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THE ISOLATION AND CHARACTERIZATION OF PHOSPHATASES FROM THE EHRLICH ASCITES TUMOR CELL LINE

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THE ISOLATION AND CHARACTERIZATION OF PHOSPHATASES FROM THE EHRLICH ASCITES TUMOR CELL LINE.

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By

INDU JAVERI ISAACS

Norman, Oklahoma

1985

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OF PHOSPHATASES FROM THE

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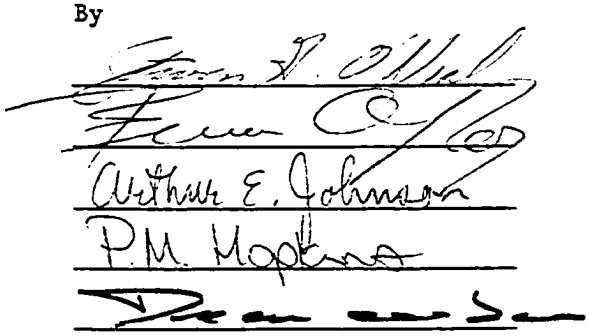
APPROVED FOR THE DEPARTMENT OF CHEMISTRY

By



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I also wish to express my sincere gratitude to my husband for his love, continual support and encouragement throughout this period of study.

Finally, but for the love and self-sacrifices of my Parents this work could not have been completed.

DEDICATION

To my husband Ben, my Parents, and my son Joshua

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LIST OF ABBREVIATIONS USED

AM	Ammonium molybdate
ATP	Adenosine triphosphate
CTP	Cytosine triphosphate
c-AMP	Cyclic-Adenosine monophosphate
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EAT cell	Ehrlich Ascites Tumor cell
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraaceticacid
EGF	Epidermal growth factor
$KH_2\ PO_4$	Potassium phosphate monobasic
MG	Malachite green
Na^{2+} $+K^{2+}$ -ATPase	Sodium-potassium adenosine triphosphate phosphatase
NEM	N-ethylmaleimide
PDGF	Platelet derived growth factor
PNPP	p-Nitrophenyl phosphate
PNPPase	p-Nitrophenyl phosphate phosphatase
RSV	Rous sarcoma virus
T	Triton x-100
TCRC-2 cell	A cell line derived from a HeLa Cell line.
TGF	Transforming growth factor
Tris	Tris(hydroxy methyl)amino methane

ABSTRACT

The presence of soluble Mg^{2+} or Mn^{2+} -dependent phosphotyrosyl phosphatase and Mg^{2+} or Mn^{2+} -dependent p-nitrophenyl phosphate phosphatase activity in Ehrlich ascites tumor cell homogenates is reported.

The crude homogenate was fractionated over Sephadex G-150 Gel filtration and DEAE- sephacel anion exchange columns, and two fractions termed Peak I and Peak II were resolved. Peak I p-nitrophenyl phosphate phosphatase and phosphotyrosyl phosphatase activities were found to be similar in that they both require essential Mg^{2+} or Mn^{2+} ions and dithiothreitol. Optimal activity was observed at neutral pH. It was further found that these two Peak I activities were inhibited by zinc and fluoride but were not inhibited by vanadate and levamisole.

Peak II was found to have low specificity toward phosphotyrosine but did contain Mg^{2+} -or Mn^{2+} - dependent p-nitrophenyl phosphate phosphatase activity. Both Peak I and Peak II p-nitrophenyl phosphate phosphatases activities were similar in that they both require essential Mg^{2+} or Mn^{2+} ion and are similarly inhibited by pyrophosphate, but are not inhibited by levamisole. However, there is evidence that these two enzymes are distinct. Differences between the two fractions with regard to optimal assay pH and reaction rates; fluoride and phosphate inhibition; and susceptibilities to inactivation by either heat,

N-ethylmaleimide, or trypsin treatment while in the presence or absence of either Mg^{2+}, Mn^{2+}, p-nitrophenylphosphate, dithiothreitol, or phosphate suggest that peak I and Peak II contain distinct Ehrlich ascites tumor cell enzymes which belong in a newly described class of phosphatases.

Chapter I

INTRODUCTION

A living system consists of an enormous number of biochemical processes that are closely interlinked. The regulation of these processes, which is important in maintaining the internal environment of the living system, is acheived by a host of regulatory mechanisms. These mechanisms can be either reversible or irreversible, and covalent or noncovalent in nature.

Some biochemical processes are activated (e.g., the proteins involved in the complement system), while others (e.g., the proteins involved in the anticoagulation pathway) are inactivated by irreversible covalent modifications of the enzymes by specific limited proteolysis of each protein's polypeptide backbone. In allostery, the interaction of a specific metabolite at a site on a protein other than the catalytic site modulates the catalytic activity of the enzyme and this is an example of a noncovalent and reversible regulatory mechanism. For example, aspartate transcarbamoylase, the enzyme that catalyzes the formation of N-carbamoyl aspartate from aspartate and carbamoyl phosphate, is allosterically feedback inhibited by cytosine triphosphate (CTP), the final product in the pathway (Gerhart, 1970).

Other biochemical processes are regulated by reversible covalent modification of specific amino acid residues. Examples of reversible

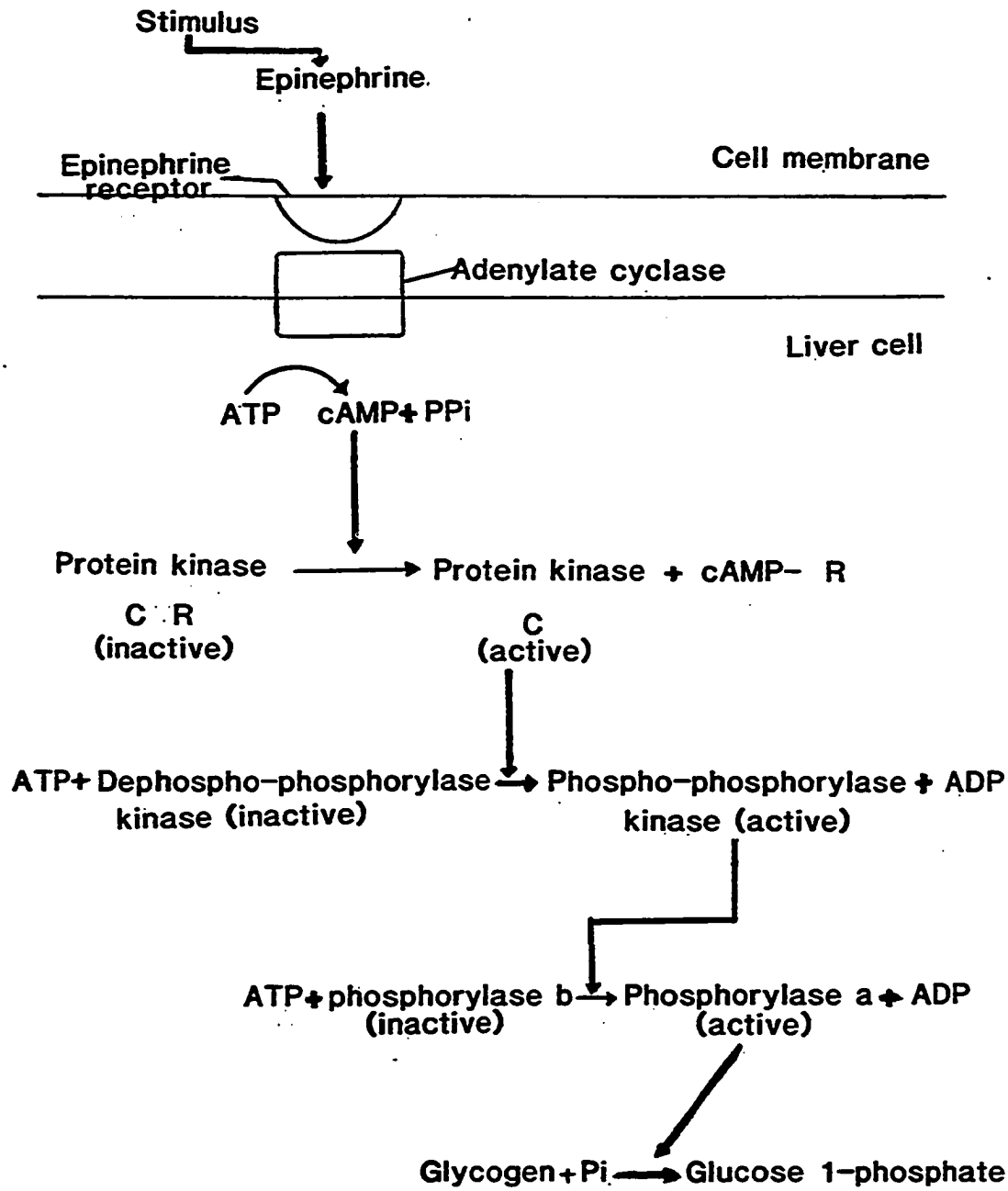
Figure 1: Regulation by phosphorylation/dephosphorylation.

Action of Epinephrine on the liver cell.

(A) Stimulation of glycogen breakdown mediated via cAMP dependent protein kinase: note the conversion of inactive form of phosphorylase to active phosphorylated form of phosphorylase.

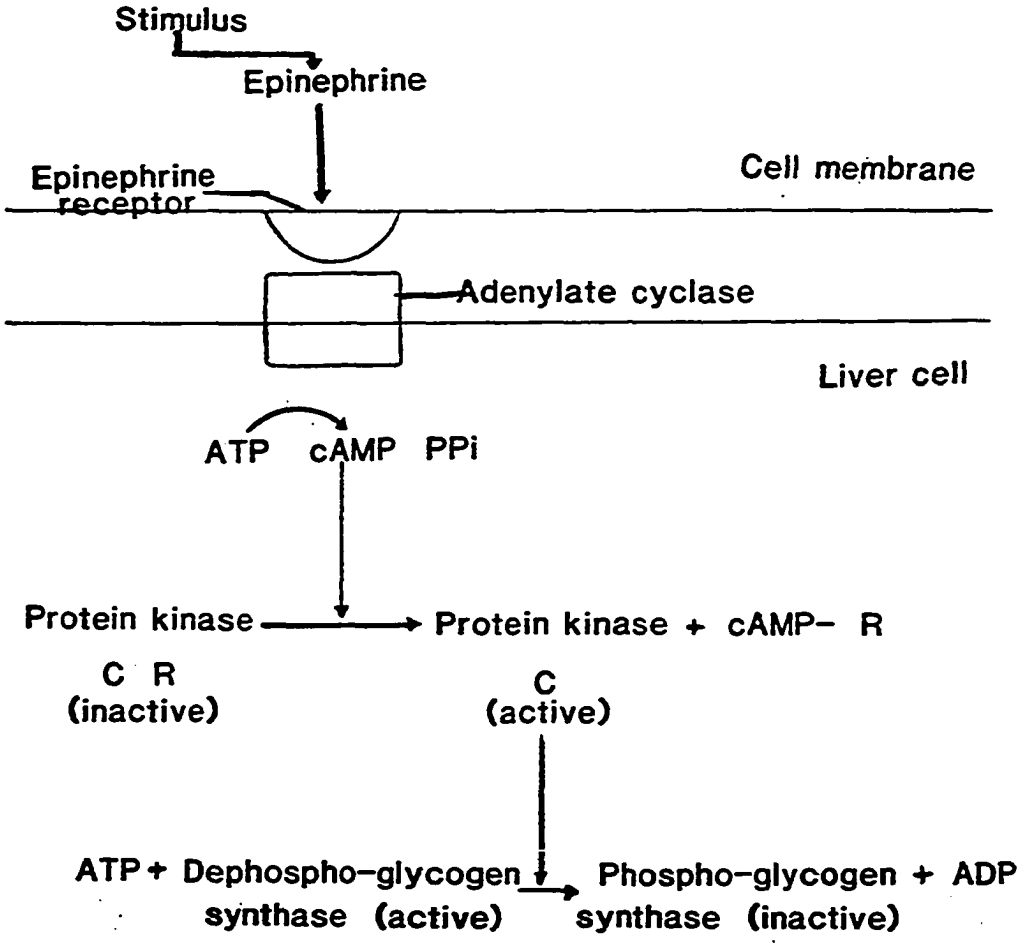
(B) Inhibition of glycogen synthesis mediated via cAMP dependent protein kinase: note the conversion of active dephospho form of glycogen synthetase to the inactive phosphorylated form of glycogen synthase.

A


Stimulus
Epinephrine
Epinephrine receptor
Cell membrane
Adenylate cyclase
Liver cell
ATP
cAMP + PPi
Protein kinase
C R
(inactive)
Protein kinase + cAMP– R
C
(active)
ATP + Dephospho–phosphorylase kinase (inactive)
Phospho–phosphorylase kinase (active) + ADP
ATP + phosphorylase b (inactive)
Phosphorylase a (active) + ADP
Glycogen + Pi
Glucose 1–phosphate

enzyme modifications include phosphorylation, methylation, acetylation and sulfation. Phosphorylation/ dephosphorylation is the most predominent and best studied. The covalent attachment of phosphate to either seryl or threonyl residues of a protein was first identified by F. Lipman and P. A. Levine in 1932. The importance of such reversible and covalent modification of proteins in cellular regulation was recognized as early as the 1950s by a series of studies carried out by investigators such as E. W. Sutherland, E. G. Krebs, E. Fisher and J. Larner. Their work clarified the role of reversible phosphorylation as a means of regulating several key enzymes in the metabolism of glycogen (Figure 1A and 1B) (Yasuitomi, N.,1984). Epinephrine (Figure 1A), which stimulates glycogen breakdown in the liver and muscle, initiates a cascade of amplification reactions. Epinephrine binds to a specific receptor site on the cell membrane, causing stimulation of the conversion of intracellular adenosine triphosphate (ATP) to 3′-5′ cyclic adenosine monophosphate (cyclic AMP) by a membrane bound adenylate cyclase. Cyclic AMP in turn activates protein kinase; this phosphorylates phosphorylase kinase at the expense of ATP, converting phosphorylase kinase into an active form which, in turn, activates glycogen phosphorylase by phosphorylation. This chain of events thus stimulates glycogen breakdown into blood glucose in the liver. Epinephrine not only stimulates the glycogen breakdown pathway but also serves to inhibit glycogen synthesis in the liver (Figure 1B). This is

B


Stimulus
Epinephrine
Epinephrine receptor
Cell membrane
Adenylate cyclase
Liver cell
ATP cAMP PPi
Protein kinase
C R
(inactive)
Protein kinase + cAMP– R
C
(active)
ATP + Dephospho–glycogen synthase (active)
Phospho–glycogen + ADP
synthase (inactive)

accomplished via the formation of cyclic AMP which promotes the protein kinase-dependent phosphorylation of the active or dephosphorylated form of glycogen synthetase to its phosphorylated inactive form.

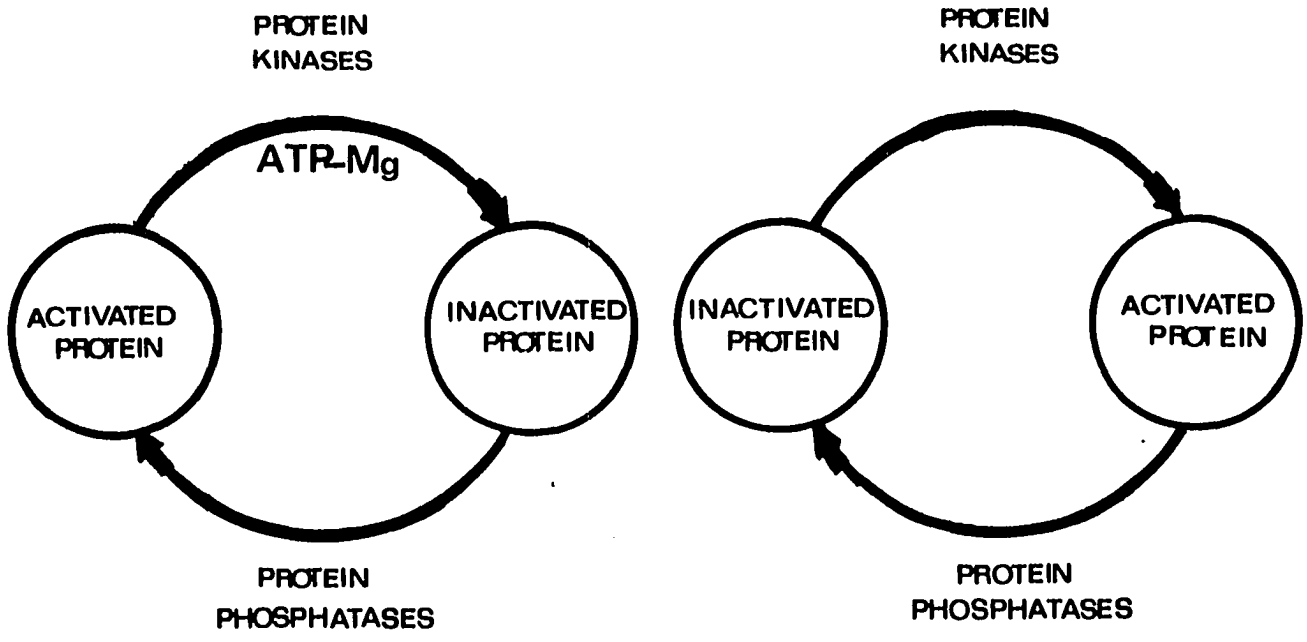
Hence, an important generality that has emerged is that enzymes in biodegradative pathways are activated by phosphorylation , whereas the enzymes in biosynthetic pathways are inactivated by phosphorylation (Figure 2) (Cohen, P., 1978 & 1982; Krebs, E., and Beavo, J., 1979; Nimmo, H., and Cohen, P., 1977; Ingerstein, T., and Cohen, P., 1977). The results also suggested that different metabolic pathways might be regulated by protein kinases and protein phosphatases. These ideas were established for protein kinases, in the late 1960s when E. G. Krebs discovered cAMP-dependent protein kinase (protein kinase A) and its definite role in signal transduction. When some hormones bind to plasma membrane-bound receptors, cAMP then acts as a secondary messenger which transmits the binding signal into the cell interior. Hormones which bind to plasma membrane-bound receptors and which stimulate cAMP production by cAMP-dependent protein kinase include, epinephrine, glucagon, gonodotropic hormones, parathyroid hormone, calicitonin and vasopressin. Clearly, as seen in Figures 1A and 1B, the effects of this secondary messenger on biosynthetic and biodegrative pathways are a consequence of the phosphorylation by cAMP dependent protein kinases of various regulatory enzymes in these pathways (Yasuitomi, N., 1984).

Most studies to date have concentrated on the pathway that involves the cAMP dependent protein kinases, enzymes that almost always phosphorylate serine residues. However, a new type of protein

Figure 2: Phosphorylation/dephosphorylation in

(A) Biosynthetic pathway.

(B) Biodegrative pathway.


PROTEIN
KINASES
ATP-Mg
ACTIVATED
PROTEIN
INACTIVATED
PROTEIN
PROTEIN
PHOSPHATASES
A
PROTEIN
KINASES
INACTIVATED
PROTEIN
ACTIVATED
PROTEIN
PROTEIN
PHOSPHATASES
B

modification, apparently involved in a number of crucial metabolic processes, was recognized only a few years ago, when Collet and Erickson, R. L. (1978) discovered that the src gene product of Rous Sarcoma Virus (RSV) $pp60^{src}$ was a protein kinase. This was subsequently identified as a tyrosine specific protein kinase (Hunter, T., (1980) , Hunter and Sefton (1980)). Cells that were transformed by RSV , as well as some other RNA tumor viruses, showed elevated levels of phosphotyrosine (Barbacid et al.,(1980), Blomberg et al., (1980), Sefton et al.,(1981)). When the RSV infected cells containing a temperature sensitive mutation in the transforming gene were shifted to a restrictive temperature for transformation, it was seen that 60% of the phosphotyrosine residues were dephosphorylated within 75 min (Sefton et al., 1980). This suggested that the level of phosphotyrosine residues in these proteins reflected on the relative activities of both phosphotyrosyl protein kinases and phosphotyrosyl protein phosphatases. It is therefore possible that the phosphorylation of the tyrosyl residues of certain proteins with regulatory functions could mediate the complex alterations in cellular metabolism that ultimately lead to a transformed phenotype.

Phosphorylation of specific tyrosine residues has also been observed in cellular regulation. For example , phosphorylation of tyrosyl residues has been seen following the addition of growth factors such as epidermal growth factor (EGF) (Carpenter et al.,1979; Cohen et al.,

1980; King et al., 1980; Ushiro and Cohen, 1980.) and platelet derived growth factor (PDGF) (Ek et al., 1982) to cell membranes. It has been seen that in A-431 human epidermoid carcinoma cells, the EGF receptor, a phosphoprotein of molecular weight 170,000, undergoes phosphorylation at tyrosine residues upon stimulation by EGF (Hunter and Cooper., 1981). The receptors for these growth factors and the proteins encoded for by the transforming genes (oncogenes) of viruses, such as RSV, have been identified as protein tyrosine kinases (Hunter and Sefton, 1980 & 1982; Ushiro and Cohen, 1980; Ek et al., 1982; Cooper et al., 1982). Phosphotyrosyl protein kinase activity has also been observed to be associated with other mitogenic peptides such as transforming growth factor (TGF) (Roberts et al., 1980) , other proteins like insulin receptor (Kasuga, M., et al., 1982; Roth, R., et al., 1983) and sodium-potassium stimulated adenosine triphosphatase (Na^+ + K^+ -ATPase) (Spector, M., et al., 1980).

Normal cells have also been seen to possess tyrosine- specific protein kinase activity. However, this activity is expressed at much lower levels than those detected in the transformed cells (Hunter and Sefton., 1980; Sefton et al., 1980). Phosphotyrosine residues account for only 0.03% of all the phosphorylated amino acids in normal cells, while phosphoserine and phosphothreonine account for the remaining 99.97% (Sefton et al., 1980; Hunter and Sefton, 1982). This tyrosyl protein kinase activity is highly conserved throughout evolution (Oppermann et al., 1976 and Bishop et al., 1980). This suggests that the phosphorylation of specific tyrosyl residues in proteins may be important in the regulation of certain essential cellular processes.

In contrast to the numerous reports dealing with the phosphorylation of specific tyrosine residues by phosphotyrosine protein kinases, there have been very few publications that have reported the results from investigations of the corresponding phosphotyrosyl protein phosphatases. In this regard, Swarup et al.,(1982), have recently demonstrated alkaline phosphatases from calf intestine, bovine liver and E. coli that dephosphorylate phosphotyrosyl histones at 5-10 times the rate observed with phosphoseryl histones. This phosphotyrosyl protein phosphatase activity was inhibited by micromolar concentrations of vanadate. It was also inhibited by phosphate and p-nitrophenylphosphate (PNPP), but was not inhibited by fluoride (F^-). These phosphatases were also seen to possess cation-independent p- nitrophenylphosphate phosphatase (PNPPase) activity.

Chernoff et al.,(1983), reported an alkaline phosphatase from bovine cardiac muscle which specifically dephosphorylated phoshotyrosyl Immunoglobulin G (IgG) . This enzyme was inhibited by zinc (Zn^{2+}) , PNPP, and phosphate (Pi) but was not inhibited by fluoride. This enzyme was also seen to possess PNPPase activity.

In light of the evidence that alkaline phosphatases are active towards phosphotyrosyl proteins, Leis and Kaplan, (1982), turned their attention toward a similar investigation of acid phosphatases and were successful in isolating an acid phosphatase from the plasma membrane of human astrocytoma that exhibited a very high specificity towards phosphotyrosine proteins. Vanadate was found to be a potent inhibitor of this phosphotyrosyl protein phosphatase. 0.5 mM vanadate inhibits about

50% of the activity. They also reported that fluoride and zinc significantly inhibited phosphoserine histone dephosphorylation but did not inhibit phosphotyrosine histone dephosphorylation by this enzyme.

Heng Chun Li., (1984) isolated an acid phosphatase from human prostrate gland which specifically dephosphorylated (32-P) labelled phosphotyrosyl casein. This phosphatase was inhibited by PNPP, molybdate, vanadate, and flouride. The purified enzyme exhibited high specificity toward phosphotyrosyl residues and little activity toward several phosphoseryl and phosphothreonyl proteins.

In addition, phosphotyrosyl protein phosphatases lacking alkaline or acid phosphatase activities have also been described. Brautigan et al., (1982), demonstrated the presence of a neutral phosphatase in membrane vesicles isolated from a number of cell lines, including the Erhlich Ascites Tumor (EAT) cell line, which dephosphorylated several phosphotyrosyl proteins. The activity of this enzyme was inhibited by micromolar concentration of zinc ion (Zn^{2+}) but was apparently not inhibited by fluoride (F^{-}). The latter is known to be a potent inhibitor of phosphoseryl protein phosphatase (Chernoff et al.,(1983)).

Foulkes et al.,(1981), described an ethylenediaminetetraacetic acid-sodium fluoride (EDTA-NaF) insensitive phosphatase activity in rat skeletal muscle and liver extracts which dephosphorylated $pp60^{src}$ 32-P labelled phosphotyrosyl-IgG. This enzyme was also inhibited by Zn^{2+}. These results suggested that the phosphotyrosyl protein phosphatase(s) present in these tissue extracts were distinct from the mammalian phosphoseryl/ phosphothreonyl protein phosphatases. Foulkes et

al.,(1983), have also isolated five phosphotyrosyl protein phosphatases from chicken brain extracts. These phosphatases were also not inhibited by EDTA or fluoride. In fact, EDTA stimulates the enzyme whereas divalent cations like Mg^{2+} and Zn^{2+} were shown to inhibit the enzyme activity. Therefore, these enzymes were similar to those isolated from rat skeletal muscle and liver extract.

We have isolated phosphatase activity from EAT cells. An investigation of phosphatase from the EAT cells is interesting because

(i) Such phosphatases may play very important roles in metabolic regulation including both cell growth and possibly cell transformation. Characterization of phosphotyrosyl phosphatases will be critical to our understanding of these processes.

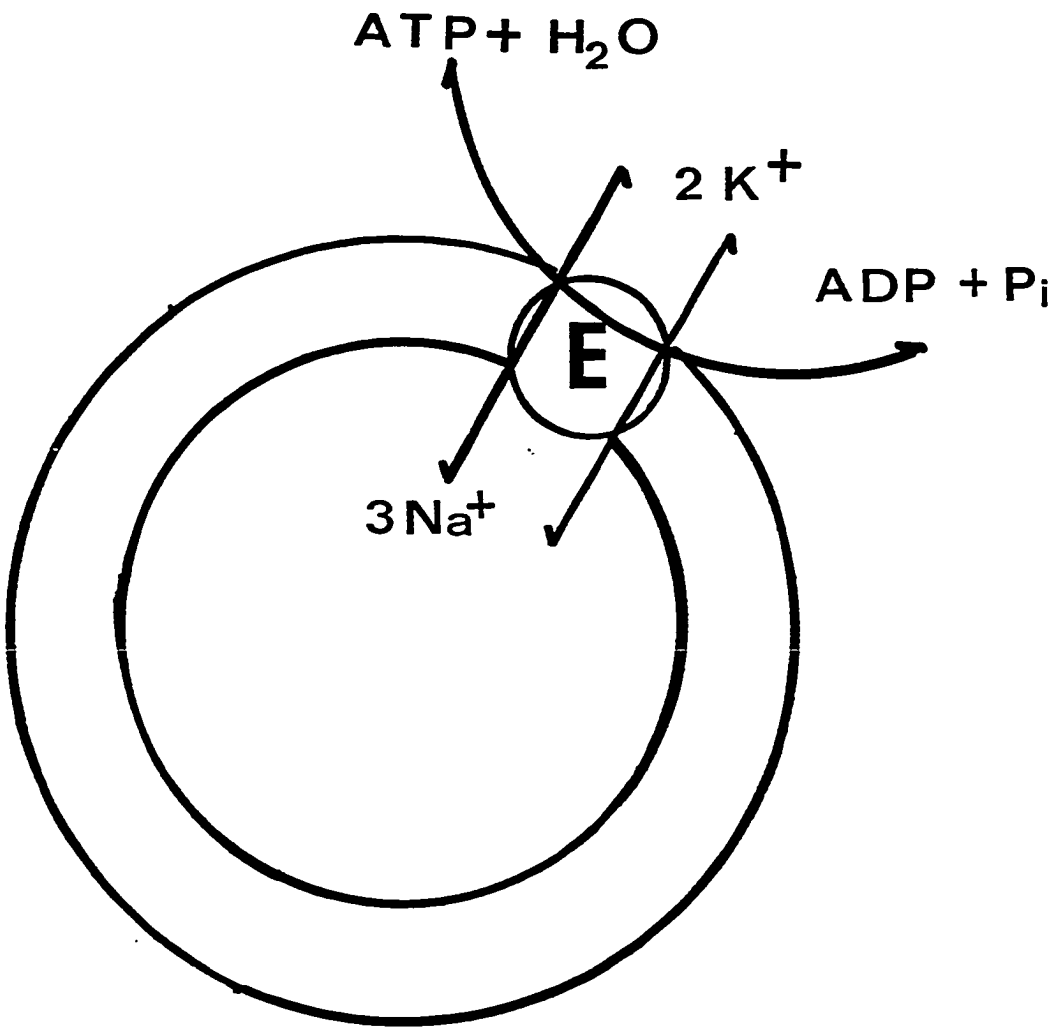
(ii) In EAT cells, it is suspected that the enzyme sodium-potasium-dependent adenosine triphosphatase (Na^+ + K^+-ATPase) does not work efficiently (Spector, M., et al., 1980). Na^+ + K^+-ATPase is the biochemical manifestation of the sodium pump. In normal cells, Na^+ + K^+-ATPase is a multisubunit enzyme. It regulates the active transport of sodium (Na^+) and (K^+) across the cell membrane (Fig 3) (Jorgensen, P.L., 1980). To facilitate this ion transport, one molecule of ATP is hydrolyzed with the concomittant efficient transport of three moles of Na^+ and two moles of K^+ across the cell membrane of normal cells. In addition , the sodium pump is involved, either directly or indirectly , in several physiological processes. These include the release and uptake of neurotransmitter (Meyer et al 1981; Vizi, 1978), the generation of the Na^+ and K^+ gradient across the cell membrane

necessary for the maintenance of the cell membrane resting potential, the control of vascular (Lang et al 1980) and visceral (Schied et al (1979) smooth muscle tone, the transport of glucose across the cell membrane (Bihler et al 1979) and the secretion of fluid in several epithelial tissues (Stewart et al., 1981).

It is clear that changes in Na^++ K^+-ATPase activity will effect several physiological processes. In a study of intact EAT cells , it was shown that Na^++ K^+-ATPase is likely to pump Na^+ ions with low efficiency. Efficiency of the pump is defined as ratio of the number of Na^+ ions pumped per mole of ATP hydrolysed. It is also known that the B subunits of Na^++ K^+-ATPase in this cell line are phosphorylated at tyrosine residues (Spector, M., et al., 1981). It is therefore possible that the phosphorylation of tyrosine residues on B subunits causes the pump to work inefficiently. This could indicate either an increase in tyrosine kinase activity in these tumor cells which increases the degree of phosphorylation of the normal substrate, or it could indicate an introduction of a mutated protein kinase with an altered substrate specificity which phosphorylates proteins which are not normally substrates. This could also indicate mutated phosphatases with altered substrate specificity or it could indicate low levels of phosphatase present in the EAT cells. Hence, in either case, isolation of phosphotyrosyl phosphatase from this cell line is important for ultimately demonstrating that phosphatase dependent dephosphorylation of the phosphorylated subunits of Na^++ K^+-ATPase would restore full Na pumping efficiency.

Figure 3: Schematic representation of active transport system for sodium and potassium ion.

E = Na^{2+}+ K^{2+}-ATPase.


ATP + H_2O
2 K^+
ADP + P_i
E
3 Na^+

The objective of my research is to isolate and characterize neutral phoshatases present in the EAT cell line. Characterization was done using the non-protein esters PNPP and L-phosphotyrosine as substrates. The ester PNPP was used because of its structural resemblance to L-phosphotyrosine (the modified amino acid residue in phosphotyrosyl proteins) (Figure 4) .

Separation of two phosphatase fractions was achieved by gel filtration and anion exchange chromatography. These two phosphatase fractions were characterized with respect to optimal assay conditions and to the effect of potential inhibitors like fluoride, phosphate, pyrophospate, zinc, vanadate and levamisole. Further characterizations were done using biochemical and chemical modifying agents namely trypsin and N- ethyl maleaimide (NEM). Thermal stability of these two phosphatase fractions was also determined .

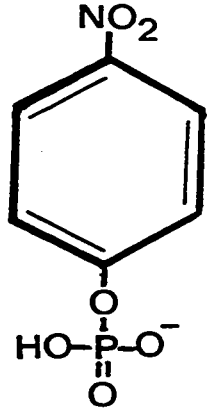
Characterization of these enzymes has shown that they are different from the ones described by other investigators, and hence could concievably belong to a new class of phosphatases (see Appendix 1).

Figure 4: Structure of:

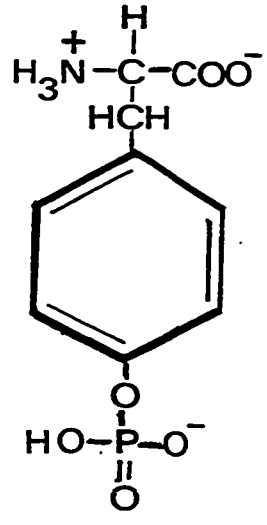
(A) p-nitrophenyl phosphate.

(B) Phosphotyrosine.

in thier partially ionized forms.



A



B

Chapter II

MATERIALS AND METHODS

2.1

MATERIALS

Sodium orthovanadate, ammonium molybdate, and sodium citrate were purchased from Fisher Scientific. Malachite green and zinc chloride were purchased from Aldrich. Gel-fitration standards were products of Bio-Rad. Iminodiacetic acid-Sepharose CL-4B was the product of Pierce Chemical Company. All other biochemicals were obtained from Sigma Chemical Company.

2.2

ISOLATION OF PNPPASES ACTIVITY FROM EAT CELLS

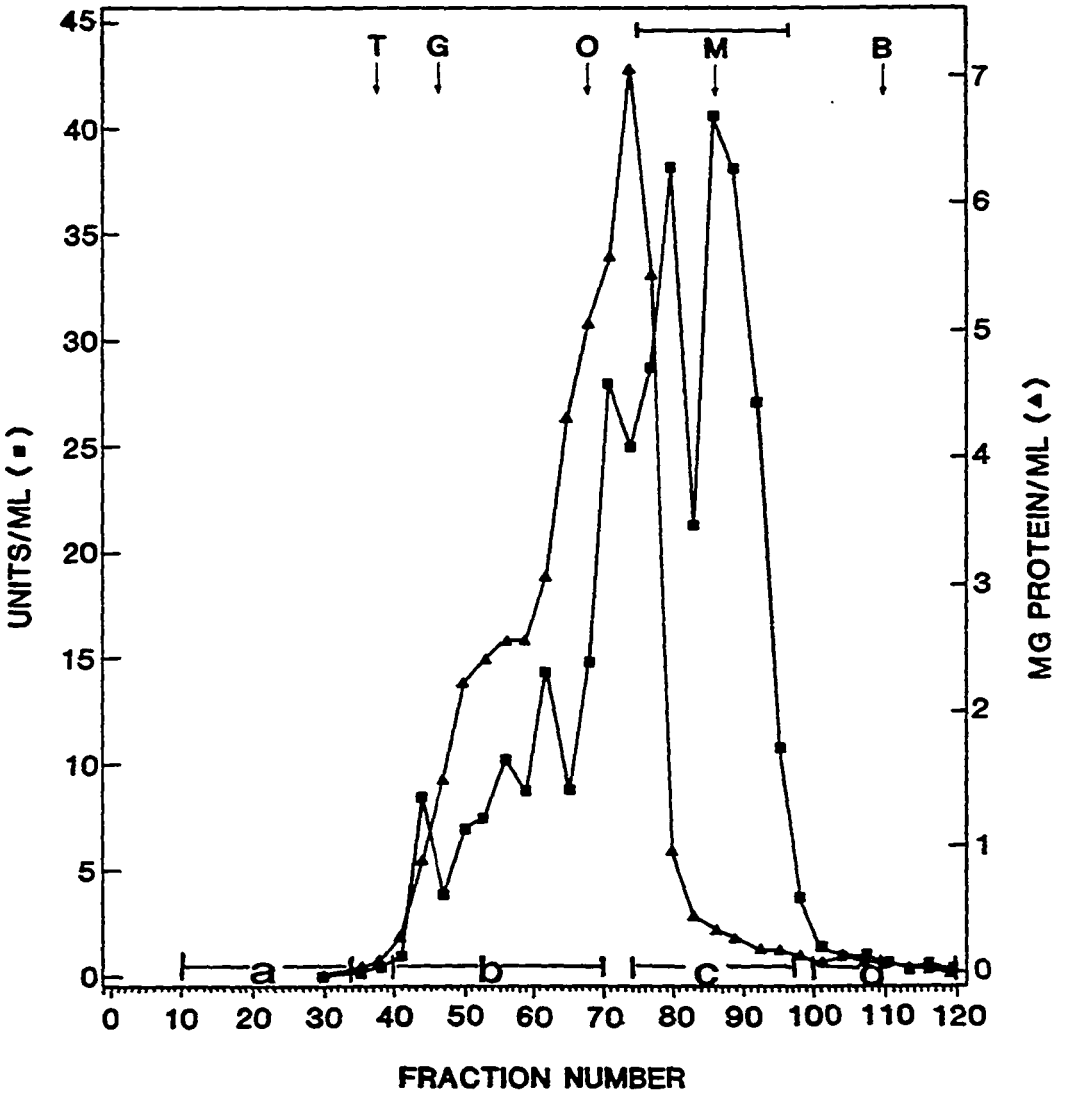
EAT cells were isolated from 9-day cultures maintained in a colony of HSD: (ICR) BR male mice (Harlan Sprague-Dawley, Inc.). Crude soluble PNPPase activity was prepared from EAT cells as follows: cells were swollen in 10 mM $CaCl_2$ at room temperature for 15 min with stirring, and incubated an additional 10 min with stirring in ice water before homogenization by 23 strokes in a tight-fitting Dounce homogenizer. Fluffy membrane fragments and unbroken cells were removed by centrifugation at 100g (average) for 1 min in a Sorvall SS-34 rotor.

Remaining membranous particles were removed by centrifugation at 17,000g (average) for 20 min. The supernatant obtained after centrifugation at 17,000g for 20 min served as the source for crude PNPPase.

The crude PNPPase was further resolved into two fractions of PNPPase activity by a two step chromatographic procedure. In the first step the crude enzyme was concentrated to 50 ml by ultrafiltration over UM 10 membranes (Amicon Corporation). The concentrate was loaded on a 4.4 x 48-cm Sephadex G-150 gel filtration column equilibrated and eluted with Buffer A (20 mM Tris-HCl, pH 7.4, 150 mM NaCl). 7 ml fractions were collected in 13 x100 mm disposable test tubes. The fractions that contained maximum PNPPase activity, 74-97, were then pooled as shown in Figure 5 and termed 'c'. The other fraction containing PNPPase activity, 40-70, was also pooled as shown in Figure 5 and termed 'b'. In addition, fractions 10-34 and 100-120 were also pooled separately and called 'a' and 'd' respectively. When these pooled fractions, 'a', 'b', and 'd' were reconstituted with fraction 'c', no stimulation in activity was observed. Hence, the pooled fraction 'c' was concentrated and equilibrated in Buffer B (20 mM Tris-HCl, pH 7.4, 50 mM NaCl) to a volume of 50 ml by ultrafiltration over PM-10 membranes (Amicon Corporation). This concentrate was then loaded onto a 1.6 x 38-cm DEAE-Sephacel anion exchange column equilibrated in Buffer B. The unbound protein was washed from the column with Buffer B until no absorbance at 280 nm was detected by an on-line UV monitor. Bound proteins were eluted in Buffer B using a linear salt gradient from 50 mM to 350 mM NaCl (600 ml total gradient volume). 7 ml fractions were

Figure 5. Sephadex G-150 elution profile for EAT cell PNPPase. The EAT cell homogenate was fractionated over Sephadex G-150. Chromatographic procedures were performed as described under Materials and Methods. The assay medium consisted of 80 mM Tris-HCl, pH 7.5, 40 mM $MgCl_2$, 20 mM PNPP, and 1 mM DTT.

For an estimation of the PNPPase molecular weights, the column was calibrated with bovine thyroglobulin (Mr 670,000) (T), bovine Y-globulin (Mr 158,000) (G), chicken ovalbumin (Mr 44,000) (O), horse myoglobulin (Mr 17,000) (M), and vitamin B12 (Mr 1350) (B). The eluted fractions were pooled as follows; Fraction 'a' 10-34; 'b' 40-70; 'c' 74-97; 'd' 100-120. The profile shown is representative of 17 separate chromatographic runs.


T
G
O
M
B
a
b
c
d
UNITS/ML (■)
MG PROTEIN/ML (▲)
FRACTION NUMBER
0
5
10
15
20
25
30
35
40
45
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
80
90
100
110
120

collected as above in an LKB Superac fraction collector. Conductivity measurements were performed on a Yellow Springs Instruments Model 31 conductivity bridge to determine the salt gradient. The fractions were assayed for PNPPase activity as described below. Two peaks of activity were obtained. Each was pooled as shown in Figure 6, and each henceforth referred to as Peak I and Peak II PNPPase in order of elution from the DEAE column. All chromatograhic steps were performed at 4 ^{o}C and at a constant flow rate of 20 ml/h using a LKB perstaltic pump.

The pooled fractions were routinely examined by native polyacrylamide gel electrophoresis using a 7 % resolving gel made as described by Davis (1964).

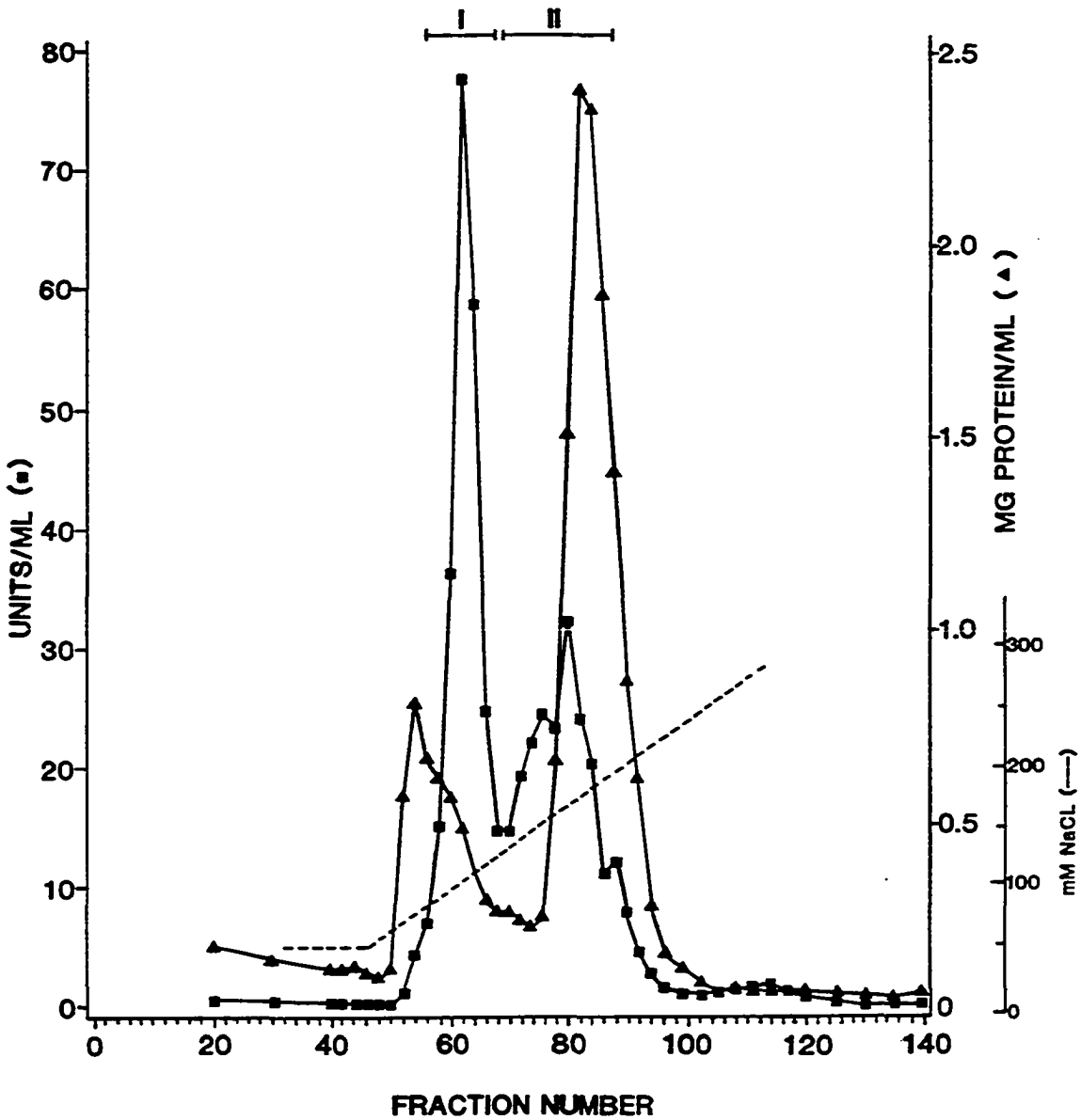
The resolving or running gel mixture was prepared by mixing the following solutions. Mix 1 part of Solution A (Tris-HCl, pH 8.9, with TEMED), 2 parts of Solution B (28% acrylamide, 0.73% bis-acrylamide), 1 part of deionized water and 4 parts of 0.14% freshly prepared ammonium persulfate solution to give a 7% resolving gel solution. This solution was poured either into 10 cm tubes or 8x8 cm slabs.

Typically, 20-30 ug of a protein sample was layered onto the polymerized gels in 20% sucrose and 0.002% bromophenol blue solution. The system was electrophoresed at 200 Volts (LKB 2310 power supply)

Following electrophoresis, the gels were fixed in a solution of 30% isopropyl alcohol and 10% acetic acid for an hour at room temperature. The fixed gels were then stained with a 0.01% Coomassie blue solution in

Figure 6. Resolution of the pooled G-150 PNPPase fractions by DEAE-Sephacel anion-exchange chromatography. Chromatographic procedures were as described under Materials and Methods. Assays were preformed as outlined in figure 9.

Fractions 57-67 and 68-87 were pooled separately and termed Peak I and Peak II respectively. The profile shown is representative of 17 separate chromatographic runs.


I
II
UNITS/ML (■)
MG PROTEIN/ML (▲)
mM NaCL (—)
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
0
0.5
1.0
1.5
2.0
2.5
0
100
200
300
0
20
40
60
80
100
120
140

10% isopropyl alcohol and 10% acetic acid overnight at room temperature. The stained gels were destained in 5% ethanol and 10% acetic acid solution in the presence of a 15 cm piece of undyed wool (to absorb the dye) until the stained protein bands were visible.

2.3

ZINC AFFINITY COLUMN CHROMATOGRAPHY

Peak I and Peak II were individually subjected to zinc affinty chromatography as described by Horlein et al.,(1982). A 5 ml bed volume of iminodiacetic acid agarose gel was used . The gel was converted to the Zn^{2+}-chelated form according to the method of Kurecki et al., (1979). This was acheived by equilibrating the column with aqueous $ZnCl_2$ (3 mg/ml, adjusted to pH 6.0 with HCl). At least 10 bed volumes of $ZnCl_2$ must pass through the column before the eluant has a pH around 6. The presence of the Zn^{2+} ion is indicated by means of $ZnCO_3$ precipitation in a Na_2CO_3 solution. The column was then equilibrated in 20 mM Tris HCl pH 7.5, and 150 mM NaCl (Buffer C). The column was washed with Buffer C and eluted step wise; first with Buffer C containing 20 mM histidine and then with Buffer C containing 60 mM histidine. The final elution buffer contained 50 mM EDTA, pH 7.0, in 0.5 M NaCl in order to remove the bound Zn^{2+} from the column and thus release any remaining tightly bound proteins. 75 drop fractions were collected by a FC-80 Gilson fraction collector.

2.4

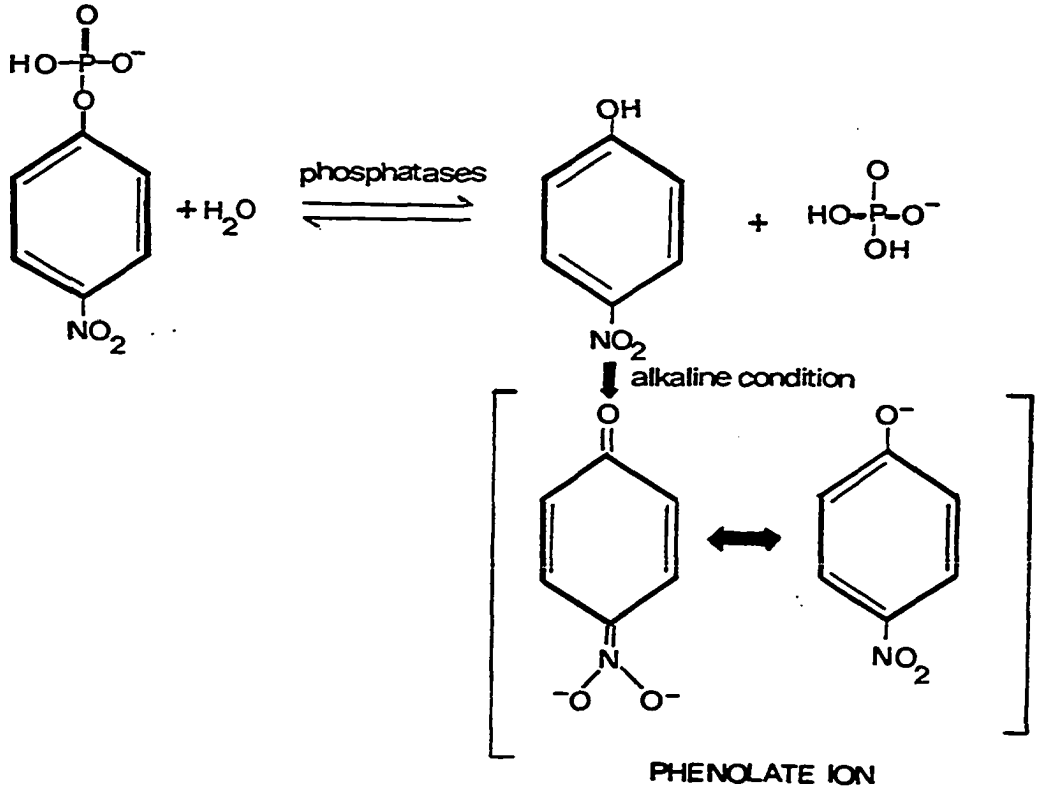
PNPPASE ACTIVITY ASSAY

Assays for PNPPase activity were performed at 37 ^{o}C for 15 min essentially as described by Li and Chan (1981), except that the buffer used was 80 mM Tris-HCl pH 7.4. This assay method makes use of an artificial substrate, p-nitrophenylphosphate, which is dephosphorylated by the phosphatases isolated. The degree of hydrolysis of the substrate is determined photometerically by measuring the amount of p-nitrophenoxide liberated in the reaction (Figure 7). p-nitrophenoxide ion absorbs strongly at 405 nm under alkaline conditions. The reaction mixture (500 ul) typically consists of 80 mM Tris-HCl, pH 7.4 buffer, 40 mM $MgCl_2$, 1 mM DTT, 15-30 ug of protein and the reaction was initiated by the addition of 20 mM PNPP. The reaction was stopped after 15 min by the addition of (500 ul) 0.1 M EDTA-Tris, pH 8.5. PNPPase activity was observed to be directly proportional to time of incubation under these conditions. The molar absorptivity for p-nitrophenoxide anion is 1.75×10^4 M^{-1} cm^{-1}. One unit of PNPPase activity is defined as that amount of enzyme which hydrolyzes 1 nmol of PNPP min^{-1} at 37 ^{o}C.

In an attempt to determine the K_m of the Peak I enzymes for the substrate PNPP, the reaction mixture (500 uls) contained either Mg^{2+} or Mn^{2+} ions, along with varying amounts of PNPP ranging from 0.05 to 25 mM.

It should be noted that 10-15 ul of the DEAE-Sephacel Peak I or Peak II fractions were used without further dialysis in the assays described.

Figure 7: Reaction catalysed by p-nitrophenyl phosphotase using PNPP as a substrate.


phosphatases
alkaline condition
PHENOLATE ION

Therefore, the assays contained an additional 24 and 32 uM NaCl if Peak I or Peak II was used as the protein source, respectively.

2.5

TRYPSINOLYSIS

20 ug of enzyme was preincubated in ice for 15 min with varying amounts of trypsin as indicated in the figure legends in a medium (350 ul) consisting of 80 mM Tris-HCl, pH 7.5. The reaction was stopped by the addition of three-fold excess of soybean trypsin inhibitor and by diluting the preincubation mixture such that the final mixture contained the complete PNPPase assay medium. The PNPPase assay was then carried out as described above.

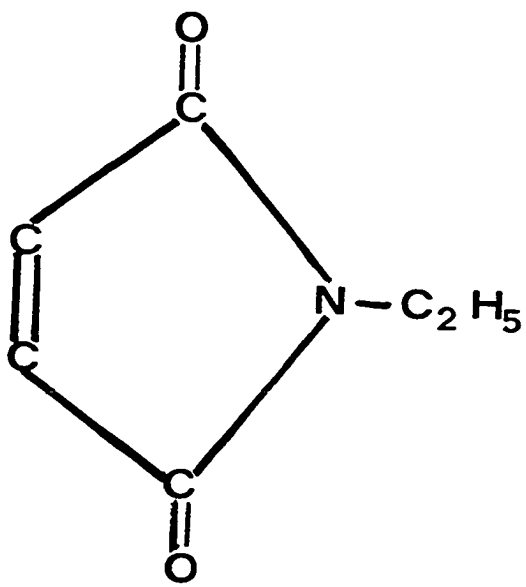
The effect of cations on trypsinolysis was studied by including the appropriate cation, either 40 mM Mg^{2+} or 250 uM Mn^{2+}, in the preincubation reaction mixture . The effect of 1 mM DTT and 20 mM PNPP was carried out similarly.

2.6

EFFECT OF A SULFHYDRYL GROUP MODIFYING REAGENT

N-ethylmaleimide (NEM) is an irreversible sulfhydryl specific group reagent (Figure 8). 20 ug of the enzyme was preincubated for 15 min on

Figure 8 Structure of N-ethyl maleaimide (NEM).


O
C
C
C
N – C2 H5
C
O

ice in a medium (350 ul) consisting of 80 mM Tris-HCl, pH 7.5, plus the indicated amount of NEM. Following the preincubation period the reaction was quenched by DTT. The preincubation reaction mixture was further diluted so that the final mixture contained the complete PNPPase assay medium (500 ul). The effect of either 40 mM Mg^{2+}, 250 uM Mn^{2+}, or 20 mM PNPP was determined by adding these in the preincubation reaction mixture.

2.7

HEAT INACTIVATION STUDIES

19- 20 ug of protein in a medium (100 ul) containing 80 mM Tris- HCl, pH 7.5, was preincubated for 10 min at different temperatures as described in the figure legends. Following the 10 min preincubation, the reaction was stopped by placing it on ice and then by diluting the preincubation reaction medium to 500 ul such that the diluted mixture contained the complete PNPPase assay medium. The effects of either 40 mM Mg^{2+}, 250 uM Mn^{2+}, 1 mM DTT, or 20 mM PNPP were determined by incubating these ligands along with the protein in the preincubation reaction medium.

2.8

PROTEIN CONCENTRATION

Protein concentration was determined by the Bradford method (Bradford, M., 1976) using crystalline bovine serum albumin as a standard.

2.9

PHOSPHOTYROSYL AND PHOSPHOSERYL PHOSPHATASE ASSAY

Phosphotyrosyl and phosphoseryl phosphatase activity were determined as described by Lanzetta etal.,(1979). This assay colorimetrically determines nanomolar amounts of inorganic Pi liberated by the action of phosphatase on phosphorylated substrates.

A typical reaction mixture contained 80 mM Tris-HCl , 20 mM $MgCl_2$, 1 mM DTT, 10 mM L-phosphotyrosine/ L- phosphoserine, and about 4 ug of protein. These were incubated for 15 min at 37 ^{o}C . 4 ug of protein was found to be optimal for the assay. 15 min incubation time was determined to be in the linear region of time course studies and this length of incubation period was used for all subsequent phosphotyrosine phosphatase assays. The reaction was stopped by the addition 0.5 ml of 0.1M EDTA-Tris pH 8.5. A 200 ul aliquot was then transferred to another test tube to which 800 ul of reagent containing 4.2% malachite green (MG), .045% ammonium molybdate (AM), 2% Triton X-100 (T) was added. (The reagent consists of a 3:1 mixture of MG and AM, plus 100 ul of T for

each 5 ml of MG/ AM solution (MG/AM/T)). After 1 min, 100 ul of 34% sodium citrate was added and mixed. This solution was read at 660 nm after allowing the color to develop for 30 min. The reagent was prepared as described by Lanzetta et al., (1979), except that 2% Triton X-100 was used instead of the detergent sterox. KH_2PO_4 was used as a standard. The amount of phosphate that was released was determined from a standard curve. The specific activity of phosphotyrosine and phosphoserine phosphatases was defined as nmoles of Pi released min^{-1} mg of $protein^{-1}$ at 37 ^{o}C.

In order to elucidate the K_m of the Peak I fraction for the substrate phosphotyrosine, the above mentioned reaction mixture contained Mg^{2+} ions along with varying amounts of the substrate.

The effects of potential inhibitors of the Peak I Mg^{2+} -dependent phosphotyrosyl phosphatase were determined by adding the inhibitor in the reaction mixture. Assays were performed as described above without preincubation.

Chapter III

RESULTS

3.1

CHARACTERIZATION OF CRUDE PNPPASE ACTIVITY

The postmicrosomal fraction from homogenates of EAT cells possess Mg^{2+}-dependent PNPPase activity as shown in Figure 9. Maximal activity was obtained in the presence of 40 mM $MgCl_2$, while half maximal activity was afforded by about 9 mM Mg^{2+} and no activity was observed in the absence of cation. Ca^{2+}, Ni^{2+} or Zn^{2+} did not substitute for Mg^{2+}, but Mn^{2+} did substitute for Mg^{2+}. About 250 uM Mn^{2+} gave maximal activity. High concentrations of Mn^{2+} proved inhibitory to the enzyme activity (Figure 10). PNPPase activity when measured at pH 7.5 in the presence of optimal 250 uM Mn^{2+} is about two fold higher than the activity measured in the presence of optimal 40 mM Mg^{2+}. 1 mM DTT stimulates the crude PNPPase activity.

Figure 9. The Mg^{2+}-dependence of PNPPase. Assays were performed as described under Materials and Methods in a medium consisting of 80 mM Tris-HCl, pH 7.5, 20 mM PNPP, 1 mM DTT, plus the indicated $MgCl_2$. The data shown is representative of three separate experiments and are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 2 %.

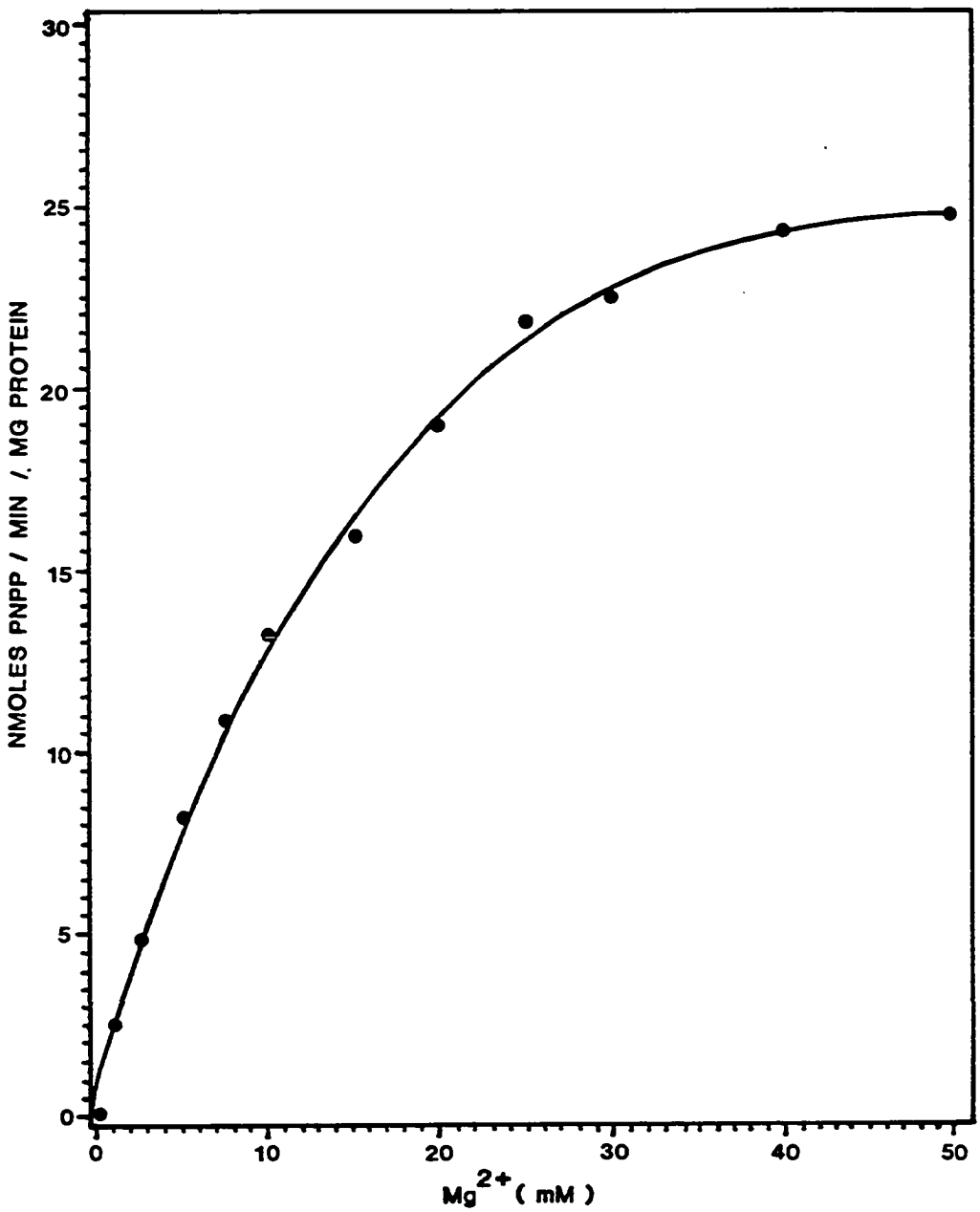
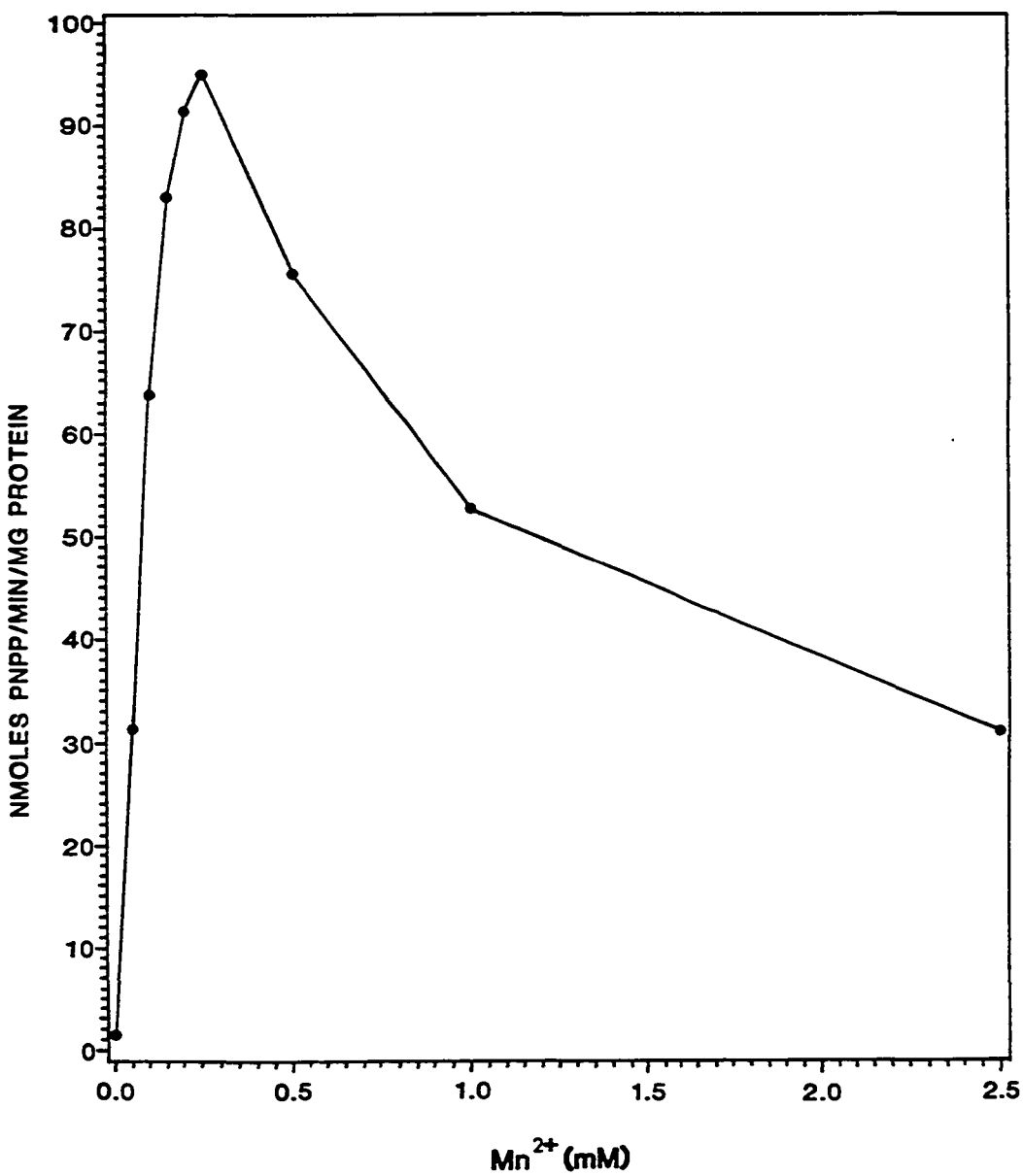

NMOLES PNPP / MIN / MG PROTEIN
30
25
20
15
10
5
0
0
10
20
30
40
50
Mg2+ (mM)

Figure 10 The Mn^{2+}-dependence of crude PNPPase. Assays were performed as in Figure 7, except that Mg^{2+} has been substituted for by the indicated concentration of Mn^{2+}. The data shown is representative of three separate experiments. Data shown are the averages of a duplicate determinations for each experiment. Duplicates in each experiment were within 3%.


NMOLES PNPP/MIN/MG PROTEIN
100
90
80
70
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
Mn2+ (mM)

3.2

ISOLATION OF TWO Mg^{2+}-DEPENDENT PNPPASE ACTIVITIES

Crude PNPPase (about 19,300 units) was concentrated and then fractionated over a Sephadex G-150 column as described under Materials and Methods. The elution profile is shown in Figure 5. The eluted fractions were assayed for PNPPase activity as described under Materials and Methods. Fractions with PNPPase activity were seen to elute at the trailing edge of the major protein peak; i.e., fractions # 74- 97 in Figure 5. For an estimation of PNPPase molecular weight, the column was calibrated with bovine thyroglobulin (Mr 670,000) (T), bovine Y-globulin (Mr 158,000) (G), chicken ovalbumin (Mr 44,000) (O), horse myoglobulin (Mr 17,000) (M) and vitamin B-12 (Mr 1350) (B). It was found that the PNPPase active fractions elute in the MW range of 15,000-30,000 Da. The reconstitution of the pooled fractions 'a', 'b' or 'd' with the pooled fraction 'c' (Figure 5) did not result in any stimulation of the observed activity. Hence, the pooled fraction 'c', that contained maximum activity, was used in the anion exchange column chromatography step using DEAE-Sephacel.

This fraction was pooled, concentrated, and equilibrated in Buffer B, and further purified by DEAE - Sephacel anion exchange chromatography (Figure 6). Most of the protein was bound to the column and did not wash out of the resin after passing several column volumes of Buffer B through the column. Bound protein was eluted from the column with a linear gradient of NaCl between 50 and 300 mM in Buffer B. The two

protein peaks showing PNPPase activity were resolved from one another and eluted at 120 mM and 160 mM salt concentration, respectively. The results are summarized in Table I. Peak I (pooled fractions 57-67) had an average specific activity of 85.4 nmoles PNPP hydrolyzed min^{-1} mg $protein^{-1}$, which represents a 3.8-fold purification over the crude postmicrosomal supernatent fraction and an 8.8% yield overall. Peak II (pooled fractions 68-87) had an average specific activity of only 17 nmol PNPP hydrolyzed min^{-1} mg $protein^{-1}$ with 7.3% yield. Neither reversing the order of the chromatograhic steps nor using DEAE-Sephacel anion exchange chromatograhy alone was effective in increasing PNPPase recovery or purification.

Figure 11 shows that the Peak I and Peak II PNPPase fractions are by no means pure at this stage of resolution. Nevertheless, the Peak I and Peak II PNPPases were used for further characterization.

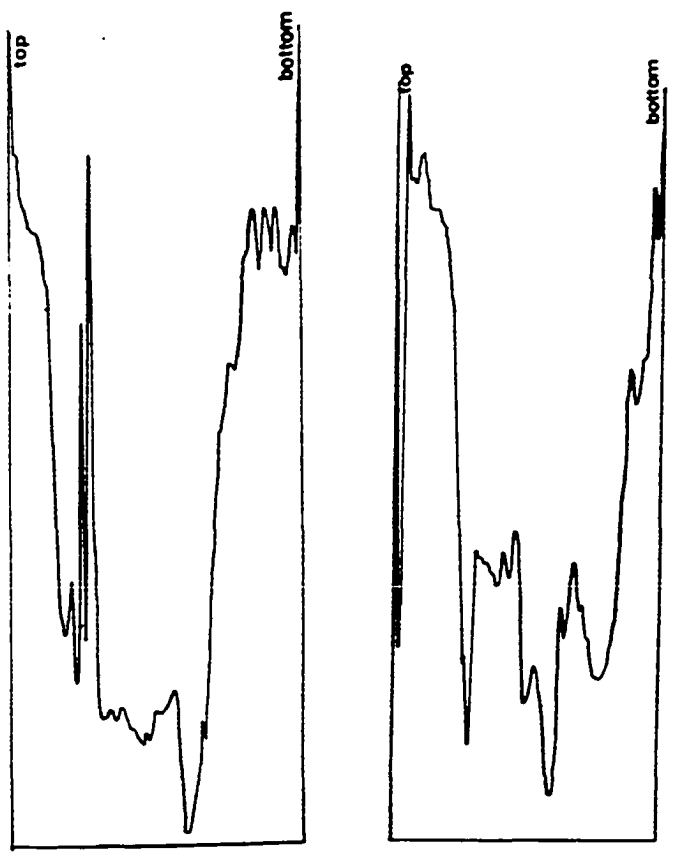
Following DEAE anion exchange chromatography, Horlein et al., (1982) subjected their first EAT cell fraction of phosphotyrosyl protein phosphatase activity to a Zn^{2+}-chelated iminodiacetic acid-agarose column chromatography step. They found that essentially all of their phosphatase activity was bound to the column and could be eluted with 20 mM histidine. Under identical conditions EAT cell Peak I PNPPase was bound to and could be eluted from a Zn^{2+}-chelated column with 20 mM histidine. The specific activity of the pooled fractions was 129 nmol PNPP hydrolyzed min^{-1} mg $protein^{-1}$, which represents a 5.8-fold purification over the crude postmicrosomal supernatant (Figure 12). On the other hand, Peak II PNPPase did not bind to the Zn^{2+} column and the

Figure 11

A Densitometer scan of Coomassie-stained, 7% native polyacrylamide gel of Peak I and II protein fractions off the DEAE-Sephacel chromatography step.

A = Peak I, B = Peak II.

top indicates the top of the gel and bottom indicates the bottom of the gel.


top
bottom
top
bottom
A
B

pooled Peak II activity which passed through the column had a Specific activity of about 95 nmol PNPP hydrolyzed min_{-1} mg $protein^{-1}$, which represents 4.3-fold purification (Figure 13). Unfortunately, both Peak I and Peak II obtained by this chromatographic procedure were extremely labile and could not be used for further characterization. In this regard, it was observed that the enzyme activities possessed a half-life of only 24 hours when storage was attempted at -80 °C. Other storage temperatures tested for possible stabilization of activity were -20 °C, 4 °C, and room temperature (22 °C). None of these storage conditions were as effective at stabilizing PNPPase as the -80 °C temperature. Hence, the Peak I and Peak II fractions obtained from an anion exchange column were used for further characterization.

Table I

SUMMARY OF THE RESOLUTION OF TWO EAT CELL PNPPASE ACTIVITIES

Fractions	Protein (mg)	PNPPase (units)	Specific activity (units/mg)	Yield (%)	Fold purification.
17,000 supernatent	855	19,300	22.6	100	1
Sephadex G-150	180	3,200	18.6	17	0.81
DEAE-sephacel Peak I	19.8	1,690	85.4	8.8	3.8
DEAE-sephacel Peak II	82.7	1,410	17.0	7.3	0.75

Footnote: This summary is representative of 17 separate chromatography runs. The results for chromatographic runs were within 10-15%.

Figure 12 Peak I : Zn^{2+}-chelated iminodiacetic acid agarose chromatography:

Elution profile of EAT cell Peak I PNPPase . Arrows A and B indicate elution of the column with 20 mM histidine and 60 mM histidine respectively. The chromatographic procedure and the assay of eluted fractions for PNPPase were performed as described under Materials and Methods. The profile shown is representative of two separate chromatographic runs using two preparations of Peak I PNPPase activity off the DEAE-Sephacel step.

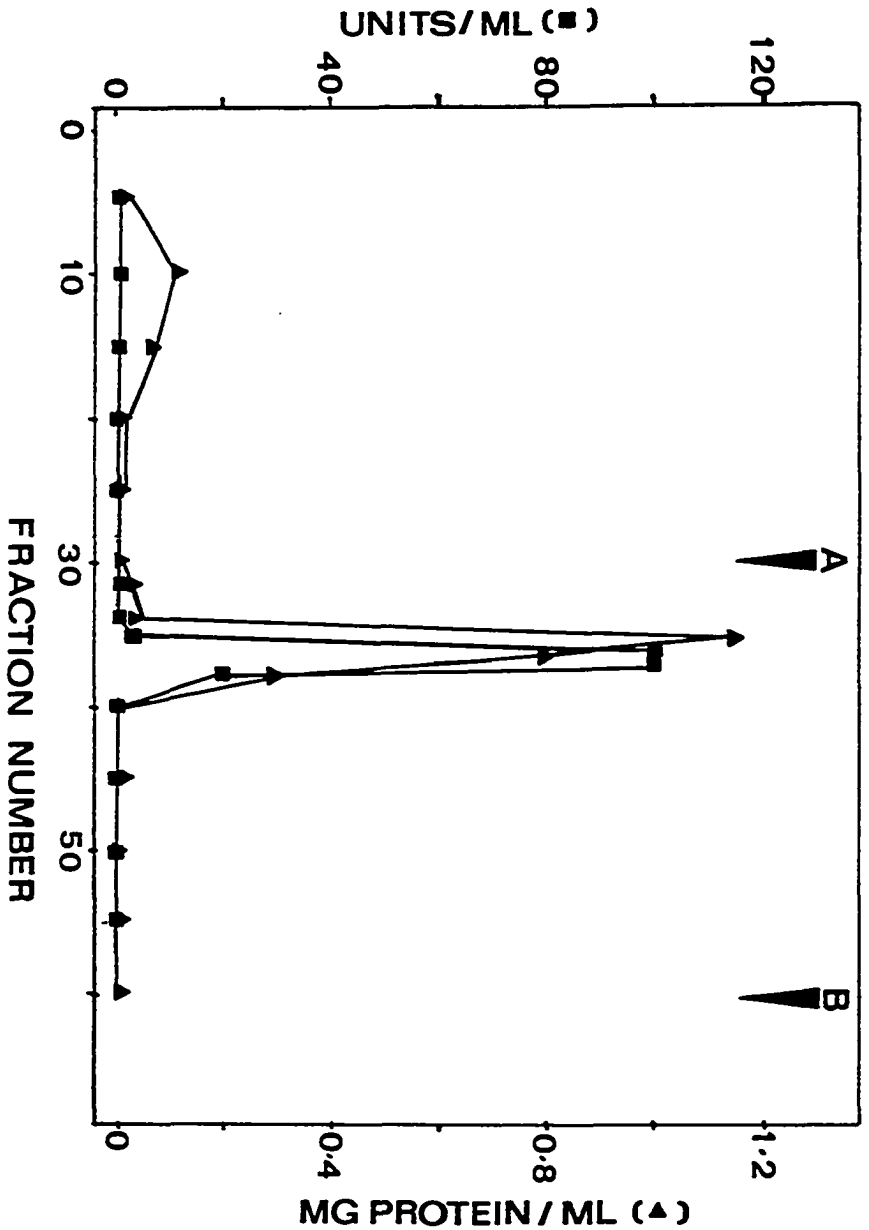
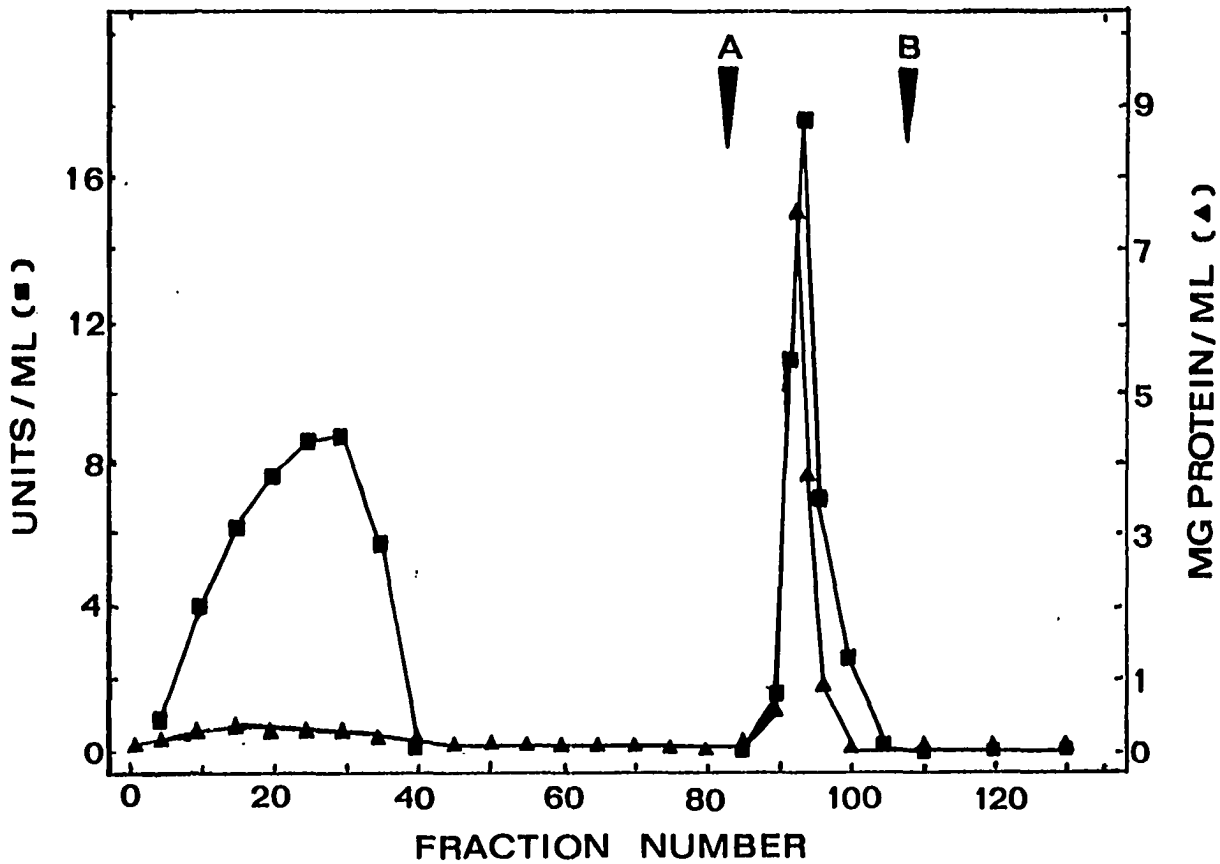

UNITS/ML (■)
0
40
80
120
FRACTION NUMBER
0
10
30
50
A
B
MG PROTEIN / ML (▲)
0
0·4
0·8
1·2

Figure 13 Peak II : Zn^{2+}-chelated iminodiacetic acid agarose chromatography:

Elution profile of EAT cells Peak II PNPPase . Arrows A and B indicate elution of the column with 20 mM histidine and 60 mM histidine respectively. The chromatographic procedure and the assay of the eluted fractions for PNPPase were performed as described under Materials and Methods. The profile shown is representative of two separate chromatographic runs using two preparations of Peak II PNPPase activity off the DEAE-Sephacel step.


A
B
UNITS/ML (■)
MG PROTEIN/ML (▲)
FRACTION NUMBER
0
4
8
12
16
0
1
3
5
7
9
0
20
40
60
80
100
120

3.3

CATION AND SULFHYDRYL GROUP REQUIREMENTS

Peak I and Peak II exhibited similar dependencies for Mg^{2+} as was observed for the crude postmicrosomal supernatant (Figure 9). 40 mM Ca^{2+}, Co^{2+}, and Zn^{2+} do not substitute for 40 mM Mg^{2+}. Mn^{2+} at 40 mM completely inhibits Peak I and Peak II Mg^{2+}-dependent PNPPases. However, low concentrations of Mn^{2+} (250 uM) can substitute for Mg^{2+}, for both the Peak I and the Peak II PNPPases. These results are the same as that observed with the crude postmicrosomal supernatant (Figure 10). The Mn^{2+}/ Mg^{2+} stimulation ratio ranges from three to five for Peak I and from two to six for Peak II, depending upon the age of the preparations. The Peak II PNPPase is more labile with time of storage than the Peak I enzyme.

These results differ considerably from those of Cathala et al (1975) who found that the bovine kidney PNPPase was equally stimulated by saturating concentrations of Mg^{2+} and Mn^{2+} at pE 8.5. Li & Chan (1981) reported that PNPPase activities from several tissue sources, including bovine brain, heart, spleen, uterus, and kidney, rabbit liver and muscle, and lobster muscle measured in the presence of saturating Mg^{2+}, are three- to six-fold higher than those measured in the presence of saturating Mn^{2+} at pH 8.5. Pato et al (1983) have isolated a Mg^{2+}-dependent phosphatase from turkey gizzard smooth muscle which was stimulated five-fold more in the presence of Mg^{2+} than by Mn^{2+}.

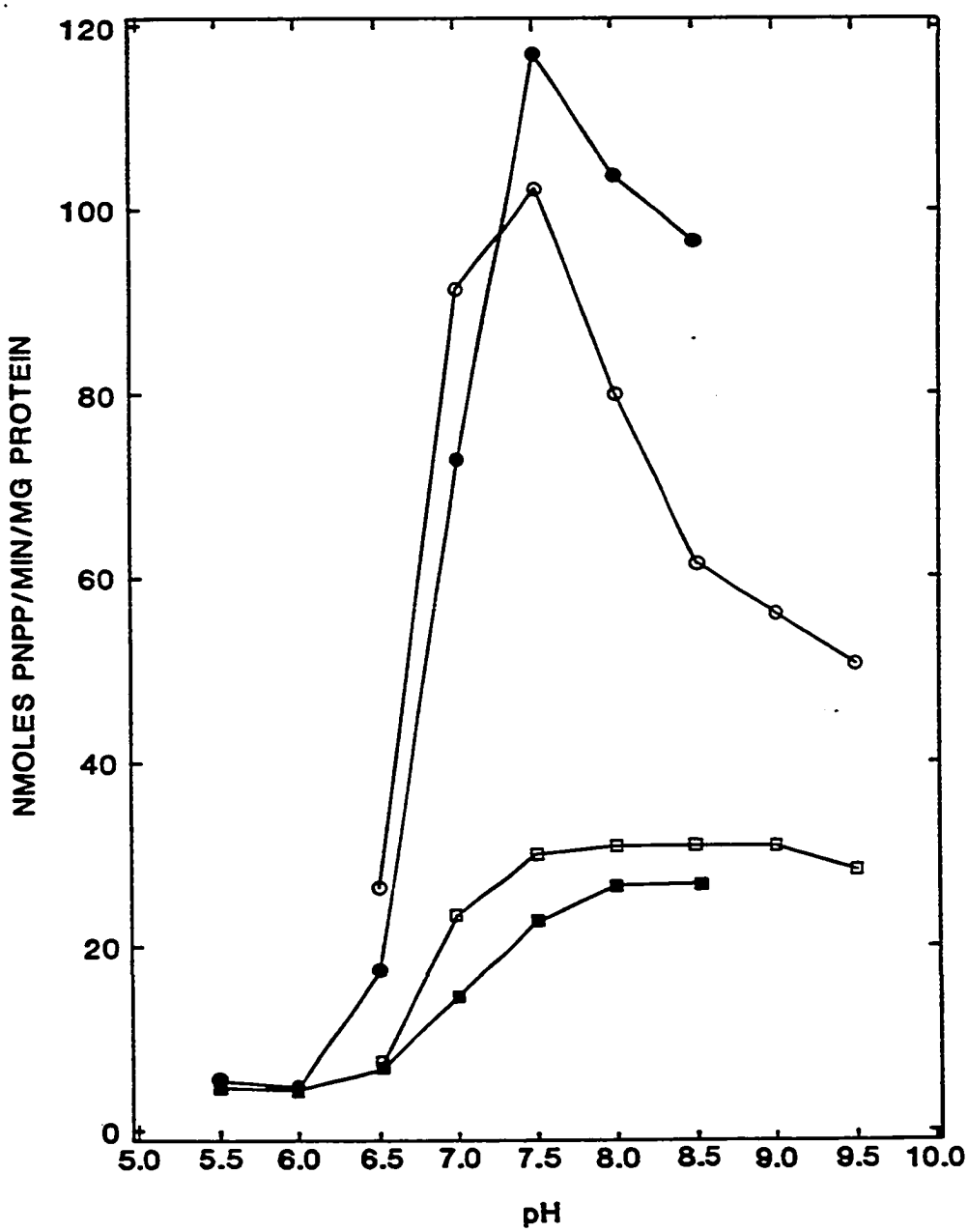
Though the 40 mM Mg^{2+}. needed for the activation of the peak I and Peak II PNPPase is much higher than the physiological Mg^{2+} concentration (about 1 mM), this large Mg^{2+} requirement (upto 100 mM) has been observed with many alkaline phosphatases (Li & Chan, 1981). However, in this case, i.e., the Peak I and Peak II PNPPases from the postmicrosomal fraction of EAT cell homogenates, the high Mg^{2+} ion concentration requirement could conceivably arise from the possible contamination of the Peak I and Peak II preparations by transfer ribonucleic acids (tRNAs). The tRNAs with their highly negative phosphate backbone could presumably act as a sink for the added Mg^{2+} ions.

Furthermore, as seen with crude PNPPase, both Peak I and Peak II PNPPases are stimulated by the sulfhydryl compound DTT.

3.4 pH PROFILE

Since PNPP is a well known substrate for both acid and alkaline phosphatases, it was interesting to determine the pH optimum for both Peak I and Peak II PNPPases. In Figure 14 it can be seen that the pH optima for Mg^{2+}-dependent Peak I and Peak II PNPPases differ. The Peak I enzyme has optimal activity in a narrow range centered at about pH 7.5 in both the imidazole-HCl and the Tris-HCl buffer systems, while the less active Peak II enzyme exhibits a much broader range of optimal activity with essentially equal rates being observed between pH 7.5 and pH 9.5 in the Tris-HCl buffer system. Peak I PNPPase has virtually the

Figure 14 pH optima for Mg^{2+}-dependent Peak I and Peak II PNPPase activities. Assays of 20 ug protein aliquots of the Peak I (○,●) and Peak II (□,■) fractions for PNPPase were performed as described in Materials and Methods at the indicated pH in an assay medium (500 ul) consisting of either 80 mM Tris - HCl (○,□) or 80 mM Imidazole-HCl (●,■), and also 40 mM $MgCl_2$, 1 mM DTT, and 20 mM PNPP. The data shown is representative of three separate experiments for each buffer system and are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 4 %.


120
100
80
60
40
20
0
NMOLES PNPP/MIN/MG PROTEIN
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

same optimal pH whether Mg^{2+} or Mn^{2+} ions were used as a stimulating cation (data not shown). The Peak II Mg^{2+}- and Mn^{2+}-dependent PNPPases were not compared. The broader pH range for Peak II PNPPase could very well be due, in part, to its contamination with Peak I PNPPase. This conclusion is supported by the observation in Figure 13 that Peak II when loaded onto a Zn-chelated column had a protein fraction with PNPPase activity which eluted under identical conditions that elute a bonafide Peak I fraction.

3.5

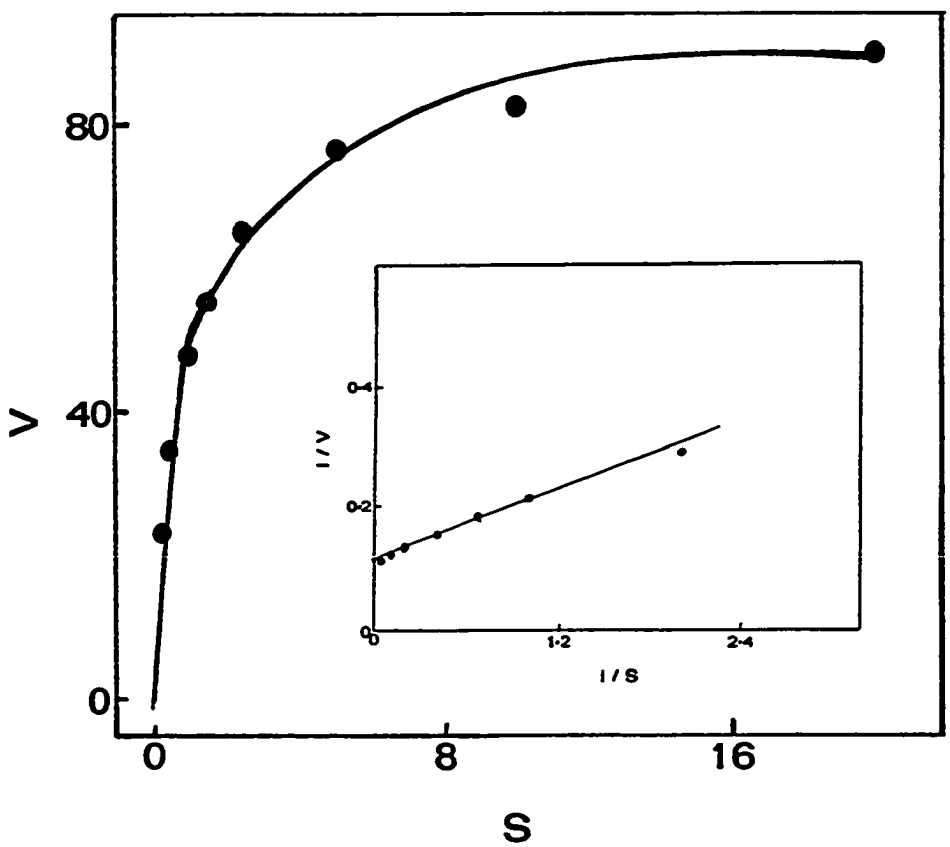
Km OF PNPP FOR PEAK I PNPPASE

The K_m of the non-protein ester, PNPP, was determined in the presence of either Mg^{2+} or Mn^{2+} ions for the Peak I fraction. In three experiments, done in the presence of Mg^{2+} ions, K_m values of 0.89, 0.61, and 0.76 mM (Figure 15) and V_{max} values of 91, 104, and 58 nmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$, were obtained respectively. For an additional experiment a variant set of results was obtained. In this latter case, the K_m in the presence of Mg^{2+} was 1.16 mM and the V_{max} was 34 nmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$.

Similarly, in three experiments, the K_m and V_{max} were determined in the presence of Mn^{2+} ions. The K_m values obtained in this case were 0.12, 0.103, and 0.09 mM (Figure 16), with a V_{max} of 307, 238, and 352 nmoles of PNPP hyrolyzed min^{-1} mg of $protein^{-1}$, respectively. For an additional experiment a variant set of results was obtained. In this

Figure 15

Saturation plot of the Peak I Mg^{2+}-dependent PNPPase activity as a function of the indicated substrate (PNPP) concentration. In this figure 'S' represents the substrate concentration in mM, whereas 'V' represents the velocity of the reaction in nmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$. The inset is a double reciprocal plot of the same data which is representative of 4 separate experiments. Duplicates in each experiment were within 3%. The data shown are the averages of a duplicate determinations for each experiment.

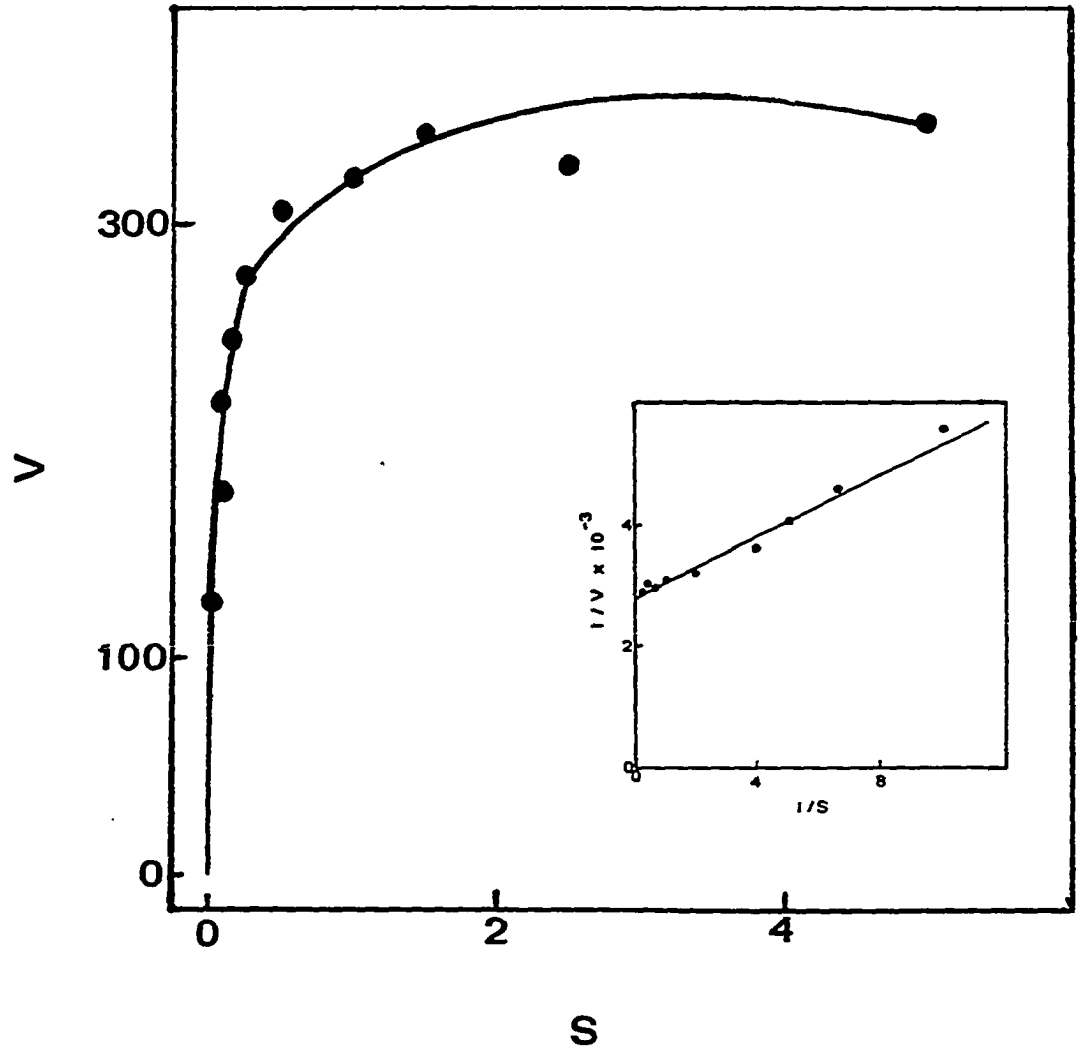

80
40
0
V
0
8
16
S
0·4
0·2
0
1/V
1·2
2·4
1/S

latter case, the K_m in the presence of Mn^{2+} was 1.05 mM and the V_{max} was 77 nmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$.

It should be noted that the V_{max} of 91 and 104 obtained for Mg^{2+}-dependent PNPPases and the 307, 238, and 352 values obtained for the Mn^{2+}-dependent PNPPases are very similar to the specific acitvity values obtained routinely for these PNPPases. However, there does exist a variation in the values measured which conceivably could arise from the differences in the different preparations of the PNPPases and/ or the age of a particular protein sample.

Figure 16

Saturation plot of the Peak I Mn^{2+}-dependent PNPPase activity as a function of the indicated substrate (PNPP) concentration. In this figure 'S' represents the substrate concentration in mM, whereas 'V' represents the velocity of the reaction in nmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$. The inset is a reciprocal plot of the same data which is representative of 4 separate experiments. Duplicates in each experiment were within 4%. The data shown are the averages of a duplicate determinations for each experiment.


300
100
0
V
0
2
4
S
1 / V x 10⁻³
4
2
0
4
8
1/S

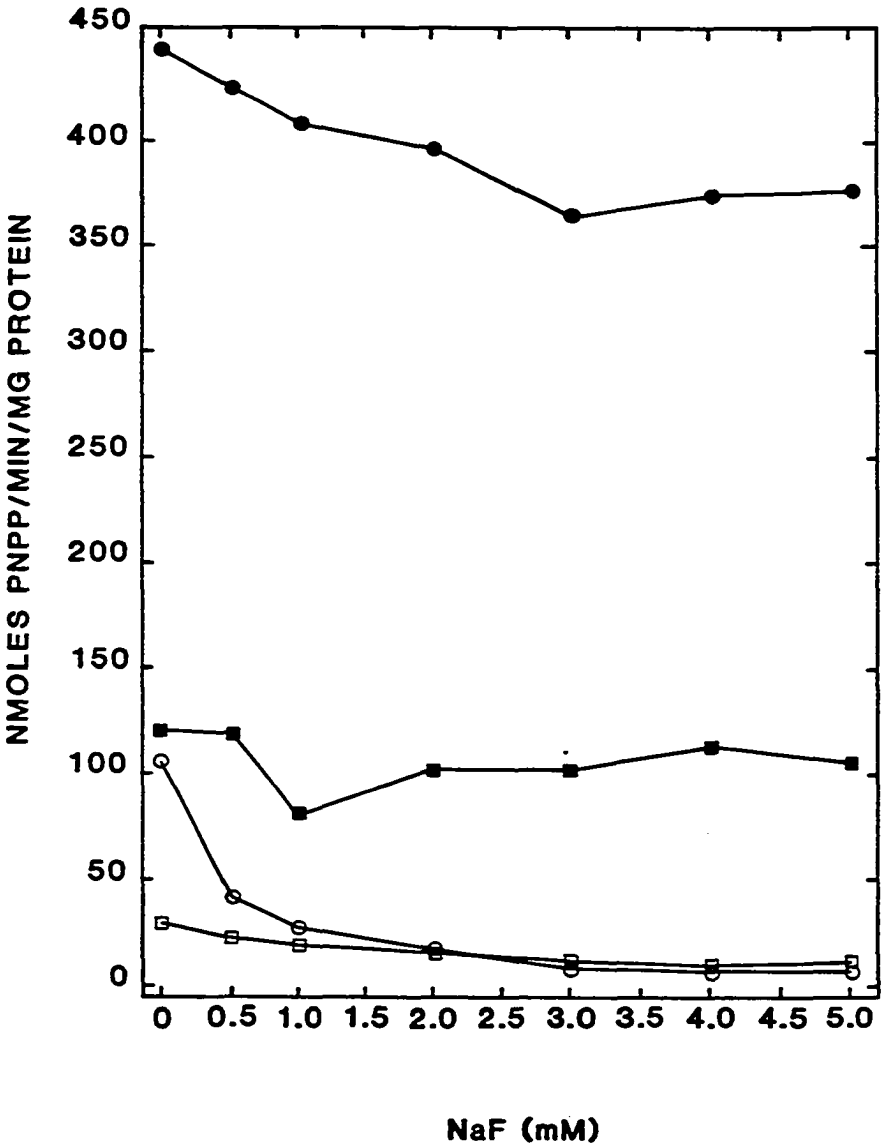
3.6 EFFECT OF INHIBITORS

3.6.1 EFFECT OF FLUORIDE

Fluoride is a known inhibitor of phosphoprotein phosphatases (Maeno & Greengard, 1972). It is most effective in inhibiting phosphoseryl protein phosphatase activity (Swarup et al., 1982). Chernoff et al., (1983), showed that phosphoseryl protein phosphatase activity is completely inhibited by NaF whereas high concentrations of NaF (50 mM) inhibit dephosphorylation by phosphotyrosyl protein phosphatase by only 50%. Similar observations that fluoride is not an effective inhibitor of phosphotyrosyl protein phosphatase activity were also made by Foulkes et al (1981,1983), Swarup et al (1982), Brautigan et al (1981), Shriner et al (1984) and Leis and Kaplan (1982).

The effects of fluoride on the EAT cell phosphatases are presented in Figure 17. Clearly, the Mg^{2+}-dependent PNPPases are far more sensitive to fluoride inhibition than the Mn^{2+}-dependent PNPPases. Compare 93% inhibition of Peak I Mg^{2+}-dependent PNPPase to 15% inhibition of Peak I Mn^{2+}-dependent PNPPase, and 61% inhibition of Peak II Mg^{2+}-dependent PNPPase to 13% inhibition of Peak II Mn^{2+}-dependent PNPPase when assayed in the presence of 5 mM fluoride. From these results it is seen that

Figure 17 Fluoride inhibition of EAT cell PNPPases. Aliquots of Peak I (15 ug protein;○) and Peak II (22 ug protein;□) fractions were assayed as described under Materials and Methods for Mg^{2+}-dependent PNPPase activity in the presence of the pH 7.5 Tris-HCl buffer system described in Figure 14 plus the indicated NaF. Mn^{2+}-dependent PNPPase activities for Peak I (2.9 ug protein;●) and Peak II (11 ug protein;■) were assayed under identical conditions except that 250 uM $MnCl_2$ substituted for $MgCl_2$. The data shown is representative of three separate experiments each for both Mg^{2+}-dependent Peak I & II PNPPase activity. The data shown are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 3 %. Similarly, for the Mn^{2+}-dependent Peak I and II PNPPase activity, the data shown is representative of four separate experiments each of the activities. The data shown are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 4 %.


450
400
350
300
250
200
150
100
50
0
NMOLES PNPP/MIN/MG PROTEIN
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
NaF (mM)

the Peak I and Peak II Mn^{2+}-dependent PNPPases are similar in that both are only slightly inhibited by fluoride. However, in the presence of 0.5 mM fluoride, Peak I Mg^{2+}-dependent PNPPase (61% inhibition) is more sensitive to fluoride than the Peak II Mg^{2+}-dependent PNPPase (25% inhibition).

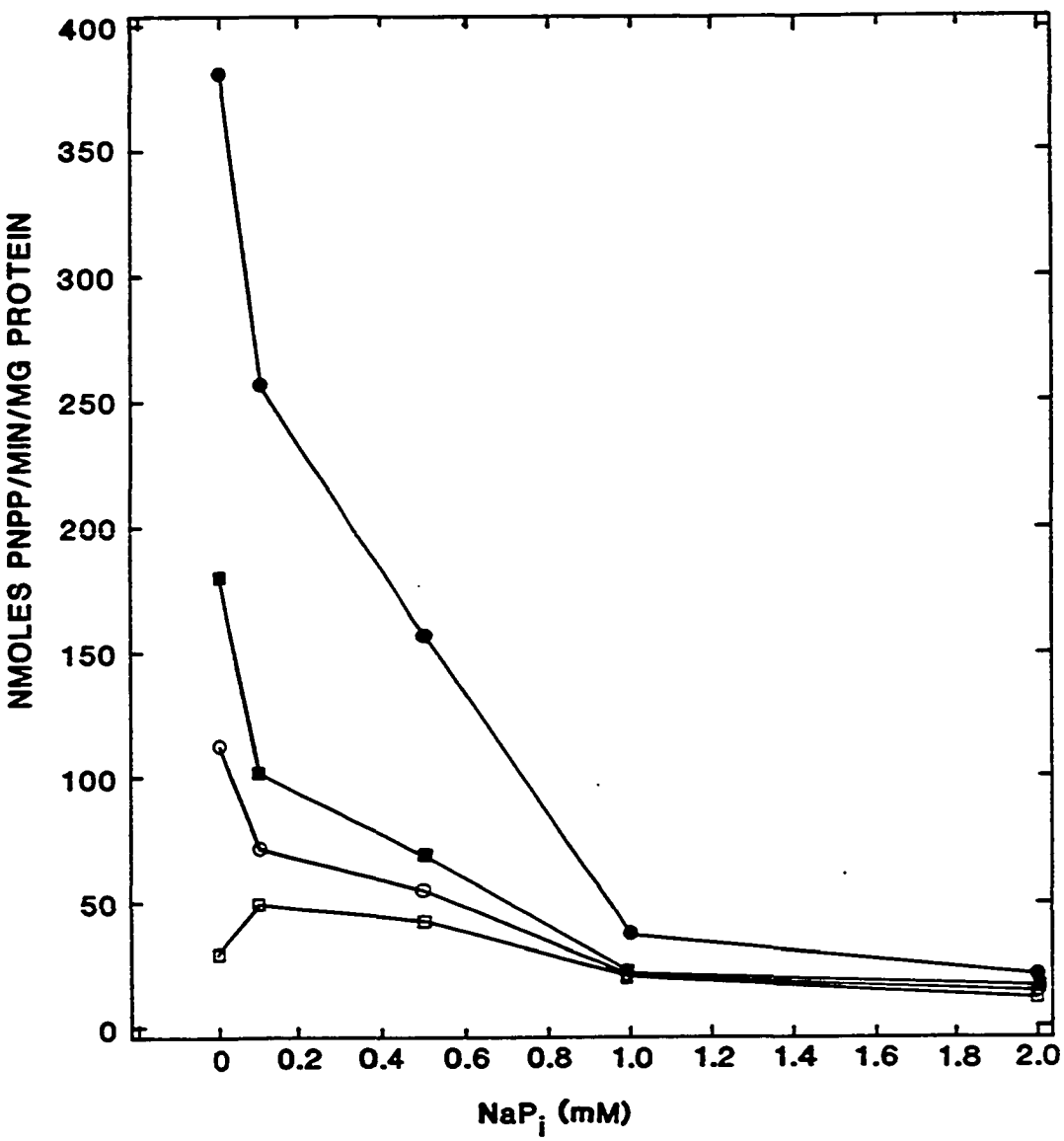
3.6.2

EFFECT OF PHOSPHATE

Inorganic phosphate (Pi) is not only a substrate and a product, but also a competitive inhibitor of alkaline phosphatases. It has been shown to be an effective inhibitor for the alkaline phoshatase associated with cardiac phosphoseryl protein phosphatase (Li, H-C., et al., 1979), and the alkaline phosphatase specific toward phosphotyrosine histone (Swarup et al., 1981). Inorganic phosphate inhibits alkaline phosphatases competitively (Moss et al., 1967; Eaton et al., 1967; Bowers et al., 1966; Torriani, A., 1960; and Hilliard et al., 1965).

We examined the effect of phosphate on Peak I and Peak II PNPPases from EAT cell and the results are presented in Figure 18. It is seen that Peak I Mg^{2+}-dependent PNPPase is more sensitive to inhibition by phosphate than the Peak II Mg^{2+}-dependent PNPPase. In fact, low concentrations of phosphate resulted in a very curious observation that Mg^{2+}-dependent Peak II PNPPase is stimulated by 0.1-0.5 mM phosphate

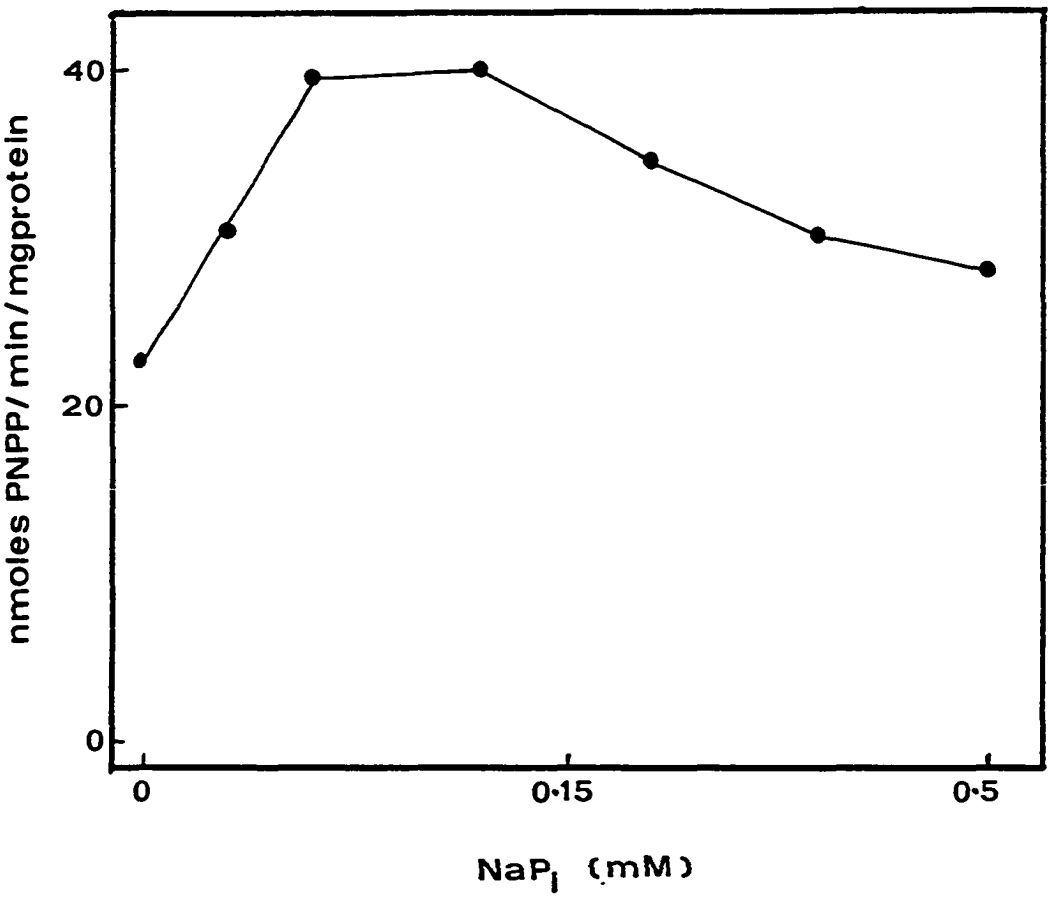
Figure 18 The effect of phosphate on EAT cell PNPPases. Aliquots of the Peak I (14.8 ug protein;○,●and Peak II (11.4 ug protein;□,■) fractions were assayed for Mg^{2+}-dependent (○,□) and Mn^{2+}-dependent (●,■) PNPPase in the presence of the indicated concentration of sodium phosphate, all as described in Figure 17. The data shown is representative of five separate experiments each for both Mg^{2+}-dependent Peak I & II PNPPase activity and are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 4 %. Similarly, for the Mn^{2+}-dependent Peak I and II PNPPase activity, the data shown is representative of four separate experiments each for the two activities. The data shown are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 3 %.


NMOLES PNPP/MIN/MG PROTEIN
400
350
300
250
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
NaP$_i$ (mM)

(Figure 19), whereas other PNPPase activities are inhibited by 30%- 45%. Maximal stimulation is about 66% and is observed at about 0.1 mM phosphate. Higher concentrations of phosphate inhibit the Peak II Mg^{2+}-dependent PNPPase, but not to as great an extent as for the other EAT cell PNPPase activities. At 2.0 mM phosphate, Peak I and II Mn^{2+}-dependent PNPPases and Peak I Mg^{2+}-dependent PNPPase are inhibited by 90-95%, while Peak II Mg^{2+}-dependent PNPPase is inhibited by 44%.

Figure 19

Peak II Mg^{2+}-dependent PNPPase activity in the presence of the indicated concentrations of Sodium Phosphate (0.05 - 0.5 mM). Data shown are the averages of a duplicate determinations, in this single experiment, which were within 2%.


40
20
0
nmoles PNPP/min/mgprotein
0
0·15
0·5
NaP$_i$ (mM)

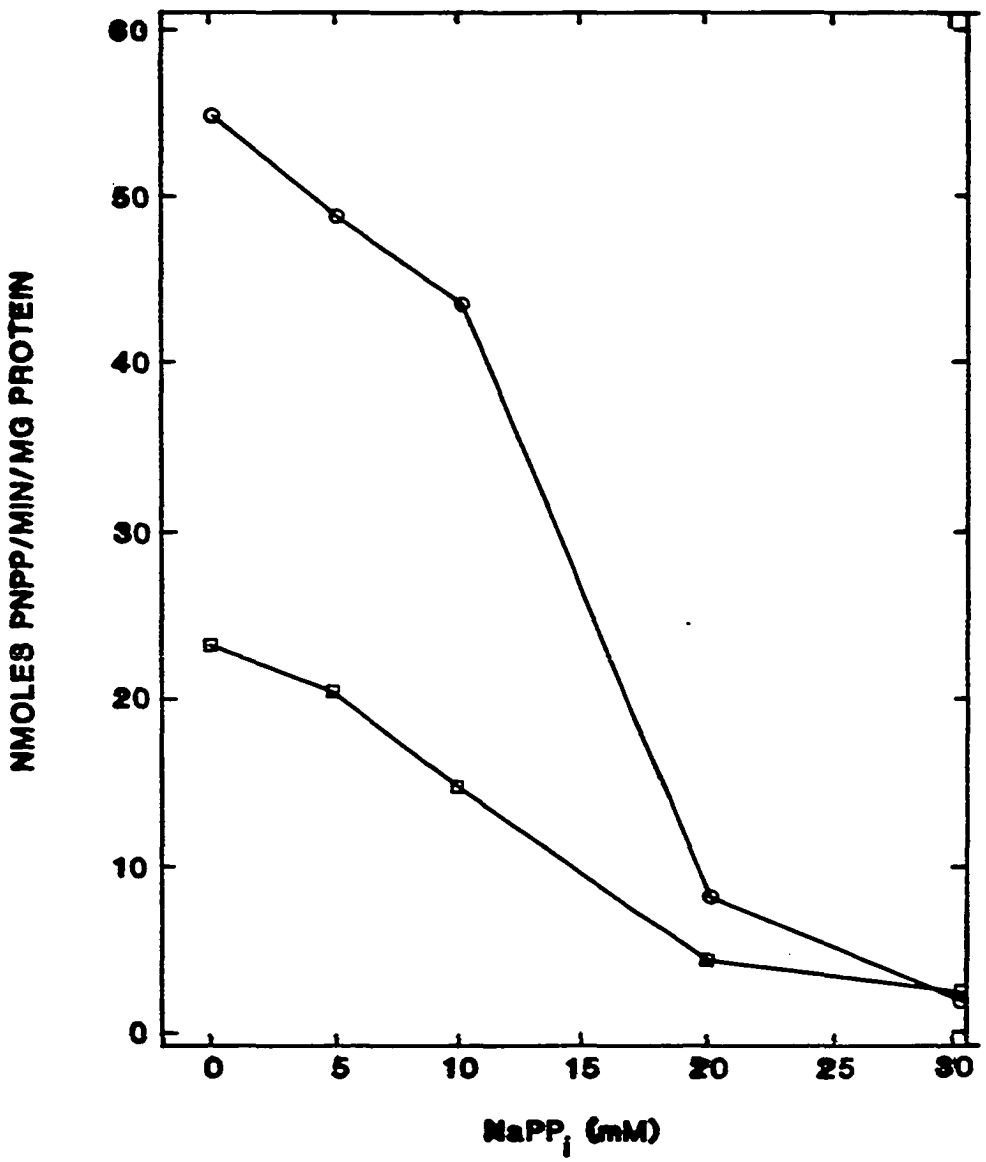
3.6.3

EFFECT OF PYROPHOSPHATE

Although there are specific pyrophosphatases (Akock & Shils, 1967; Korhonen et al., 1977; Eaton & Moss, 1967), there is good deal of evidence that the majority of nonspecific alkaline phosphatases hydrolyze pyrophosphate (PP_i) in the same manner as they hydrolyze other phosphorylated substrates. However, although it is known to be a substrate, calf intestinal alkaline phosphatase activity, (Kelly & Hamilton, 1970), and Mg^{2+}-dependent phosphatase isolated from turkey gizzard smooth muscle (Pato et al., 1983) were seen to be inhibited by PPi.

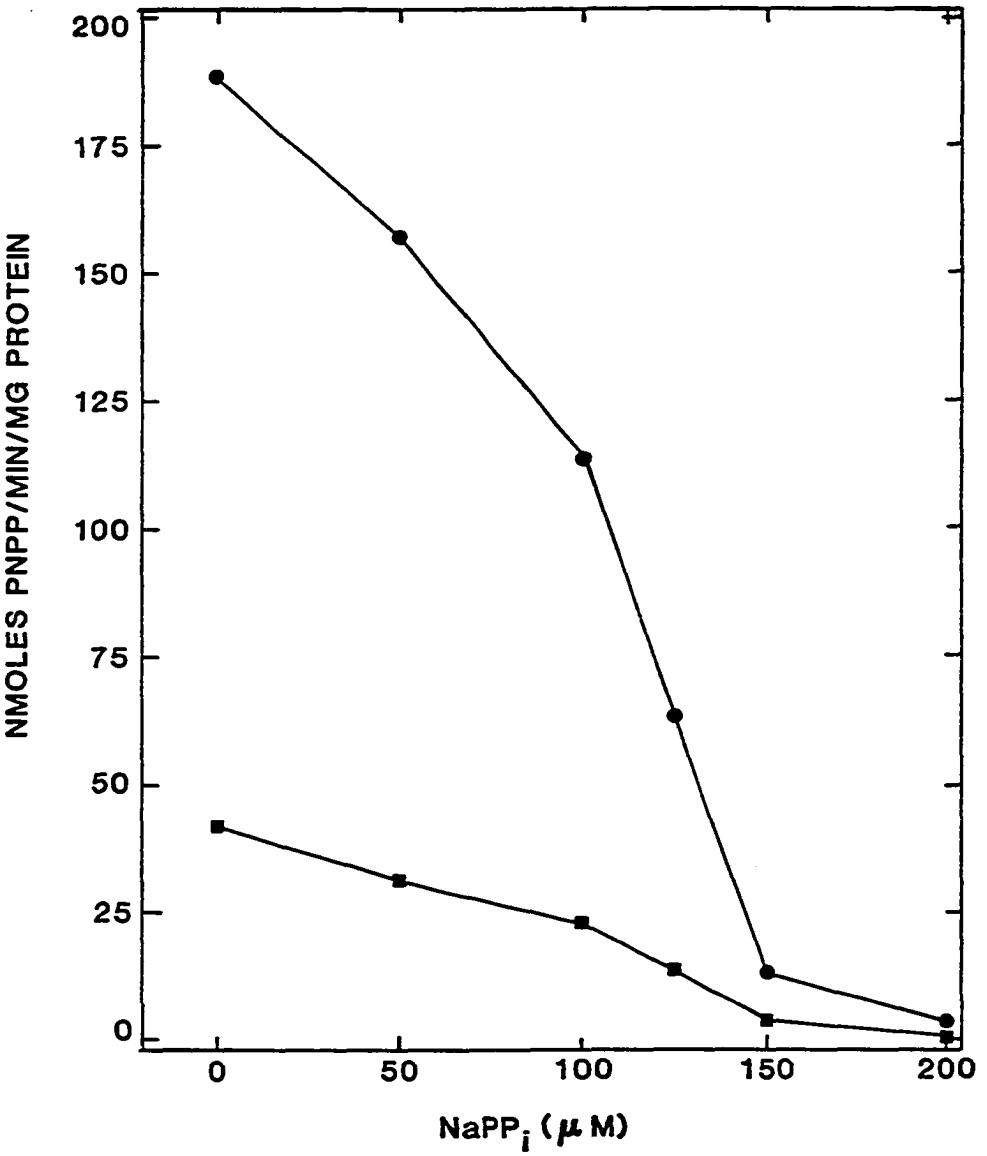
We examined the effect of pyrophosphate on Peak I and Peak II PNPPases (Figures 20 and 21). It is seen that relatively high concentrations of pyrophosphate (mM range) are required for significant inhibition of Peak I and Peak II Mg^{2+}-dependent PNPPases (Figure 20). For both Peak I and Peak II Mg^{2+}-dependent PNPPases half- maximal inhibition is obtained with 12-15 mM pyrophosphate and essentially complete inhibition is afforded by 30 mM pyrophosphate. In stark contrast to the properties of the Peak I and Peak II Mg^{2+}-dependent PNPPases, the Peak I and Peak II Mn^{2+}-dependent PNPPases are very sensitive to the inhibition by pyrophosphate. 100 uM pyrophosphate inhibits Peak I and Peak II Mn^{2+}-dependent PNPPases by about 50% (Figure

Figure 20 Pyrophosphate inhibition of EAT cell PNPPase. Aliquots of the Peak I (16 ug protein;○) and Peak II (18.8 ug protein;□) fractions were assayed for Mg^{2+}-dependent PNPPase in the presence of the indicated sodium pyrophosphate concentrations as was described in Figure 17. The data shown is representative of six separate experiments each for both Mg^{2+}-dependent Peak I & II PNPPase activities. The data shown are averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 4 %.


NMOLES PNPP/MIN/MG PROTEIN
60
50
40
30
20
10
0
0
5
10
15
20
25
30
NaPP$_i$ (mM)

21) and 200 uM pyrophosphate is required to essentially completely inhibit these activities.

Figure 21 Pyrophosphate inhibition of EAT cell PNPPases. Aliquots of the Peak I (16.3 ug protein;●) and Peak II (18.8 ug protein;■) fractions were assayed for Mn^{2+}-dependent PNPPase in the presence of the indicated sodium pyrophosphate concentrations as was described in Figure 17. For the Mn^{2+}-dependent Peak I and II PNPPase activity, the data shown is representative of five separate experiments each of the activities. The data shown are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 4 %.


NMOLES PNPP/MIN/MG PROTEIN
200
175
150
125
100
75
50
25
0
0
50
100
150
200
NaPP$_i$ (μM)

3.6.4

EFFECT OF OTHER PHOSPHORYLATED LIGANDS

The activity of the Peak I Mg^{2+}-dependent PNPPase in the presence of a number of phosphorylated compounds which might be expected to bind to the enzyme and to serve as substrates if the enzyme acts as a non-specific phosphatase was determined as shown in Table II. In each case, a partial inhibition of Peak I Mg^{2+}-dependent PNPPase was observed. The most effective inhibitors tested were ITP, GTP, NADP, 6-phosphogluconic acid, CTP, D-galatose 1-phosphate, and phosphothreonine. However, phosphotyrosine, phosphoserine, and ATP were also effective. This result indicates that either Peak I Mg^{2+}-dependent PNPPase is a nonspecific neutral phosphatase and/ or reflects on the apparent nonhomogeneity of the Peaks I and II as seen in Figure 11.

Table II

EFFECT OF PHOSPHORYLATED LIGANDS ON PEAK I Mg^{2}-DEPENDENT PNPPASE ACTIVITY

Additions[a]	Percentage control activity
None	100[b]
ITP	22.4
GTP	34.6
NADP	40.3
6-Phosphogluconic acid	45.2
CTP	49.6
x-D-Galatose 1-phosphate	50.6
x-D-Glucose 1-phosphate	52.4
Threonine-phosphate	54.3
2-Phosphogluconic acid	59.9
Tyrosine-phosphate	61.6
Serine-phosphate	65.6
ATP	70.9
Fructose 6-phosphate	74.4
Glucose 6-phosphate	93.8

Footnote:

[a] Each addition was made to a final concentration of 10 mM. Assays were performed without preincubation as in 80 mM Tris-HCl, pH 7.5, 40 mM $MgCl_2$, 1 mM DTT, 20 mM PNPP, plus the indicated additions as described under Materials and Methods.

[b] 100% control activity corresponds to 49 mmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$.

3.6.5

EFFECT OF ZINC

Martensen (1982) and Horlein et al (1982) reported that micromolar concentrations of Zn^{2+} was a potent inhibitor of EAT cell neutral phosphatases . Foulkes et al., (1981 & 1983) also reported that the phosphotyrosine protein phosphatase activities from rat skeletal muscle and liver extracts and from chicken extract were sensitive to $ZnCl_2$. In addition, Leis and Kaplan (1982), have reported a Zn^{2+}-sensitive phosphoseryl protein phosphatase activity.

We therefore investigated the effect of zinc on the EAT cell Peak I and Peak II Mg^{2+}- and Mn^{2+}-dependent PNPPases. It was seen (Table III) that in the concentration range from 0.1-0.5 mM, Zn^{2+} inhibits Peak II Mn^{2+}-dependent PNPPase more than the other EAT cell PNPPase activities. In the presence of 1.0 mM Zn^{2+}, essentially complete inhibition of Peak II PNPPase is attained, while the Peak I activities are inhibited only 63-72%. It was noted that freshly made Zn^{2+} solution is more potent than aged solution, hence fresh solutions of $ZnCl_2$ were used throughout these experiments.

Table III

$ZnCl_2$ INHIBITION OF EAT CELL PNPPASE.[a]

	$ZnCl_2$ ADDITIONS (mM)	Mg^{2+}-PNPPase	Mn^{2+}-PNPPase
		(nmoles PNPP min^{-1} mg $protein^{-1}$)	
PEAK I	0	58.9	208
	0.1	53.0	208
	0.25	52.9	192
	0.5	48.4	190
	1.0	16.4	77.1
PEAK II	0	24.5	77.1
	0.1	29.6	51.6
	0.25	27.8	34.3
	0.5	23.8	24.0
	1.0	0	1.53

[a]Aliquots of the Peak I (16.3 ug protein) and Peak II (18.8 ug protein) fractions were assayed for Mg^{2+} -dependent and Mn^{2+}-dependent PNPPase in the presence of the indicated $ZnCl_2$ concentrations as was outlined in Figure 15. The data shown here in this table is representative of three separate experiments for the Mg^{2+}-dependent PNPPase activities and five separate experiments for the corresponding Mn^{2+}-dependent activities. The data shown are the averages of a duplicate determinations for each experiment. In each experiment, duplicates were within 5 %.

3.6.6

EFFECT OF LEVAMISOLE AND VANADATE

None of the EAT cell neutral PNPPases were found to be inhibited by 1 mM levamisole, a placental type alkaline phosphatase inhibitor (Van Belle, 1972). In addition, vanadate, which inhibits human (Lopez et al., 1976) and E. coli (Seargeant & Stinson, 1979) alkaline phosphatases, human epidermoid carcinoma A-431 cell phosphotyrosyl protein phosphatase (Swarup et al., 1982a), and both phosphotyrosyl phoshatase and phosphoseryl phosphatase activities from TCRC-2 tumor cell and PNPPase from TCRC-2 tumor cells (Swarup et al., 1982b), inhibits EAT cells PNPPase no more than about 20% at a 1 mM concentration.

3.7

EFFECT OF TRYPSIN

Treatment of proteins with proteolytic enzymes like trypsin and modifying reagents like N-ethylmaleimide can result in modulation of enzymatic activity either by changes effected at the active center directly or via structural (conformational) alterations at the active center resulting from chemical changes occuring at distant sites. Hence, the effects of trypsin, which chemically alters proteins by hydrolyzing peptide bonds at lysyl or arginyl residues, and N-ethylmaleimide, which alkylates sulfhydryl residues, were determined.

The data in Figure 22 show that Peak I Mg^{2+}-dependent PNPPase is most sensitive to trypsinolysis. A maximal 75% inactivation is obtained with only 5 ug of trypsin. Greater trypsin concentrations have no additional effect on this activity (data not shown). For Peak I Mn^{2+}-dependent PNPPase, 25 ug trypsin in the preincubation mixture resulted in only 43% inactivation while this concentration of protease inactivated Peak II Mn^{2+}-dependent PNPPase by 72% and Peak II Mg^{2+}-dependent PNPPase by 78%. Greater trypsin concentrations were without additional effect. The ability of trypsin to partially inactivate each of the neutral PNPPase activities suggested that trypsinolysis could be used as as a probe to compare the effect of ligand binding to these enzymes. In Table IV, Experiment I, the effects of Mg^{2+} and Mn^{2+} on the extent of tryptic inactivation of PNPPase are compared. Neither 40 mM Mg^{2+} nor 250 uM Mn^{2+}, which optimally stimulate PNPPase activity, has an effect on the

proteolytic inactivation of Peak I PNPPase when these cations are included with trypsin in a preincubation mixture. However, Peak II Mg^{2+}-dependent PNPPase inactivation decreases from 62% in the absence of Mg^{2+} to only 11% when trypsinolysis is performed in the presence of Mg^{2+}. Only a slight protective effect against Peak II Mn^{2+}-dependent PNPPase tryptic inactivation was obtained with Mn^{2+}.

The effects of the inclusion of the substrate PNPP in the trypsinolysis preincubation mixtures are presented in Table IV, Experiment II. Protection against inactivation was observed in each case. Most affected was the Peak II Mg^{2+}-dependent PNPPase where the extent of tryptic inactivation decreased from 56% in the absence of PNPP to 15% in the presence of PNPP during preincubation. Peak II Mn^{2+}-dependent PNPPase inactivation by trypsin decreased from 55% in the absence to 26% in the presence of PNPP, and the Peak I PNPPases inactivation by trysin decreased to 10-15% in the presence of PNPP in the preincubation reaction mixture. Neither 1 mM DTT, which stimulates EAT cell PNPPase activity (Javeri et al., 1984), nor 0.2 mM phosphate, which was shown in Figure 19, above, to stimulate Peak II Mg^{2+}-dependent PNPPase, has any effect on modifying the extent of inactivation of EAT cell PNPPases by trypsin.

Figure 22 Trypsin inactivation of EAT cell PNPPases. Preincubations of aliquots of the Peak I (16.3 ug protein;○,●) and Peak II (18.8 ug protein;□,■) fractions with the indicated concentrations of trypsin were incubated on ice for 15 min as described in Materials and Methods in a medium of 390 ul consisting of 103 mM Tris-HCl, pH 7.5. Proteolytic action was quenched by three fold excess of soybean trypsin inhibitor and by dilution of the preincubation mixture such that the final 500 ul volume contained at a complete assay system appropriate for either residual Mg^{2+}-dependent (○,□) or residual Mn^{2+}-dependent (●,■) PNPPase activity determination as was described in Figure 17. The data shown is representative of five separate experiments each for both Mg^{2+}-dependent Peak I & II PNPPase activity. Duplicates within each experiment were within 5 %. Similarly, for the Mn^{2+}-dependent Peak I and II PNPPase activity, the data shown is representative of five separate experiments each of the activities. Duplicates within each experiment were within 4 %. In all cases, the data shown are the averages of a duplicate determinations for each experiment.

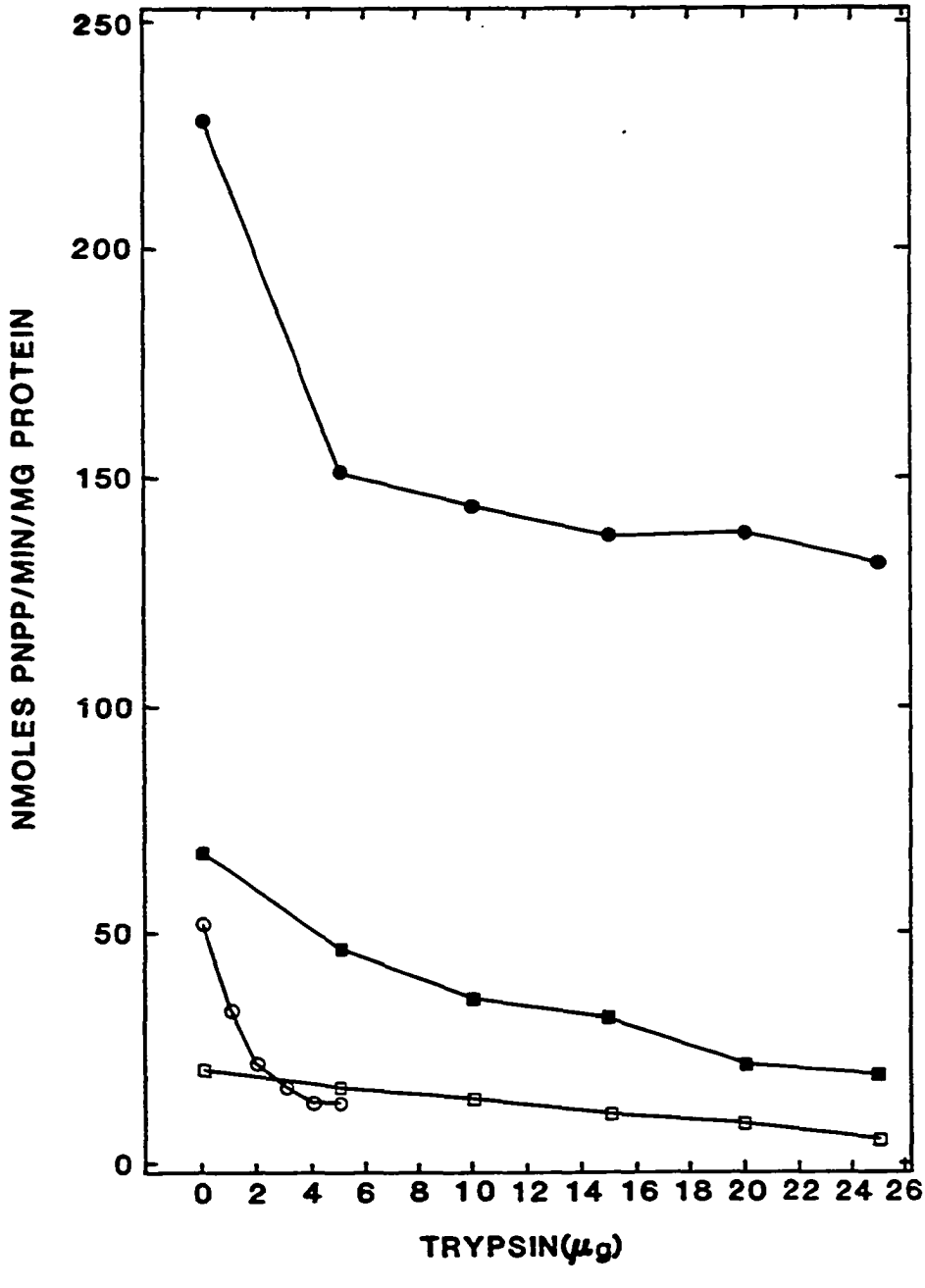

250
200
150
100
50
0
NMOLES PNPP/MIN/MG PROTEIN
0 2 4 6 8 10 12 14 16 18 20 22 24 26
TRYPSIN(μg)

Table IV

LIGAND BINDING EFFECTS ON THE TRYPTIC INACTIVATION OF EAT CELL PNPPASES.[a]

	PREINCUBATION ADDITION	Mg^{2+}-PNPPase (nmoles PNPP min^{-1} mg $protein^{-1}$)	Mn^{2+}-PNPPase (nmoles PNPP min^{-1} mg $protein^{-1}$)
EXPERIMENT I:			
PEAK I	NONE	58.1	249
	+5ug TRYPSIN	35.8	ND
	+5ug TRYPSIN + 40mM Mg^{2+}	37.6	ND
	+25ug TRYPSIN	ND	123
	+25ug TRYPSIN +250uM Mn^{2+}	ND	127
PEAK II	NONE	15.9	91.5
	+15ug TRYPSIN	6.1	47.2
	+15ug TRYPSIN + 40mM Mg^{2+}	14.2	ND
	+15ug TRYPSIN +250um mn^{2+}	ND	57.7
EXPERIMENT II:			
PEAK I	NONE	51.9	244
	+1.5ug TRYPSIN	24.9	ND
	+1.5ug TRYPSIN + 20mM PNPP	34.0	ND

	+ 25ug TRYPSIN	ND	136
	+ 25ug TRYPSIN + 20mM PNPP	ND	162
PEAK II	NONE	22.4	85.5
	+ 15ug TRYPSIN	9.91	38.6
	+ 15ug TRYPSIN + 20mM PNPP	19.1	63.2

[a] The experimental procedure were as described in figure 19, except that the indicated concentrations of either Mg^{2+}, Mn^{2+}, or PNPP were present during preincubation.

The data shown in this table, for Experiment I, is representative of three separate protection assays that were each done in duplicates. The duplicates in each experiment were within 2-3 %.

The data shown in this table, for Experiment II, is representative of three separate protection assays that were each done in duplicates. The duplicates in each experiment were within 3%. In each case, the data presented here are the averages of a duplicate determinations for each experiment.

ND = not determined

3.8

EFFECT OF N-ETHYLMALEIMIDE (NEM)

The ability of DTT to stimulate PNPPase strongly implies that one or more sulfhydryl groups may be essential for activity.

As shown in Table V, partial inactivation was observed for EAT cell Peak I and Peak II PNPPases in the presence of Mg^{2+} or Mn^{2+} when treated with 1 mM N-ethylmaleimide (NEM), an irreversible sulfhydryl group modifying reagent. Compared to the untreated controls assayed under otherwise identical conditions, Peak II Mn^{2+}-dependent PNPPase was more susceptible to NEM inactivation (83%) than Peak II Mg^{2+}-dependent PNPPase (62%), Peak I Mg^{2+}-dependent PNPPase (40%), and Peak I Mn^{2+}-dependent PNPPase (18%). The presence of either 40 mM Mg^{2+}, 250 uM Mn^{2+}, or 20 mM PNPP in the preincubation mixtures had essentially no effect on the extent of inactivation by NEM.

Table V

NEM INACTIVATION OF EAT CELLS PNPPASES.[a]

	PREINCUBATION ADDITIONS	Mg^{2+}-PNPPase (nmoles PNPP min^{-1} mg $Protein^{-1}$)	Mn^{2+}-PNPPase (nmoles PNPP min^{-1} mg $Protein^{-1}$)
PEAK I	NONE	69.3	212
	+1mM NEM	41.4	173
PEAK II	NONE	21.7	141
	+1mM NEM	8.7	23.6

[a] Aliquots (16 ug protein) of the Peak I and Peak II fractions were preincubated for 15 min on ice in a medium of 390 ul containing 103 mM Tris-HCl, pH 7.5, plus 1 mM NEM, where indicated. The sulfhydryl modification reactions were quenched by 3 mM DTT and diluting the preincubation mixture such that the final 500 ul volume contained a complete PNPPase assay system for the determination of either residual Mg^{2+}-dependent or Mn^{2+}-dependent PNPPase activity as described in Figure 15. The data shown is representative of five experiments each for the Peak I and Peak II Mg^{2+}-dependent activities; Six experiments for the Peak I Mn^{2+}-dependent activity and four experiments for the corresponding Peak II activity. The data shown are the averages of a duplicate determinations for each experiment. In all cases, duplicates in an experiment were within 4 %.

3.9

THERMOSTABILITY PROFILE

A number of tumor cell alkaline phoshatases have been characterized on the basis of their thermolability (McComb et al., 1979). The thermal stability profiles for the EAT cell PNPPases are shown in Figure 23, and these enzymes are clearly thermolabile. Peak I PNPPases are more susceptible to heat inactivation than the Peak II PNPPases with approximately 50% loss of activity occurring under our preincubation conditions at 55 ^{o}C and 60 ^{o}C, respectively. Results from a comparison of the effects of heat treatment in the presence of 40 mM Mg^{2+} or 250 uM Mn^{2+} on PNPPases are presented in Table VI. While the presence of Mg^{2+} or Mn^{2+} is shown to stabilize Peak I PNPPase at 52.5 ^{o}C, the most striking finding is that Mn^{2+} activates the Peak II Mn^{2+}-dependent PNPPase by 78% during heat treatment at 60 ^{o}C. It is also of interest that Mg^{2+} extensively destablizes peak II Mg^{2+}-dependent PNPPase during preincubation at 60 ^{o}C. Compare 49% inactivation of Peak II Mg^{2+}-dependent PNPPase in the absence of Mg^{2+} to 89% inactivation in the presence of Mg^{2+}.

Figure 23 Thermal inactivation of EAT cell PNPPases. Heat treatments were conducted by preincubating 19 ug protein aliquots of the Peak I (○,●) or Peak II (□,■) fractions in a 100 ul volume containing 80 mM Tris-HCl, pH 7.5, for 10 min at the temperatures indicated. The inactivation reactions were quenched by diluting with an appropriate PNPPase assay medium on ice as described under Materials and Methods. The mixtures were then assayed for residual Mg^{2+}-dependent (○,□) and Mn^{2+}-dependent (●,■) PNPPase as in Figure 17. The data shown is representative of three separate experiments each for both Mg^{2+}-dependent Peak I & II PNPPase activity. Duplicates within each experiment were within 5 %. Similarly, for the Mn^{2+}-dependent Peak I and II PNPPase activity, the data shown is representative of five separate experiments each of the activities. Duplicates within each experiment were within 4 %. In each case, the data shown are the averages of a duplicate determinations for each experiment.

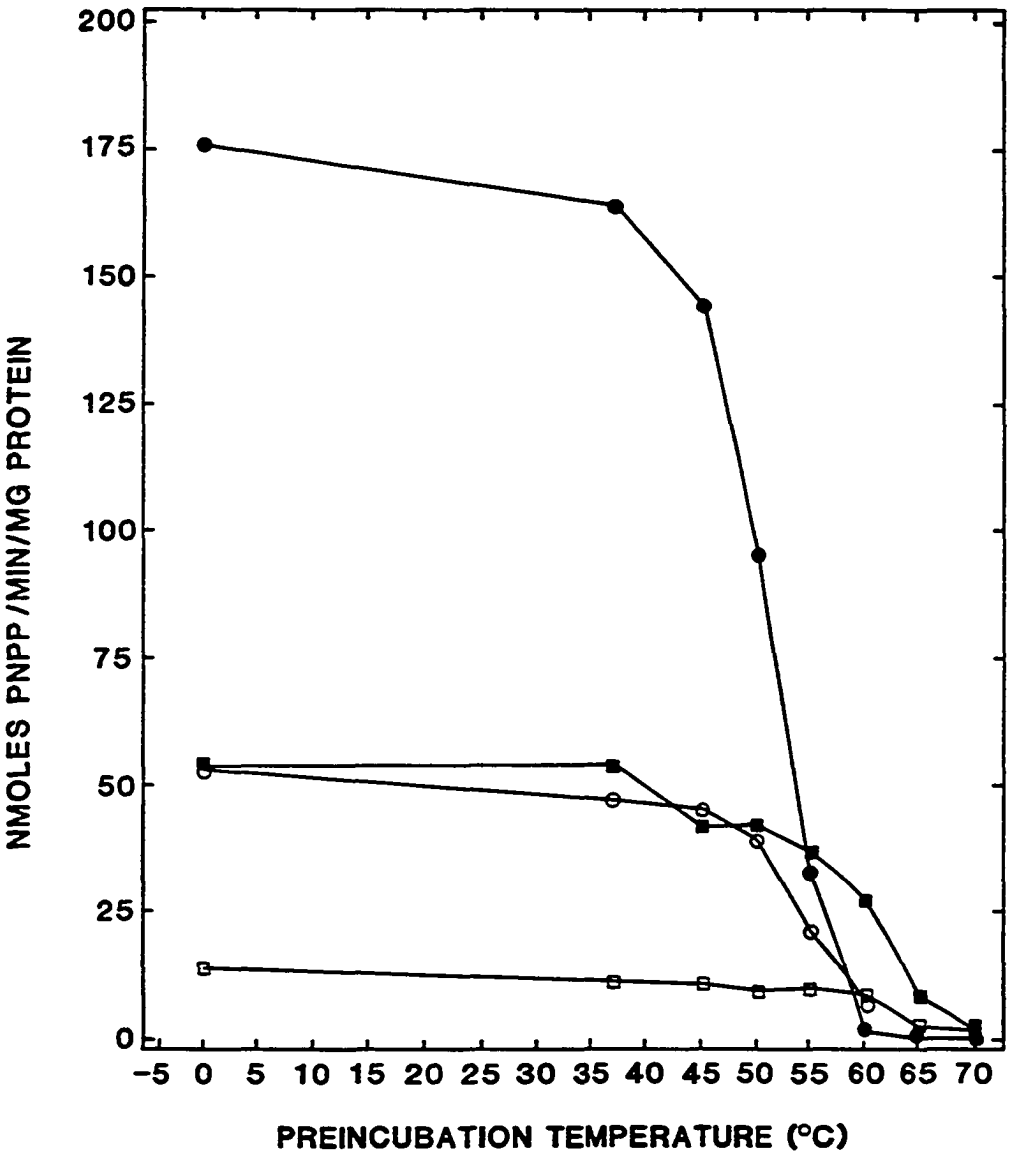

200
175
150
125
100
75
50
25
0
NMOLES PNPP/MIN/MG PROTEIN
-5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
PREINCUBATION TEMPERATURE (°C)

Table VI

CATION BINDING EFFECTS ON THE THERMAL INACTIVATION OF EAT CELL PNPPASE.[a]

	PREINCUBATION TEMPERATURE (°C)	PREINCUBATION ADDITIONS	Mg^{2+} -PNPPase (nmoles PNPP min^{-1} mg $protein^{-1}$)	Mn^{2+} -PNPPase (nmoles PNPP min^{-1} mg $protein^{-1}$)
PEAK I	0	NONE	47.8	172
	52.5	NONE	26.1	77.6
	52.5	+40mM Mg^{2+}	35.4	ND
	52.5	+250uM Mn^{2+}	ND	150
PEAK II	0	NONE	18.2	43.0
	60	NONE	9.8	24.8
	60	+40mM Mg^{2+}	1.93	ND
	60	+250uM Mn^{2+}	ND	76.6

[a] The experimental procedures followed were as described in Figure 20, except that the indicated concentrations of either Mg^{2+} or Mn^{2+} were present during preincubation. The data shown in this table is representative of four separate experiments for each of the metal ions. The data shown are the averages of a duplicate determinations for each of the experiments. The duplicates were within 3 %. ND = not determined

3.10

PHOSPHOTYROSINE & PHOSPHOSERINE AS SUBSTRATES

When phosphotyrosine and phosphoserine were used as substrates, it was seen that the Peak I fraction showed high specificity toward phosphotyrosine. The reaction was quantified by following the release of Pi by the Lanzetta method as described in Materials and Methods. Specific activity for phosphotyrosyl phosphatase was found to be 104 nmole of Pi released min^{-1} mg $protein^{-1}$. Phosphoserine did not serve as a good substrate for Peak I fraction. Specific activity was found to be about 10 nmoles of Pi released min^{-1} mg of $protein^{-1}$. These findings are similar to those of Foulkes et al., (1983), who showed that the phosphotyrosyl protein phosphatase they isolated had very high specificity toward phosphotyrosyl proteins but showed little specificity toward phosphoseryl protein, and also to those of Heng Chun Li (1984), who isolated an acid phosphatase which was found to contain high specificity toward phosphotyrosyl residues with little activity toward several phosphoseryl and phosphothreonyl proteins.

However, Peak II fraction demonstrated a very high specificity towards phosphoserine, (107 nmol of Pi released min^{-1} mg of $protein^{-1}$) and very little specificity towards phosphotyrosine as a substrate (specific activity about 25 nmol Pi released min^{-1} mg of $protein^{-1}$). The low phosphotyrosyl activity in Peak II and the phosphoseryl activity in Peak I probably result from the cross contamination of one peak with the other. This could be attributed to the fact that the proteins in Peak I

eluted from the DEAE-Sephacel column with 120 and the proteins in Peak II eluted with 160 mM NaCl. This is also borne out by the results obtained with the Zinc-chelated column (see section 3.2).

It was found that the Peak II fraction is an acid phosphatase and requires essential Mg^{2+} ion. Mn^{2+} did not substitute for Mg^{2+}. Furthermore, the activity of the Peak II fraction as a phosphoseryl phosphatase was not stimulated by sulfhydryl compounds such as DTT (results from my colleague Darrell N. Smith).

The EAT cell Peak I fraction was further characterized as a phosphotyrosyl phosphatase. In order to optimize assay conditions, titrations were performed in which phosphotyrosyl phosphatase activity was measured as a function of assay protein concentration and as a function of assay incubation time. It was found that 2-4 ug of protein and 15 min incubation time provide optimal results for this assay. It was also found that in the absence of cations Peak I did not dephosphorylate phosphotyrosine . As observed with Peak I and Peak II PNPPases, Peak I phosphotyrosyl phosphatase requires essential Mg^{2+} ion (Figure 24). 20 mM of $MgCl_2$ showed maximum stimulation in activity. However, when higher concentrations of Mg^{2+} ions were used, it gave variable results.

The K_m of the substrate phosphotyrosine, was determined in the presence of 20 mM Mg^{2+} ions (Figure 25). In four separate experiments, K_m values of 1.2, 3.5, 5.2, and 3.8 mM and V_{max} values of 104, 92, 124, and 104 nmoles Pi released min^{-1} mg of $protein^{-1}$ were obtained, respectively. In two additional experiments a variant set of results

were obtained. In this latter case, K_m values of 4.5 and 55 mM and V_{max} values of 455 and 611 nmoles of Pi released min^{-1} mg of $protein^{-1}$, were obtained, respectively. We have no way to account for the variability of these results. It should be noted that the V_{max} of 104, 92, and 124 are close to the specific activity values usually measured for this phosphotyrosyl phosphatase activity in this study.

Unlike the EAT cell PNPPases, Peak I phosphotyrosyl phosphatase requires millimolar concentration of Mn^{2+} for activity (Figure 26). Furthermore, the stimulation ratio of Mn^{2+}/ Mg^{2+}-dependent activation was seen to be 1:2. In addition, a higher concentration of protein was required for Mn^{2+}-dependent phosphotyrosyl phosphatase to hydrolyze phosphotyrosine.

Sulfhydryl group compounds are required for optimal activity by an EAT cell phosphotyrosyl protein phosphatase described by Martensen 1982, and by several alkaline phosphatases (Li and Chang, 1981). Under our assay conditions 1 mM DTT stimulates Mg^{2+}-dependent phosphotyrosyl phosphatase to about 3-5 times (Table VII). Hence, Peak I phosphotyrosyl phosphatase activity and the Peak I and Peak II PNPPase activities are similar to the EAT cell phosphotyrosyl protein phosphatase activity previously characterized by Martensen, T. M.,(1982) and Horlein et al., (1982), in that the phosphatases are neutral and thio-sensitive.

Figure 24 The Mg^{2+}-dependence of Peak I phosphotyrosyl phosphatase. Aliquots of Peak I (4 ug) were incubated for 15 min at 37°C, in a reaction mixture (500 ul) consisting of 80 mM Tris-HCl pH 7.5, 10 mM phosphotyrosine , 1 mM DTT, plus the indicated $MgCl_2$. 800 ul of MG/AM/T was added to 200 ul of the reaction mixture and the release of inorganic Pi was determined by the Lanzetta method as described under Materials and Methods. The data shown is representative of six separate experiments. The data shown are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 3-5 %.

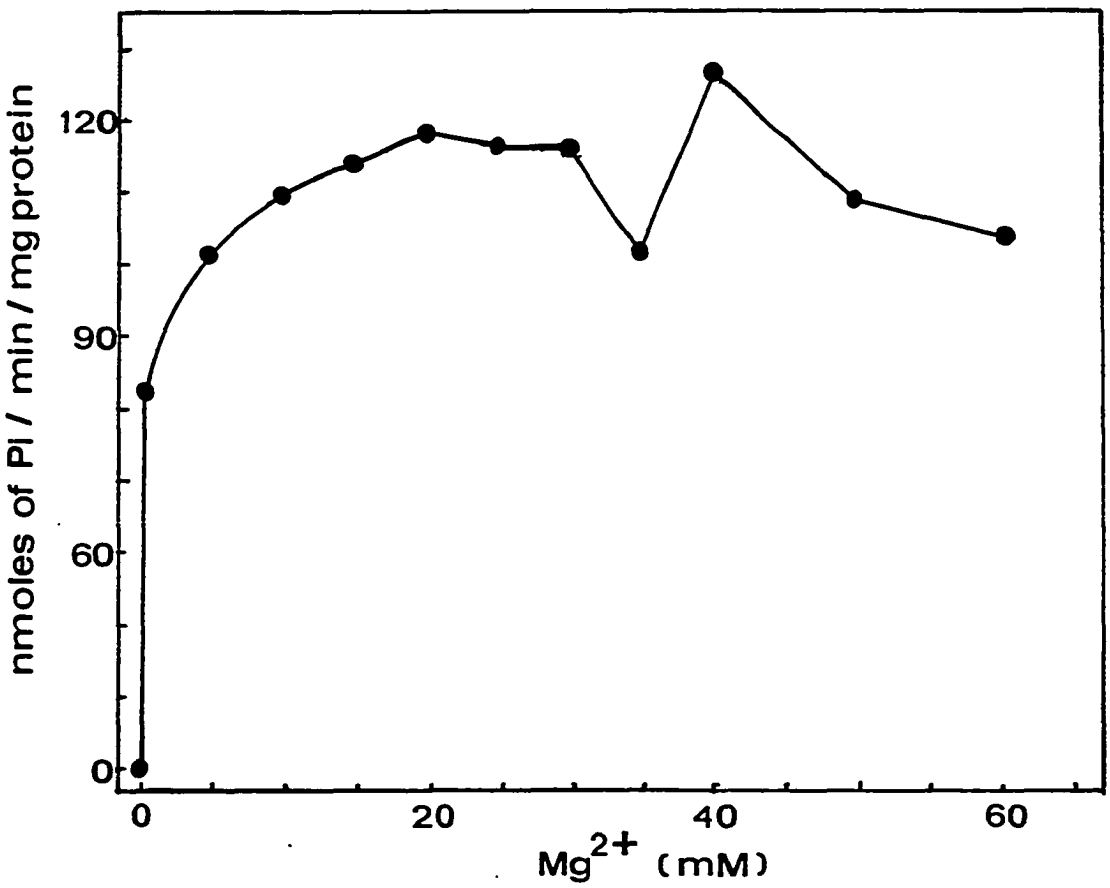

nmoles of PI / min / mg protein
120
90
60
0
0
20
40
60
Mg2+ (mM)

Figure 25

Saturation plot of the Peak I Mg^{2+}-dependent Phosphotyrosyl phosphatase activity as a function of the indicated substrate (Phosphotyrosine) concentration. In this figure 'S' represents the substrate concentration in mM, whereas 'V' represents the velocity of the reaction in nmoles of Pi released min^{-1} mg of $protein^{-1}$. The inset is a reciprocal plot of the same data which is representative of 6 separate experiments. Duplicates in each experiment were within 4%. The data shown are the averages of a duplicate determinations for each experiment.

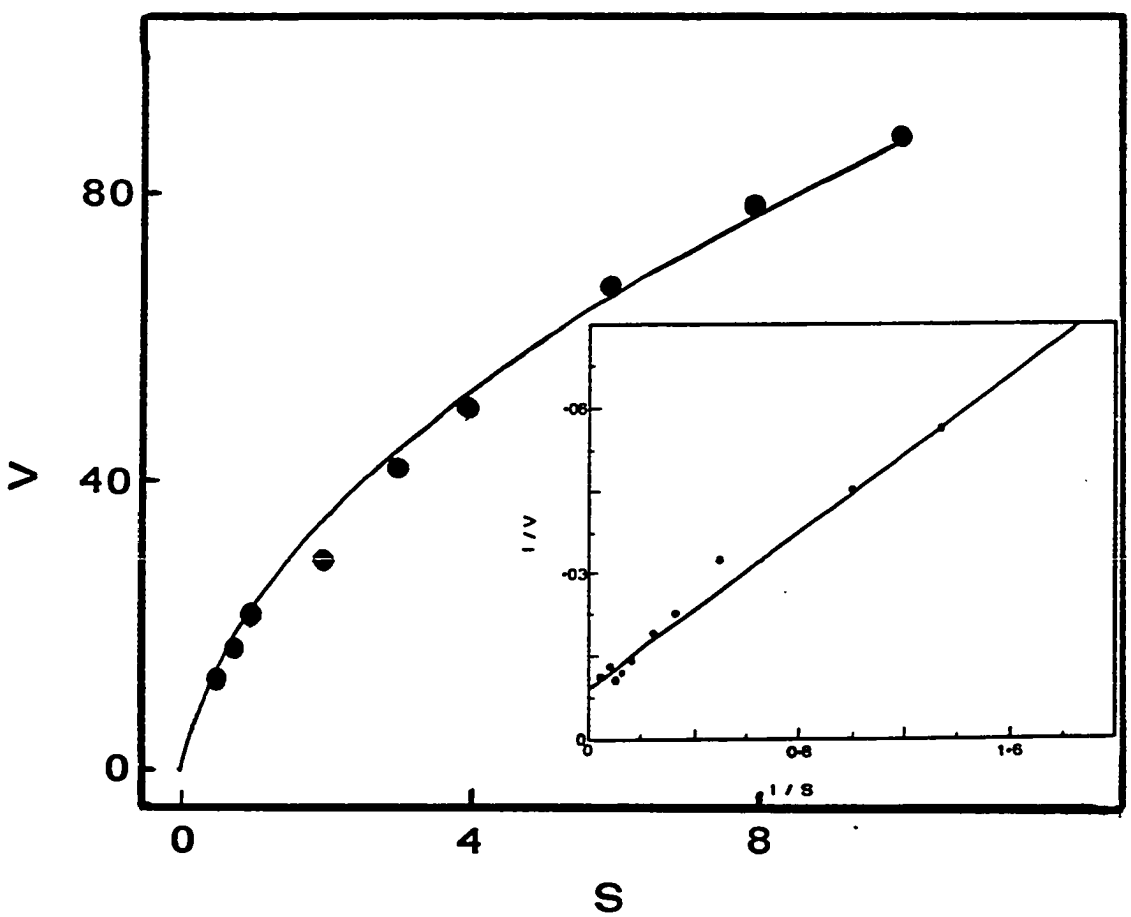

80
40
0
V
0
4
8
S
1 / V
-06
-03
0
0-6
1-6
1 / S

Figure 26 The Mn^{2+}-dependence of Peak I phosphotyrosyl phosphatase. Assays were performed as in Figure 24 except that Mg^{2+} has been substituted for the indicated concentration of Mn^{2+} and 20 ug of protein was used instead of 4 ug of protein. The data shown is representative of three separate experiments and are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 5 %.

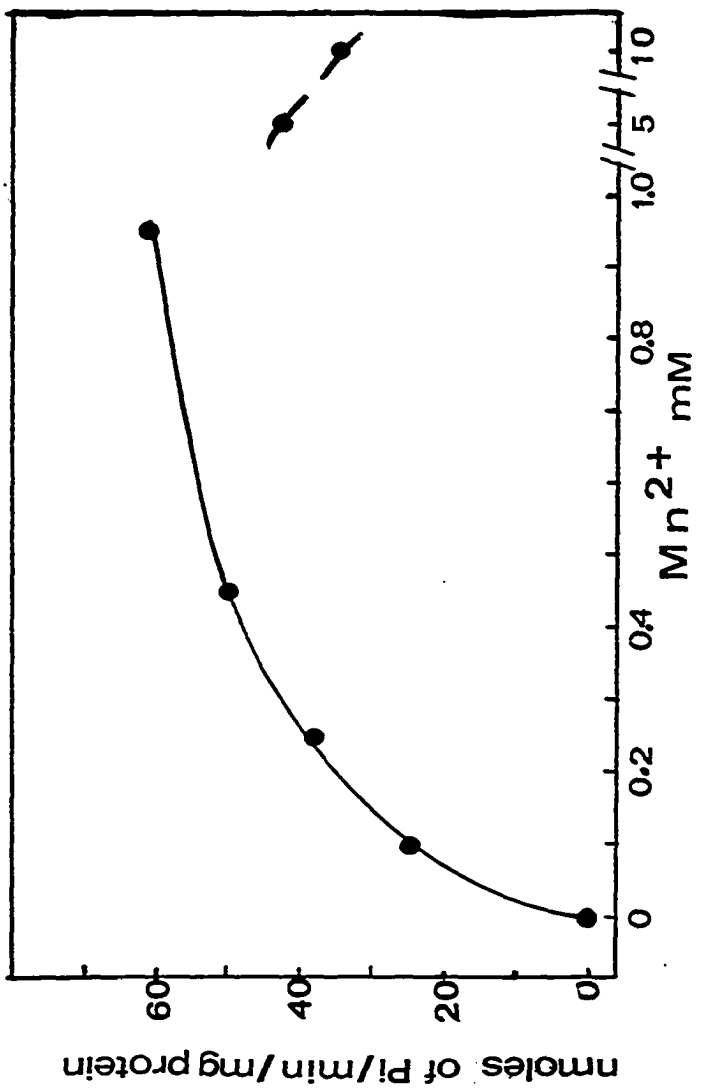

nmoles of Pi/min/mg protein
60
40
20
0
0
0.2
0.4
0.8
1.0
5
10
Mn2+ mM

Table VII

EFFECT OF DTT ON PEAK I PHOSPHOTYROSYL PHOSPHATASE.

	Specific Activity (nmole of Pi min^{-1} mg $protein^{-1}$)
-DTT	27
+DTT	85

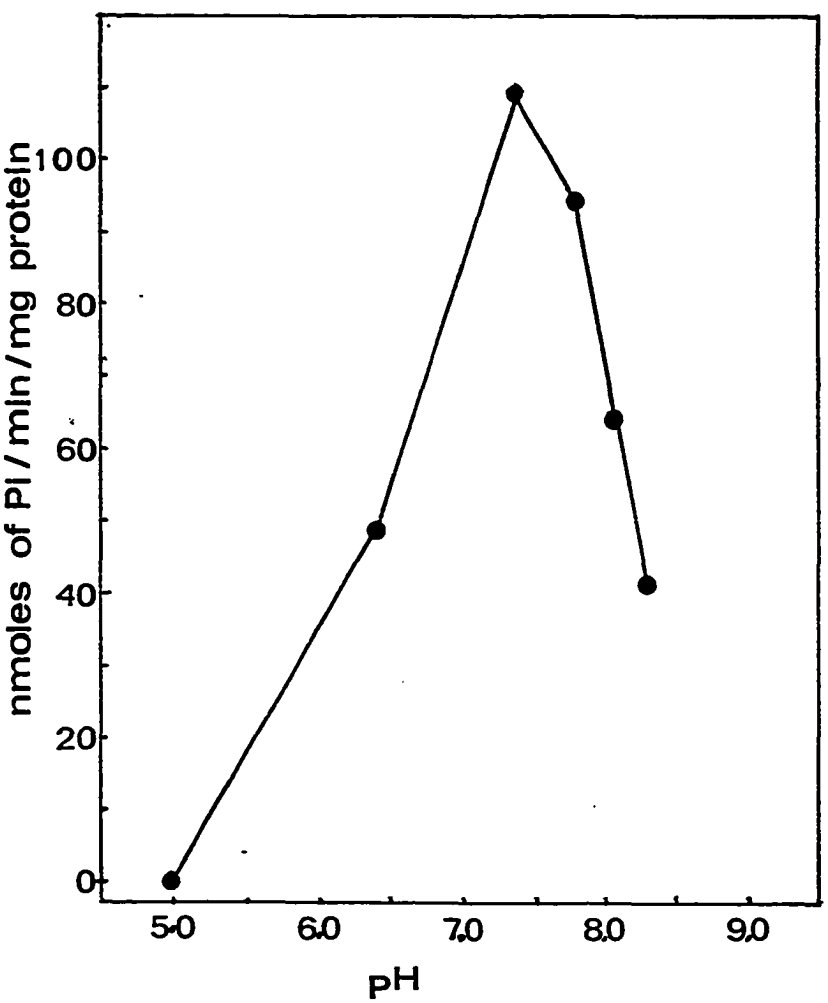
Aliquots of Peak I (4 ug) were incubated for 15 min at 37 ^{o}C in a reaction mixture (500 ul) consisting of 80 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 10 mM phosphotyrosine, in the presence and absence of 1 mM DTT. 800 ul of MG/AM/T was added to 200 ul of this reaction mixture and was assayed for the amount of inorganic Pi released in the reaction mixture by the Lanzetta Method as described under Materials and Methods. The data shown is representative of three separate experiments. The data shown are the averages of a duplicate determination for each experiment. Duplicates in each experiment were within 2 %.

3.11

pH PROFILE OF PEAK I PHOSPHOTYROSYL PHOSPHATASE

As seen in Figure 27, the pH optimum is about 7.3, indicating that the EAT cell Peak I fraction contains a neutral phosphotyrosyl phosphatase. The pH optimum was determined using a series of Tris-HCl buffers. The pH's plotted in Figure 27 were the pH's of the complete reaction mixture.

Figure 27 pH optimum for phosphotyrosyl phosphatase activity. 4 ug of Peak I was incubated for 15 min at 37 ^{o}C in an assay medium (500 ul) consisting of 80 mM Tris-HCl, at different pHs; 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, and 20 mM $MgCl_2$, 10 mM phosphotyrosine, and 1 mM DTT. The pH's plotted are the pH's of the complete reaction mixture. The release of inorganic Pi was determined as described under Figure 24. The data shown is representative of three separate experiments and are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 5 %.


nmoles of PI / min / mg protein
100
80
60
40
20
0
5.0
6.0
7.0
8.0
9.0
pH

3.12

EFFECT OF INHIBITORS ON PEAK I PHOSPHOTYROSYL PHOSPHATASE

As discussed earlier in section 3.5.6, levamisole is a common inhibitor of alkaline phosphatases (Van Belle, 1972) while vanadate is considered to be potent inhibitor of both phosphotyrosyl protein phosphatase (Swarup et al., 1982) and alkaline phosphatases (Lopez et al., 1976). These inhibitory activities are completely opposite of what we observe with Peak I phosphotyrosyl phosphatase. We found that neither 1 mM levamisole nor 1 mM vanadate inhibits the dephosphorylation of phosphotyrosine by Peak I phosphotyrosyl phosphatase.

Martensen, (1982), Foulkes et al., (1983), and Horlein et al., (1982), reported that neutral phosphotyrosyl protein phosphatases are inhibited by micromolar concentrations of $ZnCl_2$. This was also seen to be the case with our Peak I phoshotyrosyl phosphatase which was inhibited about 40% at 50 uM concentration and complete inhibition of the enzyme was seen at 1 mM $ZnCl_2$ (Table VIII).

Unlike the phosphotyrosyl protein phosphatases isolated by other workers, (Brautigan et al., 1982; Foulkes et al., 1983; & Martensen, 1982), we have seen that EAT cell Peak I phosphotyrosyl phosphatase was inhibited by fluoride. As shown in Figure 28, 250 uM of NaF inhibits about 51% of the activity.

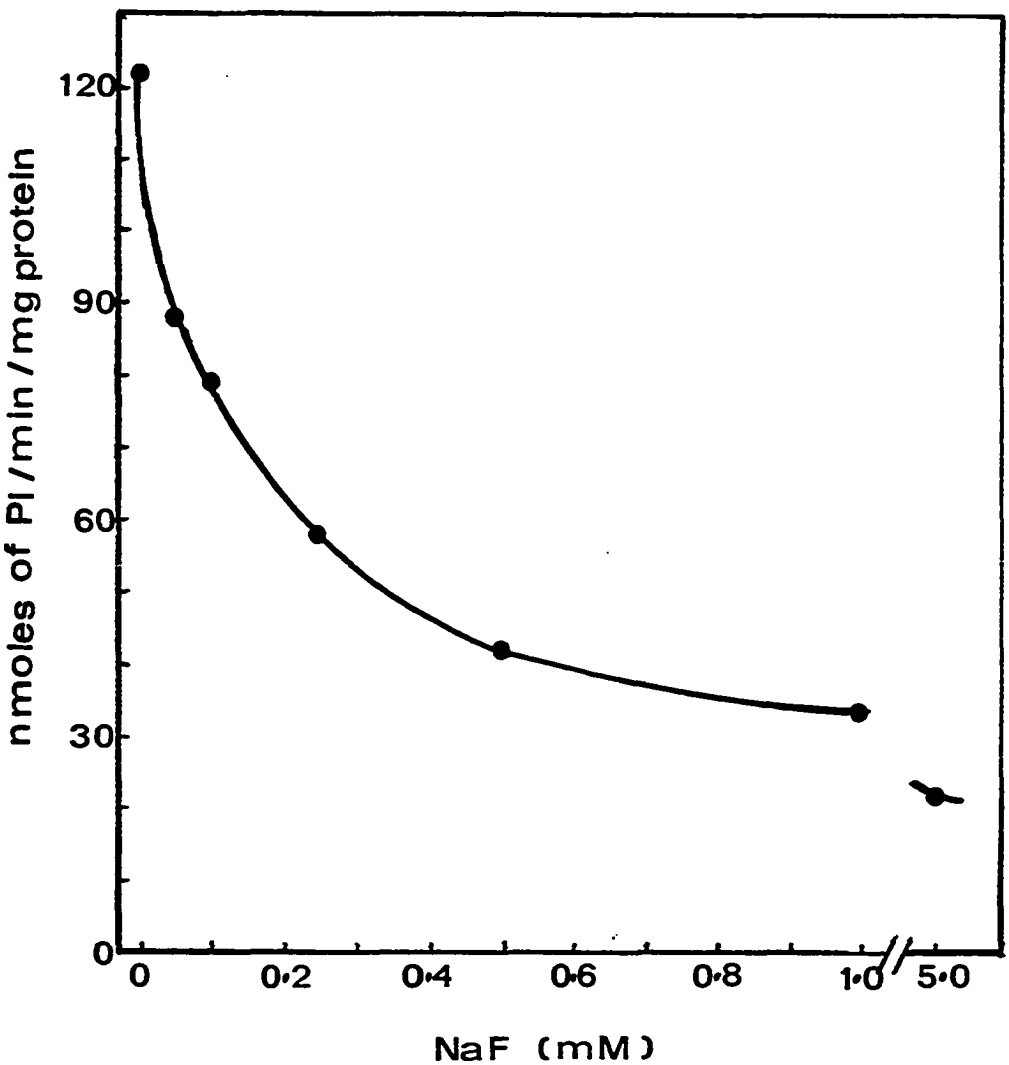
Table VIII

EFFECT OF Zn^{2+} ION ON PEAK I PHOSPHOTYROSYL PHOSPHATASE

$ZnCl_2$ (mM)	Specific activity (nmoles Pi released min^{-1} mg $protein^{-1}$)
0	93
0.05	55
0.10	30
0.25	25
0.50	17
1.00	0

Aliquot of Peak I (4 ug) were incubated for 15 min at 37°C in a reaction mixture consisting og 80 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 10 mM phosphotyrosine , 1 mM DTT and the indicated amount of $ZnCl_2$. The amount of inorganic Pi released was determined as described under Table VII. The data shown is representative of six separate experiments performed with three different preparations of the enzyme. The data shown are the averages of a duplicate determinations for each experiment. Duplicates in each of the six experiments were within 3%.

Figure 28 Fluoride inhibition of EAT cell Peak I Mg^{2+}-dependent phosphotyrosyl phosphatase. Aliquots of Peak I (4 ug) fraction were assayed as described under Materials and Methods, section 2.8, in the presence of the buffer system described in Figure 24 plus the indicated amount of NaF. The data shown is representative of six separate experiments and are the averages of a duplicate determinations for each experiment. Duplicates within each experiment were within 2 %.


nmoles of PI / min / mg protein
120
90
60
30
0
0
0·2
0·4
0·6
0·8
1·0
5·0
NaF (mM)

Chapter IV

DISCUSSION

The results presented here suggest that Mg^{2+}-dependent and Mn^{2+}-dependent phosphotyrosyl phosphatase activities and Mg^{2+}-dependent and Mn^{2+}-dependent PNPPase activities are present in the postmicrosomal supernatant of EAT cell homogenates.

Two peak fractions were resolved by a two step chromatographic procedure, gel filtration chromatography and anion exchange chromatography. These two peak fractions were initially characterized using the nonprotein ester, PNPP, as a substrate. The Peak I and the Peak II fractions were compared as having a Mg^{2+}-dependent PNPPase activity and as having a Mn^{2+}-dependent PNPPase activity. The two PNPPase fractions were found to be similar in that they both elute from a calibrated Sephadex G-150 column in the 15,000-30,000 dalton range (Figure 5), hydrolyze PNPP only in the presence of either essential Mg^{2+} or Mn^{2+} ions, have nearly identical Mn^{2+}/ Mg^{2+} stimulation ratios, and are inhibited in the same fashion by pyrophosphate (Figure 20 and Figure 21), but are not inhibited by levamisole.

The K_m of the non-protein ester PNPP, was determined in the presence of either Mg^{2+} or Mn^{2+} ions for the Peak I fraction. In three experiments, done in the presence of Mg^{2+} ions, K_m values of 0.89, 0.61, and 0.76 mM (Figure 15) and V_{max} values of 91, 104, and 58 nmoles of

PNPP hydrolyzed min^{-1} mg of $protein^{-1}$, were obtained respectively. For an additional experiment a variant set of results was obtained. In this latter case, the K_m in the presence of Mg^{2+} was 1.16 mM and the V_{max} was 34 nmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$.

Similarly, in three experiments, the K_m and V_{max} were determined in the presence of Mn^{2+} ions. The K_m values obtained in this case were 0.12, 0.103, and 0.09 mM (Figure 16), with a V_{max} of 307, 238, and 352 nmoles of PNPP hyrolyzed min^{-1} mg of $protein^{-1}$, respectively. For an additional experiment a variant set of results was obtained. In this latter case, the K_m in the presence of Mn^{2+} was 1.05 mM and the V_{max} was 77 nmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$.

It should be noted that the V_{max} of 91 and 104 nmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$ obtained for Mg^{2+}-dependent PNPPases and the 307, 238, and 352 nmoles of PNPP hydrolyzed min^{-1} mg of $protein^{-1}$ obtained for the Mn^{2+}-dependent PNPPases are very similar to the specific acitvity values obtained routinely for these PNPPases. However, there does exist a variation in the values measured which conceivably could arise from the differences in the different preparations of the PNPPases and/ or the age of a particular protein sample.

Despite these similarities there are several lines of evidence suggesting that these two PNPPase activities are distinct entities (Table IX). The most obvious difference between the two is that the Peak I Mg^{2+}- or Mn^{2+}-dependent PNPPases are always two to four times more active than the Peak II Mg^{2+}- or Mn^{2+}-dependent activities from the

Table IX

COMPARISON OF PEAK I AND PEAK II PNPPases

	PEAK I PNPPase		PEAK II PNPPase	
	Mg^{2+}-dependent	Mn^{2+}-dependent	Mg^{2+}-dependent	Mn^{2+}-dependent
Activity[a]	++	++	+	+
pH profile	7.5		broad pH range	
Fluoride[b]	--		-	
Phosphate[b]	--		-	
Trypsinolysis[c]	++	+	+	++
			better protected with Mg^{2+}	better protected with Mn^{2+}
NEM[d]	-	-	--	--
Heat Inactivation[d]	--	--	-	-
Zn^{2+}-chelated column	Binds		Does not Bind	

a = ++ = more active

b = -- = more sensitive to inhibition

c = ++ = more susceptible to proteolysis

d = -- = more sensitive to inactivation

same preparations when assayed at pH 7.5 . Also, the PNPPase activity pH profile, Figure 14, shows that the Peak II enzyme is maximally active over a relatively broad pH range in Tris-HCl while the Peak I enzyme activity decreases significantly as pH is increased above 7.5. With regard to the effects of potential inhibitors binding to the EAT cell PNPPases, it was found that the Peak I Mg^{2+}-dependent PNPPase is more sensitive to fluoride (Figure 17) and phosphate (Figure 18) inhibitions than the Peak II Mg^{2+}-dependent PNPPase. In fact, 0.1 mM phosphate actually stimulates the Peak II Mg^{2+}-dependent PNPPase by about 66% (Figure 19). Unfortunately, it is unlikely that the significance of this curious phenomenon will be revealed before the physiological role of this enzyme is determined. Additional differences between the two enzymes were indicated by the results from trypsin inactivation studies (Figure 22 and Table IV). The Peak I Mg^{2+}-dependent PNPPase is more sensitive to trypsinolysis than the Peak II Mg^{2+}-dependent PNPPase whereas the Peak II Mn^{2+}-dependent PNPPase is more sensitive to trypsin than the Peak I Mn^{2+}-dependent activity. Futhermore, the presence of Mg^{2+} or Mn^{2+} in the trypsinolysis preincubation mixture protected the Peak II enzyme, but not the Peak I PNPPase against tryptic inactivation, and the Peak II PNPPases were better protected against trypsinolysis by PNPP than the Peak I PNPPases. The Peak II PNPPases were also more susceptible to inactivation by 1 mM NEM (Table V), but less sensitive to heat inactivation (Figure 23) than the Peak I PNPPase enzyme. Even more striking differences between the two PNPPase fractions are evident from a comparison of the effects of Mg^{2+} and Mn^{2+} binding on enzyme heat

treatment (Table VI). Cations were shown to protect the Peak I PNPPases against heat inactivation while the Peak II Mg^{2+}-dependent PNPPase was destabilized by Mg^{2+} and the Peak II Mn^{2+}-dependent PNPPase was activated by Mn^{2+}. Furthermore, when Peak I and Peak II PNPPases were subjected to the Zn^{2+}-iminodiacetate agarose column (Figures 12 & 13), it was found that Peak I PNPPase fraction bound to the Zn^{2+}-chelated column and was eluted by 20 mM histidine, whereas Peak II fraction bind not bind at to the Zn^{2+}-chelated column. Although, Zn^{2+}-affinity column did not prove useful for purification, experiments with the Zn^{2+}-affinity columns were useful in revealing a specific interaction of Zn^{2+} and the Peak I fraction which was not present with the Peak II fraction. The result suggests that the Peak I fraction probably has a Zn^{2+} binding site.

Three distinct metal ion binding sites have been extensively characterized on an alkaline phosphatase from E. coli. (Coleman and Gettins, 1983), and two sets of metal ion binding sites have been shown to be present on alkaline phosphatase from bovine kidney (Cathala et al., 1975). Our results suggest that both the EAT cell PeaK I and Peak II PNPPases possess at least two distinct cation binding sites. Alternatively, the binding of Mg^{2+} or Mn^{2+} stabilize two distinct conformational states in these enzymes (Table X). Perhaps the best evidence in support of either the existence of separate binding sites for Mg^{2+} and Mn^{2+} or the existence of two distinct cation-stabilized conformational states is the fact that during the heat treatment of Peak II PNPPase at 60 ^{o}C, the presence of Mn^{2+} in the preincubation mixture

Table X

EVIDENCE TO SUPPORT TWO DIFFERENT BINDING SITES FOR Mg^{2+} AND Mn^{2+} OR THE EXISTENCE OF TWO DISTINCT CATION STABILIZED CONFORMATIONAL STATES

	PEAK I PNPPase		PEAK II PNPPase	
	Mg^{2+}-dependent	Mn^{2+}-dependent	Mg^{2+}-dependent	Mn^{2+}-dependent
HEAT INACTIVATION	presence of Mg^{2+} stabilizes	presence of Mn^{2+} stabilizes	presence of Mg^{2+} enhances inactivation	presence of Mn^{2+} activates the enzyme
FLUORIDE	more inhibited	less inhibited	more inhibited	less inhibited
PYROPHOSPHATE		100-fold more sensitive		100-fold more sensitive
NEM	more inactivation	partial inactivation	more inactivation	partial inactivation
TRYPSINOLYSIS	more sensitive	less sensitive	more sensitive Mg^{2+} provides more protection against inactivation	less sensitive Mn^{2+} provides less protection against inactivation

activates Peak II while under similar conditions the presence of Mg^{2+} enhances the thermal inactivation of the enzyme (Table VI). The comparison between Mn^{2+}-dependent and Mg^{2+}-dependent Peak I and Peak II PNPPase inhibitions by fluoride is also particularly noteworthy in ths regard (Figure 17). When either the Peak I and Peak II PNPPase enzymes are activated by Mn^{2+}, very little fluoride inhibition of PNPPase is observed, while under the same conditions the Mg^{2+}-dependent activities are considerably more inhibited. Moreover, Mn^{2+}-dependent Peak I and Peak II PNPPases are more than 100-fold more sensitive to inhibition by pyrophosphate than the same proteins assayed for Mg^{2+}-dependent activity (Figures 20 & 21). It should also be noted that NEM-treated Peak I PNPPase appears to be more completely inactivated compared to untreated controls when assayed as a Mg^{2+}-dependent enzyme than when assayed for Mn^{2+}-dependent activity (Table V). Furthermore, when aliquots of Peak I PNPPase enzyme treated with 5 ugs of trypsin are subsequently assayed for activity, the Mn^{2+}-dependent PNPPase is only 34% inactivated compared to untreated controls, while the Mg^{2+}-dependent PNPPase is about 75% inactivated (Figure 22), and that Mg^{2+} provides greater protection against Peak II PNPPase tryptic inactivation than does Mn^{2+} (Table IV).

Although it is certainly feasible that each of the EAT cell Peak I and Peak II fractions could contain distinct Mg^{2+}-dependent and Mn^{2+}-dependent PNPPases or multiple copies of each, as evidenced by the many protein bands on the 7% Davis native polyacrylamide gels (Figure 11), there are enough similarities between the two divalent cation

activated PNPPases that lead us to believe that this possibility is unlikely and that each Peak I and Peak II PNPPase contains at least one binding site each for Mg^{2+} and Mn^{2+}.

Since only partial inactivations of the EAT cells PNPPases were obtained by NEM or trypsin treatment, it could be argued that each EAT cell PNPPase fraction contains at least one PNPPase with catalytically essential sulfhydryl groups which is completely inactivated by NEM, a second enzyme which is insensitive to sulfhydryl modification, an enzyme with catalytically essential arginyl or lysyl residues which is totally inactivated by trypsinolysis , and an another enzyme which is unaffected by trypsin. If this is true, then each PNPPase fraction would contain two or more phosphatases. Alternatively, the essential arginyl or lysyl residues and sulfhdryl groups could link enzyme domains which contribute structurally toward maximal reaction rates rather than participate catalytically in the reaction mechanisms.

Therefore, these results suggest that the Peak I and the Peak II PNPPases are similar yet uniquely individual phoshatases each of which must be activated by either Mg^{2+} or Mn^{2+} binding to the enzyme. In this model, compared to the Mn^{2+} stabilized active sites, the Mg^{2+} stabilized active sites are relatively accessible to fluoride, but inaccessible to pyrophosphate for the Peak I and the Peak II PNPPases, and the Mg^{2+} stabilized enzyme is thermally destabilized in Peak II and is more sensitive to sulfhdryl group modification in Peak I than compared to the Peak II PNPPase.

When Peak I and Peak II fractions were further characterized using phosphotyrosine and phosphoserine as substrates it was found that the Peak I fraction was more active toward phosphotyrosine (104 nmole of Pi released min^{-1} mg $protein^{-1}$) than phosphoserine (10 nmoles of Pi released min^{-1} mg $protein^{-1}$) whereas the Peak II fraction was more active toward phosphoserine (107 nmole of Pi released min^{-1} mg $protein^{-1}$) than phosphotyrosine (25 nmole of Pi released min^{-1} mg $protein^{-1}$). This suggests that the Peak I fraction has phosphotyrosyl phosphatase activity and that the Peak II fraction has phosphoserine phosphatase activity. Foulkes et al., (1983), found that the phosphotyrosyl protein phosphatase they isolated in a more purified form had high specificity toward phosphotyrosyl proteins and showed little specificity toward phosphoseryl proteins. Also, Heng Chun Li et al., (1984), isolated an acid phosphatase which was found to contain high specificity toward phosphotyrosyl residues and little activity toward several phosphoseryl and phosphothreonyl proteins. Since it was seen (Figure 6) that the Peak I fraction elutes at 120 mM salt concentration and the Peak II fraction elutes at 160 mM salt concentration, the presence of low levels of phosphoseryl phosphatase in the phosphotyrosyl phosphatase fraction (Peak I), and the presence of low levels of phosphotyrosyl phosphatase in the phosphoseryl phosphatase fraction (Peak II) could be due to cross- contamination from incomplete protein resolution during DEAE-Sephacel Chromatography.

When Peak I fraction enzyme which contained more specificity toward phosphotyrosine was further characterized with respect to optimizing

Table XI

COMPARISON OF THE PROPERTIES OF THE PEAK I FRACTION AND OTHER PHOSPHATASES

	EAT CELL	OTHERS
Cations	requires essential cations, Mg^{2+} or Mn^{2+} for activity	no metal ion requirement for activity
Vanadate	No inhibition	Inhibition observed for phosphatases isolated by Swarup et al, Leis and Kaplan and Heng Chun Li et al.
Fluoride	50% inhibition by 250 uM of F	Inhibits only acid phosphatase isolated by Heng Chun Li et al.
Zinc	40% inhibition by 50 uM $ZnCl_2$	Inhibits the neutral phosphatase isolated by Brautigan et al, Foulkes et al and alkaline phosphatase by Chernoff et al.

assay conditions and the effect of potential inhibitors, it was found that this activity appears to be significantly different from the phosphotyrosyl protein phosphatases reported by other investigators, (Table XI), (Brautigan et al.,1982; Foulkes et al.,1981 & 1983; Leis et al.,1982; Chernoff et al.,1983; and Swarup et al., 1982), in that it requires essential divalent cations for activity (Figures 24 & 26). In the absence of cation no activity is observed. Vanadate, which was shown by Swarup et al .,(1982); Chernoff et al., (1983), and Heng Chun Li et al ., (1984), to be a potent inhibitor of phosphotyrosyl protein phosphatase, does not inhibit Mg^{2+}-dependent phosphotyrosyl phosphatase from EAT cell homogenate. Brautigan et al., (1982), Swarup et al., (1982), Foulkes et al., (1983), Leis et al., (1982), and Chernoff et al., (1983), reported that fluoride does not inhibit phosphotyrosyl protein phosphatases, while we found that fluoride does inhibit dephosphorylation of phosphotyrosine by the EAT cell phosphotyrosyl phosphatase (Figure 28). The phosphotyrosyl protein phosphatases described by Brautigen et al., (1982), Foulkes et al., (1983), Leis et al., (1982), and Chernoff et al., (1983), are inhibited by micromolar concentrations of $ZnCl_2$. Similarly, we found that the Mg^{2+}-dependent Peak I phosphotyrosyl phosphatase is also inhibited by $ZnCl_2$ (50 uM of $ZnCl_2$ inhibits the enzyme about 40%) (Table VIII).

The K_m of the substrate phosphotyrosine, was determined in the presence of 20 mM Mg^{2+} ions (Figure 25). In four separate experiments, K_m values of 1.2, 3.5, 5.2, and 3.8 mM and V_{max} values of 104, 92, 124, and 104 nmoles Pi released min^{-1} mg of $protein^{-1}$ were obtained,

respectively. In two additional experiments a variant set of results were obtained. In this latter case, K_m values of 4.5 and 55 mM and V_{max} values of 455 and 611 nmoles of Pi released min^{-1} mg of $protein^{-1}$, were obtained, respectively. We have no way to account for the variability of these results. It should be noted that the V_{max} of 104, 92, and 124 are very similar to the specific activity values usually measured for this phosphotyrosyl phosphatase activity in this study.

From the results it is seen that Peak I fraction contains both PNPPase and phosphotyrosyl phosphatase activities (Table XII). It is noted from the results that these two activities require essential Mg^{2+} or Mn^{2+} for their activity. However, the stimulation ratio of Mn^{2+}/ Mg^{2+} is different. While the Peak I phosphotyrosyl phosphatase stimulation ratio is 1:2 (Mn^{2+}/ Mg^{2+}), the Peak I PNPPase stimulation ratio is 2-6:1. DTT stimulates both the Peak I phosphotyrosyl phosphatase activity and the PNPPase activity, hence both are thio-sensitive enzymes. The pH profiles (Figures 14 & 27) show that both enzymes have maximal activity at neutral pH (7.3- 7.5), and that the activity decreases as the pH increases above 7.5. Furthermore, both Peak I phosphotyrosyl phosphatase and PNPPase activities are inhibited by Zn^{2+} and flouride, but are not inhibited by levamisole or vanadate. Peak I PNPPase is inhibited slightly by vanadate in the millimolar range. Chernoff et al., (1983) and Swarup et al., (1982), have reported that both PNPPase activity and phosphotyrosyl protein phosphatase activities reside in the same enzyme. The results presented in this dissertation and the fact that there exists a structural resemblance

Table XII

SUMMARY OF PEAK I FRACTION PROPERTIES

	Phosphotyrosyl activity	PNPPase activity
Mg^{2+}/Mn^{2+}	essential	essential
pH	7.3	7.5
Vanadate	No inhibition	No inhibition
Levamisole	No inhibition	No inhibition
Fluoride	Inhibition	Inhibition
Zinc	Inhibition	Inhibition

between phosphotyrosine and p-nitrophenylphosphate as seen in Figure 4, suggests that both PNPPase and phosphotyrosyl phosphatase activities of the Peak I fraction could reside in the same protein. In any event, these results strongly suggest that the EAT cell phosphatases may constitute a new class of phosphatase enzyme (see Appendix 1).

The information presented here will be useful for more effectively designing experiments which would use these phosphatases in studies specifically probing the role of phosphorylation/ dephosphorylation events in metabolic regulation and in cell transformation.

Chapter V

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APPENDIX I

DIFFERENT CLASSES OF PHOSPHATASES[a]

EC Number	Recommended Name	Reference
3.1.3.1	Alkaline phosphatase	Engstrom, (1961)
3.1.3.2	Acid phosphatase	Hollander, (1971)
3.1.3.3	Phosphoserine phosphatase	Bridgers, (1967)
3.1.3.4	Phosphatidate phosphatase	Krag & Lennarz, (1974)
3.1.3.9	Glucose-6-phosphatase	Stetten, (1970)
3.1.3.16	Phosphoprotein phosphatase	Rose & Heald, (1961)
3.1.3.17	Phosphorylase phosphatase	Hurd et al., (1966)
3.1.3.24	Sucrose phosphatase	Hawker & Hatch, (1966)
3.1.3.32	Polynucleotide 3′-phosphatase	Becker & Eurwitz, (1967)
3.1.3.41	4-Nitrophenyl phosphatase	Attias & Durand, (1973)

[a]Phosphotyrosyl protein phosphatases and our EAT cell phosphatases belong to a new class of phosphatases.