 1976) with Hepes slightly inhibitory on EAK activity but the highest EAK activity was found with bicine and glycylglycine buffers. In rat liver EAK (Weinhold and Rethy, 1974), the pH optimum was 7 to 8.5 using glycylglycine and Tris-maleate with both having equal effects on EAK activity. Kunishita *et al* (1987) found that the EAK from rat brain myelin had a pH optimum of 8.5 to 8.6 using glyclglycine and Ches with each having equal effects on EAK activity. In EAK from rat Ehrlich ascites tumour cells a pH optimum from 8.5 to 10.5 was found (Sung and Johnstone, 1967). Glycine was not inhibitory but the highest EAK activity was seen using sodium phosphate with Tris-HCl. Glycylglycine was slightly inhibitory and glycine had no effect. In soybean, it appears that glycylglycine does not inhibit EAK activity (pH optimum of 8.5) but Warfe and Harwood (1979a) did not try any buffers other than glycylglycine.

Mg^{2+} and ATP Requirements for Maximal Ethanolamine Kinase Activity

An optimum Mg^{2+} to ATP ratio of 1:1 at 5 mM was found for EAK from spinach leaves in the present study (Fig 17, Table IX, Table X). In the previous

study on spinach leaf EAK Macher and Mudd (1976) have shown that Mg^{2+}:ATP is required in a 2:1 ratio with ATP being 0.9 mM and a K_m of 63 μM for a Mg^{2+}-ATP. All investigations to date have found that EAK requires Mg^{2+} and ATP, generally at ratios of 1:1 or 2:1, except for human liver EAK where the ratio was found to be 3:2 Mg^{2+}:ATP (Draus *et al*, 1990).

The requirements for Mg^{2+} are variable. For example, in the case of rat kidney EAK, at 3 mM Mg^{2+}, a 1:1 ratio of Mg^{2+}:ATP was preferred but at 5 mM Mg^{2+}, a 2:1 ratio was preferred (Ishidate *et al*, 1985a). Also, for rat kidney EAK Mg^{2+}-ATP higher than 16 mM was inhibitory (Ishidate *et al*, 1985a). This change in ratio requirements suggests that Mg^{2+}-ATP is a substrate but free Mg^{2+} is also required in the reaction (Weinhold and Rethy, 1974). Kasinathan *et al* (1983) found two dermatophytes showing different Mg^{2+}:ATP ratio preferences with *Epidermophyton floccosum* having a 2:1 Mg^{2+}:ATP ratio and *Microsporum gypseum* a 1:1 ratio.

In this study there was not enough sample to determine the K_i values for ATP and Mg^{2+} but both ATP and Mg^{2+} seemed inhibitory when present in amounts greater than 5 mM. In Figure 17 when 10 mM $MgCl_2$ is used with ATP held at 5 mM, EAK activity decreases (-▵-) and when 10 mM ATP is used with $MgCl_2$ held at 5 mM, EAK also decreases (-▲-), as is also the case when both are at 10 mM (-▵-). This inhibition was also seen in the spinach EAK study by Macher and Mudd (1976) with the enzyme inhibited by Mg^{2+}-ATP with an apparent K_i of 6.7 mM.

Kinetic Analyses

In this study the K_m for EA was 15.5 μM and the V_{max} for the reaction was 438 mIU $\cdot$ mg^{-1} protein. This K_m value is much lower than the 42 μM obtained in the previous spinach study (Macher and Mudd, 1976). Since Macher and Mudd (1976) calculated their K_m value using the double reciprocal plot this value may be higher for that reason alone. However, the K_m calculated using the double reciprocal plot in this study would be 29.8 μM, which is still lower than Macher and Mudd's (1976) 42 μM.

The 8 μM K_m (calculated using the double reciprocal plot) for soybean EAK is much lower than that obtained for spinach EAK in this study (Warfe and Harwood, 1979a). It appears that soybean EAK has a higher affinity for EA than does spinach EAK.

Competition and Substrate Affinity

MEA and DEA competitively inhibit EAK activity with Cho not having an effect on EAK activity (Table XII). These results are in agreement with the results found in the previous spinach EAK study (Macher and Mudd, 1976). Macher and Mudd (1976) did not test MEA and DEA as substrates and in this study MEA and DEA are shown to serve as substrates for EAK, yielding a higher specific activity than EA at comparable concentrations of substrate and enzyme (Table XIII). Though Cho does not competitively inhibit EAK activity (Table XII), Cho did show some ability to be used as a substrate (Table XIII) but this activity may have been due to the presence of ChoK in the enzyme sample as discussed above. Serine did not affect

EAK activity in the present study (Table XIII). Neither serine nor tyrosine were suitable as substrates (Table XIII) reducing the possibility that EAK is a protein kinase.

The inhibition patterns of MEA, DEA and Cho are similar for both EAK and ChoK isolated from other species. In the EAK from the fat body of *Phormia regina* larvae, a similar pattern of inhibition by MEA, DEA and Cho was seen (Shelley and Hodgson, 1971). In the EAK from soybean, Cho did not compete even at 10 mM (100 fold) concentration (Warfe and Harwood, 1979a). In the EAK from *Plasmodium falciparium*, EAK showed weak inhibition by Cho. Neither Cho nor serine had an effect on EAK activity in *Culex pipiens fatigans* (Ramabrahmam and Subrahmanyam, 1981). Haubrich (1973), using [γ-^{32}P]ATP, found that rabbit brain ChoK could also use EA, MEA, and DEA as substrates. In rat liver, MEA kinase and DEA kinase are distinct enzymes from ChoK and EAK (Cao and Kanfer, 1991). In ChoK from yeast, DEA, MEA, EA, diethylethanolamine (DEEA) and monoethylethanolamine (MEEA) act as substrates with this order: Cho > DEA > DEEA > MEA > MEEA > EA and serine (0.01 M) yields no activity (Wittenberg and Kornberg, 1953).

Not only did Macher and Mudd (1976) not test substrate affinity for EAK but they did not test the effects of phosphobases on EAK activity. In this study PEA and PDEA were found to inhibit EAK activity (Table XII). The inhibition of EAK by PEA is not surprising since PEA is a product of the reaction. PEA was also found to be inhibitory to EAK in *Culex pipiens fatigans* (Ramabrahmam and Subrahmanyam, 1981) in a competitive manner. The inhibition of EAK by PDEA (Table XII) could be feed-back inhibition regulating EAK and/or perhaps having to do with a

coordination of regulation with the *N*-phosphomethyltransferases. It is interesting to note that PMEA and PCho are not inhibitory to EAK activity (Table XII). This selective behaviour between an enzyme and seemingly structurally similar metabolites is seen in the case of castor bean endosperm phosphocholine cytidylyltransferase (catalyzing the formation of CDP-choline from CTP and phosphocholine) in that this enzyme can use PMEA and PCho as substrates equally but not PEA or PDEA (Wang and Moore, 1990).

In rat liver, EAK is inhibited by PEA but is also inhibited by Cho, betaine and PCho (Brophy *et al*, 1977). In EAK from animal sources the regulation of EAK is quite different, perhaps pointing towards a different pathway from that in Figure 2 for Cho and phospholipid biosynthesis.

Effects of Ions on Ethanolamine Kinase Activity

Many ions seem to affect EAK activity that do not participate directly in the reaction. From Table XIV it appeared that NH_4^+ and PO_4^{-3} and possibly SO_4^{-2} decreased EAK activity when present at levels above 100 mM. The uncertainty involved with the inhibition of EAK by $(NH_4)_2SO_4$ is that the lower EAK activity could be caused by NH_4^+ alone or both NH_4^+ and SO_4^{-2}. An experiment that would resolve whether SO_4^{-2} is inhibitory would be to test the effects of Na_2SO_4 on EAK activity since Na^+ is not inhibitory (Table XIV). There are no other reports of these ions affecting EAK activity in other investigations.

There is a slight increase in EAK activity when 200 mM Na^+ and K^+ are added to the assay. Li^+ seemed to increase activity at 100 mM and reduce it at

200 mM (Table XIV). Also, EAK activity showed no net change when KCl was added to the assay in amounts from 8 μM to 1000 μM (Table VIII). Though the 8 μM used in Table VIII is essentially a negligible amount of KCl, a more detailed KCl requirement profile including a case without any KCl should be tried. The results from both Tables VIII and XIV suggest that EAK may not be affected by low KCl levels (less than 100 mM) and that KCl may not be required in the EAK optimized assay.

The increase in EAK activity by the alkali metal ions in spinach is not unusual for EAK enzymes. In EAK from rat Ehrlich ascites tumour cells, Na^+, K^+, Li^+ and Cs^+ increase activity (Sung and Johnstone, 1967). In EAK from rat brain myelin, activity was increased by KCl (Kunishita *et al*, 1987). This activation by these cations is not universal and in EAK from *Culex pipiens fatigans* 10 mM KCl was inhibitory (Ramabrahmam and Subrahmanyam, 1981).

KCl levels in the non-vacuolar portion of plant cells of spinach is maintained at 100 to 150 mM (Leigh and Wyn Jones, 1984) and this level increases with salinization of spinach plants as long as K^+ is available for uptake by roots (Chow *et al*, 1990). Generally enzymes that are activated by K^+ are usually activated by Rb^+ and NH_4^+ but are activated little by Na^+ and not at all by Li^+ (review: Evans and Sorger, 1966). Both Na^+ and K^+ form coordinated complexes with ATP and ADP (Melchior, 1965) but steric considerations prevent both ions together from being used by one enzyme. Cation-ATP complexes often affect enzymatic reaction rates (Lowenstein, 1960). Neither Melchior (1965) nor Lowenstein (1960) take into consideration that the concentration of K^+ needed for enzyme activation usually is

much greater than that of any substrate or cofactor (Evans and Sorger, 1966). More recently some researchers have suggested that most enzymes show similar responses to Na^+ and K^+ *in vitro* (Flowers *et al*, 1977). Perhaps the enzyme has two different sites that Na^+ and K^+ act upon. These two electrolytes often increase the K_m of an enzyme without altering V_m (Flowers *et al*, 1977). When EAK has been cloned, molecular characterization will perhaps provide some information on the mechanisms and regulatory effects of various ions on EAK activity.

Divalent Ions and Their Effect on Ethanolamine Kinase Activity

EAK uses Mg^{2+} in conjunction with ATP. When divalent ions are added to an assay containing 5 mM Mg^{2+}, EAK activity is inhibited by 5 mM of various divalent cations (Table XV) in decreasing order: $Mn^{2+} > Ca^{2+} > Co^{2+} > Ba^{2+} > Ni^{2+}$. These inhibitions occur with Mg^{2+} present so a useful experiment would be to try substituting Mg^{2+} with some of the ions to see if they would still yield EAK activity. In such an experiment EAK activity is likely to remain inhibited since even Mn^{2+}, which can often take the place of Mg^{2+} when added to the EAK assay, produces only 0.06 fold the activity. If Mn^{2+} was as effective as Mg^{2+} then the level of EAK activity should be closer to 0.90 fold (see 10 mM $MgCl_2$ data in Fig. 17).

In *Culex pipiens fatigans* Ca^{2+}, Cu^{2+}, and Ni^{2+} all inhibited the activating effect of Mg^{2+} on EAK with Mn^{2+} more stimulatory than Mg^{2+} at concentrations below 5 mM (Ramabrahmam and Subrahmanyam, 1981). In spinach EAK, Ca^{2+} was a very potent inhibitor (Macher and Mudd, 1976). For EAK from soybean, Mn^{2+} and Ca^{2+} decreased EAK activity when included in an assay along with Mg^{2+}. Mn^{2+}

is also able to substitute for Mg^{2+} yielding 0.58 fold the control EAK activity (Warfe and Harwood, 1979a). In EAK from rat Ehrlich ascites tumour cells, Ca^{2+} and Mn^{2+} were inhibitory and neither could substitute for Mg^{2+} (Sung and Johnstone, 1967).

ADP Inhibition of Ethanolamine Kinase Activity

Inhibition of EAK activity by ADP (Table XVI) is not surprising since ADP is one of the products of the reaction. Macher and Mudd (1976) also found that EAK was inhibited by ADP but at 1 fold, 2 fold and 10 fold the ATP (1.8 mM) concentration activity was 0.58, 0.48 and 0.20 fold, respectively which is less inhibition than seen in Table XVI (0.49, 0.32 and 0.07 fold, respectively). It is not known how ADP acts to decrease EAK activity or how effective an inhibitor ADP is without the determination of the K_i of EAK for ADP. In only one investigation has the kinetics of ADP inhibition of EAK been investigated and in that study ADP noncompetitively inhibited *Culex pipiens fatigans* EAK with a K_i of about 1.8 mM (Ramabrahmam and Subrahmanyam, 1981). Ramabrahmam and Subrahmanyam (1981) also found that AMP and adenosine inhibit EAK activity noncompetitively to the same extent as ADP.

Future Directions

EAK appears to have very different properties in the two plant species studied to date. The differences between EAK from spinach leaves and EAK from germinating soybean may in some cases be due to technical differences, but it seems more likely that the differences are either species or tissue specific. Warfe and

Harwood (1979a) studied EAK isolated from a non-photosynthetic tissue so it would be interesting to see how the properties of EAK from soybean leaves compare with that of EAK from spinach leaves. Comparisons cannot be made between species or tissues until the biochemical characteristics of EAK are known in great detail from at least one species. The production of purified EAK in this study will help obtain peptide sequence information for EAK and allow the use of molecular genetic techniques to provide further information. This genetic information along with the production of antibodies to spinach EAK will allow questions about the relatedness between the EAK enzymes in soybean and in spinach to be answered.

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APPENDIX

Table XVII *Details of the Crude Extract (A) and Ammonium Sulphate Fractionations*

Step[a]	[Protein]	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	$mg \cdot mL^{-1}$	*mL*	*mg*	*mIU*	$mIU \cdot mg^{-1}$ *protein*	*fold*	%
Crude (A)[b]	4.90	2170	10633	681	0.06	1	100
<2.2 M $(NH_4)_2SO_4$	36.76	315	11580	69	0.01	0.1	10
2.2-3.2 $(NH_4)_2SO_4$	7.57	110	833	941	1.13	18	138
>3.2 $(NH_4)_2SO_4$	0.02	2500	40	0	0	0	0
A.S. (A)[c]	6.93	136	942	1404	1.49	23	206

[a]see Figure 4
[b]from 917.65 g Fresh Weight tissue, 3.37 μg chlorophyll $\cdot$ mL^{-1}
[c]2.2-3.2 M $(NH_4)_2SO_4$ fraction after dialysis

Table XVIII *Details of Crude Extract (B) and Ammonium Sulphate Fractionations*

Step[a]	[Protein]	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	$mg \cdot mL^{-1}$	*mL*	*mg*	*mIU*	$mIU \cdot mg^{-1}$ *protein*	*fold*	%
Crude (B)[b]	3.24	2400	7776	1404	0.05	1	100
<2.2 M $(NH_4)_2SO_4$	10.55	640	6816	82	0.01	0.2	20
2.2-3.2 M $(NH_4)_2SO_4$	5.26	124	652	248	0.38	7	60
>3.2 M $(NH_4)_2SO_4$	0.03	2800	84	0	0	0	0
A.S. (B)[c]	7.10	160	1136	909	0.80	15	221

[a]see Figure 4
[b]from 1140.34 g Fresh Weight tissue, 3.91 μg chlorophyll $\cdot$ mL^{-1}
[c]2.2-3.2 M $(NH_4)_2SO_4$ fraction after dialysis

Table XIX *Details of Crude Extract (C) and Ammonium Sulphate Fractionations*

Step[a]	[Protein]	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	$mg \cdot mL^{-1}$	*mL*	*mg*	*mIU*	$mIU \cdot mg^{-1}$ *protein*	*fold*	%
Crude (C)[b]	4.38	2510	10994	330	0.03	1	100
<2.2 M $(NH_4)_2SO_4$	16.53	500	8265	33	0.01	33	10
2.2-3.2 M $(NH_4)_2SO_4$	5.24	172	901	198	0.22	7	60
>3.2 M $(NH_4)_2SO_4$	0.03	2840	85	0	0	0	0
A.S. (C)[c]	6.76	220	1487	595	0.40	13	180

[a]see Figure 4
[b]from 1323.68 g Fresh Weight tissue, 4.69 μg chlorophyll $\cdot$ mL^{-1}
[c]2.2-3.2 M $(NH_4)_2SO_4$ fraction after dialysis

Table XX *Details of Crude Extract (D) and Ammonium Sulphate Fractionations*

Step[a]	[Protein]	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	mg · mL^{-1}	*mL*	*mg*	*mIU*	*mIU · mg^{-1} protein*	*fold*	*%*
Crude (D)[b]	4.41	4650	20456	916	0.045	1	100
<2.2 M $(NH_4)_2SO_4$	12.93	1000	12930	91	0.007	0.2	10
2.2-3.2 M $(NH_4)_2SO_4$	10.04	150	1506	308	0.2	5	34
>3.2 M $(NH_4)_2SO_4$	0.03	5320	160	0	0	0	0
A.S. (D)[c]	11.95	221	2641	1722	0.65	14	188

[a]see Figure 4
[b]from 2029.75 g Fresh Weight tissue, 4.39 μg chlorophyll · mL^{-1}
[c]2.2-3.2 M $(NH_4)_2SO_4$ fraction after dialysis

Table XXI *Details of Crude Extract (E) and Ammonium Sulphate Fractionations*

Step[a]	[Protein]	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	mg · mL^{-1}	*mL*	*mg*	*mIU*	*mIU · mg^{-1} protein*	*fold*	*%*
Crude (E)[b]	3.49	3950	13786	1172	0.09	1	100
<2.2 M $(NH_4)_2SO_4$	18.11	650	11772	82	0.01	0.1	7
2.2-3.2 M $(NH_4)_2SO_4$	13.11	102	1337	241	0.18	2	21
>3.2 M $(NH_4)_2SO_4$	0.02	4620	92	0	0	0	0
A.S. (E)[c]	13.59	160	2174	1848	0.85	10	158

[a]see Figure 4
[b]from 1662.81 g Fresh Weight tissue, 4.45 μg chlorophyll · mL^{-1}
[c]2.2-3.2 M $(NH_4)_2SO_4$ fraction after dialysis