STUDIES ON SOME PLANT MEMBRANE GLYCOPROTEIN ENZYMES : PURIFICATION AND PROPERTIES OF 5' - NUCLEOTIDASE AND ACID PHOSPHATASE FROM THE MEMBRANES OF *ARACHIS HYPOGAEA* COTYLEDONS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, "STUDIES ON SOME PLANT MEMBRANE GLYCOPROTEIN ENZYMES : PURIFICATION AND PROPERTIES OF 5'-NUCLEOTIDASE AND ACID PHOSPHATASE FROM THE MEMBRANES OF ARACHIS HYPOGAEA COTYLEDONS" in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy in Department of Biosciences and Biotechnology, University of Roorkee, Roorkee is an authentic record of my own work carried out during a period from ~~September~~ December RM, 1983 to September, 1986, under the supervision of Professor C.B. Sharma.

The matter embodied in this thesis has not been submitted by me for the award of any other Degree.

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STUDIES ON SOME PLANT MEMBRANE GLYCOPROTEIN ENZYMES : PURIFICATION AND PROPERTIES OF 5'-NUCLEOTIDASE AND ACID PHOSPHATASE FROM THE MEMBRANES OF ARACHIS HYPOGAEA COTYLEDONS

ABSTRACT

The germinating peanut (Arachis hypogaea) cotyledon plasma membrane (PM), Golgi-apparatus (GA) and endoplasmic reticulum (ER) fractions have been shown to contain a glycoprotein enzyme, 5'-nucleotidase. The enzyme was purified from PM and GA fractions by the selective solubilization of the enzyme in a highly active and stable form with 0.5% octylglucoside in 50 mM Tris-HCl, pH 7.2, at a protein to detergent ratio of 2:3 in the presence of Mg^{2+} and EDTA followed by ion exchange chromatography on DEAE-cellulose column.

On sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) both PM-nucleotidase and the GA-nucleotidase showed a single protein band corresponding to the molecular weights of 55 K and 53.7 K daltons, respectively. The PM-enzyme had a broad pH optimum of 5.0 - 6.0. The K_m and V_{max} values were 1.0×10^{-3} M and 8.5 µmol/min/mg protein with adenosine 5'-monophosphate (AMP) as substrate, respectively. The enzyme was found to be highly specific for 5'-AMP. Other nucleotides (GMP, UMP, CMP, ADP, GDP, UDP, ATP, GTP and CTP) as well as phosphorylated sugars were not hydrolyzed. p-Nitrophenyl phosphate was hydrolyzed at relatively

much lower rate (15%) and the substrate affinity ($1/K_m$) was only one-tenth that of AMP. Accordingly, the 5'-nucleotidase was referred to as AMPase.

The enzyme was competitively inhibited by ADP ($K_i = 2.4 \times 10^{-3}$M) and was also inhibited by NaF in a non-competitive manner with a K_i value of 35×10^{-3}M. Divalent cations, Ca^{2+}, Mg^{2+}, Hg^{2+}, Zn^{2+}, Ni^{2+} and the monovalent cations, K^+, Li^+ and Na^+ had no effect on the enzyme activity.

The purified AMPase was highly unstable losing its total activity within 24 h at -20°C or 4°C. While under these conditions the crude solubilized enzyme (unpurified octylglucoside extract) was stable for several weeks indicating that some stabilizing factors, most likely phospholipids, were lost during the enzyme purification.

The plasma membrane AMPase was found to be a glycoprotein enzyme with 42.7% carbohydrate, composed of mainly D-mannose, N-acetyl-D-glucosamine and D-glucose. Evidence is presented that part of the carbohydrate moiety may be linked to the protein (apoenzyme) through the N-glycosidic linkage.

The purified Golgi-apparatus AMPase resembles closely with the PM-AMPase with only very minor differences, implicating the transport of AMPase from GA to the PM.

The microsomal membrane fraction (12,000 - 105,000 x g pellet), obtained from 2-days old germinating peanut cotyledons,

was surprizingly devoid of AMPase activity but contained sufficiently high activity of acid phosphatase. Extraction of membrane fraction with 0.5% octylglucoside at a protein to detergent ratio of 1:2 solubilized about 65% of the acid phosphatase activity from the microsomal membranes. DEAE-cellulose column chromatography yielded three acid phosphatase containing protein peaks, eluting at 50 mM (enzyme I), 100 mM (enzyme II) and 200 mM (enzyme III) NaCl gradients. The folds of purifications of the enzyme I, II and III were 69.4, 26.5 and 16.1 with 58.03, 11.9 and 7.16 per cent yields, respectively. The isoenzyme I was found to be relatively pure by polyacrylamide gel electrophoresis as in addition to a major protein band, with electrophoretic mobility of 0.23 (relative to bromophenol blue) two minor bands were also present. On SDS-gel electrophoresis, the major band splitted into a major and a minor protein bands corresponding to molecular weights of 46.7 K and 50.1 K, respectively, indicating that the enzyme may be composed of two or more subunits. Finally, the acid phosphatase isoenzyme I was obtained in pure form by preparative polyacrylamide gel electrophoresis. Like AMPase it was found to be a glycoprotein with 40% carbohydrate.

The optimum pH, K_m and V_{max} values for the hydrolysis of p-nitrophenyl phosphate were found to be 4.75, 10 mM and 5.5 μmol/min, respectively. Orthophosphate inhibited the enzyme non-competitively with K_i of 34.5 mM. In this regard the enzyme

differs from AMPase which was not inhibited by P_i. Thermodynamic parameters for the enzyme-p-nitrophenyl phosphate interaction were determined.

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TABLE OF CONTENTS

		PAGE No.
CANDIDATE'S DECLARATION		i
ABSTRACT		ii
ACKNOWLEDGEMENT		vi
LIST OF TABLES		xiii
LIST OF FIGURES		xv
LIST OF ABBREVIATIONS		xviii
1.0	INTRODUCTION	1
2.0	LITERATURE REVIEW	4
2.1	5'-Nucleotidase	4
2.1.1	Occurrence and subcellular localization	4
2.1.2	Pathways of 5'-nucleotidase action	8
2.1.3	Regulation and inhibition of 5'-nucleotidase activity	9
2.1.4	Physiological role of 5'-nucleotidase and its correlation with pathological conditions	11
2.1.5	Properties of 5'-nucleotidase	13
2.1.6	Substrate specificity	14
2.1.7	Purification of 5'-nucleotidase	16
2.2	Membrane glycoproteins	19
2.3	Intracellular transport of macromolecules with acid phosphatase as model	27
2.4	Membranes of eukaryotic cells	29
2.4.1	Markers of different plant membranes	30

2.4.1.1	Plasma membrane markers	30
2.4.1.2	Golgi-apparatus markers	31
2.4.1.3	Endoplasmic reticulum markers	32
2.4.2	Methods of isolation and purification	33
3.0	EXPERIMENTAL PROCEDURES	36
3.1	Materials	36
3.2	Methods	36
3.2.1	Germination of seeds	36
3.2.2	Methods of membrane fraction preparation	37
3.2.2.1	Preparation of crude membrane fraction	37
3.2.2.2	Preparation of pure plasma membrane fraction	38
3.2.2.3	Preparation of pure Golgi-membrane fraction	39
3.2.2.4	Preparation of ER membrane fraction	41
3.2.3	Solubilization of membrane-bound enzymes	41
3.2.3.1	Solubilization of 5'-nucleotidases	41
3.2.3.2	Solubilization of acid phosphatase	42
3.2.4	DEAE-cellulose column chromatography	42
3.2.5	Enzyme assays	43
3.2.5.1	AMPase assay	43
3.2.5.2	Acid phosphatase assay	45
3.2.5.3	Marker enzymes	45

3.2.5.3.1	Glucan synthetase II assay	45
3.2.5.3.2	Glucan synthetase I assay	46
3.2.5.3.3	Mannosyl transferase assay	47
3.2.5.3.4	Glucose-6-phosphatase assay	48
3.2.6	Protein estimation	48
3.2.7	Analysis of glycoprotein sugars	49
3.2.7.1	Removal of N-glycosidically linked oligosaccharide from glycoprotein enzyme	49
3.2.7.2	Neutral sugars	50
3.2.7.3	Analysis of sugars by HPLC	52
3.2.7.4	Estimation of total carbohydrate content	53
3.2.8	Polyacrylamide gel electrophoresis	53
3.2.8.1	Preparative polyacrylamide gel electrophoresis	53
3.2.8.2	Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	56
3.2.9	Determination of optimum pH	57
3.2.10	Determination of apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max})	58
3.2.11	Determination of Hill's coefficient (n) and binding constant (K_s)	58
3.2.12	Determination of thermodynamic parameters	59
3.2.12.1	Enthalpy change	59
3.2.12.2	Free energy change (ΔG) and entropy change (ΔS)	60

4.0	RESULTS	61
4.1	Preparation of plasma membrane, Golgi-apparatus and endoplasmic reticulum (ER) fractions from germinating peanut cotyledons	61
4.2	Distribution of marker enzymes in various membrane fractions of *Arachis hypogaea* cotyledons	66
4.3	Plasma membrane AMPase	68
4.3.1	Purification of plasma membrane AMPase	68
4.3.2	Homogeniety	81
4.3.3	Properties of the purified AMPase	84
4.3.4	Substrate specificity	84
4.3.5	Inhibitors	88
4.3.6	Stability of the purified AMPase	88
4.3.7	Evidence of N-glycosidic linkage in the glycoprotein	92
4.4	Golgi-apparatus AMPase	93
4.4.1	Subcellular distribution of AMPase in peanut cotyledons	93
4.4.2	Purification of Golgi-apparatus AMPase	96
4.4.3	Properties of purified Golgi-AMPase	100
4.4.3.1	Optimum pH	100
4.4.3.2	Kinetic parameters	100
4.4.3.3	Inhibitors	103
4.4.4	Comparison of plasma membrane and Golgi-apparatus AMPase	103

4.5	Endoplasmic reticulum AMPase	107
4.5.1	Solubilization and partial purification of AMPase	107
4.5.2	Effect of detergents on the activity of dialyzed enzyme	109
4.5.3	Properties of partially purified ER-AMPase	113
4.5.3.1	Optimum pH	113
4.5.3.2	Effect of substrate concentration	113
4.6	Membrane-bound acid phosphatase	116
4.6.1	Subcellular distribution of acid phosphatase and 5'-nucleotidase in peanut cotyledons	116
4.6.2	Activity of microsomal acid phosphatase and AMPase as a function of germination period	119
4.6.3	Solubilization of membrane-bound acid phosphatase	121
4.6.3.1	Solubilization of acid phosphatase with different detergents	121
4.6.3.2	Effect of $MgCl_2$ on the solubilization of acid phosphatase	123
4.6.3.3	Effect of protein to detergent ratio on the solubilization of microsomal acid phosphatase	123
4.6.4	Further purification	126
4.6.5	Polyacrylamide gel electrophoresis	129
4.6.6	Properties of purified acid phosphatase	132
4.6.6.1	Optimum pH	132
4.6.6.2	Kinetic and thermodynamic parameters	132

4.6.6.3	Interaction constant	140
4.6.6.4	Inhibition by phosphate	140
5.0	DISCUSSION	141
6.0	SUMMARY AND CONCLUSIONS	153
7.0	REFERENCES	161
8.0	PUBLICATIONS	
8.1	Biochimica Et Biophysica Acta (1986) in press	183
8.2	Proceedings International Seminar on Structure and Function of Enzymes (1986) in press	185
8.3	Proceedings Society of Biological Chemists,53rd Annual Meeting (1984)	186

LIST OF TABLES

		PAGE No.
Table I	Substrate specificity of 5'-nucleotidase	15
Table II	Extraction of various membranes by different detergents	17
Table III	Properties of detergents	18
Table IV	Plant glycoproteins	20
Table V	Distribution of marker enzymes in various membrane fractions of Arachis hypogaea cotyledons	67
Table VI	Purificiation of AMPase from the peanut cotyledon plasma membrane fraction	69
Table VII	Activity of AMPase and glucan synthetase II and I in plasma membrane fraction of peanut cotyledons	72
Table VIII	Efficiency of various detergents in solubilizing the membrane-bound AMPase of Arachis hypogaea L. cotyledons	74
Table IX	Effect of Mg^{2+} and EDTA on the solubilization of AMPase from the membrane fraction of peanut cotyledons	79
Table X	Substrate specificity of plasma membrane AMPase of peanut cotyledons	87
Table XI	Stability of peanut cotyledon plasma-membrane AMPase at 4^{o} and $-20^{o}C$	91
Table XII	Analysis of monosaccharides by HPLC	94
Table XIII	Subcellular distribution of AMPase in peanut cotyledons	95
Table XIV	Purification of AMPase from the peanut cotyledon Golgi-membrane fraction	97
Table XV	A comparison of properties of AMPase of plasma membrane and Golgi-apparatus membranes of peanut cotyledons	106

Table XVI	Solubilization and partial purification of endoplasmic reticulum AMPase	108
Table XVII	Loss of activity of solubilized AMPase during dialysis	110
Table XVIII	Subcellular distribution of acid phosphatase and 5'-nucleotidase in peanut cotyledons	117
Table XIX	Activity of microsomal acid phosphatase and AMPase as a function of germination period	120
Table XX	Solubilization of membrane-bound acid phosphatase with different detergents	122
Table XXI	Purification of acid phosphatase from the peanut cotyledon microsomal membrane fraction	128
Table XXII	Thermodynamic parameters for the interaction between p-nitrophenyl phosphate and acid phosphatase from peanut cotyledons at different temperatures	137

LIST OF FIGURES

		PAGE No.
Figure 1	Structures of N-linked oligosaccharides in plants	23
Figure 2	The dolichyl phosphate-linked saccharide pathway of N-glycosylation	25
Figure 3	Flow diagram for the isolation and purification of different membrane fractions from Arachis hypogaea cotyledons	63
Figure 4a)	Separation of different membrane fractions by sucrose density gradient	64
Figure 4b)	Separation of different membrane fractions by sucrose density gradient	65
Figure 5	Activity of the membrane-bound AMPase of peanut cotyledons as a function of germination period	70
Figure 6	Effect of octylglucoside on the activity of membrane bound AMPase	76
Figure 7	Solubilization of AMPase activity from the membrane fraction as a function of protein to detergent ratio	77
Figure 8	Chromatography of the dialyzed octylglucoside solubilized AMPase on DEAE-cellulose column	80
Figure 9	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis	82
Figure 10	Estimation of molecular weight of the purified peanut cotyledon plasma membrane AMPase by SDS-polyacrylamide gel electrophoresis	83
Figure 11	Effect of pH on the activity of purified plasma membrane AMPase from peanut cotyledons	85

Figure 12 Lineweaver-Burk plots of peanut cotyledon plasma membrane AMPase using AMP and p-nitrophenyl phosphate substrates 86

Figure 13 Inhibition of AMP hydrolysis by NaF 89

Figure 14 Inhibition of AMP hydrolysis by ADP 90

Figure 15 DEAE-cellulose column chromatography of the dialyzed octylglucoside solubilized Golgi-AMPase 99

Figure 16 Effect of pH on the activity of AMPase purified from Golgi-fraction of peanut cotyledons 101

Figure 17 Lineweaver-Burk plot of purified Golgi-AMPase 102

Figure 18 Inhibition of Golgi-AMPase by NaF 104

Figure 19 Inhibition of Golgi-AMPase by ADP 105

Figure 20 Effect of detergents on the activity of dialyzed ER-AMPase 111

Figure 21 Activity of endoplasmic reticulum AMPase as a function of pH 114

Figure 22 Lineweaver-Burk plots of ER-AMPase 115

Figure 23 Solubilization of membrane bound acid phosphatase as a function of Mg^{2+} concentration 124

Figure 24 Effect of protein to detergent ratio on the solubilization of acid phosphatase of microsomal membranes 125

Figure 25 Chromatography of dialyzed octylglucoside solubilized acid phosphatase on DEAE-cellulose column. 127

Figure 26 Polyacrylamide gel electrophoresis of microsomal acid phosphatase 130

Figure 27 Estimation of molecular weight of microsomal acid phosphatase by SDS-polyacrylamide gel electrophoresis 131

Figure 28 Effect of pH on the acid phosphatase activity 133

Figure 29 A) Lineweaver-Burk plots of acid phosphatase at different temperatures

B) Arrhenius plot of acid phosphatase 134

Figure 30 van't Hoff plot of acid phosphatase 136

Figure 31 Hill's plot for interaction of acid phosphatase with p-nitrophenyl phosphate 138

Figure 32 Inhibition of p-nitrophenyl phosphate hydrolysis by inorganic phosphate 139

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine-5'-monophosphate
5'-AMPase	5'-Adenosine monophosphatase (EC 3.1.3.5)
APase	Acid phosphatase (EC 3.1.3.2)
Arb	Arabinose
Asn	Asparagine
ATP	Adenosine triphosphate
CDP	Cytidine diphosphate
CHAPS	(3-[(3-Cholamidopropyl)-dimethylaminonio]-1-propane sulfonate)
CMP	Cytidine monophosphate
CTP	Cytidine triphosphate
E_a	Energy of activation
ER	Endoplasmic reticulum
ER-AMPase	Endoplasmic reticulum-adenosine monophosphatase
Fuc	Fucose
ΔG	Free energy change
GA	Golgi-apparatus
GA-AMPase	Golgi-apparatus-adenosine monophosphatase
Gal	Galactose
GDP	Guanosine diphosphate
GDP-Man	Guanosine diphosphate-mannose
Glc	Glucose
GlcNAc	N-acetylglucosamine

GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
ΔH	Enthalpy change
Hyp	Hydroxyproline
IMP	Inosine monophosphate
K_m	Michelis-Menten constant
K_s	Binding constant
Man	Mannose
n	Interaction constant
p-NP	p-Nitrophenol
p-NPP	p-Nitrophenyl phosphate
P_i	Inorganic phosphate
PM	Plasma membrane
PM-AMPase	Plasma membrane-adenosine monophosphatase
R	Gas constant
ΔS	Entropy change
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
TEMED	N,N,N',N'-Teramethyl ethylenediamine
Thr	Threonine
UDP	Uridine diphosphate
UDP-Glc	Uridine diphosphate-glucose
UMP	Uridine monophosphate
V_{max}	Maximum velocity
Xyl	Xylose

1.0 INTRODUCTION

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) is a glycoprotein enzyme associated with cell membrane and catalyses the hydrolysis of phosphomonoester bonds of nucleotides. This group of enzymes is widely distributed in animal tissues (7,65,99), plants (26,42,129) and bacteria (82,120). In most animal cells, the enzyme is located primarily in plasma membranes (56,112) and as such has been frequently used as marker enzyme in cell surface studies. The animal cell 5'-nucleotidase has been extensively studied, and implicated in a number of important cellular phenomenon including cell proliferation (81), senescence (158), malignancy (130,159), transmembrane nucleotide transport (34,53,81,175) and nucleotide pool size regulation (2). In plants, however, the enzyme, especially the plasma membrane-bound enzyme. has not been investigated in much detail or purified from different plant tissues or subcellular fractions. As a result the function of 5'-nucleotidases in plants still remains largely undefined.

It is now believed that the Golgi-apparatus is involved in the biosynthesis, modification and intracellular transport of macromolecules in both plants and animals (143,162) and it receives the biosynthetic

products from the endoplasmic reticulum (ER) which are destined for secretion or to become the part of the plasma membrane (124,156). Thus, the glycoproteins of the external surface of the plasma membranes are presumably also constructed within the ER and Golgi-vesicles. Accordingly these membrane glycoproteins must then be transported from the interior of the cell to the plasma membrane and all along the route the enzyme within the vesicles adds glycosyl units and makes other modifications. Most of the studies regarding the intracellular transport of macromolecules have, however, been carried out with animal systems and only little work has been done with the plant cells. One of the reasons for this laps has been the difficulty in preparation of various membrane fractions in pure form from plant cells since specific markers for different plant membrane fractions are not known.

In view of the above it was considered worthwhile to prepare plasma-membrane, Golgi-apparatus and ER fractions in pure or highly enriched form and isolate the glycoprotein enzymes (5'-nucleotidase and acid phosphatase), which are associated with the cell surface, from the different membrane fractions which could serve as model glycoproteins for studying the intracellular transport of macromolecules in plant

cells. Such a study will also be useful in understanding the process of storage of seed proteins, which are mostly glycoproteins.

The present investigation on the membrane - bound 5'-nucleotidase and acid phosphatase was undertaken with the following objectives:

(1) To obtain sufficient amount of pure or highly enriched plasma membrane, Golgi-apparatus and ER fractions of peanut cotyledons.

(2) To purify and characterize the AMPase from the different membrane fractions.

(3) To compare the properties of AMPase of plasma membranes and Golgi-apparatus and to find out whether plasma membrane and Golgi-apparatus enzymes are same or different.

(4) To purify and characterize the membrane-bound acid phosphatase of peanut cotyledons, which represent a different physiological state of the seed than the AMPase.

2.0 LITERATURE REVIEW

2.1 5'-Nucleotidase

2.1.1 Occurrence and subcellular localisation

5'-Nucleotidases (5'-nucleotide phosphohydrolase, EC 3.1.3.5) catalyze the dephosphorylation of 5'-nucleotides to produce nucleosides and phosphates. The enzyme has been extensively studied in various tissues of different origin. Among the animal tissues, rat heart (7,35,126), chicken liver (72), rat liver (56,116,155), bovine tissue (65,154), lymphocyte (59,121), bull seminal plasma (51,99) and blood (13, 174) have been studied by different workers. But a very little information is available about the enzyme in plants. Kohn and Reis (82) first demonstrated the enzyme in various bacteria and the enzyme was later purified and characterized from E. coli (120,121). Takei (163) purified and studied the 5'-nucleotidase from yeast.

5'-Nucleotidase is largely and primarily located in the plasma membrane (56,127). Avruch and Wallach (6) demonstrated a 12- to 16-fold enrichment of 5'-nucleotidase over the homogenate. Similarly, large activity in plasma membrane and much lower in microsomal preparation was found in rat liver tissue (45). The enzyme

always shows low enrichment in organelle other than plasma membrane (127,149). In microsomal membrane fractions, large activity resides in the plasma membrane fragment of microsomal preparation (153). Due to its enrichment in plasma membrane, the enzyme is by far the most commonly determined plasma membrane marker and has been used extensively in various studies (12,32,37,47,112,168). DePierre and Karnovsky (33) reviewed 5'-nucleotidase as plasma membrane marker.

In membranes the activity of 5'-nucleotidase is demonstrated at the external surface only. Thus, it is an Ecto-enzyme (36,169,181,182). Various workers confirmed the extracellular localization using following approaches: (i) no significant increase in rate of hydrolysis of nucleotides by disrupted cell fractions over that of intact cells suggests its extracellular nature (22,60) and the ability of intact cells to hydrolyze extracellular AMP confirms its ectoenzyme nature (149,151,152), (ii) concentration of inorganic phosphate produced by the enzymatic action was found to be 18 to 100 times greater in the extracellular medium than in the intracellular melieu (33), and (iii) the release of 5'-nucleotidase into solution during osmotic shock (Neu 147). He demonstrated that

the growth of bacteria occurred rapidly in the presence of AMP and explained that surface activity of 5'-nucleotidase provided carbon and phosphorus by hydrolysis of AMP.

Contrary to above facts, Lamer et al. (84) showed two sites of 5'-nucleotidase, intra-and extracellular, in rat heart. Structurally, transmembrane nature of the enzyme was established. Due to its surface localization, Wallach and Knufferman (195) visualized its probable function as transfer of nucleotides across the plasma membrane.

Besides plasma membrane, 5'-nucleotidase is also located in various subcellular organelle. Microsomal 5'-nucleotidase has been reported by several workers (55,173,180). The findings in this context are not very clear. In one case it was found that most of the microsomal activity is associated with vesicles derived from the plasma membrane (178). On the other hand, Song et al. (153) demonstrated the activity in heavier fraction, i.e., membranes with attached ribosomes (rough endoplasmic reticulum). Similarly, ER nucleotidase was confirmed by separating three forms of the enzyme by sucrose density gradient; two forms in lighter fraction and one in heavier fraction (37). Many others have also established the presence of

5'-nucleotidase associated with endoplasmic reticulum (41,179). In addition the enzyme has been shown to exist in lysosomes and Golgi-apparatus (4,49) also.

Whereas, membrane-bound enzyme regulates the extracellular nucleotides and phosphates, degradation of intracellular phosphate is regulated by the cytoplasmic-5'-nucleotidase (43). The work of different workers shows that cytosol 5'-nucleotidase is different kinetically from the membrane-bound enzyme (28). Since, this enzyme has preference for IMP over AMP, the degradation of AMP proceed to IMP (73,171). Product inhibition of the enzyme by inosine confirms the action of cytosol-5'-nucleotidase on IMP (183).

From the above literature survey it is evident that the enzyme has been most extensively investigated in animals and in contrast very little work has been carried out with the membrane-bound 5'-nucleotidases of higher plants. Recently, Polya (129) has studied the 5'-nucleotidase activity from potato and Carter and Tipton (26) have purified and characterized a 5'-nucleotidase from Zea mays microsomes. However, the microsomal fraction used by these workers was a mixed membrane preparation containing plasma membranes, Golgi-apparatus and endoplasmic reticulum (ER), the assessment of intracellular localization of AMPase is

not possible. The enzyme has also been reported to be enriched in plasma membranes of the fungus Phycomyces blakeslecanus (77).

2.1.2 Pathways of 5'-nucleotidase action

As a simple case, 5'-nucleotidase acts on adenosine 5'-monophosphate (5'-AMP) and adenosine is produced. Adenosine may further be degraded to inosine by adenosine deaminase. Another route, where 5'-nucleotidases have preference for IMP over AMP is also present. AMP is first converted into IMP and then 5'-nucleotidase acts on IMP to produce inosine. Both routes are present in living systems and extent of each reaction depends upon the physiological conditions and requirements. The whole mechanism is regulated by the enzymatic machinery consisting 5'-nucleotidases and deaminases. Cytosol-5'-nucleotidase of rat liver (71) cells hydrolyse IMP more rapidly than 5'-AMP and AMP-hydrolysing activity was less than 10% of AMP-deaminase. In such cases the degradation of AMP occurs by later route. 5'-nucleotidase, adenosine kinase and adenosine deaminase, etc., work in close association and a substrate cycle between AMP and adenosine is produced (2). Comparative activities of these enzymes thus provide the regulatory mechanism of the AMP metabolism.

2.1.3 Regulation and inhibition of 5'-nucleotidase activity

A small change in activity of the enzymes produces significant changes in the metabolism. Activities of adenosine kinase or deaminase are inversely correlated with the phosphohydrolases. Magnitude of phosphohydrolase activity is indicative of anaerobic nature of muscle (2). Both in animals and plants, these enzymes depend upon O_2/CO_2 gas phase, at least partially (174). Thus, under anoxic conditions decreased O_2 tension affects the enzyme activity. Besides O_2/CO_2 some other biochemical factors regulate these enzymes as well. For example, ATP is a major stimulator of AMP-deaminase and P_i works as inhibitor for it. In anoxic and starved conditions, IMP pathway is followed by degradation of AMP and ATP is lost rapidly due to starved conditions with lot of P_i generation. Under such conditions, AMP is accumulated as both ATP and P_i factors work on the system simultaneously. Similar is the case with 5'-nucleotidase. This enzyme is inhibited by various nucleotides. Of these ATP is a strong inhibitor and its effect has been studied in details (9,23,84). 5'-nucleotidase from rat cerebelum gets activated by Mg^{2+}, Co^{2+} and Mn^{2+}. Sullivan and Alpers (157) demonstrated the regulation of 5'-nucleotidase by ADP, ATP and Mg^{2+}. According to them ADP was more inhibitory

than ATP and Mg^{2+} ions were required for activity. Mg^{2+} relieved the inhibition caused by nucleotides completely by forming a nucleotide-magnesium complex. Similarly, inorganic phosphate removes the inhibition of ATP in sheep brain but does not affect the enzyme itself (69). Gibbson and Drummond (60) studied the combined effect of Mg^{2+} and ATP and showed that ATP with equal concentration of Mg^{2+} has little stimulating effect at low substrate concentrations, but inhibits the 5'-nucleotidase at high substrate concentration. When ATP is in excess of Mg^{2+}, it strongly inhibits the enzyme at high substrate concentrations. This report clearly demonstrated the combined effect of ATP, Mg^{2+} and substrate concentrations on the enzyme. Malol (109) showed modification in activity by divalent cations and nucleotides. Regulation of 5'-nucleotidase by cyclic nucleotides was suggested by Polya (128). Thus, it appears that in vivo, several factors, including inhibitors, stimulators, physiological effectors, etc., act on the enzymatic machinery collectively and produce a cumulative effect. Concanavalin A (Con A) is also described as a potent inhibitor by several workers (24,134,151). The inhibitory action of Con A may be attributed to the interaction with carbohydrate moiety (mannose and galactose) and suggests that the enzyme is a glycoprotein.

2.1.4 Physiological role of 5'-nucleotidase and it's correlation with pathological conditions

One of the important functions of 5'-nucleotidase is its participation in the phenomena of senescence and cell division. It was found that 5'-nucleotidase activity increases in the cell with age. Specific activity increases from 0.29 to 1.37 μmol/hr/10^6 cells from 3 weeks to 2 years old rabbit chondryocyte (185). Sun and Holland (159,160) showed that in permanent lymphoid cell lines, there is no increase in 5'-nucleotidase activity during cell ageing. Rapid proliferation is characteristic of young cells and almost no proliferation of senescent cells. 5'-nucleotidase increases 10-folds in senescent human embryonic lung from young stage (158). Klaushoffer and Bock (81) found a direct relationship between cell proliferation and level of the 5'-nucleotidase activity.

An interesting observation is that 5'-nucleotidase is either absent or is present in very low level in malignant cells (27,130). Malignant cells are characterized by enlarged nucleus, which occupies almost the whole cell, leaving behind little cytoplasm. Since, the nuclear materials are nucleic acids and nucleotides are their building blocks, low activity of 5'-nucleotidase to some extent explains the increase in nucleus size. Thus, study of enzymes of

purine pathway may provide useful information about malignancy (15).

Accumulation of toxic concentrations of ATP and other nucleotides and nucleosides occurs in malignant cells due to decreased 5'-nucleotidase activity (25). Wortman (184) showed that T-lymphoblasts are much more sensitive to deoxyadenosine toxicity than β-lymphoblasts. Another important feature of cancerous cells is their abnormal proliferative pattern. Rapid proliferation may be attributed to the increased DNA material and decreased 5'-nucleotidase activity. Liberman and Gordan Smith (101) correlated the myoproliferative and lymphoproliferative disorders in acute leukemia and Hodgkin's disease with 5'-nucleotidase activity. An inhibitor protein, which inhibits 5'-nucleotidase of normal cells, was found in homogenates of different kinds of human leukemic cells (159).

Thus, in animal systems 5'-nucleotidases have been found to affect various biological functions in animals, like regulation of blood flow (9), transmembrane nucleotide transport (34,53,81,175) and regulation of nucleotide pool size (2). A correlation between enzyme activity and several pathological conditions has been established which includes increase in

enzymatic level with senescence (158,159) and decrease with proliferation (160), malignancy (15,27,130), etc. But in plants only some possible functions of this enzyme have been anticipated so far. One of the main functions envisaged for AMPase in plants is to maintain the nucleotide level since it has been shown that the ratio of the high energy nucleotides (ATP and ADP) to the total nucleotide pool (ATP, ADP, AMP) remains constant even with the drop in nucleotide pool size.

2.1.5 Properties of 5'-nucleotidase

In general both plant and animal 5'-nucleotidases usually possess a broad pH-optimum (26,163) varying from pH 5.0 to 8.0 and in most cases Mg^{2+} ions were found to effect the optimum pH (22,99). The enzyme shows low K_m values with purine nucleotides (19,23) and the high K_m values with pyrimidine nucleotides (26,163). However, higher K_m values for purine nucleotides than pyrimidine nucleotides for cytosol and microsomal 5'-nucleotidase have been reported (55,56). Molecular weight of 5'-nucleotidase is reported to be about 150,000 daltons from various sources (47). In some cases 50,000 dalton units were obtained by SDS-PAGE but the enzyme molecule was usually found to be composed of two or more such units (26,129).

2.1.6 Substrate specificity

In general 5'-nucleotidases show a broad substrate specificity. They hydrolyse various nucleotides, phosphorylated sugars and p-nitrophenyl phosphate, but the degree of hydrolysis vary from substrate to substrate and from source to source of the enzyme. Table I shows the relative activity of 5'-nucleotidase on various substrates. A special mention is to be made of a plant nucleotidase, purified by Carter and Tipton (26) from the microsomes of Zea mays seedlings. It was shown that the enzyme has stronger affinity for purine nucleoside monophosphates than for pyrimidine nucleotides and for the membrane-bound 5'-nucleotidases, reported thus far 5'-AMP is the preferred substrate. However, for the cytosol 5'-nucleotidase the preferred substrate is IMP (73,171). It has been suggested that the membrane-bound 5'-nucleotidase and cytosol 5'-nucleotidase follow two different pathways for degradation of AMP. It has been observed that a varying, but significant, amount of non-specific phosphatase activity is always associated with 5'-nucleotidases. For instance, potato 5'-nucleotidase (129) contains very high activity towards p-nitrophenyl phosphate and the Zea mays 5'-nucleotidase (26) besides catalyzing the hydrolysis of AMP also

TABLE I SUBSTRATE SPECIFICITY OF 5'-NUCLEOTIDASE

Substrate	5'-nucleotidase activity (%)		
	Animals		Plants
	Rat heart (156)	Mouse liver (47)	Zea mays (26)
Adenosine 5'-monophosphate	100	100	100
Uridine 5'-monophosphate	81	105	71
Cytidine 5'-monophosphate	75	69.5	46
Guanosine 5'-monophosphate	53	88.0	116
Ionosine 5'-monophosphate	53	-	99
Glucose-6-phosphate	3.8	-	-
p-Nitrophenyl phosphate	-	-	3.0
β-Glycerol phosphate	3.3	-	1.0

catalyse the hydrolysis of 5'-GMP, 5'-UMP and 5'-IMP with nearly equal efficiency (Table I). The hydrolysis of 5-CMP was about 50% of the AMPase activity. Identical results have been obtained with animal 5'-nucleotidases (47,156).

2.1.7 Purification of 5'-nucleotidase

Nucleotidases have been purified from a number of sources mainly from animal tissues. Since the enzyme is membrane-bound, various procedures have been used to release the enzyme from the membranes including the use of detergents as solubilizing agents of the membrane proteins (66). Table II lists various detergents which have been used to solubilized membrane proteins from different sources and their properties are summarized in Table III (67). In general, digitonin, CHAPS and octylglucoside have enjoyed popularity for achieving solubilization and preserving function even with difficult cases. Zwittergent 3-14 was the detergent most capable of solubilizing 5'-nucleotidase (9). There is no definite rule as far as the effectiveness of the solublizing agent is concerned. It is only the hit and trial method that one has to use in order to find a suitable solubilizing agent. The best solubilizing agents may be those which are capable of removing the membrane glycoproteins in active form.

TABLE II EXTRACTION OF VARIOUS MEMBRANES BY DIFFERENT DETERGENTS

Source	Membrane-fraction	Detergent used	Reference
Beef heart	Mitochondrial	Lysolecithin	141
Saccharomyces cerevisiae (yeast)	Mixed	Nonidet P-40	146
Chicken liver	Microsomal	Triton X-100	1
Corn root	Plasma membrane	Triton X-100	41
Ascite cells	Microsomal	Triton X-100, phospholipase	21
Rat heart	Sarcolema	Phospholipase C	126
Zea mays	Microsomes	Zwittergent 3-12	26
Micrococus lysodeikticus	Cell membranes	Deoxycholate	142
Mouse liver	Plasma membrane	Sarcosyl-Tris buffer	47

TABLE III PROPERTIES OF DETERGENTS (67)[a]

Property	Sodium cholate	CHAPS	BIGCHAP	Digitonin	Zwitter-gent 3-14	Octyl gluco-side	Triton X-100	Lubrol PX
Monomer molecular weight	431	615	862	1229	364	292	650	582
Micelle molecular weight	1700	6150	6900, 13,800	70,000	30,000	8,000	90,000	64,000
Critical micelle concentration								
% (w/v)	0.36	0.49	0.12	-	0.011	0.73	0.02	0.006
(mM)	8.00	1.4	1.4	-	0.3	25.0	0.3	0.1
Dialyzability	+	+	+	-	-	+	-	-
Suitability for "charge fractionation"	-	+	+	+	+	+	+	+
Binds divalent cations	+	-	-	-	-	-	-	-
Significant A_{280}	-	-	±	-	-	-	+	-
Interference with protein assays	-	-	+	-	-	-	+	+

[a]For detail see reference no. 67.

2.2 Membrane glycoproteins

Glycoproteins are important component of membranes, in which the sugar moiety is firmly attached to the polypeptide by covalent linkage. These biomolecules are considered to be the major evolutionary component in transition from pro- to eukaryotes as the paucity of prokaryotic glycoproteins and ubiquity of the eukaryotic glycoproteins was observed. A great deal of information is available concerning animal glycoproteins (83a) but considerably less is known about plant glycoproteins (44,148). However, the significance of some plant glycoproteins such as lectins or seed proteins is now receiving much attention (86). Higher plant glycoproteins include lectins, enzymes, reserve polymers, structural proteins, toxins, etc. (47). A brief account of these is summarized in the Table IV (144a).

There are vast diversities in the structure of glycoproteins. However, some basic similarities have been observed. The sugar unit is attached to the polypeptide by the following commonly occurring linkages: (i) N-glycosidic linkage between amide nitrogen of asparagine and C-1 of N-acetyl glucosamine (Asn-GlcNAc) (105), (ii) O-glycosidic linkage between hydroxyproline, serine or threonine residue and a sugar

TABLE IV PLANT GLYCOPROTEINS (144a)

General type	Examples	Plant source	Type of linkage	Sugars present[a]
Lectins	Soybean agglutinin	Soybean seed	N-glycosidic to Asn	GlcNAc, Man
	Potato lectin	Potato tuber	O-glycosidic to Hyp/Ser	Arb, Gal
Enzymes	Bromelain	Pineapple stem	N-glycosidic to Asn	GlcNAc, Man
	Ficin	Ficus latex	-do-	GlcNAc, Fuc, Gal
	Peroxidase	Horse raddish root	-do-	GlcNAc, Man
Reserve proteins	Vicilin	Various legumes	N-glycosidic to Asn	GlcNAc, Man
	Legume	-do-	-do-	-do-
	7S-protein	Soybean seed	-do-	-do-
	Glycoprotein II	Phaseolus vulgaris seed	-do-	-do-
Toxins	Ricin D	Ricinus seed	-do-	-do-
	Mitogen	Wisteria seed	-do-	GlcNAc, Man, Fuc (Arb, Xyl)
Primer proteins	Glucoprotein starch primer	Potato tuber	Uncertain	Glc
	Glucoprotein cellulose primer	Prototheca	-do-	-do-

Contd.....

Table IV Contd.

Structural polymers	Hydroxyproline-rich glycoproteins (extensin)	Sycamore Tomato Tobacco Runnerbean	O-glycosidic -do- -do- -do-	Arb, Gal -do- -do- -do-
Proteoglycans	Water soluble glycoproteins	Vicia faba leaves	O-glycosidic to Hyp	Arb, Gal
	Intracellular glycoproteins	Tobacco	-do-	-do-
	Extracellular glycoproteins	Tobacco	-do-	-do-
	Arabino-galactan peptide	Corn pericarp, wheat endosperm, various seeds	-do-	-do-
Slimes	Slime-polysaccharides	Zea mays	O-glycosidic to Thr	Xyl

[a]N-acetylglucosamine, GlcNAc; mannose, Man; arabinose, Arb; galactose, Gal; xylose, Xyl; and Fucose, Fuc.

commonly, L-arabinose or D-galactose. This group can further be divided into alkali labile D-galactose to serine or threonine linkage (88) and alkali stable bond involving L-arabinose or D-galactose and hydroxyproline (85,114).

Glycosylated hydroxyproline seems to be unique to the plant kingdom (111). N-glycosidic Asn-GlcNAc linked glycoproteins are the most commonly found type in membranes (133) and possess an identical core region composed of three mannose and two N-acetylglucosamine residues (Fig. 1D) (20). This structure has been derived from animal cells but is probably similar in plant cells also. Further elongation of this oligosaccharide chain results basically in two types - the 'high mannose' and the 'complex' types. Plants have been shown to have the high mannose type of glycoproteins, namely - soybean lectin (104), vicillin (46) and legumin (10). Fig. 1 shows the oligosaccharide chains of some GlcNAc— Asparagine linked glycoproteins.

The synthesis of glycoproteins starts like other proteins on ribosomes. A specific amino acid region, the "Signal sequence", provides the attachment point of mRNA/ribosomes/nascent polypeptide complex to the membranes (16). This nascent polypeptide, led by

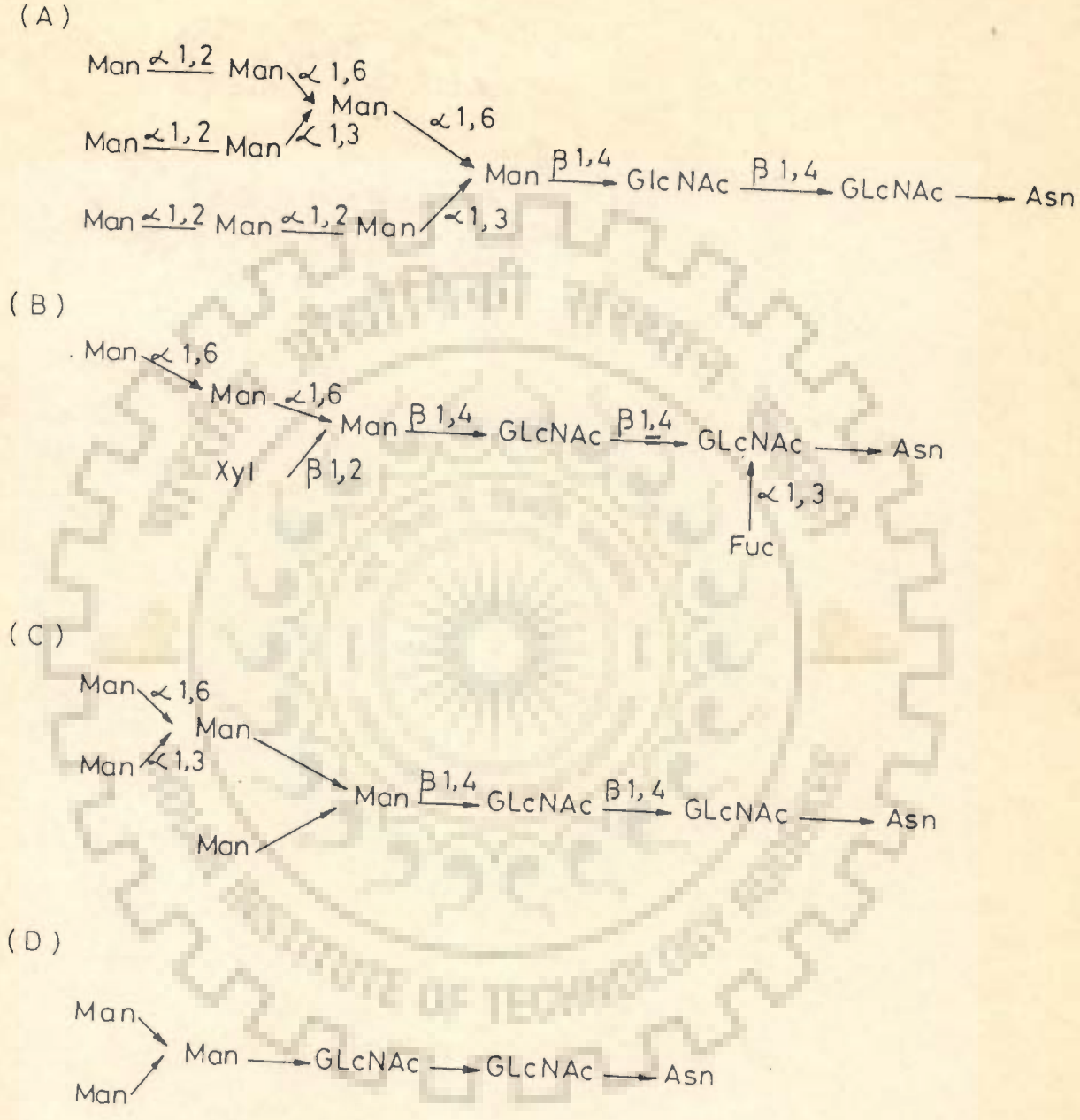
Fig. 1 Structures of N-linked oligosaccharides in plants.

A) Soybean agglutinin

B) Bromelain

C) Taka-amylase

D) Common core region


(A)
Man α 1,2 Man α 1,6
Man
Man α 1,2 Man α 1,3
α 1,6
Man β 1,4 GlcNAc β 1,4 GLcNAc Asn
Man α 1,2 Man α 1,2 Man α 1,3
(B)
Man α 1,6
Man α 1,6
Man β 1,4 GLcNAc β 1,4 GLcNAc Asn
Xyl β 1,2
α 1,3
Fuc
(C)
Man α 1,6
Man
Man α 1,3
Man β 1,4 GLcNAc β 1,4 GLcNAc Asn
Man
(D)
Man
Man GLcNAc GLcNAc Asn
Man

signal sequence passes to the cisternal space of the ER membrane compartment (102). The first step in synthesis of Asn-GlcNAc linked oligosaccharide is transfer of core structure (Glc_3-Man_9-$GlcNAc_2$) from dolichol lipid carrier to the nascent polypeptide. The role of lipid-linked oligosaccharide as intermediate is well established (29,145,146). The details of the dolichol phosphate-linked saccharyl pathway for N-glycosation are shown in Fig. 2 (95).

It has been demonstrated that the core glycosylation occurs as a cotranslational event (80,106,167) which is applied only to the initial transfer in endoplasmic reticulum (20). Leavitt et al. (96) have suggested the possibility that the core glycosylation may function as 'secretion signal' for the exit of the proteins from RER.

Post-translational processing of N-linked oligosaccharide that involves removal of certain glycosyl residues as well as addition of new ones and initiation of O-linked oligosaccharides occur on Golgi-apparatus (143,162). Regulation and processing is controlled both by the specificity of glycosidases and the use of specific glycosyl transferase steps to trigger further glycosidase activities (161). Some phosphorylated sugars may act as a selective 'retention

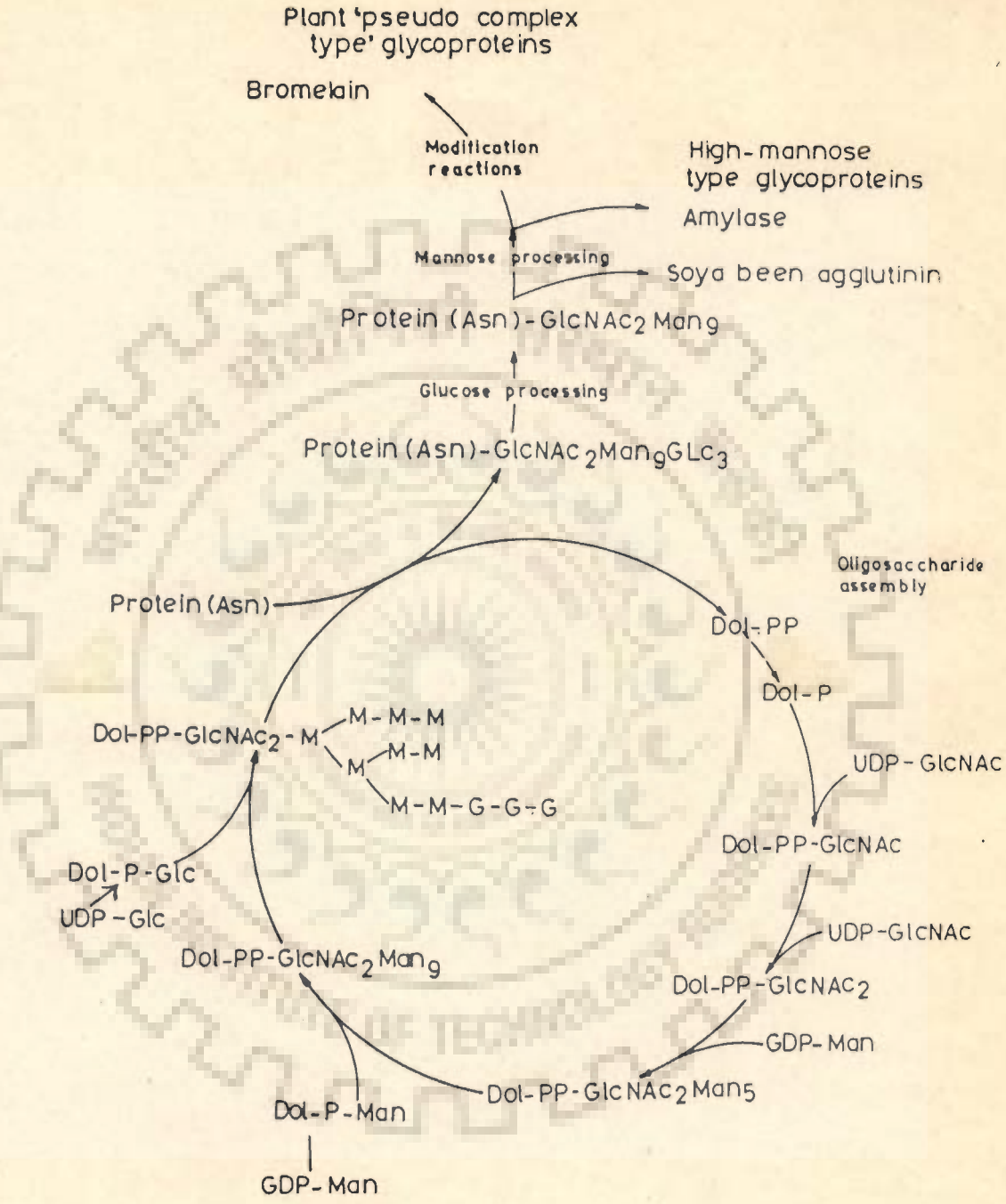


FIG.2. THE DOLICHYL PHOSPHATE-LINKED SACCHARIDE PATHWAY OF N-GLYCOSYLATION

signal' for the stay of molecule in the particular organelle (79).

The involvement of membrane glycoproteins in eukaryotes is of extreme importance as the inclusion of glycosyl moieties on these proteins and lipids makes the membrane selective and flexible molecular melieu which can regulate functions not only via lipid-lipid, lipid-protein or protein-protein interactions but also via carbohydrate-protein and carbohydrate-lipid interactions. The assymetry, fundamental feature of membranes that pertain in all components of membrane, appear to be absolute for carbohydrate and protein (58,150). The carbohydrate moieties are noncytoplasmic and are situated on the outer side of integral protein moieties (5,95).

The biological significance of sugar units are apparent but the exact functions are still obscure, except possibly for some animal glycoproteins. There is a large body of data to show that this extra non-amino acid information (i.e. sugars) is often crucial, especially in the functions that involve recognition aspects such as mating, pollen incompatibility in higher plants, etc. (86). The other possible important functions include recognition of related cell (31), transport of macromolecules (48), stabilization of

tertiary structure of protein moiety (176) and *in vivo* localization of glycoprotein (93).

2.3 Intracellular transport of macromolecules with acid phosphatase as model

Most work concerning the transport of secretory materials has been carried out in fungi, along with the deposition of cell wall material in budding yeast or filamentous fungi (144b). Phosphatases are in abundance in cell wall and were among the first wall enzymes to be studied (89). Like several other secretary glycoproteins, acid phosphatase was found to be associated to various membranes during their transport. This include endoplasmic reticulum, vesicles derived from ER, proliferating ER, Golgi-apparatus, central vacuole, etc. (172). In filamentous fungi, the material of apical wall synthesis is first secreted in ER (62). The budding process in yeast is reported to be initiated with vesiculation of ER (115). These vesicles contain cell wall material and are termed as 'glucnase vesicles' due to their content in exo (1→3)-β-glucnase (112). The 'glucnase vesicles' were found to contain acid phosphatase (103). Acid phosphatase has been used as a secretory marker in *S. cerevisiae* (yeast) to deduce a precise picture of intracellular transport

(103,172) as it was reported to be associated with ER, flat vesicles, Golgi like structure and nuclear membranes. In yeast cells, acid phosphatase was found to reside on the outer side of the cell membrane (113, 177). An external acid phosphatase in S. cerevisiae was shown to contain 50% carbohydrate and was actively secreted by the protoplasts (135). Glycosylated forms of this enzyme associated to membranes and the cell lysate, which appear to be precursors of external enzyme, were analysed by isopycnic density gradient. Carbohydrate content of membrane-bound enzyme was shown to be less than external acid phosphatase and only a fraction of membrane bound enzyme attained the buoyant density of external enzyme (17), while the carbohydrate content of soluble internal form was similar to the external enzyme. This seems to be relevant, as vacuoles may function as a storage compartment containing finished products which are released in lysate during fractionation (144b). The same was observed in other secretory enzymes like α-galactosidase and invertase (170). It may be explained by assuming that the membrane-bound enzymes like acid phosphatase, invertase, etc. are being completed by addition of mannosyl residue to the inner core (144b), since mannosyl transferases are

also membrane-bound enzymes (11). In view of the above it would be reasonable to presume that plasma membrane-bound 5'-nucleotidase should follow the same pathway as has been found in the case of other membrane-bound enzymes. A study of the carbohydrate structure of 5'-nucleotidase enzymes, present in ER, Golgi-apparatus and plasma membranes, will be useful in understanding the mechanism of the intracellular transport of proteins destined to become plasma membranes proteins.

2.4 Membranes of eukaryotic cells

Eukaryotic cell membranes are highly developed with compartmentation of functions in different sub-cellular organelles and consist of nuclear envelop, endoplasmic reticulum, Golgi-apparatus, plasma-membrane, lysosomes, etc. The plasma membrane, besides providing the boundary of protoplast, forms a selective barrier of the cell.

Among eukaryotic cells, animal cell membranes have been investigated quite extensively and are fairly well characterized. However, relatively little work has been done on the molecular characterization of plant cell membranes. One of the main reasons has been the paucity of convenient and reliable methods for the isolation of different plant cell membranes.

In the following section the methodology including the specific marker enzymes that have been frequently used to isolate plasma membranes, Golgi-apparatus and microsome (endoplasmic reticulum) has been reviewed.

2.4.1 Markers of different plant membranes

2.4.1.1 Plasma membrane markers

Phosphphotungstic acid chromic acid stain (PTAC) is quite specific and has been used frequently to identify the plasma membranes (137). Use of many enzymic markers (K^+-ATPase and glucan synthetase) is based on correlation of these enzymes with PTAC stain (97). K^+-ATPase and glucan synthetase-II activities have been correlated with the physiological functions of plant plasma membranes (64,108) and thus have been used as markers. Some reports are available showing different distribution of the two enzymes in the cell which raise doubts about their use. Some other methods like NPA (Naphthylphthalamic acid) binding (75) and use of monoclonal antibodies (123) have also been employed. However, since no single marker has yet been found to be plant plasma membrane specific, a combination of different markers is generally applied to ensure high degree of purity of the membrane preparations.

2.4.1.2 Golgi-apparatus markers

Golgi-apparatus is a distinguished part of cell due to its morphology, which consists of a stack of cisternae having localized swelling and isolated vesicles at the edges of cisternae. It forms an important component of the biosynthetic pathway in which it receives the products from endoplasmic reticulum (ER) which are destined for secretion or arrival at the plasma membranes (124,156). The typical morphology of the organelle has been used for its identification during purification (116). Use of glycosyl transferases, enzymes responsible for addition of terminal sugars to glycoproteins, as marker was suggested (156), however the glycosyl transferases were found to be very poor markers. As an alternative, inosine-diphosphatase (IDPase) activity has been used as a specific marker, although it has not been used very frequently in plant cell fractionation studies. β-Glucan synthetase-I activity was found to be important for identification of Golgi-apparatus (66) and a good correlation between IDPase and glucan synthetase I distribution was observed. Recently, the use of antibody raised against purified Golgi-apparatus from animals has been suggested in radioimmuno assay for identification (66). The method, however, is yet to be developed as

a routine standard preparation method of Golgi-membranes. Another possibility which may be useful is the use of a monensin, an inhibitor of intracellular transport of material from Golgi-apparatus to the plasma membrane, in the isolation of Golgi-apparatus by measuring the accumulation of certain materials (61). Further investigations in this direction may be highly rewarding.

2.4.1.3 Endoplasmic reliculum markers

Endoplasmic reticulum forms a network in the cell and is of two types, rough and smooth endoplasmic reticulum featured by the presence and absence of ribosomes attached to its surface, respectively. Electrone-transport enzymes NADH- and NADPH-cytochrome c-reductase are reliable and popular markers for microsomes. The enzymes responsible for synthesis of phospholipids may also be used as marker (66) though they are not exclusively localized on its surface. Utilization of Mg^{2+}-induced density shift is particularly useful for rough endoplasmic reticulum (RER) membrane identification (107). However, the purity of microsomes should be ascertained carefully as many artifacts function during its isolation.

2.4.2 Methods of isolation and purification

An uptodate account of isolation of membranes and organelles from plant cells has been compiled by Moore (63). A survey of the literature shows that to obtain subcellular membrane fractions from plant tissues in active and pure form is comparatively difficult due to presence of rigid cell wall and lack of convenient markers. However, the following general method is commonly used with modifications as per requirement. The tissue is homogenized under mild conditions with appropriate buffer medium. To prevent much fragmentation and inactivation of subcellular organelle, gentle methods of homogenisation like rajor blade chopping, pestle and mortar and glass teflon homogenisers are used. The medium is usually Tris-buffer, pH 7 to 8, containing sucrose (250 mM) as osmoticum to preserve the activity of fractions (41). Various other protectants and activators have also been used according to the requirements. For example, EDTA is usually added in the buffer during plasma membrane fractionation to chelate divalent cations and to convert rough endoplasmic reticulum into the smooth one (97). Mg^{2+} is employed to obtain the intact ribosomes as it reduces the ribonuclease activity. Similarly, presence of a stabilizing agent glutaral-

dehyde produce active Golgi-fraction.

Besides, some other methods have also been utilized, like treatment with cellulase, which softens the cell wall and thus makes the disruption milder and easy. Dissolution of cell wall by hydrolytic enzyme action is an important technique to avoid the inactivation due to strong forces (92). However, use of this technique was limited to yeast and bacteria and is not commonly employed for higher plants.

The cell wall free homogenate is fractionated by differential centrifugation. As a general guideline, low speed centrifugation (150-2500 x g) sediments heavy particles like nuclei, while mitochondria and chloroplasts are pelleted at 10,000 x g. At higher centrifugal force (100,000 x g or above) a pellet of microsomal membrane fraction is obtained which consists of plasma membranes, endoplasmic reticulum and Golgi-apparatus (18). However, the technique was proved to be poor as the fractions yielded were contaminated by other organelles.

Perhaps the most important technique used for isolation of different membrane-fractions is density gradient centrifugation. Several materials like sucrose, serum albumin, Ficoll (98), and silica gel (108) have been used to prepare density gradient.

The technique emerged as a good tool and has been employed in various forms to isolate and purify different subcellular fractions. A 20-50% sucrose range has been used (18,63,110) for purification of plasma membranes from the fractions obtained by differential centrifugation. Usually the microsomal fraction, 100,000 x g pellet, is used for this purpose (41) but other fractions like mitochondrial and even total homogenate were also loaded on the density gradient. Plasma membranes stay at 1.14 - 1.17 g/cm^3 density (97). Recently, the plasma membranes have been purified from Zea mays roots (41,98).

Root tips are the best source of Golgi-apparatus. Golgi-apparatus forms a band at 1.12 - 1.15 g/cm^3 density (119). With the help of precisely prepared density gradients together with marker enzymes it is possible to prepare highly enriched, if not absolutely pure, membrane fractions from plant sources. In the present investigation we have employed such approach to prepare plasma membrane, Golgi-apparatus and endoplasmic reticulum fractions from peanut cotyledons.

3.0 EXPERIMENTAL PROCEDURES

3.1 Materials

Adenosine 5'-monophosphate (AMP) and other 5'-ribonucleotides, glucose-6-phosphate, glucose-1-phosphate, p-nitrophenyl phosphate, used as substrates, were purchased from Pierce Chemical Co. (U.S.A.). Tris, n-octyl-β-D-glucopyranoside, Triton X-100, sodium dodecyl sulfate (SDS), sodium deoxycholate, taurooleonyl cholate, Nonidet NP-40, UDP-glucose were obtained from Sigma. Acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethyl ethylenediamine, and DEAE-cellulose (Servacel DEAE 23 SH) were obtained from Serva (FRG). The protein standards for molecular weight determination were obtained from Bio-Rad (U.S.A.). Radioactive UDP-[^{14}C] glucose (260 Ci/mol) and GDP-[^{14}C]-mannose were (307 Ci/mol) purchased from the Radiochemical Centre, Amersham (U.K.). All other chemicals were reagent grade from various commercial sources. Peanut seeds (large variety) were purchased from the local seed stores.

3.2 Methods

3.2.1 Germination of seeds

Peanut seeds (Arachis hypogaea L.) were germinated in dark at 35°C under asceptic conditions. Surface sterelization of about 200 healthy seeds was

done by treatment with 0.1% NaOCl for 5 minutes, followed by thorough washing with water to remove the hypochlorite completely. Seeds were then spread on 4 layers of cheese cloth in a tray and kept in the germinator at 35°C temperature with 100% humidity. Throughout the germination period the seeds were washed twice a day with water to prevent any fungal infection.

3.2.2 Methods of membrane fraction preparation

3.2.2.1 Preparation of crude membrane fraction

Unless stated otherwise all operations were carried out at 0-4°C. Crude microsomal membrane fraction was prepared by differential centrifugation, as described by Bonner (18). Approximately 200 cotyledons (80-100 g fresh weight) of 7-days old germinating seeds were excised, washed and suspended in 400 ml homogenizing buffer consisting of 50 mM Tris-HCl, pH 7.2, 0.25 M sucrose, 3 mM EDTA and 0.04% β-mercaptoethanol. It was homogenized by three 30 seconds bursts, in a Waring Blender and the homogenate was filtered through 4-layers of cheesecloth. The filtrate was then successively centrifuged at 12,000 x g for 20 min and 105,000 x g for 60 min. The 12,000 x g to 105,000 x g membrane pellet, containing ER, Golgi-apparatus and

the plasma membrane fractions, was suspended in homogenizing buffer and centrifuged at 105,000 x g for 60 min. The membrane fraction thus obtained was then suspended in 2 ml 50 mM Tris-HCl buffer, pH 7.2 and 0.04% β-mercaptoethanol and used for a number of preliminary experiments.

3.2.2.2 Preparation of pure plasma membrane fraction

For the separation of plasma membrane from Golgi-apparatus and ER membrane fractions, method of DuPont *et al*. (41) was employed. The mixed membrane fraction (12,000 x g to 105,000 x g pellet) was suspended in 4 ml of 25 mM Tris-HCl, pH 7.2, containing 20% (w/w) sucrose and 1 mM β-mercaptoethanol, layered carefully over 5 ml 34% (w/w) sucrose in the same buffer and centrifuged at 80,000 x g for 90 min. The clear sucrose layers were removed carefully by aspiration. The microsomal membrane fraction plus Golgi-apparatus remaining at the interface of 34% and 20% sucrose and the pellet containing plasma membrane (41) were recovered, suspended in 1 ml of the same buffer containing 20% sucrose and 1 mM β-mercaptoethanol. The protein concentration and enzyme activity were determined immediately. The total protein recovered in the plasma membrane pellet varied between 16-18 mg per 100 g fresh weight of cotyledons.

Alternatively the plasma membrane vesicles were separated from other membranes by a discontinuous sucrose gradient centrifugation method as described by Hall (63). The discontinuous gradients were prepared in 38 ml tubes by carefully layering 4 ml of 45% (w/w) sucrose and 6.4 ml each of 37, 34, 30, 25 and 20% sucrose. Two ml (ca 25 mg protein) were carefully layered on to the gradient and the tubes centrifuged at 95,000 x g for 2 h in SW-27 rotor. Visible bands of membranes were removed with a Pasteur pipette. The plasma membrane fraction (the 45/37% interface band) corresponding to an equilibrium density in sucrose of 1.15 - 1.17 g/cm^3 was identified by the presence of marker enzyme glucan synthetase II (63).

The plasma membranes prepared by either of the methods, DuPont *et al.* (41) or of Hall (63), were comparable as judged by the specific activity of AMPase as well as the activity of glucan synthetase II and I (Table VII). Because of the simple operation, unless mentioned otherwise, method of DuPont *et al.* (41) was used to prepare the high AMPase activity plasma membranes.

3.2.2.3 Preparation of pure Golgi-membrane fraction

The plasma membranes were removed from the crude microsomal membrane fraction (12,000 x g to 105,000xg

pellet) by step gradient of 34/20% (w/w) sucrose as described under 3.2.2.2. The membrane fraction staying at 34/20% sucrose gradient interface was removed carefully with the help of Pasteur pipette and fractionated further into the Golgi-apparatus and ER by sucrose-density gradient method described by Green (61) with slight modifications as described below.

The 34/20% sucrose gradient interface membrane fraction was diluted with equal volumes of homogenizing buffer and pelleted by centrifugation at 105,000 x g for 60 min. The pellet was suspended in 4 ml of 25 mM Tris-HCl, pH 7.2, containing 20% sucrose and 1 mM β-mercaptoethanol. This was then floated carefully on a sucrose gradient system prepared by layering of 7 ml of each of 43%, 37% and 25% sucrose solution successively in the same buffer. The system was immediately centrifuged at 105,000 x g for 3 h. A major band was obtained at 37/25 per cent sucrose gradient interface. The supernatant part was removed carefully without disturbing the interface and saved for the preparation of ER. The membrane fraction at the interface was removed carefully as before. This fraction represented the Golgi-apparatus membrane system as the activity of the marker enzyme, glucan synthetase I, was exclusively localized in this fraction. This fraction was used in all the experiments concerning Golgi-apparatus.

3.2.2.4 Preparation of ER membrane fraction

The 25% supernatant part, obtained after 43/37/25/20 per cent sucrose density gradient centrifugation during Golgi-apparatus purification (3.2.2.3), was removed carefully. This fraction was diluted with equal volumes of 25 mM Tris-HCl buffer, pH 7.2, and centrifuged at 105,000 x g for 60 minutes. The pellet (ER) was suspended in homogenizing buffer and used as enriched endoplasmic reticulum (ER) membrane fraction. The enrichment fold was determined by assaying the mannosyl transferase activity in different membrane fractions, relative to the enzyme activity present in crude membrane fraction.

3.2.3 Solubilization of membrane-bound enzymes

3.2.3.1 Solubilization of 5'-nucleotidases

All steps of solubilization and purification were carried out at 0-4°C. The membrane fraction was suspended (30 mg/ml) in 50 mM Tris-HCl buffer, pH 7.2, containing 0.25 M sucrose, 0.04% (v/v) β-mercaptoethanol, 20 mM $MgCl_2$ and 5 mM EDTA with a Teflon tissue homogenizer. To 1 ml suspension (30 mg protein) was added dropwise with stirring 11 ml of 0.6% (w/v) n-octyl-β-D-glucoside in the same buffer so that the final detergent concentration and the protein to

detergent ratio were 0.5% and 2:3, respectively. The enzyme was solubilized by gentle agitation on a Vortex mixer for 2 min followed by three strokes in a Teflon homogenizer and then immediately centrifuged at 105,000 x g for 60 min to separate the soluble enzyme from the insoluble material.

3.2.3.2 Solubilization of acid phosphatase

Two ml of membrane fraction (21.5 mg protein) suspended in 50 mM Tris-HCl, pH 7.2, containing 0.25M sucrose, 0.04% (v/v) β-mercaptoethanol, 20 mM $MgCl_2$ and 5 mM EDTA, were mixed with 6.6 ml of 0.65% detergent (octylglucoside). The final detergent concentration and protein to detergent ratio were 0.5% and 1:2, respectively. The enzyme was solubilized by stirring the mixture gently and intermittently on a Vortex mixer for 2 minutes, followed by three strokes in Teflon homogenizer. The homogenate was then immediately centrifuged at 105,000 x g for 60 min and the supernatant was used as solubilized enzyme for further purification and analysis.

3.2.4 DEAE-cellulose column chromatography

The solubilized enzyme was dialyzed 6 h against 100 volumes of 10 mM Tris-HCl buffer, pH 7.2. The

dialyzed enzyme was applied on a pre-cooled DEAE-cellulose column (1.5 x 10 cm) which was previously equilibriated with the same buffer. The column was washed with 20 ml buffer to remove unabsorbed proteins. The absorbed proteins were eluted by a linear gradient from 0 to 300 mM NaCl using a single mixing container with 50 ml 10 mM Tris-HCl buffer (pH 7.2) and reservoir with 50 ml of the same buffer containing 300 mM NaCl. Alternatively, the batchwise elution was carried out using successively 15 ml of 50 mM, 100 mM, 200 mM and 300 mM of NaCl gradient in equilibriating buffer. Fractions (1 ml) were collected at the flow rate of 0.2 ml/min. Aliquots (0.2 ml) from every other fraction were analyzed for protein content and enzyme activity. The peak enzyme containing fractions were pooled, concentrated by ultrafilteration and analyzed.

3.2.5 Enzyme assays

3.2.5.1 AMPase assay

5'-Nucleotidase activity was measured with 5'-AMP as substrate as described by Riemer and Widnell (132) with slight modifications. The standard reaction mixture, unless stated otherwise, contained (1 to 2.5 mM AMP), 50 mM sodium acetate buffer, pH 5.0 and 0.1 ml of the enzyme preparation (8-70 μg protein, depending upon the form of enzyme assayed)

which was added last, in a total volume of 1.0 ml; control incubations contained no substrate. Incubation was carried out at 30°C for 15 min and the enzyme activity was terminated by adding 0.5 ml cold 20% trichloroacetic acid. Protein was removed by centrifugation and P_i was determined in the supernatant fluid by the procedure of Fiske and SubbaRow (52). Under the assay conditions P_i released was linear with time upto 60 min and enzyme concentration provided that not more than half of the substrate was hydrolyzed at the longest time interval (data not shown). The specific activity of AMPase corresponds to the μmoles of P_i liberated by dephosphorylation of 5'-AMP per min per mg protein under assay conditions. When detergents especially Triton X-100 and Nonidet NP-40 were present in the assay system the P_i was determined by the modified Fiske and SubbaRow method as described by Dulley (39). In this method P_i analyses were done in the presence of 3% SDS which eliminated interference due to Triton X-100 and other detergents.

Alternatively, the enzyme was assayed spectrophotometrically in the presence of an excess of adenosine deaminase by coupling the reaction of 5'-nucleotidase to the deamination of adenosine as described by Ipata (69). Results of both methods were comparable within experimental error.

3.2.5.2 Acid phosphatase assay

Acid phosphatase activity was measured by a slightly modified method as described by Odds and Hierholzer (125) using p-nitrophenyl phosphate as substrate. The incubation mixture (1 ml), contained 50-100 µg enzyme protein, 2.5 mM p-nitrophenyl phosphate, 50 mM acetate buffer, pH 5.0. Reaction was terminated after 15 minutes incubation at 30°C by addition of 1.5 ml of 4% Na_2CO_3. The absorbance of yellow colour of p-nitrophenol released was measured at 420 nm against the control, to which enzyme had been added after terminating the reaction. Specific activity was expressed as µmol p-nitrophenol produced per minute, per milligram protein.

3.2.5.3 Marker enzymes

3.2.5.3.1 Glucan synthetase II assay

The glucan synthetase II activity was measured as described by Ray (131) using high concentration of UDP-glucose, in the absence of Mg^{2+}. Plasma membrane fraction (100 µg protein) was incubated in 0.1 ml of reaction mixture containing 50 mM Tris-HCl, pH 7.5, 0.1 µCi of UDP-[^{14}C] glucose (260 Ci/mol) and 0.5 mM unlabelled UDP-glucose at 25°C for 20 min. The reaction was terminated by the addition of 1 ml 70% (v/v)

ethanol, 50 µl 50 mM $MgCl_2$ and 50 µl boiled plasma membranes (0.2 to 0.3 mg protein) as a carrier for the labelled products. The mixture was immediately boiled for 1 min, and after standing over-night at 4°C, was centrifuged at 1500 x g for 5 min. Precipitated particulate material was washed 4 x with 70% ethanol to remove all unreacted radioactive substrate and ethanol soluble byproducts. The washed precipitate was suspended in scintillation fluid (dioxane cocktail containing 10% naphthalene and 0.5% PPO in dioxane) and radioactivity measured in Beckman LS 1801 liquid scintillation counter.

3.2.5.3.2 Glucan synthetase I assay

Glucan synthetase I activity was measured as described by Green (61) using low concentration of UDP-glucose and 10 mM Mg^{2+}. The reaction mixture contained 100 µg membrane protein, 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 10 mM cellobiose, 4 mM EDTA, 2 mM β-mercaptoethanol and 0.1 µCi of UDP-[^{14}C]-glucose (6 nmol) in a total volume of 0.1 ml. The reaction was terminated by heating the mixture to 95°C followed by addition of 10 mg of powdered cellulose as carrier for newly synthesized β-glucans. The precipitate was collected by centrifugation and

washed sequentially 3 x with 1 ml of hot distilled water, 1 x with $CHCl_3/CH_3OH$ (1:2, v/v), 1 x with methanol and 2 x with 1N NaOH. The alkali insoluble residue was rinsed with 1 ml distilled water and the radioactivity was measured as mentioned above.

3.2.5.3.3 Mannosyl transferase assay

To measure the mannosyl transfer from GDP-[^{14}C]-mannose to endogeneous as well exogenous lipid acceptor (dolichol phosphate) the method of Lehle et al. (94) was used. The reaction mixture consisted of 50 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$, 5 mM $MgCl_2$ and 0.1 µCi GDP-[^{14}C]-mannose. 100-200 µg protein (particulate membrane fraction) was incubated with the reaction mixture in a total volume of 70 µl. For the incorporation of mannose to the exogeneous lipid acceptor, 10 µl of dolichol monophosphate (10 µg) was mixed with 10 µl of 0.1M Mg-EDTA and dried under nitrogen. The dried lipid was dispersed with 10 µl of 5% Nonidet. This lipid was then incubated with the reaction mixture as described above. The reaction was stopped after 30 min by adding 2 ml of chloroform/methanol (3:2, v/v). The precipitated protein was separated by centrifugation and the soluble portion was partitioned with 0.4 ml of 4 mM $MgCl_2$ solution. After thorough mixing

the phases were separated by centrifugation. Upper aqueous phase was discarded and the lipid phase was washed by the method of Folch et al. (54). The washed lipid was dried in a vial and the radioactivity was measured after suspending the dried material in scintillation fluid (dioxane cocktail). Blank was prepared under identical conditions and the enzyme protein was added after terminating the reaction.

3.2.5.3.4 Glucose-6-phosphatase assay

The activity of glucose-6-phosphatase was assayed as described by Arnoson (3). The reaction mixture contained 1 mM acetate buffer pH 5.0, 10 mM glucose-6-phosphate in a total volume of 1 ml. The reaction mixture was incubated with membrane fraction at 35°C for 15 min. The liberated P_i was determined by Fiske and SubbaRow method (52).

3.2.6 Protein estimation

Protein was estimated by the method of Lowry et al. (50) with slight modifications using bovine serum albumin as standard. β-mercaptoethanol was removed by heating the protein samples at 60°C for 30-60 minutes in a water bath, before performing Lowry method (164). Since the solubilized enzyme contained 0.5% octylglucoside, all samples (including

standard proteins) were normalized with regard to detergent and the estimation was done in the presence of 0.1% SDS (40).

3.2.7 Analysis of glycoprotein sugars

3.2.7.1 Removal of N-glycosidically linked oligosaccharide from glycoprotein enzyme

The glycoprotein sample (200-500 µg) was treated with 0.5-5 units N-acetyl-β-D-glucosaminidase (Sigma Chemicals Co.) in 0.1M sodium citrate buffer, pH 7.4. Incubation was carried out at 37°C for 20 h. The reaction was stopped by placing the reaction vessel in boiling water bath for 5-10 minutes followed by the addition of 10% trichloroacetic acid. After cooling the denatured protein precipitate was separated from the soluble part by centrifugation at 50,000 x g for 60 min. The precipitate was washed two times with 5% trichloroacetic acid and the supernatants from various runs were pooled. The carbohydrate content in both precipitate and the supernatant were determined. The decrease in the carbohydrate content of the acid precipitated protein and the corresponding increase in the carbohydrate content of the supernatant indicated the removal of carbohydrate by endo-N-acetyl-β-D-glucosaminidase.

3.2.7.2 Neutral sugars

Oligosaccharide, released by endo-N-acetyl-β-D-glucosaminidase treatment, was hydrolyzed as described by Tashiro and Trevithick (165) with slight modification. Oligosaccharide sample (200-500 µg) was hydrolyzed with 0.5 ml of 4N HCl for 6 h in a sealed tube at 100°C. The hydrolysate was evaporated to dryness under the stream of nitrogen many times to remove remaining HCl. The resultant residue was dissolved in 10 ml water and treated with mixed bed resin, Dowex 50-X8 (H^+) and Dowex-1(Cl^-) or BioRad ion-retardation resin. The resin was removed by filteration through G-4 glass filter and washed two times with 10 ml water. The combined filterate was evaporated to dryness in vacuo. This fraction represents the neutral carbohydrate. The resin after removal of neutral carbohydrates was packed in a column and eluted with 50 ml of 2N HCl. The eluted solution contained amino acids and hexosamine. Both carbohydrate and hexosamine fractions were analysed qualitatively by chromatography on Whatman No. 1 paper with ethylacetate-pyridine-water (10:4:3, v/v) and was also analysed by HPLC. To determine the amount of degradation of neutral sugars, during the course of hydrolysis, 0.5 mg of enzyme plus 50,000 cpm of [^{14}C]-mannose

was hydrolysed and tested as described above. After paper chromatography the mannose was eluted with water directly into the counting vial, dried and counted in 5 ml dioxane cocktail fluid with Beckman LS-1801 liquid scintillation counter. The radioactivity thus eluted was compared to that of [^{14}C] -mannose subjected to all but acid hydrolyzing conditions. The ratio of acid hydrolyzed [^{14}C] -mannose to unhydrolyzed [^{14}C] -mannose yielded an estimate of per cent degradation of mannose (140). The correction factor was applied to both glucose and mannose. For hexosamines, 0.5 mg glycoprotein sample plus 50,000 cpm of [^{14}C] -N-acetyl-glucosamine was hydrolyzed with 4N HCl at 100^{o}C as before. The hydrolysate was dried under vacuum many times to remove the remaining HCl. The dried sample was redissolved in 1 ml of water and the drying step was repeated three times to ensure complete removal of HCl. The amino sugar was absorbed on Dowex 50-X8 (H^+) column (1 x 10 cm) which had been previously washed with HCl, followed by deionised water. The column was washed with 25 ml of water to remove neutral carbo-hydrates. Then the column was washed with 50 ml 2N HCl to obtain amino sugar. The amino sugar was quali-tatively analysed by paper chromatography in n-butanol-pyridine-water (6:4:3, v/v) (140). The sugar spots were located both by staining with silver and by

radioactivity on the chromatogram. The comparison of radioactivity of the hydrolyzed [^{14}C] -N-acetylglucosamine with unhydrolyzed, yielded the extent of degradation of hexosamine during the hydrolysis. The correction was applied.

3.2.7.3 Analysis of sugars by HPLC

The high performance liquid chromatography was carried out with Shimadzu LC-4A HPLC equipped with a uv spectrophotometric detector SPD-2AS and a chromatopac C-R2AX data processing system. The sugar samples were prepared as described by Jentoft (78). The hexosamines were reacetylated by dissolving the samples in 0.1 ml dry methanol followed by addition of 40 µl pyridine and 40 µl of acetic anhydride and incubating the samples for 1 h at room temperature. The samples were dried under the stream of nitrogen. 40 µl of toluene were added and samples were redried and dissolved in water or CH_3CN:H_2O (90:10), v/v. Sample (10 µl) was injected to a 25 cm, 5 µm Zorbax C-18 column. The column was eluted with 90% acetonitrile (CH_3CN) for 30 minutes at a flow rate of 0.5 ml/min. The retention times of the sugars in acid hydrolyzate were compared with those of standard sugar samples. The radioactive standard sugars were also used as references to be

doubly sure of the identity of the monosaccharides obtained after hydrolysis of glycoproteins.

3.2.7.4 Estimation of total carbohydrate content

Total carbohydrate content was measured by phenol/sulphuric acid method (38). To a 2 ml aqueous solution (ca 20-75 µg carbohydrate) 50 µl of 90% phenol solution and 5 ml of concentrated H_2SO_4 were added, followed by quick stirring on Vortex. The mixture was incubated at room temperature for 30 min and the absorbance of the orange colour was measured at 485 nm against the blank, prepared without sample. Amount of carbohydrate was computed from the standard curve of glucose, prepared under identical conditions.

3.2.8 Polyacrylamide gel electrophoresis

3.2.8.1 Preparative polyacrylamide gel electrophoresis

The procedure of Gabriel (57) was followed for discontinuous polyacrylamide gel electrophoresis either using gel rods (9 cm length x 0.5 cm diameter) or gel slabs (length x breadth x thickness : 14 x 14 x 0.2 cm) with ten wells of about 1 cm width with 2 mm spacing in between two wells. Various solutions used were as following :

Stock solutions

Solution-A

1N HCl	48 ml
Tris	36.3 g
TEMED	0.23 ml

Total volume made to 100 ml with distilled water. Final pH of solution was 8.9. For determining the electrophoretic mobility, 0.5 M phosphate buffer, pH 7.4, was used instead of Tris-buffer.

Solution-B

Acrylamide	60 g
N,N'-methylene-bis-acrylamide	0.6 g

Total volume made to 100 ml with distilled water.

Solution-C

Ammonium persulfate	140 mg/100 ml distilled water

Working solution for preparation of gel slab :

Solutions	7.5% gel
Solution-A	1.0 volume
Solution-B	1.0 volume
Solution-C	4.0 volumes
Water	2.0 volumes

Running buffer

25 mM Tris-glycine buffer, pH 8.3 was used for preparative electrophoresis. In the case of experiments concerning electrophoretic mobility, 0.05 M phosphate buffer, pH 7.5 was used.

Samples of enzymes (100 µg protein) from DEAE-cellulose column were layered gently in the wells of slab gel and allowed to concentrate at a constant current of 10 mA per slab electrophoresis unit. The current was later increased to 25 mA per slab. Electrophoresis was carried out at 4 ± 2°C till the tracking dye bromophenol blue reached the base of the gel. The gel strips on extreme left and right, containing protein bands, were cut and stained by immersing in 0.5% Coomassie Blue in acetic acid-methanol-water (7:45:50, v/v/v) for 6-8 hours at room temperature. The gels were destained by electrophoretic destaining using a solution of 25% methanol, 10% acetic acid and distilled water in a total volume of 2.4 litres. The destaining was achieved by applying 24 volts for about 45 min. The stained strips were used for location of protein band on the unstained gel slab. The protein band was cut and homogenized in sodium acetate buffer, pH 5.0. The homogenate was centrifuged and the process was repeated three times to ensure complete extraction

of enzyme from polyacrylamide gel. The homogenate was freeze dried and used for determining the activity.

3.2.8.2 Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis in the presence of SDS was carried out by the procedure described by Laemmli (83b). 10% polyacrylamide gel columns were prepared as follows : 9 ml aqueous solution containing 1.98 g acrylamide and 74 mg N,N'-methylene-bis acrylamide was mixed with 7 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.2% SDS, 1 ml of 0.35% ammonium-persulfate solution and 2.96 ml distilled water. The mixture was deareated under suction for 30 min. To this mixture 0.04 ml TEMED was added and the solution was immediately poured in the vertically fixed glass tubes (9 cm length x 0.5 cm diameter) upto equal height. A drop of buffer was layered slowly on the top of the gelating solution to prevent the formation of concave surface. Gels were allowed to polymerize for 2 hours.

Enzyme samples were dialyzed against distilled water and dried. Residual proteins were dissolved in 0.01 M phosphate buffer, pH 7.4, containing 1% SDS, 1% β-mercaptoethanol and were heated for 5 minutes in the boiling water. Reference proteins, used for

molecular weight determination, were also subjected to the same treatment. Protein samples (40-100 µg proteins) were layered on gels through the electrophoresis buffer (0.05 M phosphate buffer, pH 7.5, containing 0.1% SDS) in 40% sucrose solution. Gels were run at a constant current of 5 mA per tube for 2.5 h at room temperature. Protein bands were located by staining the gels with 0.5% Coomassie Brilliant blue in water/methanol/acetic acid solution (50:45:7, v/v/v) for 6-8 h and were destained with 7% acetic acid. Mobilities were determined relative to bromophenol dye.

3.2.9 Determination of optimum pH

A fixed amount of enzyme protein (15 µg) was incubated with 2.5 mM p-nitrophenyl phosphate/AMP at 30°C for 15 min in a suitable buffer medium of varying pH. The buffers used were 50 mM acetate buffer pH 3.0 to 5.0 and 50 mM Tris-HCl, pH 6.0 to 8.0. Enzyme activity was assayed as described before, under enzyme assay. Control assays at each pH were run simultaneously. The results were plotted as pH versus per cent of maximum activity.

3.2.10 Determination of apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max})

Lineweaver-Burk plots were employed to determine the values of V_{max} and K_m. The enzyme activity was measured at five different levels of substrate concentration, by the method described under enzyme assay. Care was taken that levels of substrate concentration used were such as to give from partially to fully saturated enzyme system. Linear plots of reciprocals of velocity (1/v) versus reciprocals of substrate concentration (1/S) were obtained. The intercepts on X-axis and y-axis were equal to $-1/K_m$ and $1/V_{max}$, respectively.

3.2.11 Determination of Hill's coefficient (n) and binding constant (K_s)

Interaction coefficient or Hill's coefficient (n) for the interaction between p-nitrophenyl phosphate and the enzyme was determined by Hill's equation. The logarithmic form of the equation is given below :

$$\log \frac{v}{V_{max} - v} = n \log [S] - \log K_s$$

where v is the rate of reaction at a substrate concentration[S] ; V_{max} is the reaction rate when the

enzyme is fully saturated; n is interaction coefficient (number of substrate molecules bound to enzyme molecules) and K_s is the binding constant.

Plots of log[S] versus log $\frac{v}{V_{max} - v}$ show a linear relationship. The slope of the curve equals to n and log K_s is the intercept on ordinate where log[S] = 0, i.e., at 1 mM substrate concentration(166).

3.2.12 Determination of thermodynamic parameters

Thermodynamic parameters, energy of activation (E_a), enthalpy change (ΔH) and entropy change (ΔS) were determined as described by Irshad and Sharma (70), using K_m and V_{max} values obtained from the Lineweaver-Burk plots at different temperatures. The K_m was assumed to represent the association constant for the formation of active enzyme-substrate complex. E_a was determined from the slope of Arrhenius plot, prepared by plotting log V_{max} versus 1/T absolute, which equals to $-E_a/2.3R$, where E_a is energy of activation and R is gas constant.

3.2.12.1 Enthalpy change

The value of ΔH for the enzyme catalyzed reaction was calculated according to van't Hoff equation:

$$\log_{10} K = A - \frac{\Delta H}{2.3\ RT}$$

log K_m was plotted against 1/T absolute. Value of ΔH was calculated from the slope of the curve, which is equal to $-\Delta H/2.3\ RT$.

3.2.12.2 Free energy change (ΔG) and entropy change (ΔS)

ΔG for the enzyme catalyzed hydrolysis of p-nitrophenyl phosphate was estimated according to the following equation.

$$\Delta G = -2.303\ RT \log_{10} K_m$$

where R is gas constant, T is absolute temperature, K_m is Michaelis-Menten constant assuming that it represents association constant for the formation of enzyme-substrate complex. Finally entropy change (ΔS) for the formation of above complex was calculated according to the following equation :

$$\Delta G = \Delta H - T\Delta S$$

4.0 RESULTS

4.1 Preparation of plasma membrane, Golgi-apparatus, and endoplasmic reticulum (ER) fractions from germinating peanut cotyledons

Figure 3 shows the flow chart for the preparation of plasma membrane and Golgi-apparatus from the peanut cotyledons. The cell free extract was prepared in a medium containing 0.25 M sucrose, 3 mM EDTA and 0.04% β-mercaptoethanol without $MgCl_2$. Under these conditions the ribosomes remained detached from the rough endoplasmic reticulum and the integrity of plasma membrane as well as other organelles was maintained (97). The centrifugation of the cell free extract at 12,000 x g removed mitochondria, nuclei, etc. This fraction was discarded. The supernatant when centrifuged at 105,000 x g for 60 min resulted in a crude membrane pellet which mainly consisted of plasma membranes, Golgi-apparatus and endoplasmic reticulum. The supernatant (cytosol fraction) was discarded.

The plasma membrane fraction was separated from the Golgi-apparatus and ER by the step density gradient centrifugation. The details of the steps involved are shown in Fig. 4(a). The first step involved a two step sucrose gradient (34/20%, w/w) in 25 mM Tris-HCl,

pH 7.2, containing 1 mM β-mercaptoethanol. On centrifugation at 80,000 x g for 90 minutes, the crude membrane fraction (12,000-105,000 x g pellet) was fractionated into three clear bands (Fig. 4a, step II), (i) the pellet representing the plasma membrane, (ii) a band at 34/20% interface representing mainly Golgi and ER with some contamination of plasma membrane, and (iii) a diffused band near the top containing ER and lipids. These bands were carefully separated.

The 34/20% interface band was subjected to a step-wise sucrose gradient (43/37/30/25/20%) centrifugation (Fig. 4b, step III). A major membrane band at the 37/25% interface and a minor band at 43/37% interface were obtained (Fig. 4b, step IV). The former represents the Golgi-fraction where as the later is a plasma-membrane which was present as contamination in Golgi-fraction.

The supernatant above the 37/25% interface, containing mostly ER, was removed and diluted with 50 mM Tris-HCl, pH 7.2, to adjust the sucrose concentration to 12% and then centrifuged at 105,000 x g for 120 minutes. The pellet formed represented the ER fraction.

Fig. 3 Flow diagram for the isolation and purification of different membrane fractions from Arachis hypogaea cotyledons. Unless stated otherwise, all steps were carried out at $4^{\circ}C$.

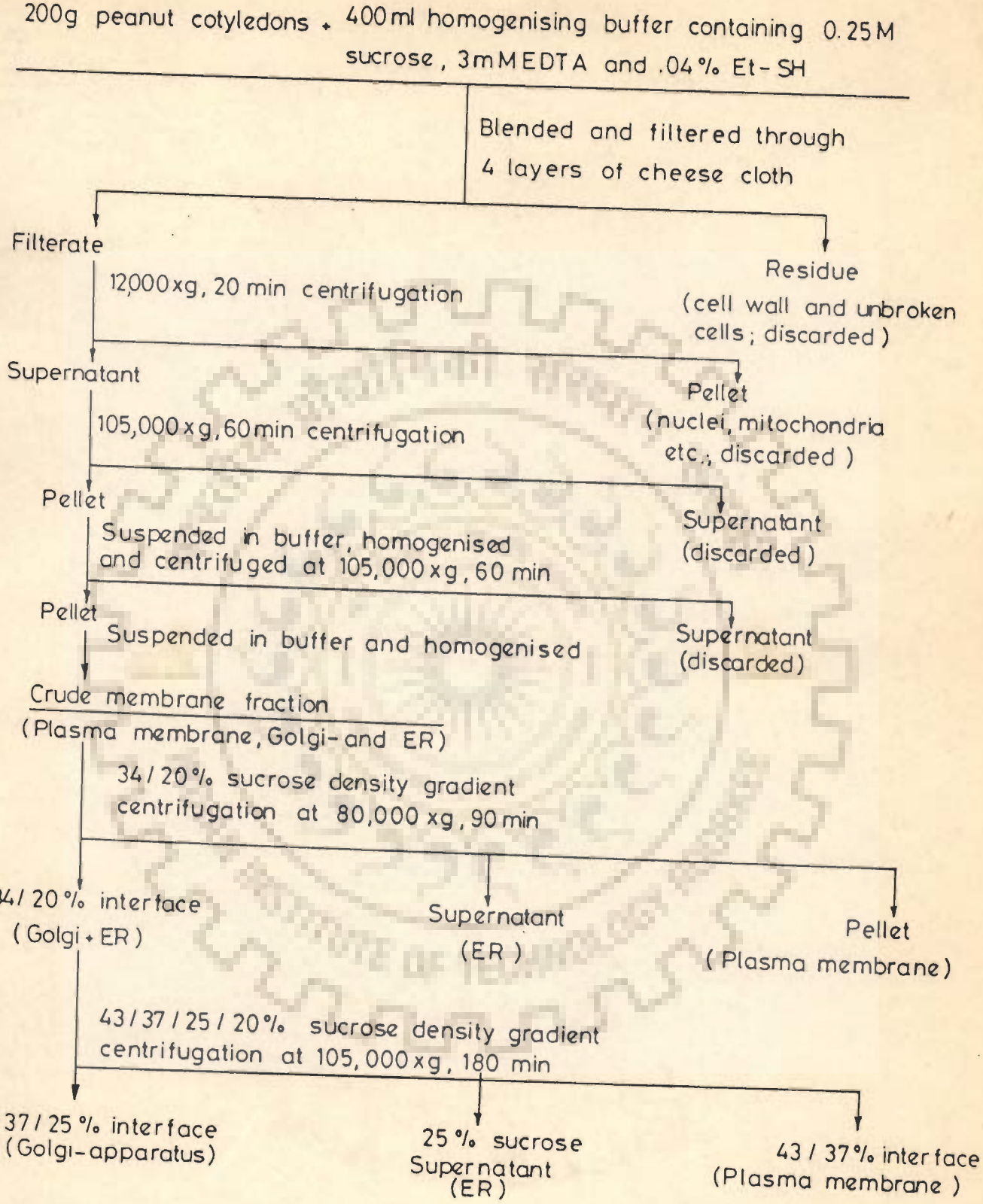

200g peanut cotyledons + 400ml homogenising buffer containing 0.25M sucrose, 3mM EDTA and .04% Et-SH
Blended and filtered through 4 layers of cheese cloth
Filterate
Residue (cell wall and unbroken cells; discarded)
12,000xg, 20 min centrifugation
Supernatant
Pellet (nuclei, mitochondria etc.; discarded)
105,000xg, 60 min centrifugation
Pellet
Supernatant (discarded)
Suspended in buffer, homogenised and centrifuged at 105,000xg, 60 min
Pellet
Supernatant (discarded)
Suspended in buffer and homogenised
Crude membrane fraction (Plasma membrane, Golgi- and ER)
34/20% sucrose density gradient centrifugation at 80,000xg, 90 min
34/20% interface (Golgi + ER)
Supernatant (ER)
Pellet (Plasma membrane)
43/37/25/20% sucrose density gradient centrifugation at 105,000xg, 180 min
37/25% interface (Golgi-apparatus)
25% sucrose Supernatant (ER)
43/37% interface (Plasma membrane)

Fig. 4a) Separation of different membrane fractions by step-wise sucrose density gradient centrifugation.

Step I Layering of crude membrane fraction in 20% sucrose on 34% sucrose buffer solution.

Step II Separation of plasma membrane, Golgi and ER fractions after 80,000 x g, 90 min centrifugation. Purification of plasma membranes by 45/37/34/30/25/20% sucrose density gradient centrifugation.

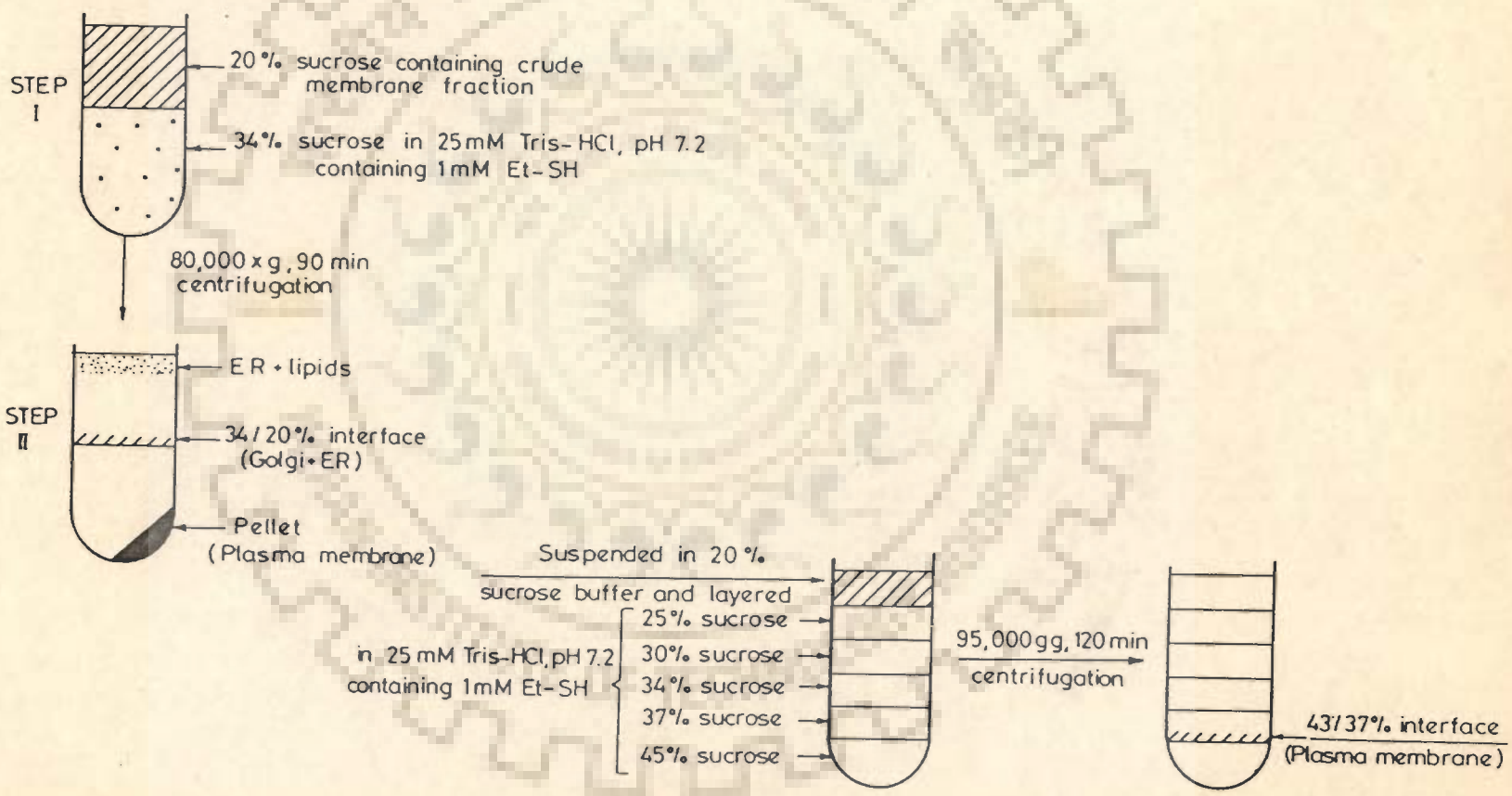
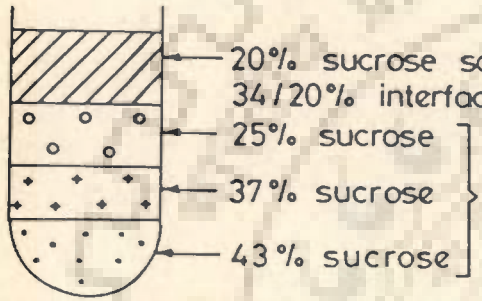

STEP I
20% sucrose containing crude membrane fraction
34% sucrose in 25mM Tris-HCl, pH 7.2 containing 1mM Et-SH
80,000 x g, 90 min centrifugation
STEP II
E R + lipids
34/20% interface (Golgi+ER)
Pellet (Plasma membrane)
Suspended in 20% sucrose buffer and layered in 25 mM Tris-HCl, pH 7.2 containing 1mM Et-SH
25% sucrose
30% sucrose
34% sucrose
37% sucrose
45% sucrose
95,000 g g, 120 min centrifugation
43/37% interface (Plasma membrane)

Fig. 4b) Separation of different membrane fractions by step-wise sucrose density gradient centrifugation.

Step III Layering of 34/20% interface membrane fraction (Fig. 4a, step II) on a 43/37/25% sucrose gradient system.

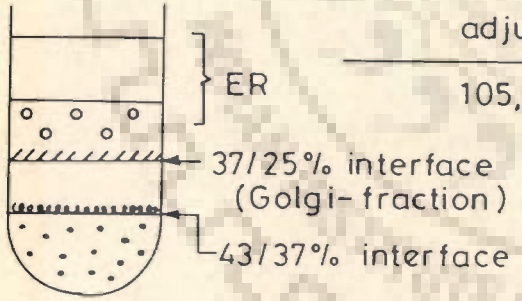
Step IV Separation of Golgi-membrane fraction (37/25% interface) and endoplasmic reticulum (25% supernatant) after 105,000 x g, 180 min centrifugation.

STEP III



105,000xg, 180 min centrifugation

STEP IV



(residual plasma membranes)

adjust sucrose to 12%,

105,000xg 60min centrifugation

Pellet (ER)

4.2 Distribution of marker enzymes in various membrane fractions of *Arachis hypogaea* cotyledons

Table V summarizes the distribution of glucan synthetase II, glucan synthetase I, mannosyl transferase and glucose-6-phosphatase used as the marker enzymes for the identification of plasma membranes, Golgi-apparatus and ER. From these results it is apparent that the plasma membrane fraction so obtained, has been enriched approximately 11-folds relative to the crude membrane fraction and that it is almost free from the Golgi-apparatus and ER contamination. Similarly Golgi-apparatus has been enriched nearly 3-folds and does not show any significant cross contamination either by the plasma membranes or ER. The ER fraction likewise shows maximum activity of the marker enzymes mannosyl transferase and glucose-6-phosphatase. In fact, it shows more than 6-folds enrichment relative to the crude membrane fraction. This fraction is free from plasma membranes. It also appears to be free from Golgi-apparatus. Thus, it may be concluded that by using differential centrifugation together with density gradient centrifugation and marker enzymes it was entirely possible to obtain sufficiently pure plasma membrane, Golgi-apparatus and ER fractions for the preparation of membrane-bound AMPase.

TABLE V DISTRIBUTION OF MARKER ENZYMES IN VARIOUS MEMBRANE FRACTIONS OF *ARACHIS HYPOGAEA* COTYLEDONS.

Membrane fraction	Activity of marker enzymes (radioactivity incorporated/mg)			Glucose-6-phosphatase activity (µmol P_i/min/mg protein)
	Glucan synthetase I cpm x 10^{-3}	Glucan synthetase II cpm x 10^{-3}	Mannosyl transferase[a] cpm x 10^{-3}	
Crude membranes (12,000-105,000 x g pellet)	6.30	8.10	61.00 (212.88)	21.3[b]
Plasma membrane	1.23	88.00	1.68 (3.08)	4.16
Golgi-apparatus	17.35	1.60	7.20 (18.66)	10.60
Endoplasmic reticulum	1.97	3.22	380.00 (533.12)	30.00

[a]Data were obtained with endogeneous as well as exogenous lipid acceptor (dolichol monophosphate) with GDP-[^{14}C]-mannose as donor substrate; values in parentheses were obtained with dolichol monophosphate as exogenous acceptor of mannose units.

[b]The activity observed was higher than the actual glucose-6-phosphatase activity, as the enzyme preparation contains other non-specific phosphatases also.

4.3 Plasma membrane AMPase

4.3.1 Purification of plasma membrane AMPase

Table VI summarizes the purification of the AMPase from peanut cotyledon plasma membranes. One of the important steps of the purification scheme is the preparation of high specific activity plasma membrane fraction. Results given in Fig. 5 show that AMPase was not present in the membrane fraction of dormant seeds. It was emerged only after 48 h imbibition of seeds in water at 35°C. However, after the initial lag period was over, there was a sudden and sharp rise in the activity of membrane-bound AMPase reaching a maximum level between 6th and 7th day of germination. Thereafter, the enzyme activity declined very rapidly. For instance, after 12th day of germination only one-fifth of the maximum activity was found in the membrane fraction. Thus, 7-days-old germinating cotyledons were used for the preparation of high specific activity plasma membrane fraction for the purification of AMPase.

The crude membrane fraction (12,000-105,000 x g pellet) was found to contain high levels of AMPase activity with a specific activity of 26 μmol P_i/min/mg protein. This membrane fraction was used as the basis

TABLE VI PURIFICATION OF AMPase FROM THE PEANUT COTYLEDON PLASMA MEMBRANE FRACTION

The plasma membrane fraction was prepared from 7-day-old germinating cotyledons (100 g fresh weight) and extracted once with 0.5% detergent (final concentration) at a protein/detergent ratio of 2:3 in the presence of 20 mM $MgCl_2$ and 5 mM EDTA as described under Experimental procedures. The supernatant fraction after 105,000 x g centrifugation represented the solubilized enzyme. The values of total protein, total enzyme activity and per cent yield in DEAE-cellulose peaks I, II and III (Fig. 8) represent a pool of only top three peak fractions from each peak.

Fraction	Total protein (mg)	Total AMPase activity (μmol P_i/min)	AMPase specific activity (μmol P_i/min/mg protein)	Purification (fold)	Yield (%)
Crude membrane fraction (12,000-105,000 x g pellet)	92.00	2399.4	26.0	-	100
Plasma membrane	16.28	1400.0	86.0	3.3	58.35
Octylglucoside extract (105,000 x g supernatant)	4.16	665.0	159.9	6.15	27.72
DEAE-cellulose					
Peak I	0.16	112.0	700.0	26.92	4.66
Peak II	0.15	51.6	344.0	13.23	2.15
Peak III	0.04	3.4	85.0	3.27	0.14

Fig. 5 Activity of the membrane-bound AMPase of peanut cotyledons as a function of germination period. Seeds were germinated in dark at 35°C for the indicated periods. Fifty cotyledons were excised, membrane fractions (12,000 to 105,000 x g pellet) were prepared and the enzyme activity was determined as described under Experimental procedures. The maximum activity was observed between 6th and 7th day of germination.

SPECIFIC ACTIVITY (—●—)
(μ moles P_i Liberated/min/mg)
TOTAL ACTIVITY, μ moles/min/Cotyledon x10^{-3} (—○—)
PROTEIN, mg/Cotyledon, (—△—)
GERMINATION PERIOD, DAYS
0
2
4
6
8
10
12
4
8
12
16
20
24
28
32
0.5
1.0
1.5
2.0
1
2
3
4
5
6
7
8

of purification since the crude homogenate contained a large amount of non-specific acid phosphatase activity. A further purification of the plasma membrane fraction was achieved by centrifugation in sucrose step gradients (20% w/w and 34% w/w) at 80,000 x g for 90 min. The pellet so obtained was found to contain high specific activity of AMPase (86 μmol P_i/min/mg protein) giving a purification of 3.3 folds over the crude membrane fraction. Comparable results were obtained (Table VII) when the plasma membrane fraction was separated from other membranes by discontinuous sucrose gradient as described by Hall (63) using glucan synthetase II as the marker enzyme (131). That this fraction represents mainly plasma membranes was indicated by the fact that it exhibited very high activity of the marker enzyme glucan synthetase II, with almost no activity of glucan synthetase I, a marker enzyme for the Golgi-apparatus (61) (Table V). These results indicated further that the plasma membrane fraction was free from Golgi bodies contamination. In all experiments, unless noted otherwise, this membrane fraction was used for solubilization and purification of AMPase.

The next step involved using a detergent which would solubilize the membrane-bound enzyme in stable

TABLE VII ACTIVITY OF AMPase AND GLUCAN SYNTHETASE II AND I IN PLASMA MEMBRANE FRACTION OF PEANUT COTYLEDONS

Plasma membrane preparations I and II were obtained by the methods of DuPont *et al.* (41) and Hall (63), respectively. Glucan synthetase II and I, used as marker enzymes for plasma membranes and Golgi-apparatus fractions, respectively, were assayed as described under Experimental procedures.

Fraction	AMPase activity (μmol P_i/ min/mg)	Glucan synthetase II activity (radio-activity incorpor-ated), cpm	Glucan synthetase I activity (radio-activity incorpor-ated), cpm
Blank	-	214 $\pm$ 16	66 $\pm$ 6
Plasma membrane prep. I	86.0	28,318 $\pm$ 356	69 $\pm$ 10
Plasma membrane prep. II	88.2	34,420 $\pm$ 370	75 $\pm$ 10

and highly active form. Table VIII shows the solubilizing efficiency of five different detergents for the enzyme. The results indicated that of the detergents used octylglucoside solubilized the enzyme most effectively in a highly active form. Nearly one-third of the total activity was released from the membranes into the solution under very mild conditions while at the same time only less than 10% of the total membrane protein was solubilized. The selective solubilization of AMPase by octylglucoside from the plasma membranes resulted in 6.15 folds purification. On the other hand sodium deoxycholate and taurocholate were found to be very poor in solubilizing AMPase, although the amount of protein solubilized by various detergents including the octylglucoside was comparable.

Since the critical micellar concentration of octylglucoside was much higher (7.5 mg/ml) compared to Triton X-100 or Nonidet (138), it was possible to remove the octylglucoside by dialysis before ion exchange chromatography to avoid interference in the purification procedure. In addition, the octylglucoside solubilized enzyme was found stable and could be stored at $-20^{\circ}C$ or $4^{\circ}C$ for several days without any appreciable loss of activity (Table XI).

TABLE VIII EFFICIENCY OF VARIOUS DETERGENTS IN SOLUBILIZING THE MEMBRANE-BOUND AMPase OF *ARACHIS HYPOGAEA* L. COTYLEDONS

The membrane fraction was suspended in assay buffer containing an amount of detergent to be used to give a protein to detergent ratio of 2:3 corresponding to 0.5% detergent (final concentration). The mixture was gently agitated on a Vortex mixer for 2 min and centrifuged at 105,000 x g for 60 min. The enzyme activity in the supernatant fraction was assayed as described in Experimental procedures and expressed as μmol of P_i released per min.

Fraction	Total protein (mg)	Total AMPase activity (μmol P_i/ min)	Specific activity (μmol P_i/ min/mg protein)	Protein solubilized (%)	AMPase activity solubilized (%)
Membrane fraction (12,000-105,000 x g pellet)	17.5	346.5	19.8	-	-
Detergent extract (105,000 x g supernatant)					
Sodium deoxycholate	1.56	31.8	20.4	8.9	9.15
Taurocholate	1.43	43.7	30.6	8.1	12.61
Triton X-100	1.65	71.3	43.2	9.4	20.57
Nonidet P-40	1.61	78.1	48.5	9.2	22.54
Octylglucoside	1.71	106.5	61.9	9.8	30.73

The effect of octylglucoside concentration on the activity of the membrane-bound AMPase is shown in Fig. 6. From these results it can be seen that percentage of AMPase activity remaining in the presence of detergent is nearly constant (88-94%) over the detergent range of 0.25 - 0.5%, while above or below this range the enzyme activity remaining was less than 50%. These results further indicate that the enzyme is fairly stable in the presence of 0.25 - 0.5% detergent concentration. Since protein to detergent ratio may be a critical factor in selectively solubilizing the membrane-bound enzyme, the effect of detergent concentration was investigated in terms of protein to detergent ratio using a fixed detergent concentration of 0.5% at which the enzyme showed maximum stability. The data (Fig. 7) show that a protein to detergent ratio of 2:3 is optimum for the solubilization of AMPase from the plasma membranes. At this concentration nearly 35% activity and about 11.9% protein were solubilized. The specific activity of the solubilized enzyme was increased 2.96 folds over the membrane-bound enzyme. Thus, octylglucoside concentration of 0.5% corresponding to protein to detergent ratio of 2:3, which consistently resulted in the solubilization of AMPase with high specific activity for AMP hydrolysis, was employed in further studies.

Fig. 6 Effect of octylglucoside on the activity of membrane-bound AMPase. Enzyme assays were carried out in the presence of indicated amounts of the detergent as described under Experimental procedures. The results are expressed as specific activity (μmol P_i/min/mg) and per cent of total activity remaining.

SPECIFIC ACTIVITY OF AMPase (●—●)
(μ moles Pi liberated / min/mg)
1.20
1.00
0.80
0.60
0.40
0.20
0
0.2
0.4
0.6
0.8
OCTYLGLUCOSIDE CONCENTRATION (%)
% TOTAL ACTIVITY REMAINING (○—○)
120
100
80
60
40
20

Fig. 7 Solubilization of AMPase activity from the membrane fraction as a function of protein to detergent ratio. The protein to detergent ratio was adjusted to 1:1, 1:1.5, 1:2 and 1:3. The final concentration of the detergent was maintained at 0.5%. The solubilization of AMPase and enzyme assays were carried out exactly as described under Experimental procedures. The results are expressed as soluble AMPase specific activity, per cent of total activity and per cent of total protein solubilized.

SOLUBLE AMPase SPECIFIC ACTIVITY (●—●)
(μmoles Pi Liberated / min / mg)
85
80
75
70
65
60
55
60
50
40
30
20
10
% OF TOTAL ACTIVITY SOLUBLISED(○—○)
% OF TOTAL PROTEIN SOLUBLISED(▲—▲)
1:1
1:2
1:3
PROTEIN:DETERGENT (w/w)

Magnesium and EDTA were found to be essential for the selective solubilization of AMPase (Table IX). It was found that $MgCl_2$ together with EDTA helps in selective solubilization of AMPase by octylglucoside mainly by suppressing the solubilization of membrane proteins other than AMPase. On the basis of the above mentioned results the optimum and essential requirements for the selective solubilization of AMPase from peanut cotyledon membranes are as follows : 50 mM Tris-HCl, pH 7.2; octylglucoside, 5 mg/ml; membrane fraction, 3.33 mg/ml; EDTA, 5 mM and $MgCl_2$, 20 mM.

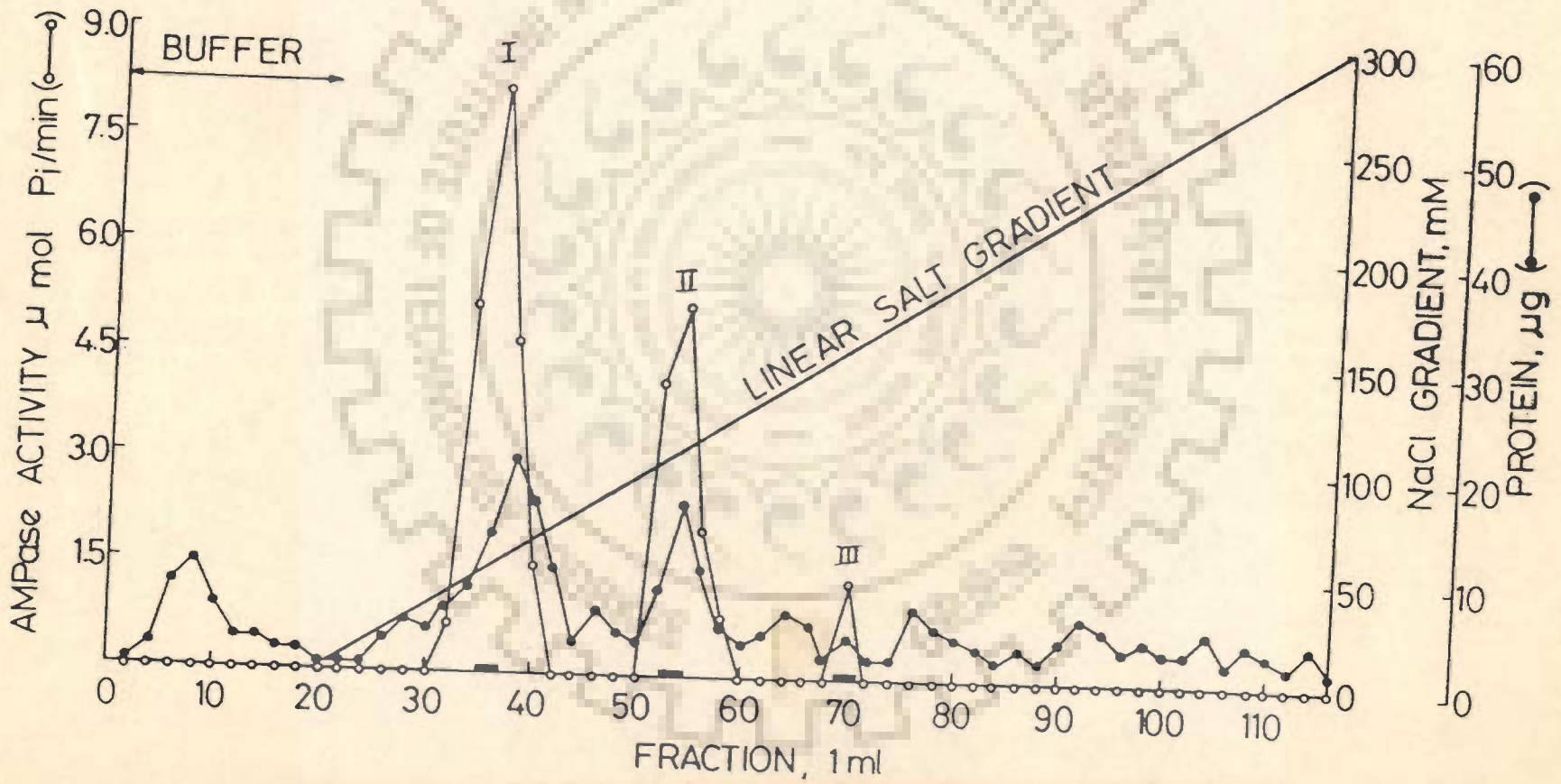
The final purification step involved DEAE-cellulose column chromatography. Fig. 8 illustrates the elution pattern of the dialysed octylglucoside solubilized enzyme on a DEAE-cellulose column. The enzyme activity was eluted in three peaks (I, II and III) eluting at 50, 107 and 155 mM salt gradient, respectively. Peak I was the major peak and contained most of the total activity recovered. As can be seen from the data in Table VI,the total activity recovered in three peaks accounts for only 25% of the total activity loaded on the column. The likely reasons for the low recovery may be as following; firstly, unlike the crude octylglucoside extract the DEAE fractions are highly unstable and become totally

TABLE IX EFFECT OF Mg^{2+} AND EDTA ON THE SOLUBILIZATION OF AMPase FROM THE MEMBRANE FRACTION OF PEANUT COTYLEDONS

Membrane fraction (12,000 - 105,000 x g pellet, 3.33 mg/ml) was extracted with octylglucoside (5 mg/ml) without and with $MgCl_2$ and EDTA exactly as described under Experimental procedures. The homogenate was centrifuged at 105,000 x g for 60 min. The supernatant fraction was used as solubilized enzyme. The AMPase activity was assayed as described under Experimental procedures. Values are average of three separate experiments.

Conditions of solubilization		Protein solubilized (%)	AMPase activity solubilized (%)	AMPase specific activity (μmol P_i/min/mg protein)
EDTA (mM) +	$MgCl_2$ (mM)			
0.0	0.0	28.0 ± 2	23.0 ± 2	19.3 ± 1
0.0	20.0	14.0 ± 2	27.4 ± 2	45.8 ± 2.5
5.0	0.0	9.2 ± 1	10.5 ± 1	26.8 ± 2
5.0	5.0	8.6 ± 1	7.7 ± 1	21.0 ± 2
5.0	10.0	8.0 ± 1	13.9 ± 1	40.7 ± 2
5.0	20.0	8.9 ± 0.5	34.9 ± 2.5	86.1 ± 3
5.0	30.0	8.8 ± 1	18.1 ± 1	48.2 ± 2

Fig. 8 Chromatography of the dialyzed octylglucoside solubilized AMPase (105,000 x g supernatant) on DEAE-cellulose column. A 9 ml sample (4.16 mg protein) was loaded on the column (1.5 x 10 cm), and the absorbed proteins were then eluted by a linear gradient of sodium chloride (0 - 300 mM) Tris-HCl, pH 7.2, as described in the text. A flow rate of 12 ml per hour was used and fractions (1 ml) were collected. Aliquots (0.2 ml) from every other tube were analyzed for AMPase activity and protein content as described in the text. The fractions indicated by the bar (▬) were pooled and used for further studies. Fraction I (35 to 37) was found pure by SDS polyacrylamide gel electrophoresis.


AMPase ACTIVITY μ mol Pi/min
9.0
7.5
6.0
4.5
3.0
1.5
BUFFER
I
II
III
LINEAR SALT GRADIENT
NaCl GRADIENT, mM
300
250
200
150
100
50
0
PROTEIN, μg
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
FRACTION, 1 ml

inactive within 24 h at 4°C or -20°C (Table XI), and secondly in order to avoid overlapping of other protein peaks only top three peak fractions of each peak were pooled and a large portion of the enzyme activity had to be discarded. The specific activity, purification and yield of the enzyme in peak I were 700 μmol P_i/min/mg protein, 26.92 folds and 4.66%, respectively. The actual yield, however, would be greater as the starting crude preparation of membrane contains an unknown amount of other nonspecific phosphatases. Recently, Carter and Tipton (26) have reported that only one-fourth of the total phosphatase activity in Zea mays microsomes was 5'-nucleotidase activity.

4.3.2 Homogeneity

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified enzyme (Peak I) in the presence of β-mercaptoethanol resulted in a single protein band (Fig. 9). The apparent molecular weight of this band was found to be 55 K (Fig. 10). Since the electrophoresis was performed under completely dissociated conditions, the peanut cotyledon plasma membrane AMPase appears to have only a single polypeptide chain. However, presence of more

Fig. 9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples from various stages of AMPase purification samples were prepared and handled as described under Experimental procedures. Samples and amounts loaded were : M, plasma membrane fraction, 100 μg protein; S.E., solubilized enzyme (dialyzed 105,000 x g supernatant), 80 μg protein; Frac. I, DEAE-cellulose fraction I, 40 μg of protein; and Std., standards (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme, 5 μg each); M.W., molecular weight standards. Fraction I protein band corresponds to molecular weight of 55 K. The direction of electrophoresis was top to bottom.

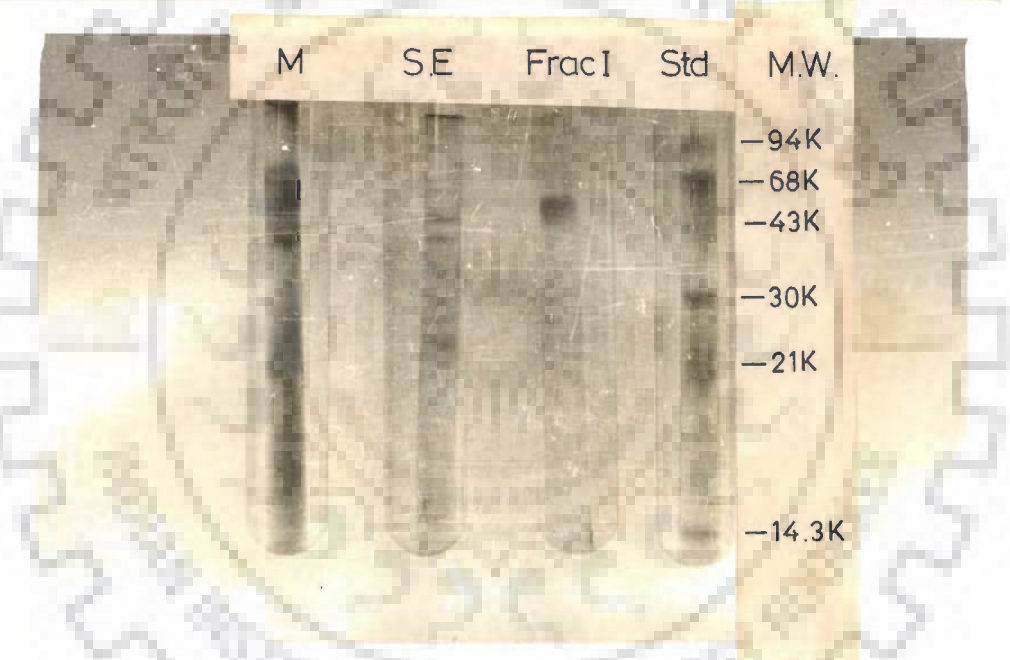
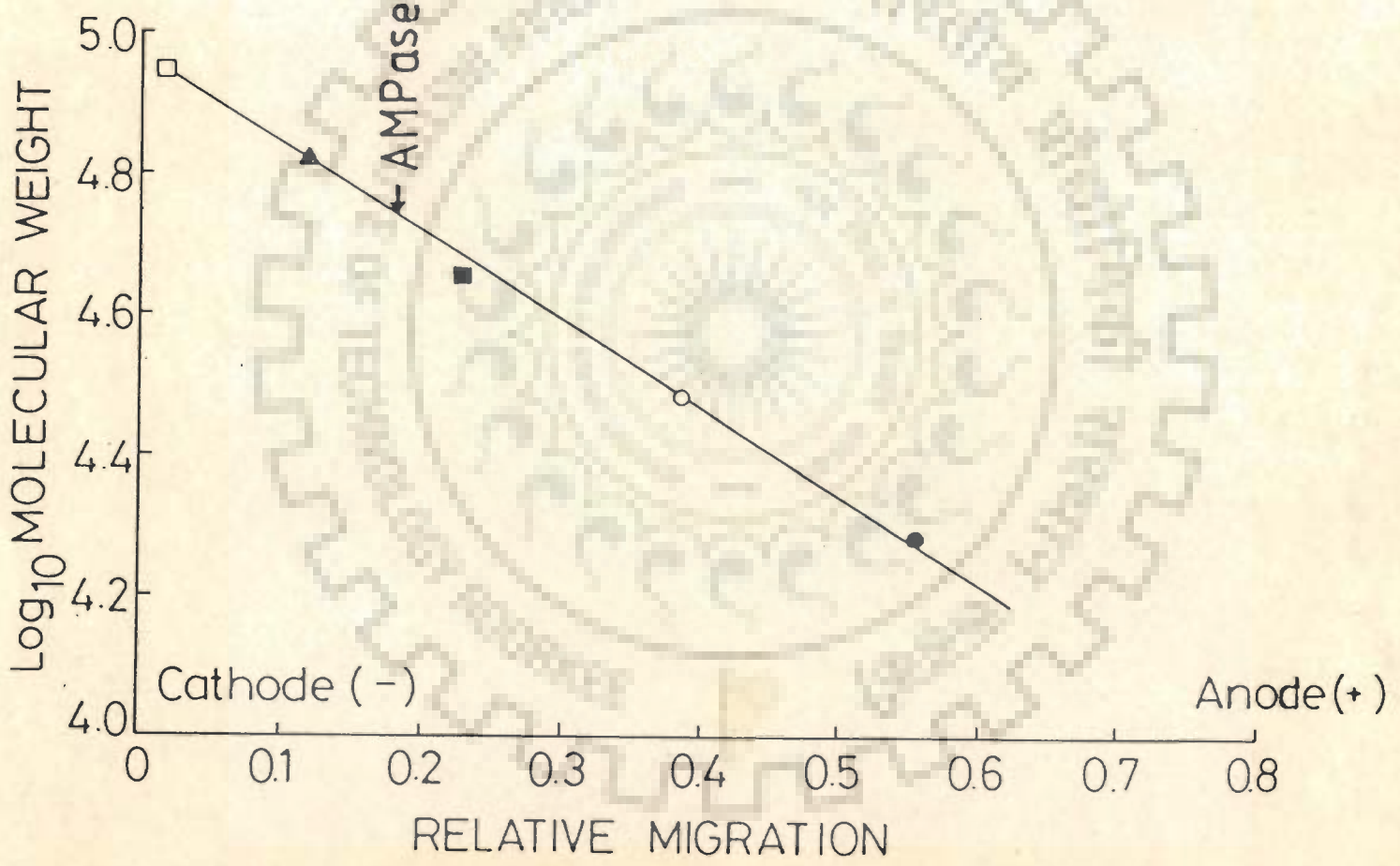

M
S.E
Frac I
Std
M.W.
—94K
—68K
—43K
—30K
—21K
—14.3K

Fig. 10 Estimation of the molecular weight of the purified peanut cotyledon plasma membrane AMPase by SDS-polyacrylamide gel electrophoresis. The standard proteins used were treated in the same manner as AMPase was treated. Electrophoresis was performed on 10% polyacrylamide gel under fully dissociating conditions as described under Experimental procedures. Mobilities were determined relative to the migration of bromophenol blue as tracking dye. □, phosphorylase b (M.W. 94,000); ▲, bovine serum albumin (M.W. 68,000); ■, ovalbumin (M.W. 43,000); o, carbonic anhydrase (M.W. 30,000); and ●, soybean trypsin inhibitor (M.W. 21,000). The molecular weight of the purified AMPase, as computed from the calibration plot of $\log_{10}$ molecular weight versus relative mobility of standard proteins, was found to be 55 K daltons. The calculations were based on average of 3 independent electrophoretic runs.


5.0
4.8
4.6
4.4
4.2
4.0
Log10 MOLECULAR WEIGHT
AMPase
Cathode (-)
Anode (+)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
RELATIVE MIGRATION

than one polypeptide chain of exactly the same size can not be completely ruled out at the moment.

4.3.3 Properties of the purified AMPase

The purified AMPase shows a broad pH-optimum range of pH 5.0 to 6.0 for the hydrolysis of AMP (Fig. 11). The enzyme activity declined rapidly below pH 4 and above 6.5. The dependence of AMPase on AMP is shown in Fig. 12. The K_m value of the enzyme for AMP was found to be 1.0×10^{-3} M with a V_{max} of 8.47 μmol P_i/min/mg protein.

4.3.4 Substrate specificity

The substrates tested for the hydrolysis by AMPase are listed in Table X. The results show that the specific activity of the purified enzyme was clearly specific for AMP. Other nucleoside phosphates as well as phosphorylated sugars were not hydrolyzed. Although p-nitrophenyl phosphate was hydrolyzed, its rate of hydrolysis was only 15% and the substrate affinity, $1/K_m$, was one-tenth that of AMP (Fig. 12), indicating that p-nitrophenyl phosphate was an extremely poor substrate for the plasma membrane AMPase. Since the enzyme was found homogeneous by SDS-PAGE, it appears unlikely that the

Fig. 11 Effect of pH on the activity of purified plasma membrane AMPase from peanut cotyledons. The enzyme activity was measured in the standard assay system except that the buffer and pH were varied. Buffers used were 50 mM acetate buffer, pH 3 - 5.5 and 50 mM Tris-HCl, pH 6 - 8.

AMPase ACTIVITY (μmoles Pi /min)
8
7
6
5
4
3
2
3
4
5
6
7
8
9
pH

Fig. 12 Lineweaver-Burk plots of peanut cotyledon plasma membrane AMPase using AMP and p-nitrophenyl phosphate (p-NPP) substrates in 50 mM sodium acetate buffer, pH 5.0. Standard assays with varying amounts of substrate were carried out for 10 min with 10 µg enzyme. K_m and V_{max} values were computed from the intercepts on X-axis and Y-axis, respectively.

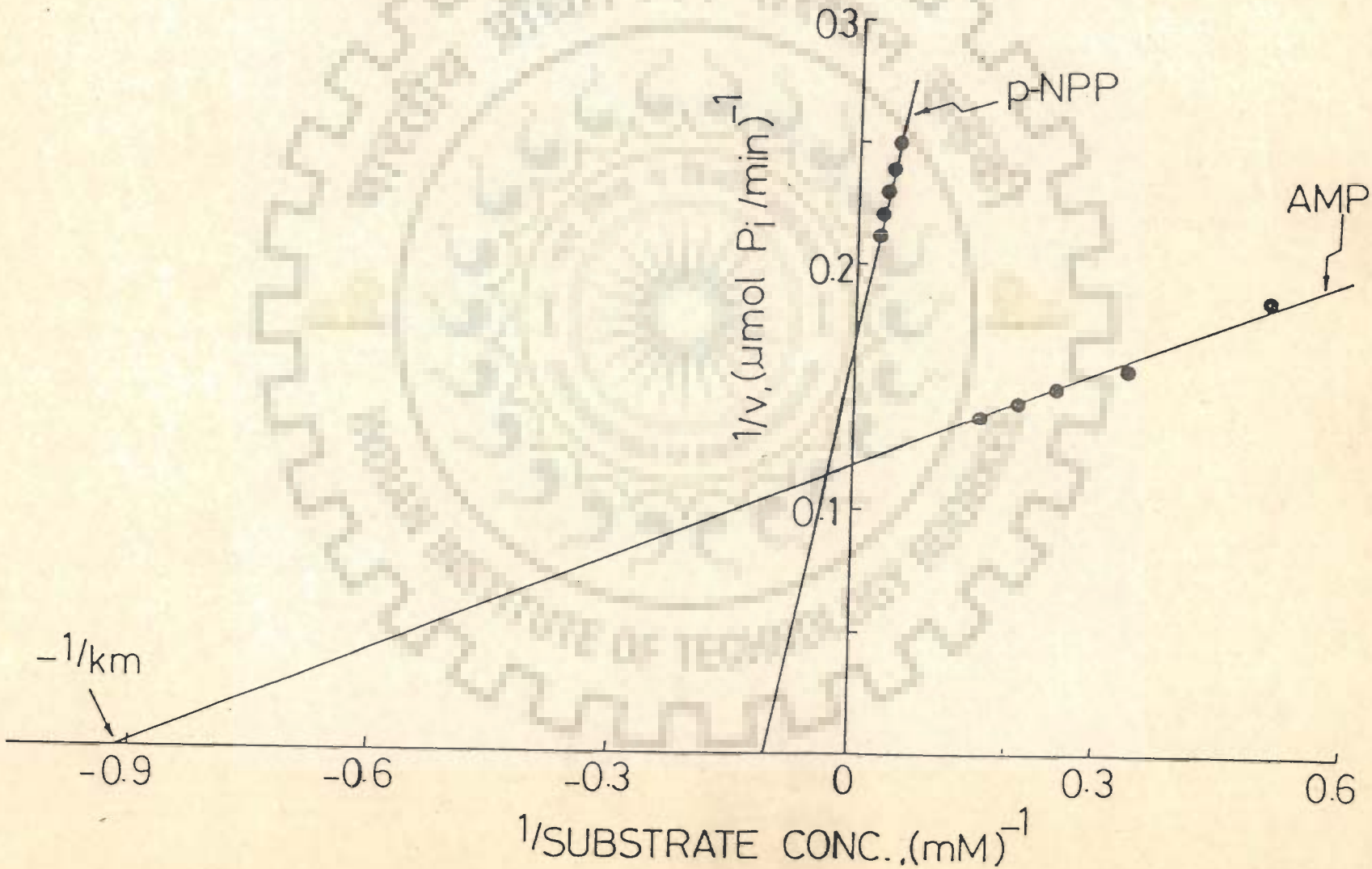

0.3
0.2
0.1
p-NPP
AMP
1/v, (μmol Pi /min)⁻¹
-1/km
-0.9
-0.6
-0.3
0
0.3
0.6
1/SUBSTRATE CONC., (mM)⁻¹

TABLE X SUBSTRATE SPECIFICITY OF PLASMA MEMBRANE AMPase OF PEANUT COTYLEDONS

Enzyme assays were carried out using 2.5 mM substrate concentration as described under Experimental procedures.

Substrate	Membrane-bound AMPase activity		Purified AMPase activity	
	(μmol P_i/mg/min)	%	(μmol P_i/mg/min)	%
Adenosine 5'-monophosphate	86.0	100	700.0	100
Guanosine 5'-monophosphate	-	-	-	
Cytidine 5'-monophosphate	-	-	-	
Uridine 5'-monophosphate	-	-	-	
Adenosine 5'-diphosphate	18.7	21.7	-	
Guanosine 5'-diphosphate	-	-	-	
Uridine 5'-diphosphate	-	-	-	
Adenosine 5'-triphosphate	53.7	62.4	-	
Cytidine 5'-triphosphate	-	-	-	
Guanosine 5'-triphosphate	-	-	-	
Glucose-6-phosphate	34.4	40.0	-	
Glucose-1-phosphate	19.8	23.0	-	
p-Nitrophenyl phosphate	33.2	38.6	105.6	15.08

p-nitrophenyl phosphate hydrolysis was due to contamination of some other nonspecific acid phosphatase.

4.3.5 Inhibitors

NaF and ADP were found to inhibit the activity of AMPase. The inhibition by NaF was of the non-competitive type (Fig. 13) with a K_i value of 35 mM. In contrast the ADP inhibitory action was of the competitive type (Fig. 14) with a K_i of 2.40 mM indicating that ADP is a stronger inhibitor of AMPase.

The activity of the purified AMPase, under assay conditions used, was unaffected by the following cations (as chlorides except Zn^{2+} as sulfate) at 10 mM final concentration : Mg^{2+}, Ca^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, K^{+}, Li^{+} and Na^{+}. No effect of Hg^{2+} ions on the purified AMPase suggests that SH-groups of cysteine residues are not involved in the interaction between substrate and the enzyme.

4.3.6 Stability of the purified AMPase

Table XI shows the results of the per cent stability of the membrane-bound, unpurified octyl-glucoside extract and the purified AMPase at $-20^{o}C$ and $4^{o}C$. It can be seen that the purified enzyme was

Fig. 13 Inhibition of AMP hydrolysis by NaF. Hydrolysis was determined and kinetic parameters were estimated using varying amounts of AMP in the presence of 0, 10, 20 and 30 mM NaF as described under Experimental procedures. (A), Lineweaver-Burk plots showing non-competitive type of inhibition of peanut cotyledon plasma membrane AMPase by NaF. (B), Replot of slopes of Lineweaver-Burk plots versus NaF concentration. The intercept on X-axis equals the value of $-K_i$.

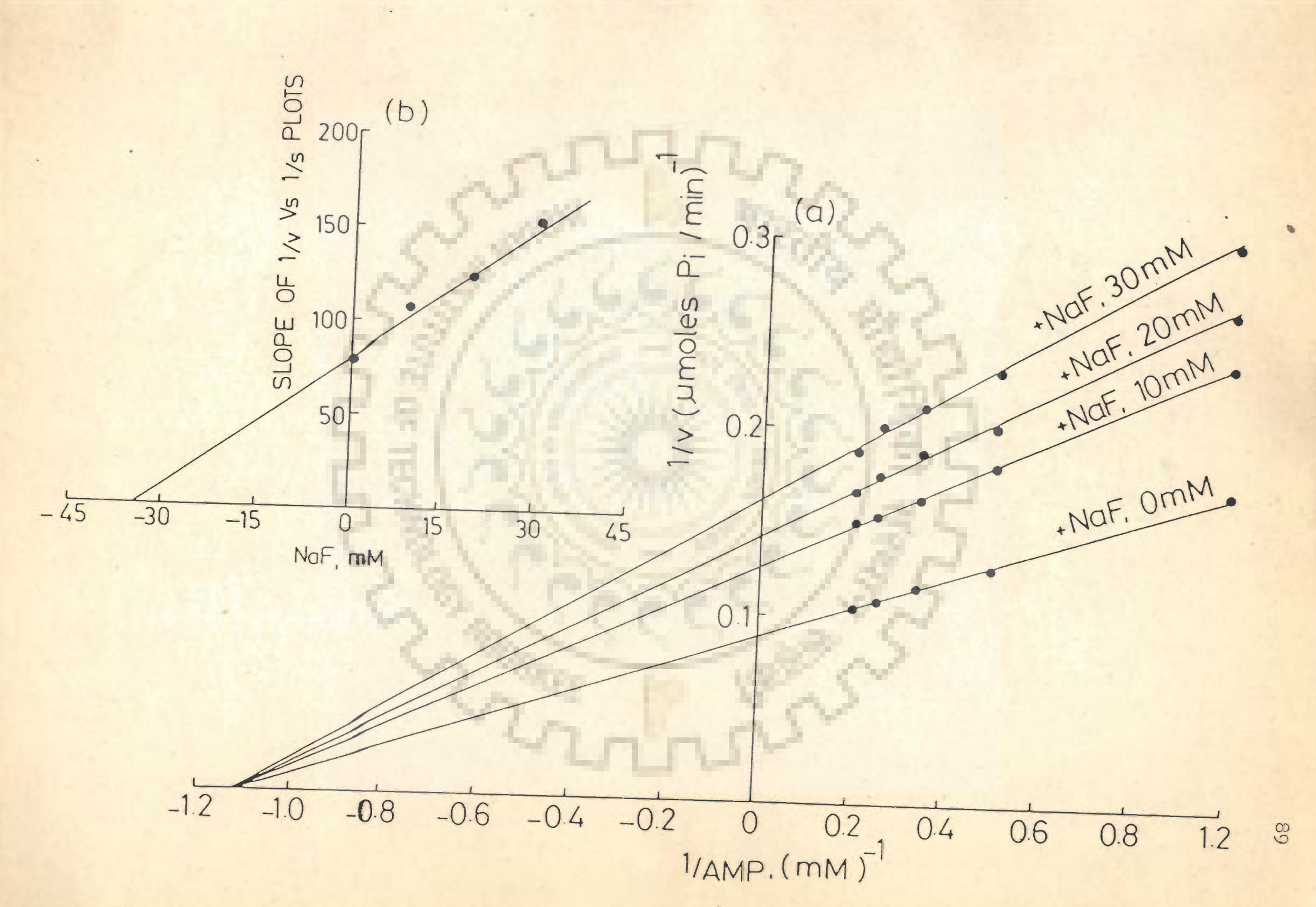

(b)
SLOPE OF 1/v Vs 1/s PLOTS
200
150
100
50
-45
-30
-15
0
15
30
45
NaF, mM
(a)
1/v (μmoles Pi /min)^-1
0.3
0.2
0.1
+NaF, 30mM
+NaF, 20mM
+NaF, 10mM
+NaF, 0mM
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.2
1/AMP. (mM)^-1

Fig. 14 Inhibition of AMP hydrolysis by ADP. Enzyme assays were carried out using varying amounts AMP in the presence of 0, 1, 2.5 and 3.5 mM ADP. (A), Lineweaver-Burk plots of AMP hydrolysis showing competitive type of inhibition. (B), Replot of slopes of Lineweaver-Burk plots versus ADP concentration. The intercept on X-axis equals the value of $-K_i$.

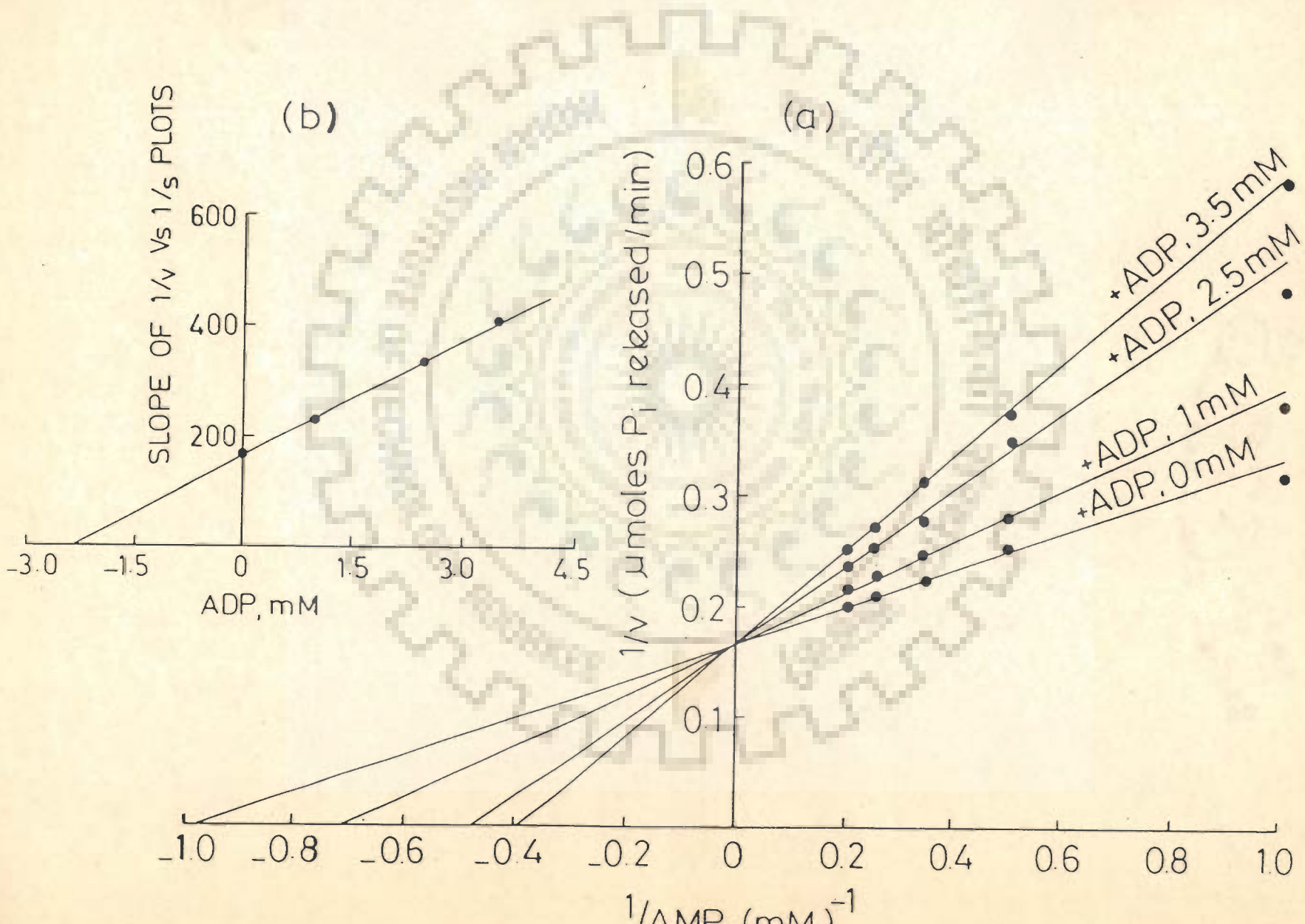

(b)
SLOPE OF 1/v Vs 1/s PLOTS
600
400
200
-3.0
-1.5
0
1.5
3.0
4.5
ADP, mM
(a)
1/v (µmoles Pi released/min)
0.6
0.5
0.4
0.3
0.2
0.1
+ADP, 3.5 mM
+ADP, 2.5 mM
+ADP, 1 mM
+ADP, 0 mM
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
1/AMP (mM)-1

TABLE XI STABILITY OF PEANUT COTYLEDON PLASMA MEMBRANE AMPase AT 4°C AND -20°C.

The membrane-bound, solubilized (octylglucoside extract) and purified enzyme preparations were stored for varying time periods at indicated temperatures and then assayed in the routine way as described in Experimental procedures.

Period of storage (days)	Per cent residual activity of AMPase					
	Membrane-bound enzyme		Solubilized enzyme		Purified enzyme	
	-20°C	4°C	-20°C	4°C	-20°C	4°C
0	100	100	100	100	100	100
1	98.5	98.2	99.0	98.1	-	-
2	86.1	96.4	98.8	97.4	-	-
4	80.0	92.0	98.5	93.7	-	-
8	76.5	85.3	97.2	80.0	-	-
10	70.3	80.0	96.5	78.1	-	-
15	64.7	70.0	88.0	71.0	-	-

completely inactivated within 24 h when stored either at -20°C or 4°C. In contrast, however, both membrane-bound and the unpurified octylglucoside solubilized enzymes were fairly stable for several days under such conditions. In fact, the solubilized enzyme (unpurified) could be stored at -20°C for 10 days without any significant loss of activity (Table XI), indicating that some stabilizing factors, probably phospholipids, which were responsible for the stability of the unpurified detergent solubilized enzyme, were lost during purification on DEAE-cellulose.

The purified AMPase was found to contain 42.7% (w/w) carbohydrate and, therefore, appears to be a glycoprotein like other 5'-nucleotidases (47,134).

4.3.7 Evidence of N-glucosidic linkage in the glycoprotein

The treatment of the glycoprotein AMPase with the enzyme endo-N-acetyl-β-D-glucosaminidase, which cleaves the glycosidic bond between two adjacent N-acetylglucosamine residues (91), releases nearly 80% the carbohydrate present in the glycoprotein. These results suggest that the oligossacharide is attached to protein via chitobiose linkage. The analysis of carbohydrate moiety by HPLC shows that the only sugars present in the glycoprotein were

N-acetylglucosamine and mannose (Table XII). A rough estimate shows that the molar ratio between N-acetyl-glucosamine and mannose is 2:22, indicating that the N-glycosidically linked oligosaccharide is of the 'high mannose' type, containing about 22 mannose units. These results are only tentative and more precise estimations have to be made in order to get correct picture of the size of oligosaccharides. The results of the glycoprotein hydrolysis also show the presence of glucose which was not found in the oligosaccharide released by endo-N-acetyl-β-D-glucosaminidase. These results suggest that glucose may be attached through O-glycosidic linkage with serine or threonine amino acid residues of polypeptide chain.

4.4 Golgi-apparatus AMPase

4.4.1 Subcellular distribution of AMPase in peanut cotyledons

Table XIII shows the distribution pattern of AMPase in various membrane fractions in germinating peanut cotyledons. The total crude membrane fraction (12,000 - 105,000 x g pellet), which contained mainly plasma membrane, Golgi apparatus and ER, was used as the starting material for the separation of various membrane fractions. From the data it can be seen that approximately 58% of the total precipitable AMPase

TABLE XII ANALYSIS OF MONOSACCHARIDES BY HPLC

Hydrolyzed glycoprotein samples, prepared as described in Experimental procedures, were injected on Shimadzu LC-4A system on Zorbax C-18 column. The fractionation was performed with the mobile phase of acetonitrile water (90:10, v/v) at a flow rate of 1.5 ml/min. The monosaccharide units were detected at 195 nm wavelength and K' values were calculated with respect to the injection peak time (K' value = Retention time/ injection peak).

Reference compound	Standard sample		Hydrolyzed sample	
	Retention time (min)	K' value	Retention time (min)	K' value
N-Acetylglucosamine	10.77	5.66	10.82	5.64
Mannose	5.7	3.0	5.74	2.99
Glucose	4.45	2.34	4.50	2.35

TABLE XIII SUBCELLULAR DISTRIBUTION OF AMPase IN PEANUT COTYLEDONS

Fraction[a]	Total protein (mg)	Total AMPase activity[b]		AMPase specific activity (μmol P_i/ min/mg)
		(μmol/min)	(%)	
Total 12,000-105,000xg pellet	92	2399.4	100	26.6
Plasma membrane	16.2	1400.0	58.34	86.0
Golgi-apparatus	14.9	565.7	23.57	37.9
ER	11.1	298.3	12.43	26.87

[a]Various centrifugal fractions were prepared as described in Experimental procedures.

[b]Represents 200 seven-days germinating cotyledons.

activity was found to be associated with plasma membranes. The enzyme from these membranes was purified and results are described in 4.3. The Golgi-apparatus contained nearly 23.7% of the total precipitable activity. This fraction appeared to be free from plasma membranes since it did not show any significant activity of glucan synthetase II, the marker enzyme for plasma membranes. It was therefore reasonable to presume that about 23% of total AMPase activity was associated with Golgi-apparatus. The ER which stayed in the 25% sucrose supernatant phase accounted for only 12.4% of the total precipitable activity. In terms of specific activity the plasma membrane fraction showed the highest activity (86 μmol P_i/min/mg) followed by Golgi-apparatus (37.9 μmol P_i/min/mg) and ER (26.87 μmol/min/mg). As mentioned earlier the enzyme present in the Golgi-apparatus may be a precursor of the enzyme found in the plasma membranes. In order to test this hypothesis the enzyme has been purified, characterized and compared with that of plasma membrane AMPase.

4.4.2 Purification of Golgi-apparatus AMPase

Table XIV summarizes the purification data of the Golgi-apparatus AMPase. The separation of Golgi-fraction from the crude membranes (12,000-105,000 x g

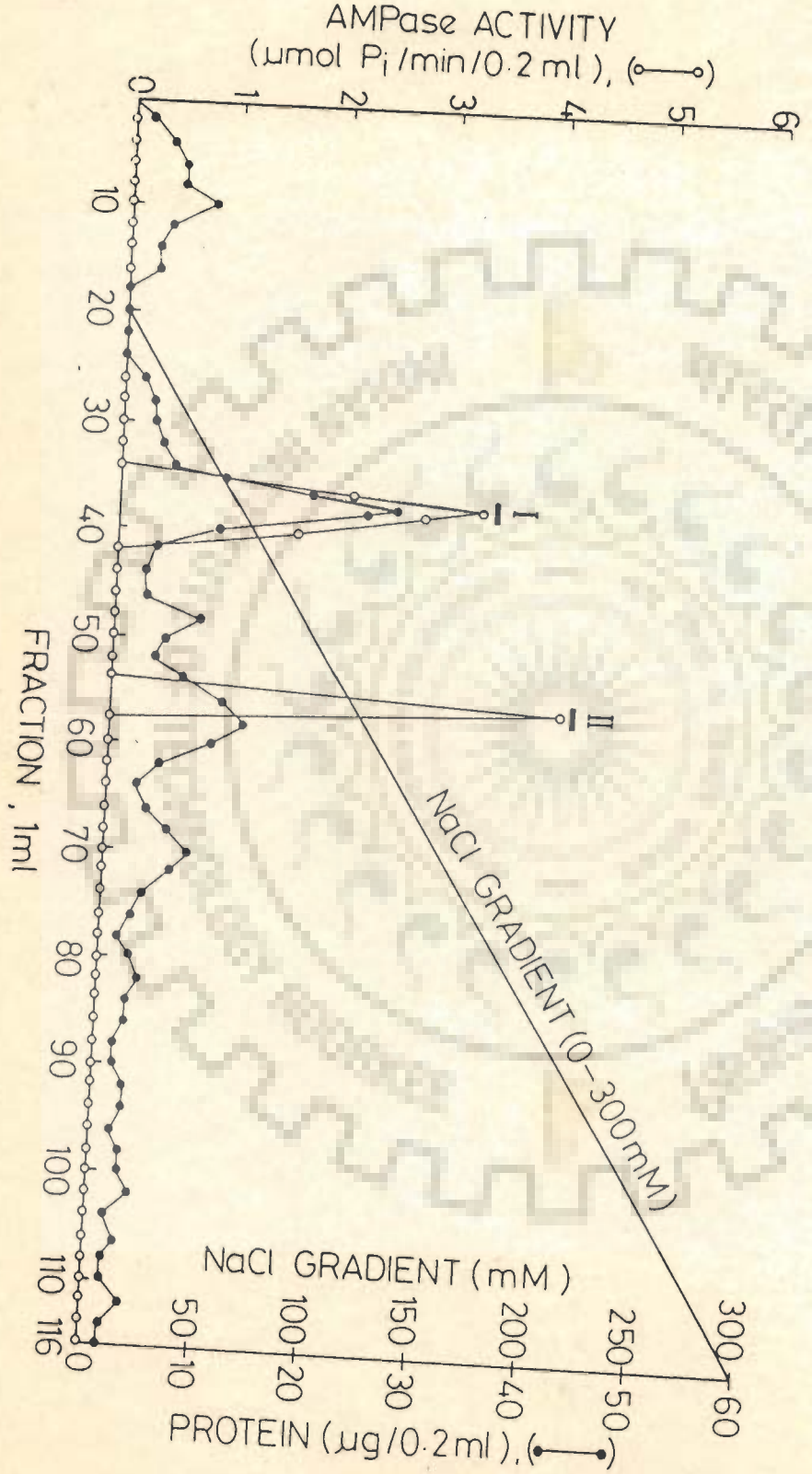
TABLE XIV PURIFICATION OF AMPase FROM THE PEANUT COTYLEDON GOLGI-MEMBRANE FRACTION

Golgi-apparatus membranes were obtained from the crude particulate fraction (12,000 - 105,000 x g) as described under Experimental procedures. The purified Golgi-membranes (3.33 mg protein/ml) was extracted with 0.5% octylglucoside in presence of 20 mM $MgCl_2$ and 5 mM EDTA, followed by 105,000 x g centrifugation. The supernatant fraction was used as the solubilized form of enzyme. DEAE cellulose peak I and II represent the pool of only top three fractions of each peak. The purification and specific activity data were computed relative to the crude membrane fraction.

Fraction	Total protein (mg)	Total AMPase activity (μmol P_i/ min)	AMPase specific activity (μmol P_i/ min/mg protein)	Purification (fold)	Yield (%)
Crude membrane fraction (12,000-105,000 x g pellet)	127.1	3128.5	24.6	-	100.00
Golgi-fraction (37/25% sucrose interface)	24.3	740.4	30.47	1.24	23.70
Octylglucoside extract (105,000 x g supernatant)	3.2	240.6	74.5	3.02	7.70
DEAE-Cellulose					
Peak I	0.271	45.49	167.74	6.81	1.4
Peak II	0.232	27.68	119.35	4.85	0.9

pellet) resulted in 1.24 folds purification relative to the crude membrane fraction, with a yield of 23.7%. The solubilization of membrane bound enzyme by octyl-glucoside extract further increased the purification by 3-folds, the yield at this stage was 7.7% with respect to the crude membrane fraction. However, if the Golgi-fraction is taken as the starting material, yield would then become about 32.5 per cent. This value closely ressembles with the solubilization data of the plasma membranes. Final purification step, as in the case of plasma membranes, was ion-exchange chromatography on DEAE-cellulose column. The elution profile of the AMPase activity and the protein are shown in Fig. 15. Two major peaks containing AMPase activity were eluted at 55 mM and 107 mM NaCl gradient. The third enzyme containing peak, which was present in the elution profile of plasma membrane AMPase (Fig. 8), was absent. On the basis of the peak area it can be seen that peak I accounted for most of the enzyme activity eluted from the column. As in the case of plasma membranes, peak I fraction was found to be homogeneous by polyacrylamide gel electrophoresis. The second enzyme peak was heavily contaminated by other proteins. The purification folds of enzyme I and enzyme II, relative to crude membrane

Fig. 15 DEAE-cellulose column chromatography of the dialyzed, octylglucoside solubilized Golgi-AMPase. A 14 ml sample (ca 6.5 mg protein) was loaded on the column. The absorbed proteins were eluted by a linear gradient of NaCl (0-300 mM) in 10 mM Tris-HCl, pH 7.2. Fractions of 1 ml were collected at the flow rate of 0.2 ml/min and aliquots (0.2 ml) from every other fraction were analysed for protein content and AMPase activity. The fractions indicated by bar (—) were pooled and used for further studies.


AMPase ACTIVITY
(μmol Pi /min/0·2 ml), (o—o)
0
1
2
3
4
5
6
10
20
30
40
50
60
70
80
90
100
110
116
FRACTION , 1ml
I
II
NaCl GRADIENT (0–300 mM)
NaCl GRADIENT (mM)
0
50
100
150
200
250
300
10
20
30
40
50
60
PROTEIN (μg/0·2 ml), (o—o)

fraction, were 6.8 and 4.8 with per cent yield of 1.4 and 0.9%, respectively. The actual purification fold and per cent yield will obviously be more than the observed values because of the presence of other non-specific phosphohydrolases in the crude membrane fraction as well as in the Golgi-apparatus fraction for that matter. In any case the enzyme present in DEAE-cellulose peak I was pure and on SDS-PAGE gave a tight protein band corresponding to the molecular weight of 53.7 K (Fig. 10).

4.4.3 Properties of purified Golgi-AMPase

4.4.3.1 Optimum pH

The activity of Golgi-apparatus AMPase as a function of pH is shown in Fig. 16. The results show that pH-optimum of the Golgi-AMPase is between pH 5.0 to 5.5.

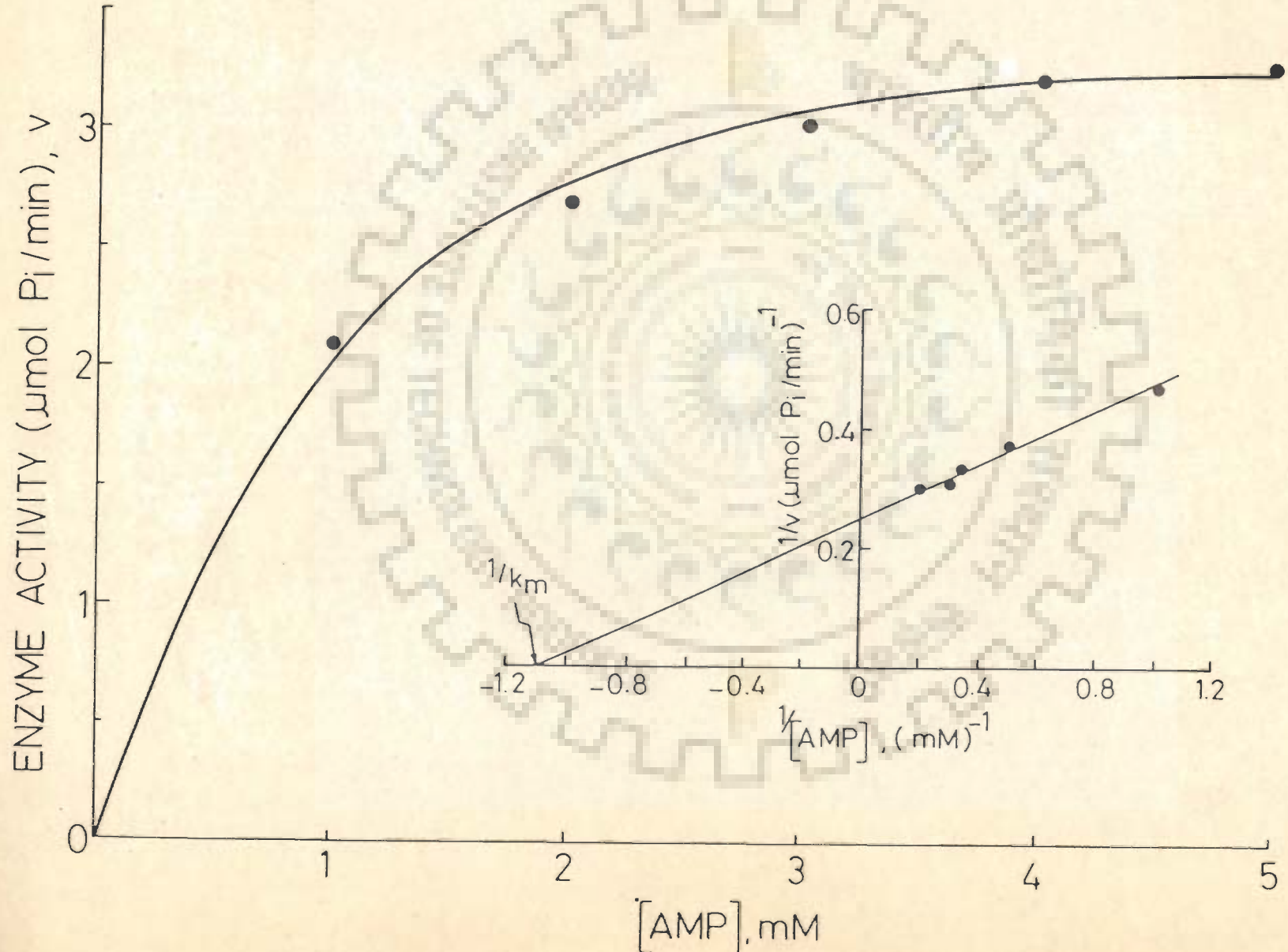
4.4.3.2 Kinetic parameters

Figure 17 shows the Lineweaver-Burk plot of 1/v versus 1/S, with AMP as substrate. The linear plot was obtained which intersected at X-axis and Y-axis giving a value of K_m and V_{max} of 0.9×10^{-3} M and 4 µmol/min, respectively. From the plot between the enzyme activity and the substrate concentration

Fig. 16 Effect of pH on the activity of AMPase purified from Golgi-fraction of peanut cotyledons. The rate of hydrolysis of AMP by purified enzyme fraction was measured by standard enzyme assay at varied pH. The buffers used were 50 mM acetate buffer, pH 3.0 to 5.5 and 50 mM Tris-HCl, pH 6.0 to 8.0.

AMPase ACTIVITY (μmol Pi / min)
7.5
6.0
4.5
3.0
1.5
0
2
3
4
5
6
7
8
pH

Fig. 17 Lineweaver-Burk plot of purified Golgi-AMPase. Enzyme assay with varying amounts of substrate, AMP and fixed amount of enzyme protein was carried out as described under Experimental procedures. K_m and V_{max} values were computed from the intercept on X-axis and Y-axis, respectively.


ENZYME ACTIVITY (μmol Pi/min), v
0
1
2
3
1
2
3
4
5
[AMP], mM
1/v (μmol Pi/min)⁻¹
0.6
0.4
0.2
1/km
-1.2
-0.8
-0.4
0
0.4
0.8
1.2
1/[AMP], (mM)⁻¹

a rectangular hyperbolic curve was obtained indicating that enzyme followed Michalies-Menten kinetics.

4.4.3.3 Inhibitors

NaF was found to inhibit the AMPase non-competitively (Fig. 18A). The replot of the slopes of Lineweaver-Burk plots versus inhibitor concentration (Fig. 18B) was linear intersecting on x-axis at a point corresponding to K_i value of 41 mM concentration. Results given in Fig. 19(A) show that the inhibition of AMP by ADP is of the competitive type. The K_i value as determined by the replot of slopes of Lineweaver-Burk plots versus ADP concentration came to be 3.4 mM. These results clearly indicate that ADP is a much stronger inhibitor of AMPase than NaF.

4.4.4 Comparison of plasma membrane and Golgi-apparatus AMPase

Table XV shows a comparison of the properties of purified AMPase from plasma membrane and Golgi-apparatus. The optimum pH of plasma membrane enzyme (pH 5 to 6) is relatively broader than the Golgi-apparatus enzyme (pH 5.0 to 5.5). The inhibition by ADP and NaF, molecular weight and K_m values were comparable. The V_{max} value of Golgi-apparatus (4 μmol/min) is about half that of plasma membrane AMPase (8.7 μmol/min). The elution pattern of enzyme

Fig. 18 Inhibition of Golgi-AMPase by NaF. Enzymatic hydrolysis of AMP was measured using varying concentration of substrate (AMP) in the presence of 0, 10, 20 and 30 mM NaF by the standard enzyme assay.

A) Lineweaver-Burk plots of peanut cotyledon Golgi-AMPase showing non-competitive type of inhibition by NaF.

B) Replot of slopes of Lineweaver-Burk plots versus NaF concentration. The intercept on X-axis equals the value of $-K_i$.

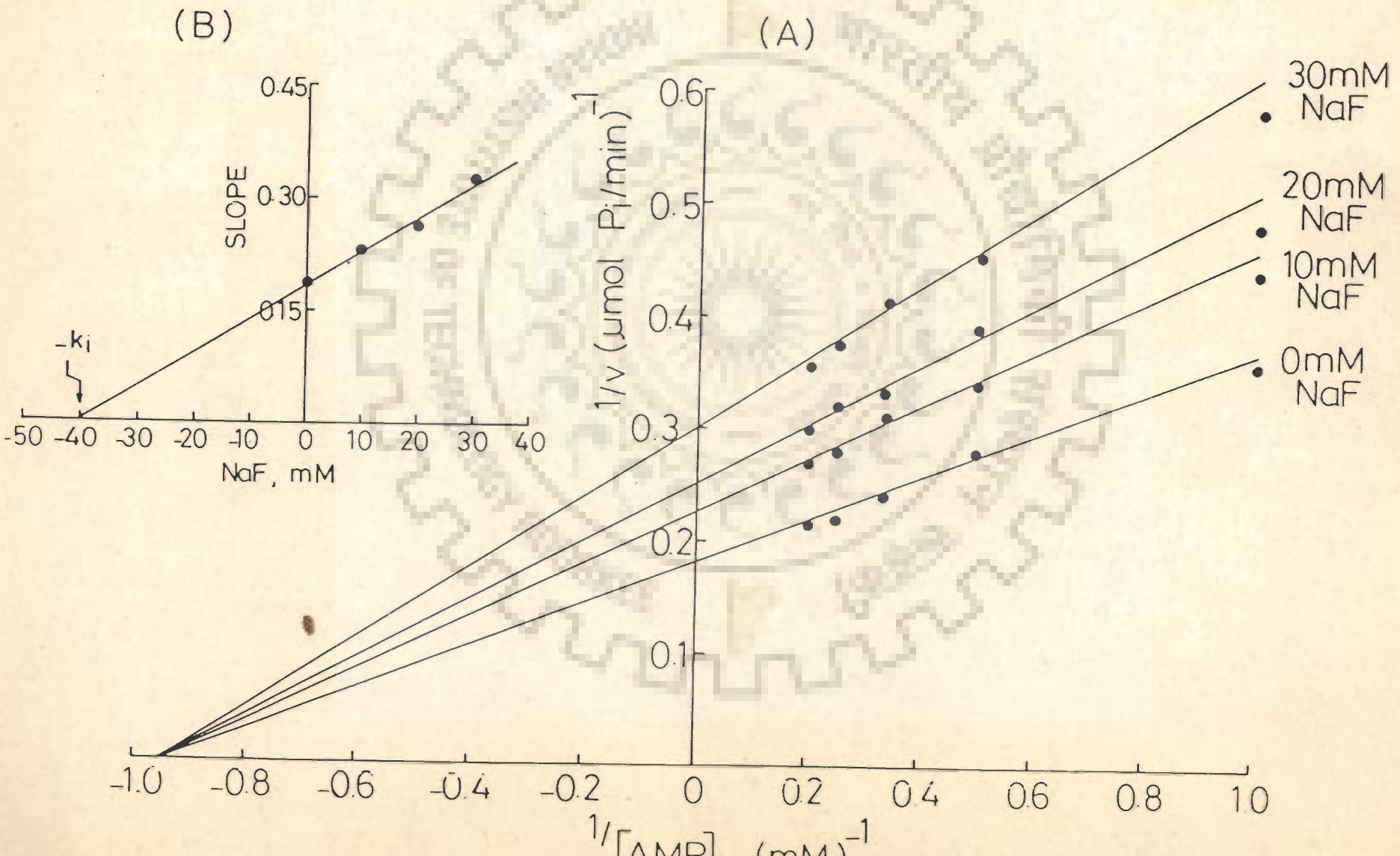

(B)
0.45
0.30
0.15
SLOPE
-ki
-50 -40 -30 -20 -10 0 10 20 30 40
NaF, mM
(A)
0.6
0.5
0.4
0.3
0.2
0.1
1/v (μmol Pi/min)-1
30mM NaF
20mM NaF
10mM NaF
0mM NaF
-1.0 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1.0
1/[AMP] (mM)-1

Fig. 19 Inhibition of Golgi-AMPase by ADP. Enzyme activity was determined using varying amounts of AMP in the presence of 0, 1, 2.5 and 3.5 mM ADP.

A) Lineweaver-Burk plots of AMP hydrolysis, showing competitive type of inhibition.

B) Replot of slopes of Lineweaver-Burk plots versus ADP-concentration.

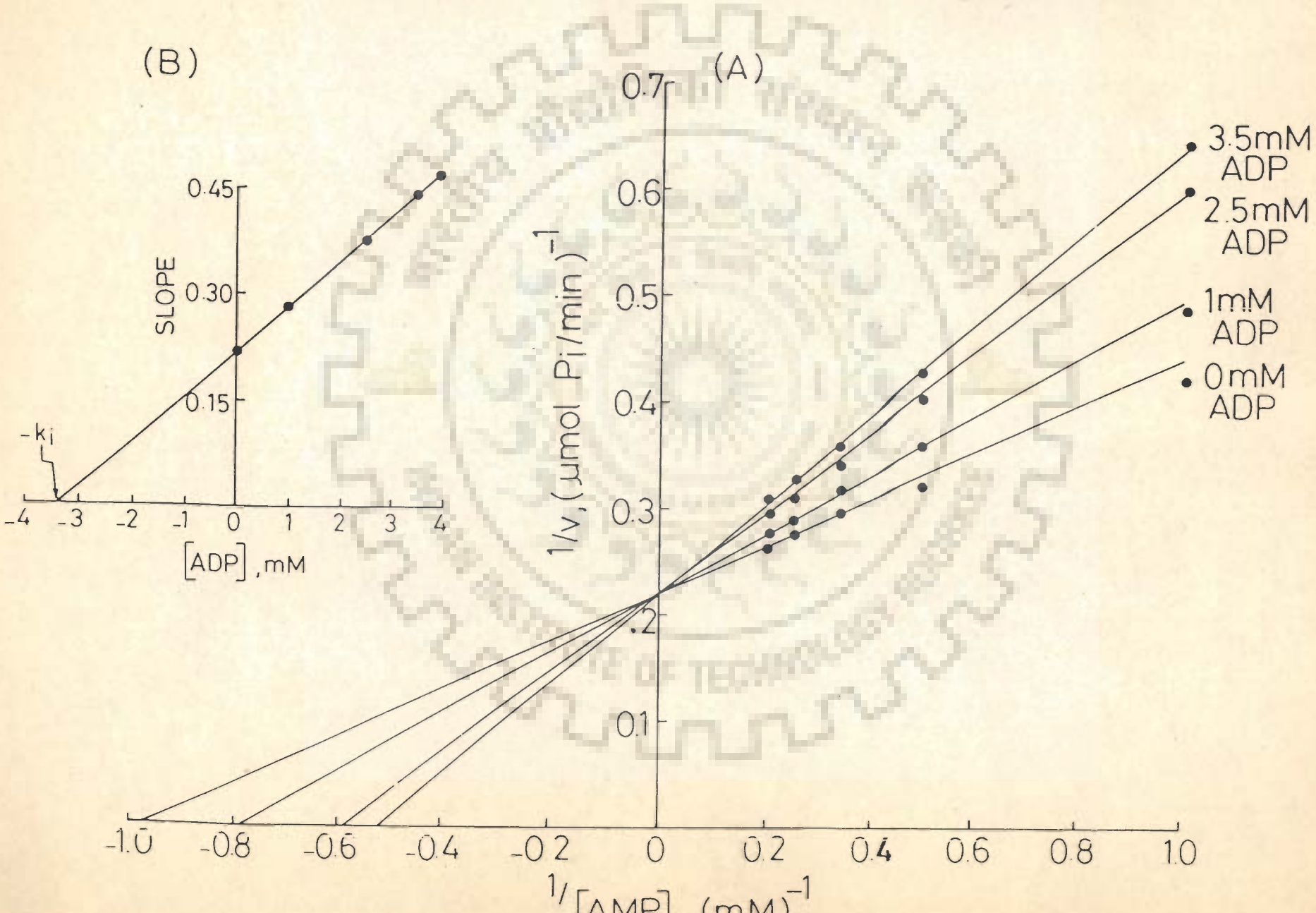

(B)
0.45
0.30
0.15
SLOPE
-ki
-4 -3 -2 -1 0 1 2 3 4
[ADP], mM
(A)
0.7
0.6
0.5
0.4
0.3
.2
0.1
1/v, (μmol Pi/min)⁻¹
3.5mM ADP
2.5mM ADP
1mM ADP
0mM ADP
-1.0 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1.0
1/[AMP], (mM)⁻¹

TABLE XV A COMPARISON OF PROPERTIES OF AMPase OF PLASMA MEMBRANE AND GOLGI-APPARATUS MEMBRANES OF PEANUT COTYLEDONS

Properties	Purified 5'-AMPase	
	Plasma membrane	Golgi-apparatus
pH-optimum	5 - 6	5 - 5.5
K_m	1.08 mM	0.91 mM
V_{max}	8.47 μmol/min	4.00 μmol/min
Inhibition by ADP	Competitive (K_i = 2.4 mM)	Competitive (K_i = 3.4 mM)
Inhibition by NaF	Non-competitive (K_i = 35 mM)	Non-competitive (K_i = 41 mM)
Molecular weight	55 K daltons	53.7 K daltons
Carbohydrate	42.7%	38.46%
Electrophoretic mobility	0.18	0.18
Elution from DEAE-cellulose column	Peak I (50 mM); Peak II (107 mM); Peak III(155 mM)	Peak I (55 mM); Peak II (107 mM)
Component monosaccharides	GlcNAc, Man and Glc	GlcNAc, Man and Glc[a]

[a]GlcNAc = N-acetylglucosamine; Man = mannose; Glc = glucose.

peak I and II from the DEAE cellulose column are identical but in the case of Golgi-apparatus enzyme the third peak, which eluted at 155 mM NaCl gradient, was missing. Some differences in the properties of plasma membrane and Golgi-apparatus enzyme were also noticed. For example, the carbohydrate contents were somewhat lower than the plasma membrane enzyme, although the component monosaccharides forming the oligosaccharide moiety of the enzymes appeared to be identical. On the basis of these results it appears, that the Golgi-apparatus AMPase is the precursor of the plasma-membrane AMPase and that some modification of the Golgi-AMPase has occurred before becoming a part of the plasma membrane.

4.5 Endoplasmic reticulum AMPase

4.5.1 Solubilization and partial purification of AMPase

The AMPase was solubilized from the enriched ER fraction by treatment with 0.5% octylglucoside in presence of $MgCl_2$ and EDTA. The conditions of solubilization were exactly those used for the solubilization of AMPase from plasma membrane and Golgi-apparatus. The results are shown in Table XVI. The specific activity of the solubilized enzyme was found to increase from 22.7 μmol P_i/min/mg to

TABLE XVI SOLUBILIZATION AND PARTIAL PURIFICATION OF ENDOPLASMIC RETICULUM AMPase

The endoplasmic reticulum membrane fraction was treated with 0.5% octylglucoside at protein to detergent ratio of 2:3 in the presence of Mg^{2+} and EDTA, as described under Experimental procedures. The supernatant, obtained after centrifugation at 105,000 x g for 60 min, was tested for protein content and AMPase activity.

Fraction	Total protein (mg)	Total AMPase activity (μmol P_i/ min)	AMPase specific activity (μmol P_i/ min/mg protein)	Purification (fold)	Yield (%)
Endoplasmic reticulum	6.6	150.0	22.7	1	-
Octylglucoside extract (105,000 x g supernatant)	0.77	90.0	117.0	5.15	60.0

117.0 μmol P_i/min/mg giving a purification fold of 5.15, with a yield of 60%. Compared to plasma membrane and Golgi-AMPase, the solubilization of the endoplasmic reticulum AMPase was almost two times more.

Results given in Table XVII show the stability of the enzyme during dialysis as compared with those of plasma membrane and Golgi-apparatus AMPase. These results indicate that unlike the plasma membrane and Golgi-apparatus AMPase, the ER enzyme was highly unstable and lost 40% activity within 6 h during dialysis at 4°C. On further dialysis for 12 h the enzyme was rendered almost completely inactive. In contrast, after 12 h of dialysis the plasma membrane and Golgi-AMPase retained nearly three fourth of the total activity. These results indicate that the ER-AMPase when reaches into Golgi-apparatus and finally to plasma membrane, it gets modified and as a result becomes more stable than the ER-enzyme.

4.5.2 Effect of detergents on the activity of dialysed enzyme

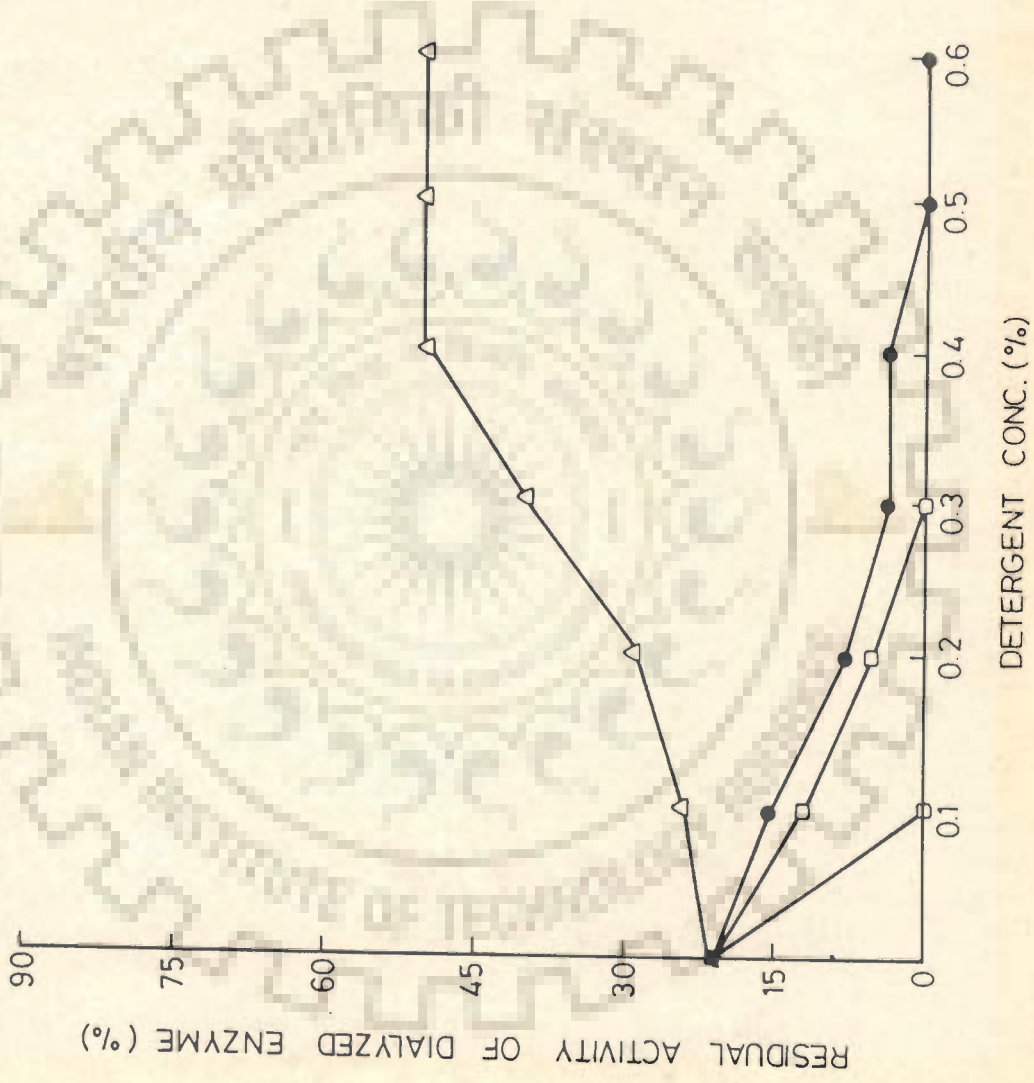
Results shown in Fig. 20 show that the activity of the dialysed ER-AMPase can be partially recovered by adding back octylglucoside. For instance, after 4 h of dialysis the residual activity of the enzyme was only 44%. But when octylglucoside was added to the

TABLE XVII LOSS OF ACTIVITY OF SOLUBILIZED AMPase DURING DIALYSIS

The solubilized enzyme fraction was dialyzed against 10 mM Tris-HCl, pH 7.2, at 4°C. The dialyzed enzyme was tested for the AMPase activity after the specified period, as described under Experimental procedures.

Solubilized enzyme fraction	Residual activity (%)			
	Dialysis period (h)			
	0	4	6	12
Plasma membrane	100	98.6	98	78
Golgi-apparatus	100	99.0	97	74
Endoplasmic reticulum	100	44.0	20.75	15

Fig. 20 Effect of detergents on the activity of dialyzed ER-AMPase. The solubilized ER AMPase was dialyzed against 10 mM Tris-HCl, pH 7.2, for 6 h. The dialyzed enzyme was assayed for AMPase activity in the presence of different concentrations of the detergents, SDS, Triton X-100, Nonidet P-40 and octylglucoside. The results are expressed as residual activity of AMPase in the presence of, Triton X-100, ■—■; Nonidet, □—□; SDS, o-o; and octylglucoside, Δ-Δ. The activity of dialyzed AMPase was 20.75% of the undialyzed enzyme.


RESIDUAL ACTIVITY OF DIALYZED ENZYME (%)
90
75
60
45
30
15
0
0.1
0.2
0.3
0.4
0.5
0.6
DETERGENT CONC. (%)

enzyme reaction mixture, the activity was increased upto 73% of the original activity (undialyzed enzyme) i.e., nearly 30%. When the enzyme was dialysed for 6 h the residual activity was 20.75%. This activity was raised to 50% by the addition of 0.4% octylglucoside. Beyond this limit the activity could not be raised by increasing the detergent concentration. The other detergents, namely Triton X-100, Nonidet P40 and SDS were found to be inhibitory even at very low concentration. For instance Triton X-100 inactivated the dialyzed enzyme at a final concentration of 0.1%. Thus in the presence of octylglucoside the endoplasmic reticulum AMPase not only remains active but also regains part of the activity lost during dialysis. These results are different than those of obtained with either plasma membrane AMPase or Golgi-AMPase, which were quite stable even after the detergent had been removed by dialysis. On the basis of these observations it seems possible that ER-AMPase could be further purified in the presence of octylglucoside. At the moment, however, all attempts to purify the enzyme were unsuccessful because of the loss of activity of the enzyme in the absence of detergent. Therefore the properties of the partially purified enzyme are reported here. However, in future attempt

will be made to purify the enzyme in the presence of suitable amount of octylglucoside.

4.5.3 Properties of partially purified ER-AMPase

4.5.3.1 Optimum pH

Figure 21 shows the enzyme activity as a function of pH. The enzyme shows two pH-optima, one at pH 4.5 and the other at pH 6.0. The pH profiles of the solubilized as well as membrane bound enzymes were identical. Since in the case of plasma membrane as well as Golgi-apparatus multiple form of AMPase were found, it is likely that the two pH-optima found in the case of ER-enzyme indicate the presence of at least two AMPase form in ER. Although the pH-optima of ER enzyme are somewhat different from the plasma membrane and Golgi-apparatus AMPase, the pH-optimum range is identical.

4.5.3.2 Effect of substrate concentration

Figure 22 shows the Lineweaver-Burk plots of the membrane-bound and the solubilized ER-AMPase. The K_m and V_{max} values of the solubilized enzyme with AMP as substrate were found to be 10.0×10^{-4}M and 232 µmol P_i/min/mg, respectively. The K_m and V_{max} values for the membrane bound enzyme were found to be

Fig. 21 Activity of endoplasmic reticulum AMPase as a function of pH. The enzyme activity was measured at varied pH in the standard assay system. The buffer used were 50 mM acetate buffer, pH 3.0 to 5.5 and 50 mM Tris-HCl, pH 6.0 to 8.0. ●-●, membrane bound AMPase; o-o, solubilized AMPase activity.

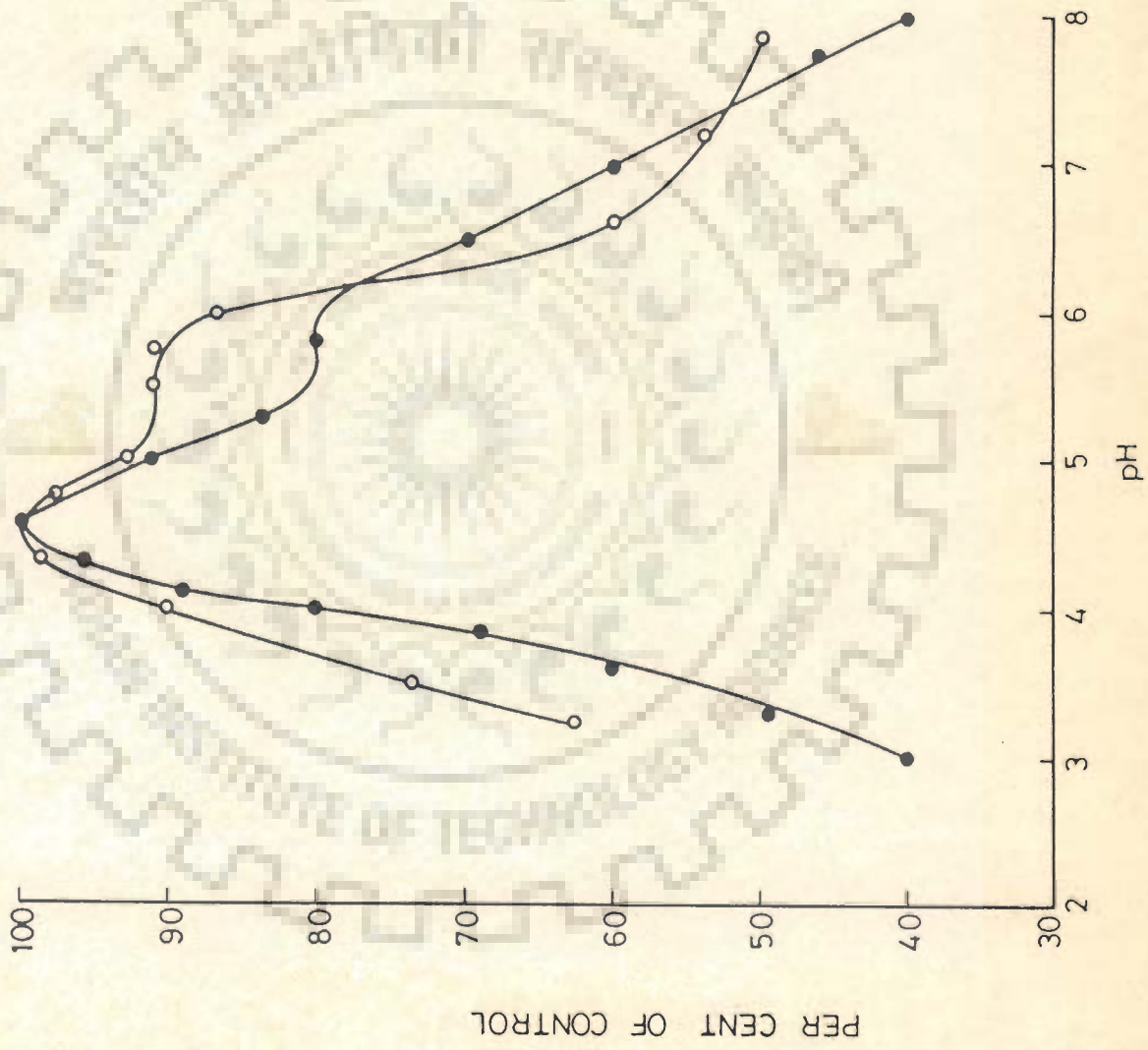
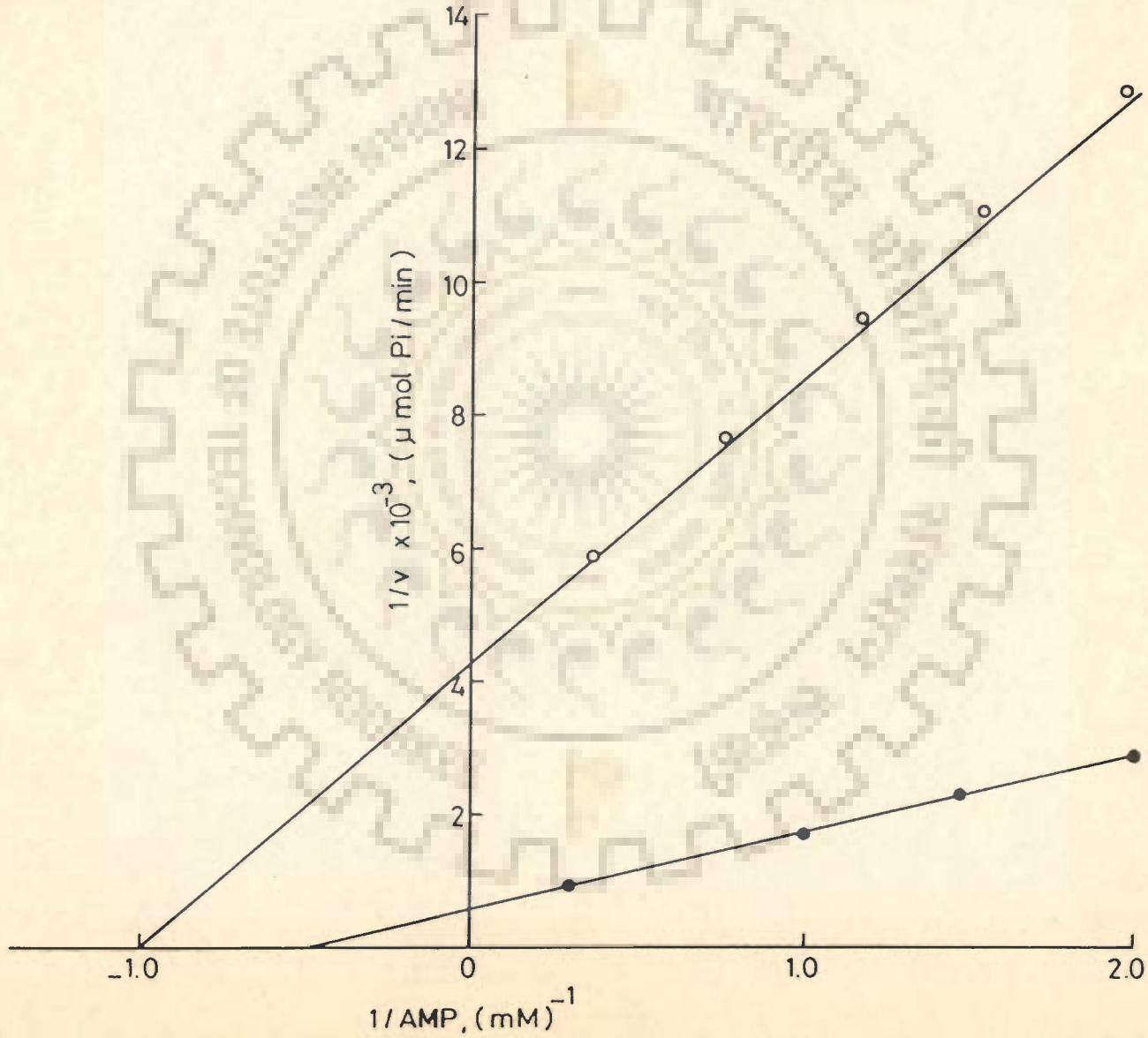

PER CENT OF CONTROL
100
90
80
70
60
50
40
30
2
3
4
5
6
7
8
pH

Fig. 22 Lineweaver-Burk plots of ER-AMPase. Standard assays with varying amounts of AMP were carried out for 15 min with fixed amount of enzyme. K_m and V_{max} values were computed from the intercept on X-axis and Y-axis, respectively. ●—●, membrane bound; and o—o, solubilized AMPase.


14
12
10
8
6
4
2
1/v x10^{-3}, (μ mol Pi/min)
-1.0
0
1.0
2.0
1/AMP, (mM)$^{-1}$

20.0×10^{-4}M and 1600 µmol/min/mg. There seems to be some change in affinity ($1/K_m$) of the solubilized enzyme towards the substrate. For instance, the affinity of the solubilized enzyme is two times more than the membrane bound enzyme. Since the ER AMPase could not be purified further, it has not been possible to compare its properties with the purified plasma membrane and Golgi-AMPase. Nevertheless the information regarding the stability of enzyme is important as it suggests that ER AMPase has been modified in Golgi-apparatus and plasma membrane.

4.6 Membrane-bound acid phosphatase

4.6.1 Subcellular distribution of acid phosphatase and 5'-nucleotidase in peanut cotyledons

A comparison of the cellular distribution of acid phosphatase and 5'-nucleotidase (AMPase) in the germinating cotyledons of peanut is shown in Table XVIII. The subcellular fractions were collected by differential centrifugation corresponding to particulate fractions at 12,000 x g and 105,000 x g. The 12,000 x g pellet represents mainly mitochondria and has been found to contain only 7.7% of the total acid phosphatase activity. The 105,000 x g pellet which represents the microsomal fraction was found to contain nearly 27.7% of the total phosphatase activity.

TABLE XVIII SUBCELLULAR DISTRIBUTION OF ACID PHOSPHATASE AND 5'-NUCLEOTIDASE IN PEANUT COTYLEDONS

Seven-days germinating cotyledons (100 g) were homogenized in 50 mM Tris-HCl buffer, pH 7.2, containing, 3 mM EDTA, 0.25 M sucrose and 0.04% β-mercaptoethanol and various subcellular fractions were prepared by differential centrifugation as described under Experimental procedures. Acid phosphatase and 5'-nucleotidase activity was measured using p-nitrophenyl phosphate and 5'-AMP as substrates, respectively. p-NP is p-nitrophenyl phosphate. Total enzyme activity was expressed as per 50 cotyledons.

Fraction	Acid phosphatase		AMPase	
	Total activity (μmol p-NP/ min)	Specific activity (μmol p-NP/ min/mg protein)	Total activity (μmol P_i/ min)	Specific activity (μmol P_i/min/ mg protein)
12,000 x g pellet (mitochondria etc.)	484.6	38.8	953.3	75.66
105,000 x g pellet (microsomal frac.)	1733.3	27.7	1658.2	26.58
105,000 x g supernatant (soluble frac.)	4025.7	21.3	4290.3	35.80

The 105,000 x g supernatant fraction (cytosol) contains the highest amount of acid phosphatase activity accounting for nearly two-third of total acid phosphatase activity in 7 days germinating cotyledons. Thus, significant amount of acid phosphatase activity is associated with microsomal membrane fraction. Although the specific activity of 12,000 x g pellet was found to be highest (38.8 μmol/min/mg), the microsomal membrane with specific activity 27.7 μmol/min/mg was used as a source of enzyme because the later contained nearly four times more activity than the former. The subcellular distribution of 5'-nucleotidase activity is somewhat different than that of acid phosphatase in 7 days old germinating cotyledons. For instance, the specific activity in 12,000 x g pellet was found to be five times greater than that of microsomal 5'-nucleotidase. Moreover, the total activity of 5'-nucleotidase in both 12,000 x g pellet and 105,000 x g pellet was nearly comparable. The soluble fraction represented nearly two-third of the total nucleotidase activity present in cotyledons. Thus, microsomal fraction contains significant amount of both acid phosphatase as well as AMPase activity. At the moment it is not certain whether or not acid phosphatase and AMPase activities represent the same enzyme protein.

4.6.2 Activity of microsomal acid phosphatase and AMPase as a function of germination period

The changes in the levels of microsomal acid phosphatase and AMPase during germination period (0-12 days) were investigated in order to find out if the developmental pattern of these enzymes were different. The results given in Table XIX clearly indicate that the 2 days old germinating cotyledons show nearly 40% of the maximum acid phosphatase activity found in 6 days old germinating cotyledons. On the other hand, the 5'-nucleotidase activity was found to be completely absent from 2 days old germinating peanut cotyledons indicating that AMPase activity emerged only after 48 h of imbibition of seeds. This physiological state of the seed is of interest from the point of view of preparing the microsomal membranes which should apparently be free from 5'-nucleotidase activity. Thus, advantage was taken of this observation to prepare microsomal membrane fraction only from 2 days old germinating seedlings for solubilization and purification of membrane-bound acid phosphatase. It may also be pointed out here that the most active physiological state of germinating peanut cotyledons, in terms of acid phosphatase and AMPase activity levels, is between 6th and 7th day of

TABLE XIX ACTIVITY OF MICROSOMAL ACID PHOSPHATASE AND AMPase AS A FUNCTION OF GERMINATION PERIOD

Seeds were germinated at 35°C in dark. 200 cotyledons of different germination stages were excised and microsomal membrane fractions were prepared by differential centrifugation as described under Experimental procedures. Protein content and enzyme activity were determined by standard assay procedures. Total enzyme activity was expressed as per 50 cotyledons.

Germination period (days)	Protein (mg)	Acid phosphatase		AMPase	
		Total activity (μmol p-NP/ min)	Specific activity (μmol p-NP/ min/mg protein)	Total activity (μmol P_i/ min)	Specific activity (μmol P_i/ min/mg protein)
0	192.0	307.2	1.6	-	-
2	144.0	633.6	4.4	-	-
4	96.0	556.8	5.8	528.0	5.5
6	95.5	1591.9	16.67	1652.15	17.3
7	70.28	1274.0	18.20	1670.80	23.6
8	67.7	1299.8	19.20	846.25	12.5
12	44.0	1016.4	23.10	299.20	6.8

germination. At this stage both acid phosphatase and AMPase show maximum activity. Since, acid phosphatase and AMPase are difficult to separate from each other by routine chromatographic procedures, the use of 2 days old germinating cotyledons constituted a very useful and important step in the purification of membrane-bound acid phosphatase. The difference in the developmental pattern of acid phosphatase and AMPase in peanut cotyledons also suggests that the two enzymes are different from one another. Working on this observation we attempted to solubilize, purify and characterize the acid phosphatase activity from microsomal membrane fraction.

4.6.3 Solubilization of membrane-bound acid phosphatase

4.6.3.1 Solubilization of acid phosphatase with different detergents

One of the pre-requisites for solubilizing the membrane bound enzyme is to use a detergent, which would solubilize the enzyme in active and stable form. Data summarized in Table XX indicate that of the five detergents (deoxycholate, taurocholate, Triton X-100, Nonidet P-40 and octylglucoside), octylglucoside was clearly the best detergent to solubilize the acid phosphatase from microsomal membrane fractions. Under the conditions described,

TABLE XX SOLUBILIZATION OF MEMBRANE-BOUND ACID PHOSPHATASE WITH DIFFERENT DETERGENTS

For solubilization of the membrane-bound acid phosphatase, microsomal membrane fraction (5.10 mg protein), was treated with 0.5% detergent corresponding to a protein to detergent ratio of 1:2 in presence of 20 mM $MgCl_2$ and 5 mM EDTA, followed by centrifugation at 105,000 x g for 60 min. The supernatant was assayed for enzyme activity as described in Experimental procedures. Total activity is expressed as µmol p-NP formed per minute.

Fraction	Specific activity (µmol p-NP/ min/mg protein)	Activity solubilized (%)	Protein solubilized (%)
Membrane fraction (12,000-105,000 x g pellet)	5.10	-	-
Detergent extract (105,000 x g supernatant)			
Deoxycholate	2.21	4.19	8.74
Taurocholate	3.09	5.37	8.34
Triton X-100	14.96	30.01	9.42
Nonidet P-40	15.00	29.36	9.20
Octylglucoside	31.30	65.57	9.82

nearly two-third of the total activity was solubilized and there was a six folds increase in the specific activity. It is interesting to note that the amount of protein solubilized by different detergents was comparable.

4.6.3.2 Effect of $MgCl_2$ on the solubilization of acid phosphatase

Mg^{2+} was found to have marked effect on solubilization of microsomal acid phosphatase by octylglucoside (Fig. 23). The results indicate further that in the presence of 20 mM $MgCl_2$, the total enzyme activity solubilized increases by 3 folds and there was about 2.5 folds increase in the specific activity of the enzyme. The solubilization was therefore carried out in the presence of 20 mM $MgCl_2$.

4.6.3.3 Effect of protein to detergent ratio on the solubilization of microsomal acid phosphatase activity

The results shown in Fig. 24, demonstrate that by increasing the concentration of detergent, the per cent solubilization of total acid phosphatase activity also increased. But the specific activity of the solubilized enzyme was found to be maximum when the protein to detergent ratio was 1:2. Below and above this ratio, there was a marked decrease

Fig. 23 Solubilization of membrane bound acid phosphatase as a function of Mg^{2+} concentration. Solubilization was carried out exactly as described under Experimental procedures. 0.5% final concentration of detergent at the protein to detergent ratio of 1:2, in the presence of 5 mM EDTA and indicated amounts of Mg^{2+} were utilized for solubilization. Results are expressed as; ●—●, specific activity of octylglucoside soluble fraction; ■—■, per cent of total activity solubilized by octylglucoside.

SPECIFIC ACTIVITY (µmol pNP / min /mg), (●—●)
5
10
15
20
25
30
35
0
10
20
30
MgCl2 (mM)
OCTYL GLUCOSIDE EXTRACT
15
30
45
60
75
90
105
PER CENT ACTIVITY SOLUBILISED, (■—■)

Fig. 24 Effect of protein to detergent ratio on the solubilization of acid phosphatase of microsomal membranes. At 0.5% of final concentration of detergent, protein to detergent ratio was adjusted to 1:1, 1:1.5, 1:2 and 1:3. Solubilization was carried out as described under Experimental procedures at 5 mM EDTA and 20 mM $MgCl_2$. Results are expressed as; ●—●, specific activity of the solubilized fraction; ■—■, per cent of total activity solubilized.

SPECIFIC ACTIVITY (μmol pNP/min/mg) (●—●)
PER CENT ACTIVITY SOLUBILISED (●—●)
PROTEIN-DETERGENT RATIO
OCTYLGLUCOSIDE EXTRACT
35
30
25
20
15
10
5
0
105
90
75
60
45
30
15
1:1
1:1.5
1:2
1:3

in the specific activity. Thus, on the basis of these results the optimum conditions for solubilization of microsomal acid phosphatase may be summarized as below : 50 mM Tris-HCl, pH 7.2, 0.5% octylglucoside (corresponding to protein to detergent ratio of 1:2), 20 mM $MgCl_2$ and 5 mM EDTA. Under these conditions nearly 74% of the total microsomal membrane acid phosphatase activity was solubilized with a purification folds of 6.7.

4.6.4 Further purification

The solubilized enzyme was dialyzed overnight against 100 volumes of 10 mM Tris-HCl buffer, pH 7.2. The same buffer was used to equilibriate the DEAE-cellulose column used for fractionation of the solubilized enzyme. The elution profile of the acid phosphatase activity and the protein from the DEAE-column are shown in Fig. 25. It can be seen that a large amount of acid phosphatase activity as well as protein was eluted with the front. When the column was washed by a stepwise gradient of NaCl, three acid phosphatase containing peaks were obtained at 50 mM, 100 mM and 200 mM salt gradients. Top three fractions in each peak were pooled. The purification folds of peak I, peak II and peak III were 69.4, 26.47 and 16.1 with yields of 58, 12 and 7 per cent,

Fig. 25 Chromatography of dialyzed octylglucoside solubilized acid phosphatase (105,000 x g supernatant) on DEAE-cellulose column. A 10 ml sample (5 mg protein) was loaded on the column (1.5 x 10 cm), and the absorbed protein was eluted by passing successively 50 mM, 100 mM, 200 mM and 300 mM NaCl (15 ml each) in 10 mM Tris-HCl, pH 7.2, through the column. Fractions (1 ml) were collected at a flow rate of 0.2 ml/min and from every other fraction, aliquots (0.2 ml) were tested for protein content and acid phosphatase activity. Fractions indicated by bar (▬) were pooled and used for further studies.

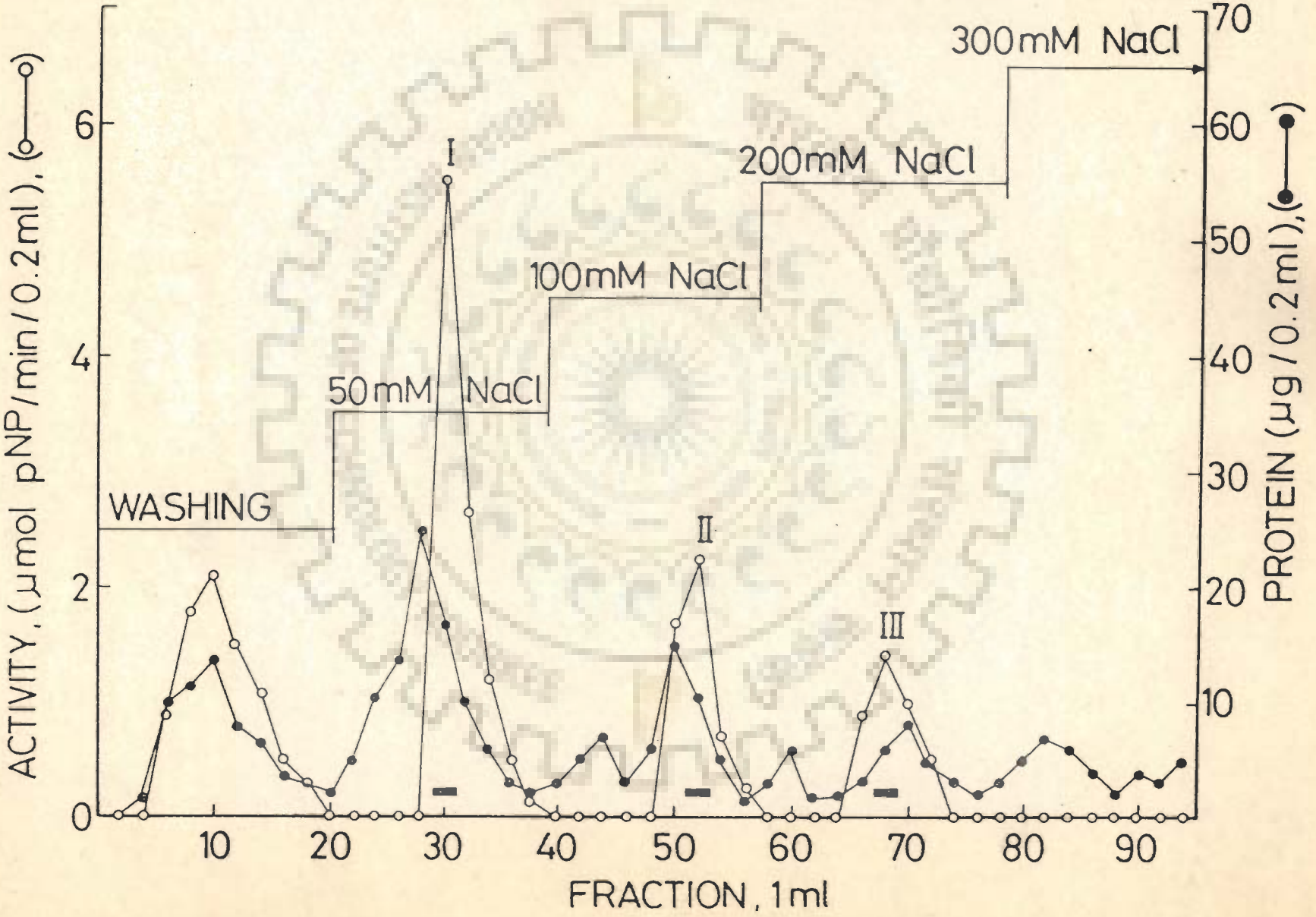

ACTIVITY, (μmol pNP/min/0.2ml), (o—o)
PROTEIN (μg/0.2ml), (●—●)
FRACTION, 1ml
WASHING
50mM NaCl
100mM NaCl
200mM NaCl
300mM NaCl
I
II
III
0
2
4
6
10
20
30
40
50
60
70
80
90

TABLE XXI PURIFICATION OF ACID PHOSPHATASE FROM THE PEANUT COTYLEDON MICROSOMAL MEMBRANE FRACTION

The microsomal membrane fraction (10.00 mg/ml), prepared from 2 days old germinating cotyledons (about 80-100 g fresh weight) of peanut, was extracted with 0.5% octylglucoside in the presence of 20 mM $MgCl_2$ and 5 mM EDTA as described under Experimental procedures and then centrifuged at 105,000 x g for 60 min. The supernatant fraction was used as soluble enzyme for further purification and analysis. DEAE-cellulose peaks I, II and III represent the pool of only top three fractions of each peak.

Fraction	Total protein (mg)	Total activity (μmol p-NP/ min)	Specific activity (μmol p-NP/ min/mg protein)	Purification (fold)	Yield (%)
Microsomal membrane fraction (12,000-105,000 x g pellet)	150.5	662.2	4.4	-	100.0
Octylglucoside extract (105,000 x g supernatant)	16.5	491.7	29.8	6.7	74.34
DEAE-cellulose					
Peak I	1.26	384.8	305.4	69.40	58.03
Peak II	0.68	79.22	116.50	26.47	11.90
Peak III	0.67	47.43	70.80	16.10	7.16
Preparative gel electrophoresis of peak I	0.8	264.0	330.0	75.0	39.86

respectively (Table XXI). The maximum purification of 69.4 folds was achieved in peak I. The emergence of three different peaks of acid phosphatase activity indicates the presence of multiple forms of acid phosphatase.

4.6.5 Polyacrylamide gel electrophoresis

Figure 26 shows the polyacrylamide gel electrophoresis of different fractions with and without SDS. As can be seen, the crude detergent extract representing the solubilized enzyme contained a large number of protein bands. Of the DEAE-cellulose fractions, peak I was found to be relatively pure as one major protein band corresponding to molecular weight of 46.7 K (Fig. 27) was found with three minor bands. A comparison of the electrophoretic pattern with and without SDS shows that the major protein band splits into two protein bands, one major and one minor corresponding to molecular weights of 46.7 K and 50.1 K. These results were interpreted to mean that the acid phosphatase may be composed of at least of two subunits. However, this aspect needs further studies, including the reconstitution of the enzyme. Based on intensity of the bands, it appears that the impurities are relatively minor. The enzyme was further purified by preparative polyacrylamide gel

Fig. 26 Polyacrylamide gel electrophoresis of microsomal acid phosphatase. Electrophoresis was carried out with and without SDS on 7.5 and 10% gels, respectively, by the method described in Experimental procedures. Samples were: S.E., solubilized enzyme (60-80 μg protein); I, DEAE-cellulose peak I (40-60 μg protein); II, DEAE-cellulose peak II (50 μg protein); III, DEAE-cellulose peak III (55 μg protein); Std., standards (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme (5 μg each); M.W., molecular weight standards. Direction of electrophoresis was from top to bottom.

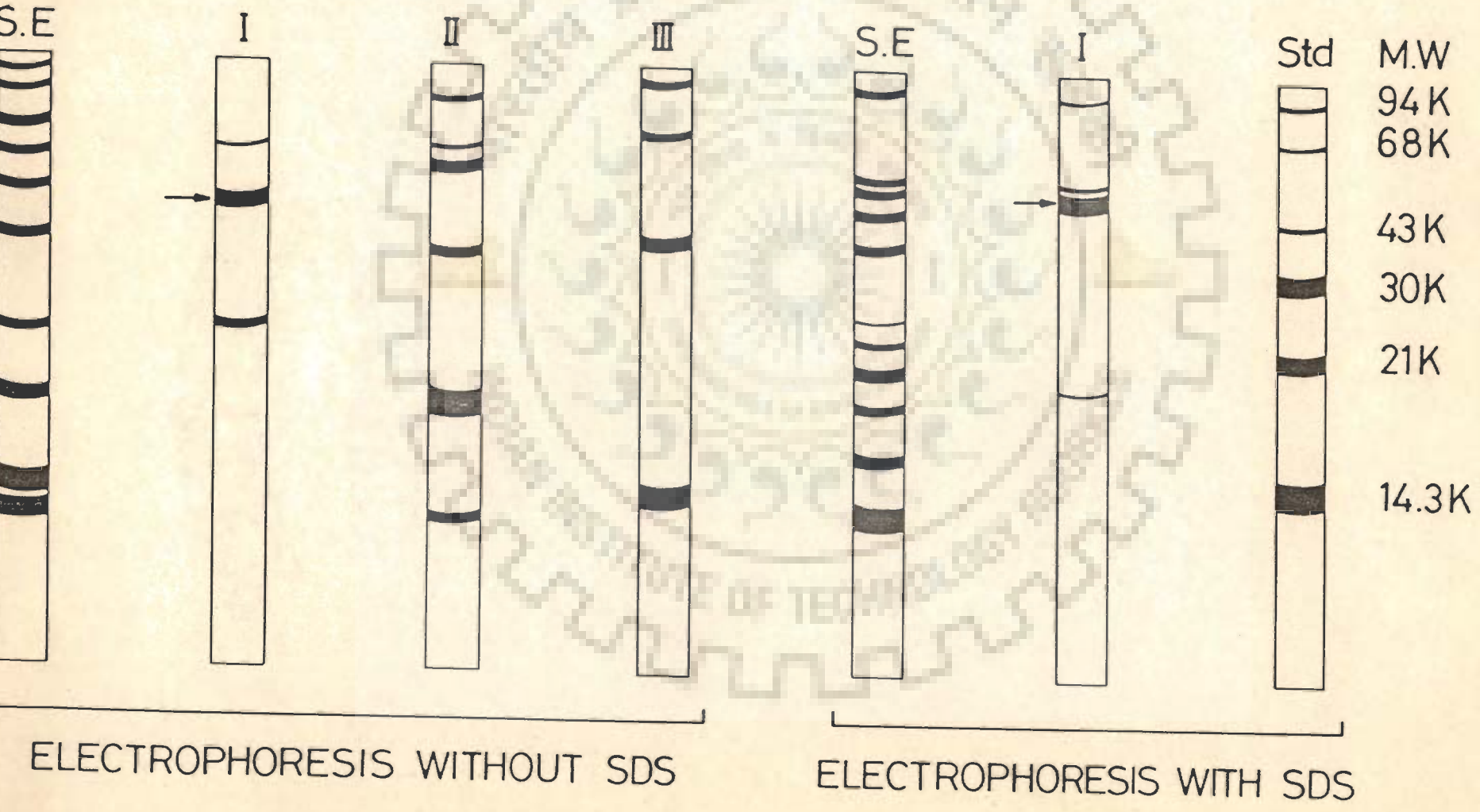
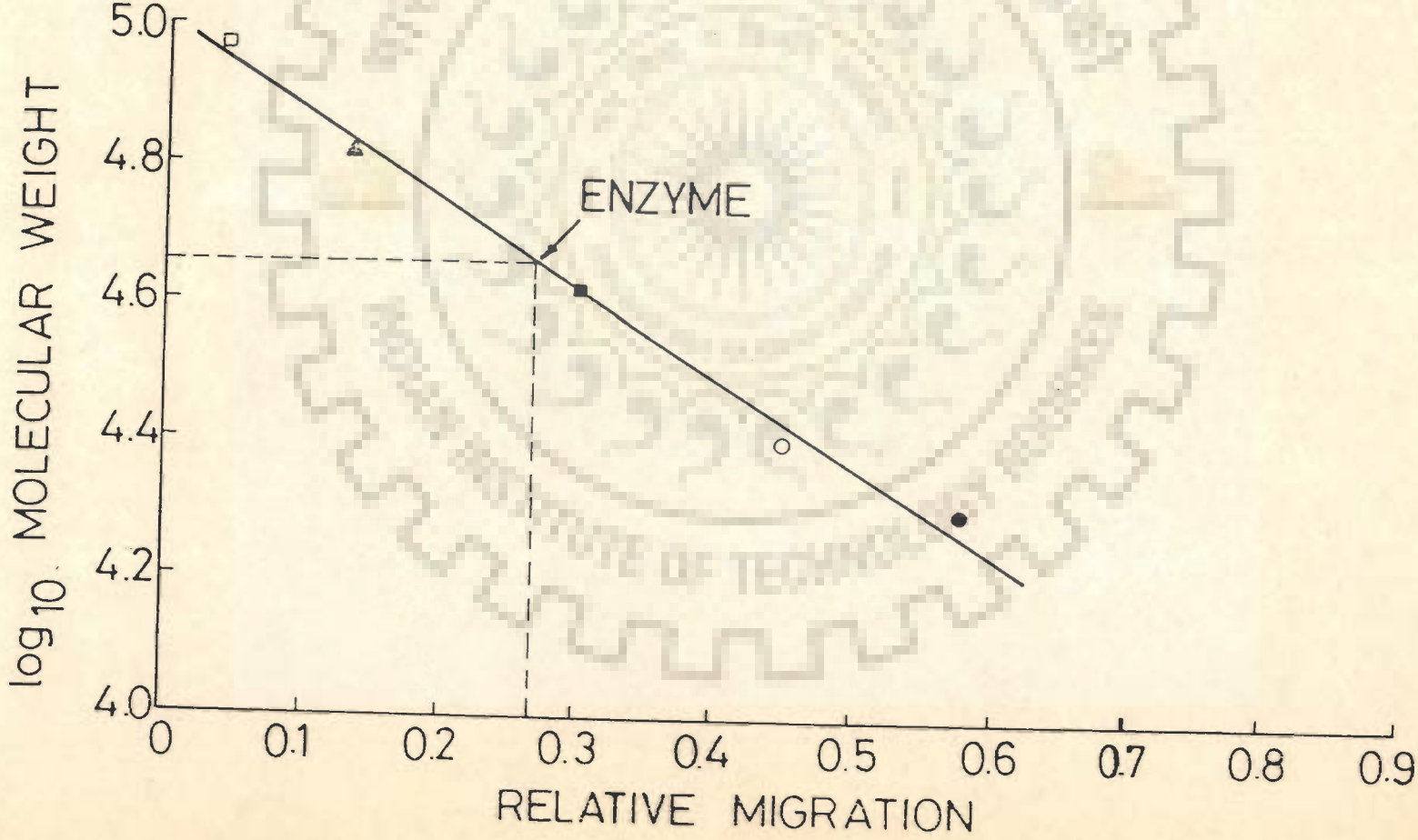

S.E
I
II
III
S.E
I
Std
M.W
94K
68K
43K
30K
21K
14.3K
ELECTROPHORESIS WITHOUT SDS
ELECTROPHORESIS WITH SDS

Fig. 27 Estimation of molecular weight of microsomal acid phosphatase by SDS-polyacrylamide gel electrophoresis. The standard proteins were treated in the same manner as sample protein was treated. Electrophoresis was carried out on 10% gel under dissociating conditions as described under Experimental procedures. Mobilities were determined relative of migration of bromophenol blue, as tracking dye, □ , phosphorylase b (M.W. 94,000); ▲, bovine serum albumin (M.W. 68,000); ■ , ovalbumin (M.W. 43,000); o, carbonic anhydrase (M.W. 30,000); and ●, soybean trypsin inhibitor (M.W. 21,000).


5.0
4.8
4.6
4.4
4.2
4.0
log10 MOLECULAR WEIGHT
ENZYME
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
RELATIVE MIGRATION

electrophoresis. This step of purification yielded a pure acid phosphatase preparation with 75 folds purification over the membrane bound enzyme with about 40% yield. Most of the characterization of the enzyme was done with this purified enzyme preparation.

4.6.6 Properties of purified acid phosphatase

4.6.6.1 Optimum pH

Figure 28 shows the optimum pH profiles of the membrane bound (Fig. 28A), solubilized (Fig. 28B) and purified (Fig. 28C) acid phosphatase. The membrane bound enzyme shows optimum pH 5.0 with a sharp decline in activity above this pH. However, the difference in the activity between pH 4.0 and 5.0 was rather small. Both solubilized and purified enzymes showed pH-optima curves sharply peaked at pH 5.0 and pH 4.75, respectively. In both the cases, the activity declines sharply above and below the pH-optima.

4.6.6.2 Kinetic and thermodynamic parameters

Figure 29 shows the Lineweaver-Burk plots at different temperatures. At reaction temperature ($30^{o}C$) the K_m and V_{max} values for the p-nitrophenyl phosphate were found to be 10 mM and 5.5 μmol/min, respectively. From these results it can be seen that both K_m and V_{max} values increase with increasing

Fig. 28 Effect of pH on the acid phosphatase activity. The enzyme activity was measured as described in assay under identical conditions except that of buffer and pH. Acetate buffer, pH 3.0 - 5.5 and Tris-HCl, pH 6.0 - 7.0 were used. Optimum pH of A) membrane bond, B) solubilized enzyme and C) purified enzyme.

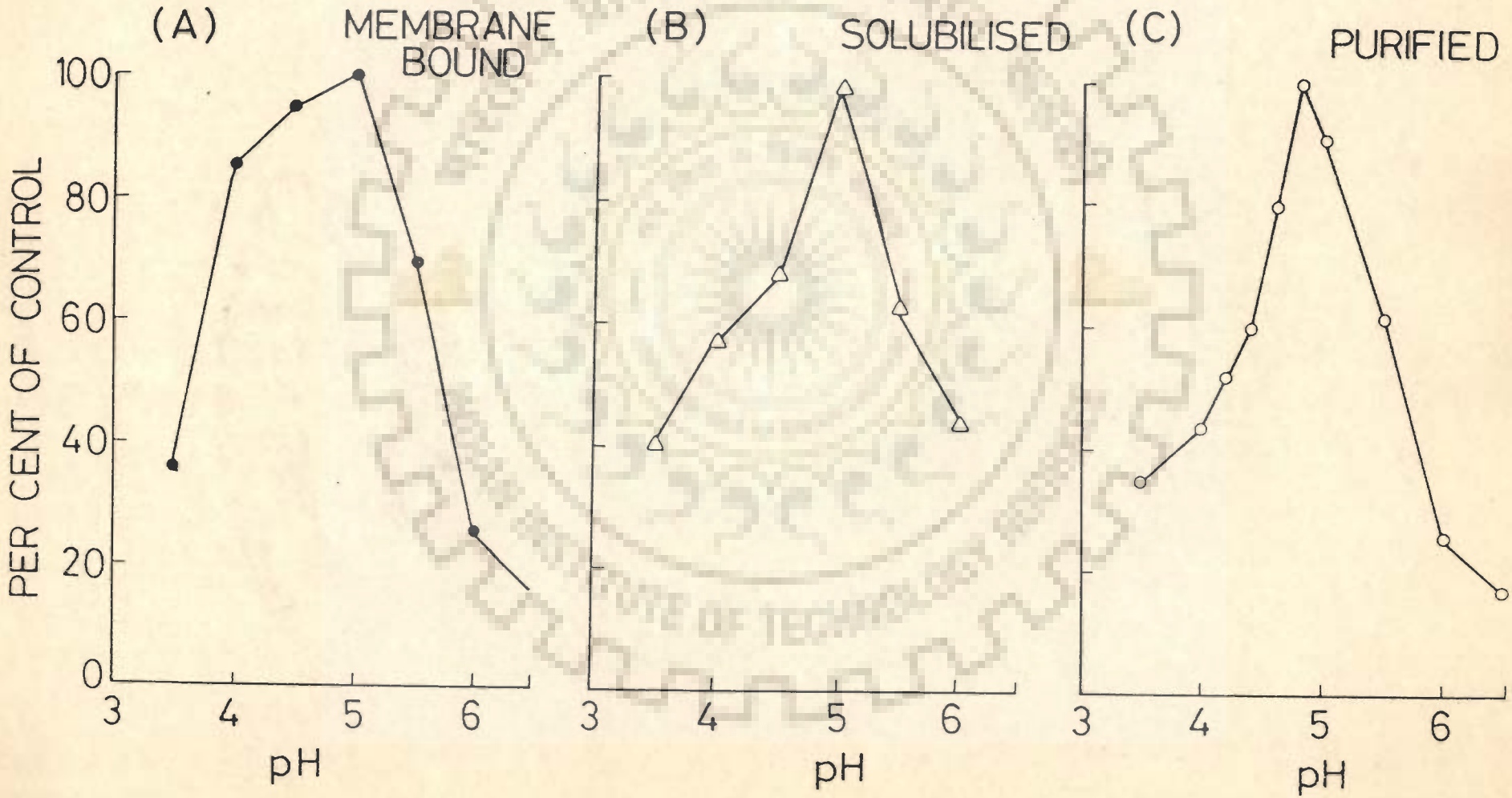
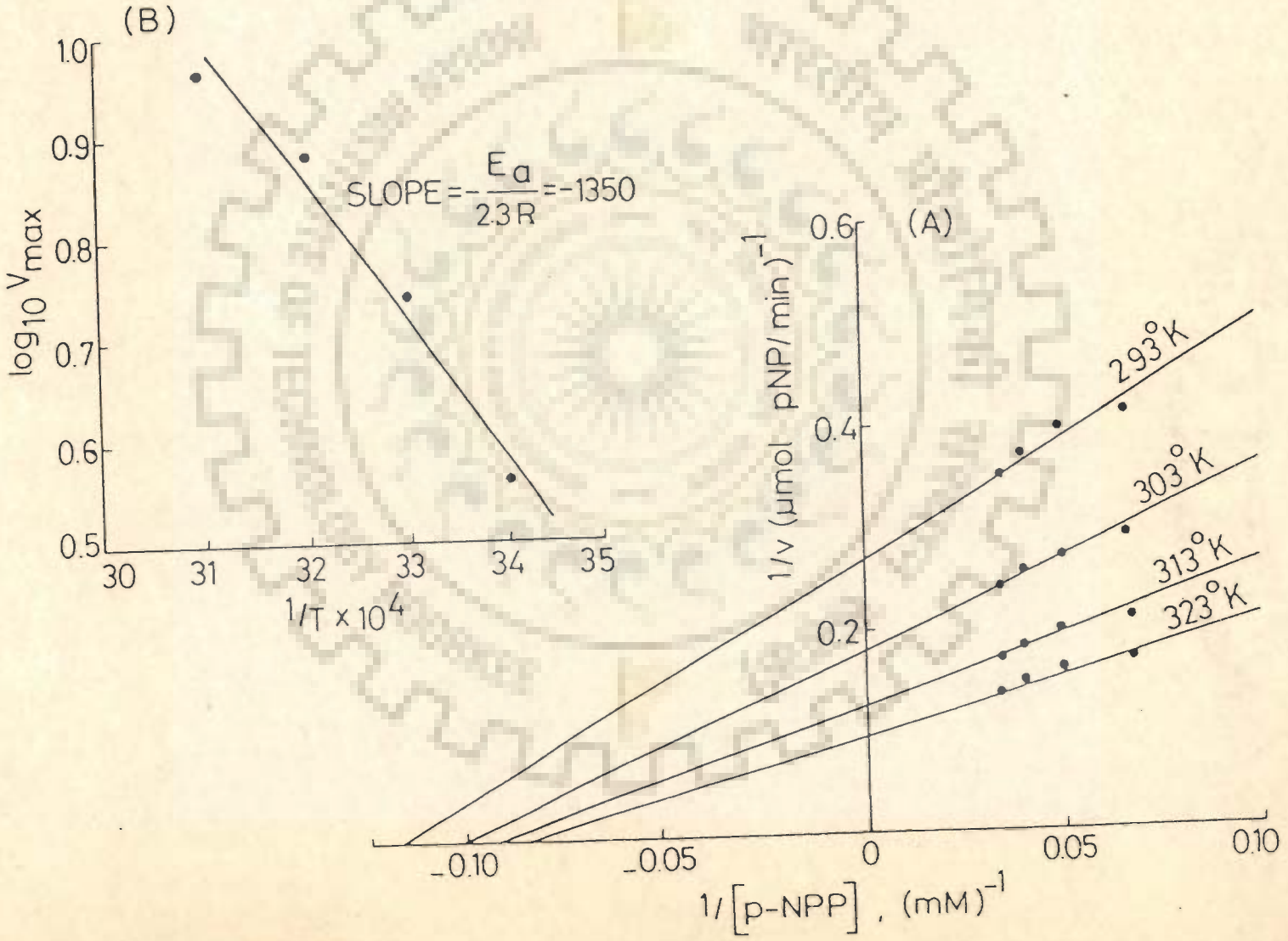

(A)
MEMBRANE BOUND
(B)
SOLUBILISED
(C)
PURIFIED
PER CENT OF CONTROL
100
80
60
40
20
0
3
4
5
6
pH

Fig. 29 A) Lineweaver-Burk plots of acid phosphatase at different temperatures. Enzyme was assayed at four different concentrations of p-NP (20-40 mM) under the standard assay conditions at four temperatures (20-50^{o}C). 1/v were plotted against 1/S at each temperature.

B) Arrhenius plot of acid phosphatase. $\log_{10} V_{max}$, obtained from the Figure 29A), was plotted against corresponding absolute temperature (^{o}K). Value of 'activation energy' was calculated from the slope of curve, which is equal to $-E_a/2.3R$.


(B)
1.0
0.9
0.8
0.7
0.6
0.5
$\log_{10} V_{max}$
SLOPE = $-\frac{E_a}{2.3R}$ = -1350
30
31
32
33
34
35
$1/T \times 10^4$
(A)
0.6
0.4
0.2
$1/v$ (µmol pNP/min)$^{-1}$
293°K
303°K
313°K
323°K
-0.10
-0.05
0
0.05
0.10
$1/[p\text{-NPP}]$, $(mM)^{-1}$

temperature between 20 to 50°C. A linear Arrhenius plot of 1/T versus $\log_{10} V_{max}$ (Fig. 29) was obtained with a slope of -1350. The E_a, as calculated from the slope was found to be 6155 Kcal/mol.

Assuming that K_m values at different temperatures represent the association constant for the formation of enzyme-substrate complex, the thermodynamic parameter ΔH, as computed from a plot of 1/T versus pK_m (Fig. 30), was found to be 4.25 Kcal/mol. The positive ΔH indicates that the reaction between acid phosphatase and p-nitrophenyl phosphate is endothermic.

Table XXII shows the thermodynamic parameters, ΔG and ΔS for the interaction between p-nitrophenyl phosphate and acid phosphatase at different temperatures. From the results, it can be seen that ΔG does not change significantly with temperature in the temperature range of 20°C - 50°C. The positive ΔG value once again indicates that the reaction is endogenic. Since, ΔS is a measure of the change in orderliness, the relatively low positive ΔS values suggest that unfolding of the polypeptide chain into a less ordered reaction has not occurred, that is no inactivation has taken place due to unfolding of the polypeptide chain.

Fig. 30 van't Hoff plot of acid phosphatase. Values of pK_m obtained from the Figure 29A) were plotted against reciprocals of corresponding absolute temperature. Slope of the curve is equal to $-\Delta H/2.3R$.

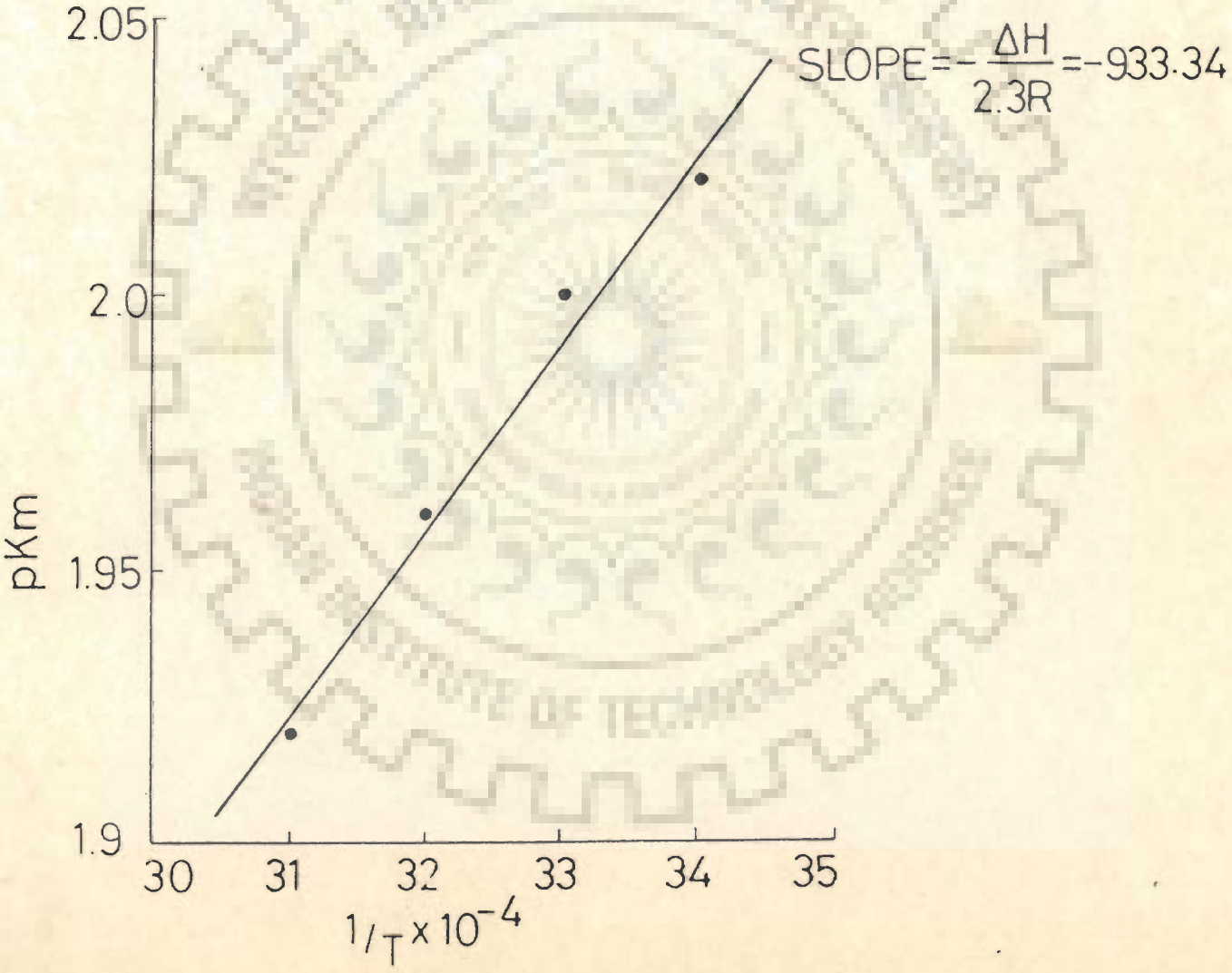

2.05
2.0
1.95
1.9
pKm
30
31
32
33
34
35
1/T x 10^-4
SLOPE = -ΔH/2.3R = -933.34

TABLE XXII THERMODYNAMIC PARAMETERS FOR THE INTERACTION BETWEEN p-NITROPHENYL PHOSPHATE AND ACID PHOSPHATASE FROM PEANUT COTYLEDONS AT DIFFERENT TEMPERATURES

Enthalpy change (ΔH) was calculated by van't Hoff plot; free energy change (ΔG) and entropy change (ΔS) were calculated by following equations :

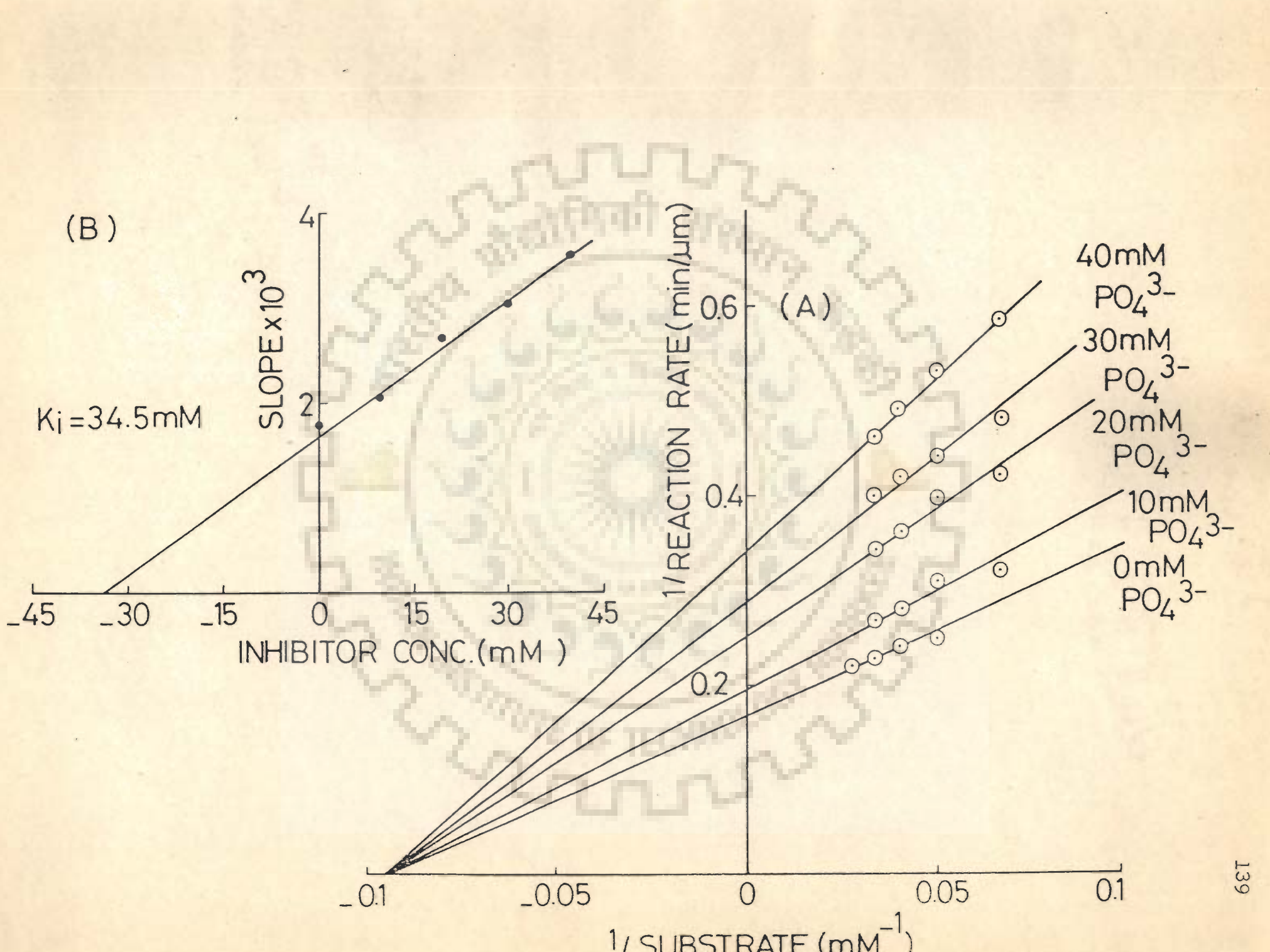
$\Delta G = -2.3RT \log_{10} K_m$ and $\Delta S = \frac{\Delta H - \Delta G}{T}$, respectively.

Temperature (^{o}C)	ΔG (Kcal/mol)	ΔS (Cal/deg/mol)
20	2.945	4.47
30	2.769	4.90
40	2.803	4.64
50	2.834	4.40

Fig. 31 Hill's plot for interaction of acid phosphatase with p-nitrophenyl phosphate. Curves were obtained by ploting $\log \frac{v}{V_{max} - v}$ against log[S] and the number of interacting substrate molecule per enzyme molecule was determined from the slope of curve. Intercept of curve on Y-axis, where log p-NPP = 0, is equal to $\log K_s$. n, interaction constant represents the slope of curve.

SLOPE =n=0.95
0.8
0.6
0.4
0.2
_2.0
_1.5
_1.0
0.5
0.0
$\log_{10}\left[\frac{v}{V_{max}-v}\right]$
$\log_{10}$ [pNP -P]

Fig. 32 Inhibition of p-nitrophenyl phosphate hydrolysis by inorganic phosphate. Enzyme assay was carried out using varying amounts of p-nitrophenyl phosphate in the presence of 0, 10, 20, 30 and 40 mM sodium monophosphate as described under Experimental procedures. (A) Lineweaver-Burk plots showing non-competitive type of inhibition (B) Replot of slopes of Lineweaver-Burk plots versus inhibitor concentration. The intercept on X-axis equals the value of $-K_i$.


(B)
Ki=34.5mM
SLOPE x 10^3
4
2
-45
-30
-15
0
15
30
45
INHIBITOR CONC.(mM)
(A)
1/REACTION RATE(min/μm)
0.6
0.4
0.2
40mM PO4 3-
30mM PO4 3-
20mM PO4 3-
10mM PO4 3-
0mM PO4 3-
-0.1
-0.05
0
0.05
0.1
1/SUBSTRATE (mM^-1)

4.6.6.3 Interaction constant

Figure 31 shows the double logarithmic plot of p-nitrophenyl phosphate concentration versus $(v/V_{max} - v)$. The linear plot with a slope of 0.95 shows that one mol of substrate interacts with one mol of enzyme to form an active enzyme-substrate complex and that the phenomenon of co-operativity is not present (136).

4.6.6.4 Inhibition by phosphate

The inhibition of acid phosphatase by ortho-phosphate (PO_4^{3-}) ion is shown in Fig. 32. The Lineweaver-Burk plots in the presence of different phosphate ion concentrations show a linear relationship, intersecting the x-axis at a common plot. From the pattern of Lineweaver-Burk plots it is apparent that the inhibition of acid phosphatase by phosphate ions is of the non-competitive type. Figure 32(B) shows the replot of slopes of Lineweaver-Burk plots in Figure 32(A) versus inhibitor concentration (phosphate ions), the intersection on x-axis gave the value of K_i 34.5 mM.

5.0 DISCUSSION

The present work describes the purification and properties of AMPase from the plasma membranes prepared from the germinating peanut cotyledons. This perhaps, is the first report that provides the purification of an AMP-specific 5'-nucleotidase from the purified plasma membrane fraction of a plant storage tissue. The purification described here is relatively simple involving three steps, namely preparation of high specific activity plasma membrane fraction, selective solubilization of AMPase activity by octylglucoside in presence of appropriate amount of Mg^{2+} and EDTA, and finally the DEAE-cellulose column chromatography.

The 7-days-old germinating peanut cotyledons provided the most suitable source for the preparation of high specific activity plasma membrane fraction as at this stage of germination not only the total activity but also the specific activity of the enzyme was maximum. The plasma membrane fraction, obtained by the method described, was of high purity as judged by the high specific activity of glucan synthetase II, a marker enzyme for the plant plasma membranes (63,131) and absence of glucan synthetase I activity, a marker enzyme for Golgi-apparatus (61). Since the plasma membrane fraction was prepared in the presence of EDTA-

without Mg^{2+} ions, the membrane fraction was also considered free from rough endoplasmic reticulum, a common contaminant of plasma membranes (63). Furthermore, presence of EDTA is also known to maintain the integral structure of plasma membranes (63).

The enzyme is tightly bound to the membrane and can only be released by treatment with detergents. The presence of Mg^{2+} and EDTA in the solubilizing medium was essential for the selective solubilization of the plasma membrane-bound AMPase, since these markedly suppressed the solubilization of membrane proteins except AMPase (Table IX). Although 35% solubilization of AMPase activity of plasma membrane may appear somewhat low, it is considered quite significant in view of the fact that the membrane fraction has a number of nonspecific phosphomonoesterases which as per one estimate accounts for as much as three-fourth of total phosphatase activity (26).

Generally, detergent treatment destroys the hydrophobic environment of membrane structure and releases the proteins in the hydrophilic medium. Surprisingly, this change in natural environment did not render the enzyme inactive. Obviously, some stabilizing factors, most probably phospholipids, were released from the plasma membranes along with the

solubilized AMPase in solution. Since the solubilized enzyme stays active even after removal of detergent by dialysis, the stabilizing factors may be of large molecular weight such as protein or protein-lipid complex. That stabilizing factors may indeed be present in the octylglucoside extract (unpurified solubilized AMPase) was indicated by the fact that the purified enzyme obtained from DEAE-cellulose column was rendered highly unstable, losing its total activity within 24 h at -20°C. As mentioned earlier the low enzyme recovery may largely be attributed to the removal of stabilizing factors during the purification step involving ion exchange chromatography on DEAE-cellulose.

The purified peanut cotyledon plasma membrane AMPase described here is comparable with the 5'-nucleotidase recently purified from the microsomal fraction of Zea mays seedling shoots (26) in regard to the following properties. It is a glycoprotein with 42.7% carbohydrate content, apparent molecular weight of 55 K, broad pH-optimum, and no effect of some common divalent and monovalent cations. However, there are some striking differences in the properties of the two enzymes, especially the subunit composition, stability and substrate specificity. For instance, on the basis of SDS-PAGE data the Zea mays microsomal enzyme (26)

has been reported to be composed of two subunits of molecular weights 24,500 and 25,500. On the contrary, the purified peanut cotyledon plasma membrane AMPase shows only one protein band corresponding to 55 K on SDS-PAGE under completely dissociated conditions indicating the presence of only single polypeptide unit. The Zea mays microsomal enzyme shows greater stability than the peanut plasma membrane AMPase. In fact, former is stable at -20°C for one month without any significant loss of activity, but the latter is rendered totally inactive within 24 h at -20°C. Finally, unlike other 5'-nucleotidases which also show sufficient hydrolytic activity towards other nucleotides (26, 47,157), the peanut enzyme is highly specific for 5'-AMP with no hydrolysis of other nucleoside phosphates or phosphorylated sugars tested (Table X). Thus, in view of the above results, the peanut cotyledon plasma membrane AMPase appears to be quite different from those found in Zea mays microsomes (26) and other plant sources (128,129). This is not unusual, however, as characteristics of enzyme vary from source to source.

Sullivan and Alpers (157) demonstrated the regulation of 5'-nucleotidase activity by adenosine nucleotides in rat heart and that ADP was a more potent

inhibitor than ATP. We also found that ADP is a strong competitive inhibitor of plasma membrane AMPase (K_i, 2.4 x 10^{-3}M). In agreement with the above it is suggested that ADP may be involved in the regulation of AMPase activity in plasma membranes of germinating peanut cotyledons and consequently helps in regulating adenine nucleotide (ATP + ADP + AMP) pool size to keep the ratio of ATP + ADP (high energy nucleotides) to the total adenine nucleotide pool size in the cell constant. The physiological function of plasma membrane AMPase in plants still remains to be studied, however.

It is generally accepted that proteins destined for export are synthesized on the rough endoplasmic reticulum (RER), vectorially discharged into the lumen of RER, translocated to Golgi in vesicles, move through various lamellae of the Golgi stack and finally are packaged in secretory vesicles for transport to plasma membranes (76). Since AMPase is found in plasma membranes, and it has also been shown to be present in microsomal fraction (26), it may serve as a model for studying the intracellular transport of macromolecules in plants.

It is now well established that the Golgi-membrane complex, functions in the intracellular

transport of macromolecules (117). It then follows that the AMPase, which is found to be associated with the plasma membranes of peanut cotyledons, could be present in the Golgi-apparatus. The results described in 4.4, clearly suggest that this, indeed, is the case. The approach used was as follow : firstly to prepare pure Golgi-apparatus fraction free from plasma membrane vesicles, and secondly to isolate pure enzyme and compare its properties with that of plasma membrane AMPase. The first step was achieved by precisely monitoring the sucrose density gradient as to separate the plasma membranes and ER fractions from the Golgi-bodies and by assaying the activities of glucan synthetase II and glucan synthetase I, the marker enzymes for plasma membranes and Golgi-apparatus, respectively. The Golgi-apparatus forms a band at the 37/25% (w/w) sucrose interface, corresponding to the bouyant density of 1.12-1.15 g/cm^3. These results were in agreement with those reported by Green (61). Virtually, no glucan synthetase II activity was detected in Golgi-apparatus (Table V) indicating further that the membrane fraction was free from the plasma membranes. We believe that the fraction obtained after purification contains all membranous elements of the Golgi-apparatus as no attempt was made to sub-fractionate the mixture of

elements that comprise the organelle. However, presence of some contamination of microsomes (ER) can not be completely ruled out, since relatively small activity of dolicholmonophosphate-GDP-mannose: mannosyl transferase and glucose-6-phosphatase were found in Golgi-apparatus band (Table V). The presence of relatively small amount of ER in the Golgi-apparatus is unlikely to make any difference in the overall conclusions drawn from the results regarding the possible mode of translocation of AMPase from the rough endoplasmic reticulum (RER) to the plasma membranes as the essential prerequisite for the above study is to have Golgi-apparatus free from plasma membranes.

The conditions of solubilization and scheme of purification of Golgi-apparatus AMPase were kept exactly the same as used in the case of plasma membranes in order to avoid any modification of the enzyme during the above mentioned steps. As expected the patterns of solubilization and elution from DEAE-cellulose of both Golgi-apparatus and plasma membrane AMPase were same except that third AMPase peak, which eluted at 155 mM NaCl gradient from the plasma membranes, was absent in the Golgi-apparatus. The presence of multiple forms of 5'-nucleotidase with low and high K_m values in microsomal membrane fraction

were also reported by Doss and Carraway (36). In addition kinetic properties, response to metal ions, inhibitors(NaF and ADP), optimum pH, electrophoretic mobility, stability, molecular weight and subunit structure were comparable (Table XIV). Minor difference was observed, however, in carbohydrate content. The difference in carbohydrate content, although small, is significant. This is not considered unusual as processing of carbohydrate moiety of glycoprotein also takes place in Golgi-apparatus (143,162). These results were interpreted to mean, but by no means prove, that the AMPase present in the Golgi-apparatus represent the same enzyme with slight modification as found in the plasma membranes.

The results of the endo-N-acetyl-β-D-glucosaminidase treatment of purified AMPase clearly show that oligosaccharide-peptide linkage is glucosaminyl-asparagine linkage. Thus, the enzyme appears to be a true glycoprotein. Many glycoproteins from various resources have mannose and N-acetylglucosamine-containing oligosaccharide chains (104). The structure of these sugar chains is such that the mannose is attached to an asparagine residue of the peptide via a chitobiose units (20). The plasma membrane AMPase reported here is similar in that the chains contain

only mannose and glucosamine. Since the carbohydrate content of purified glycoprotein AMPase is about 42%, it is most likely that several N-glycosidic linkages may be present per molecule of the enzyme. As pointed out earlier, more research work is needed to establish the oligosaccharide structure of the glycoprotein enzyme. Work in this direction is in progress.

The present study indicates that AMPase may provide a model system for studying the intracellular transport of macromolecules. Immunological studies using antibodies raised against the purified AMPase from both plasma membranes and Golgi-apparatus will further demonstrate the relationship between the Golgi-apparatus and plasma membrane AMPase.

Endoplasmic reticulum membrane fraction accounted for about 12% of membrane bound AMPase activity and it's solubilization resulted 60% yield. But the enzyme could not be purified because the solubilized enzyme becomes inactive in the absence of the detergent, octylglucoside. It seems that the change in environment, from hydrophobic to hydrophylic, on solubilization proves unfavourable for the enzyme. The assumption was supported by the fact that the enzyme activity of the dialyzed enzyme was partially (73%) recovered by octylglucoside suppliment and that this was not the

the case with the purified plasma membrane or Golgi-apparatus AMPase. Due to the inability to purify the ER-AMPase, comparison of the enzyme with that of plasma membrane and Golgi-AMPase was not possible.

The purpose to purify acid phosphatase was to characterize the peanut cotyledon microsomal acid phosphatase devoid of 5'-nucleotidase activity. One of the major difficulties was the separation of 5'-nucleotidase from the acid phosphatase present in the microsomes of plants since even the apparently purified 5'-nucleotidase preparations (homogeneous by PAGE) were found to show acid phosphatase activity (26). Our reports also support these findings and we attributed the acid phosphatase activity shown by purified AMPase to the non-specific action of AMPase towards p-nitrophenyl phosphate. The unequivocal answer may be provided by the immunological studies using antibodies raised against the purified acid phosphatase devoid of 5'-nucleotidase activity. The present study will be useful in this regard. The question, however, is that whether or not the acid phosphatase devoid of AMPase activity was present in the microsomal fractions. The results described showed that the microsomal membrane fraction, prepared either from the dormant peanut seeds or from 48 h germinating

cotyledons, contained no 5'-nucleotidase activity but were rich in acid phosphatase activity. Thus, we used microsomal membrane fraction of 2 days old germinating peanut cotyledons as the starting material for the isolation of an acid phosphatase which was devoid of 5'-nucleotidase activity.

The acid phosphatase activity was selectively solubilized with the help of octylglucoside in the presence of Mg^{2+} and EDTA. The detergent extract contained solubilized enzyme in the stable form. The removal of detergent by dialysis did not result in any inactivation of the enzyme.

There are certain properties which distinguish the acid phosphatase from 5'-nucleotidase. For instance, 5'-nucleotidase was not inhibited by phosphate ions whereas the acid phosphatase like any other phosphatase (125) was inhibited. Another striking difference was in the pH-optima of acid phosphatase and AMPase. The former showed a sharp optimum pH at pH 5.0 and there was sharp decline in activity with slight change in pH while AMPase had a broad pH range of 5 to 6.

In most of the cases, the acid phosphatase inhibition by orthophosphate ions is of the competitive type. In contrast, we found that the peanut

microsomal acid phosphatase was inhibited non-competitively by PO_4^{3-}. At the moment, it is not certain if the non-competitive type of inhibition of acid phosphatase by phosphate ions is a characteristic property of the microsomal enzyme.

The function of membrane-bound acid phosphatase in plants is not clear. The non-specific acid phosphatases present in the cytosol are known to be involved in the mobilization of stored phosphorus from germinating cotyledons to other anatomical parts of the plants for its early use and growth (64b). In corn roots it was shown that phosphatases probably hydrolyze and solubilize organic soil macromolecules (28). What role microsomal acid phosphatases play, is still a matter of speculation. The inhibition of enzyme by phosphate ions suggests that the level of inorganic phosphate may be involved in the regulation of non-specific acid phosphatase activity. Further studies would be necessary to understand the role of the microsomal acid phosphatase in seed metabolism.

6.0 SUMMARY AND CONCLUSIONS

6.1 Membrane-bound glycoprotein enzymes, phosphohydrolases

The membrane-bound glycoprotein enzymes, 5'-nucleotidase (5'-nucleotide phosphohydrolase, EC 3.1.3.5) and acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2), of peanut cotyledons have been investigated with a view to characterize them, understand their physiological function and phenomenon of intracellular transport of proteins in plant cells. The main approach adopted involved the following : (i) preparation of different membrane fractions (plasma membrane, PM; Golgi-apparatus, GA; and endoplasmic reticulum, ER) in highly enriched form, (ii) solubilization and purification of the membrane-bound glycoprotein enzymes, 5'-nucleotidase and acid phosphatase, and (iii) characterization and comparison of properties of the enzyme present in different membrane fractions.

6.1.1 Membrane-bound 5'-nucleotidase

The changes in the level of the activity of membrane-bound 5'-nucleotidase of peanut cotyledons as a function of germination period indicated that the enzyme emerged only after 48 h of imbibition of seeds at 35°C. Following this dormant period the total and

specific activities of the adenosine 5'-monophosphatase (AMPase) increased rapidly reaching a maximum level on 7th day of germination. At this physiological stage nearly one-fourth of the total AMPase activity was associated with the 12,000 - 105,000 x g pellet containing PM, GA and ER.

The highly enriched PM, GA and ER fractions of peanut cotyledons were obtained by using sucrose density gradient centrifugation together with specific marker enzymes. As judged by the level of activity of various marker enzymes in different membrane fractions, the PM was found to be free from GA and ER contamination. GA fraction, however, seemed to contain some ER, but was free from the PM contamination. Of the total membrane-bound AMPase, the PM, GA and ER accounted for 58.3, 23.6 and 12.4 per cent, respectively.

Extraction of the PM, GA and ER fractions with a detergent buffer containing 0.5% octylglucoside (corresponding to protein to detergent ratio of 2:3), 50 mM Tris-HCl, pH 7.2, 20 mM $MgCl_2$, 5 mM EDTA, 250 mM sucrose and 0.04% β-mercaptoethanol solubilized about 35, 32 and 60% of the AMPase activity, respectively. Other detergents, namely Triton X-100, Nonidet P-40, deoxycholate and taurocholate were found unsatisfactory. The presence of Mg^{2+} and EDTA was essential

for the selective solubilization of AMPase. The specific activity of the octylglucoside solubilized AMPase from PM, GA and ER fractions increased 6.15, 3.0 and 5.1 folds, respectively. The solubilized enzyme from the PM and GA were stable for one month at -20°C and the detergent could be removed by dialysis without any significant loss of enzyme activity. In contrast, however, the solubilized enzyme from ER was rendered inactive on removal of detergent by dialysis and could not be purified further, therefore.

The solubilized AMPase was purified further by DEAE-cellulose chromatography. The PM and GA enzymes were separated in three and two isoenzymes of AMPase, respectively. The major peak (peak I) containing AMPase, which eluted at 50 mM salt concentration in both cases, was found to be homogeneous by SDS-gel electrophoresis. The purification of the PM-AMPase and GA-AMPase was 26.9 and 6.8 folds, relative to the crude membrane fraction, respectively. The actual purification fold will be more since the crude membrane fraction contains large amount of other non-specific phosphatases. This is perhaps the first report on the purification of an AMP-specific 5'-nucleotidase from the PM and GA of plant cotyledons.

The purified PM-AMPase showed a broad optimum pH of 5 to 6. The K_m value of the enzyme for AMP was found to be 1.0×10^{-3}M with a V_{max} of 8.47 µmol P_i/min/mg protein. Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Hg^{2+}, Li^{2+} and K^{+} ions had no effect on the activity of AMPase. The enzyme was inhibited by ADP and NaF in a competitive and non-competitive manner with K_i values of 2.4 mM and 35 mM, respectively. Interestingly, unlike acid phosphatase, the AMPase was not inhibited by orthophosphate.

The enzyme was highly specific for AMP. Other nucleoside 5'-phosphates and phosphorylated sugars were not hydrolyzed. p-Nitrophenyl phosphate was hydrolyzed but only to a very small extent (15%). The K_m value for p-NPP was nearly ten times higher than that of AMP, indicating that the former was a very poor substrate for purified AMPase.

The purified enzyme was found to be highly unstable losing its total activity within 24 h at -20°C. In contrast, the membrane-bound as well as the octylglucoside solubilized enzyme were quite stable at -20°C. These results suggested that some stabilizing factors, most likely phospholipids, were present both in the membranes and the solubilized enzyme, which were removed during purification on DEAE-cellulose column.

The molecular weight of the enzyme, as determined by SDS.-gel electrophoresis under completely dissociating conditions, was estimated to be 55 K. Unlike other 5'-nucleotidases, the enzyme appeared to be composed of a single polypeptide chain.

The plasma membrane AMPase is a glycoprotein containing 42.7% (w/w) carbohydrate. The qualitative analysis of the purified glycoprotein enzyme carbohydrates by HPLC showed mannose, N-acetylglucosamine and glucose to be the main component monosaccharides of the oligosaccharide moiety indicating that the latter is of the high mannose type. The removal of the major carbohydrate content from the glycoprotein by endo-H enzyme suggested the presence of the N-glycosidic linkage(s) between the amide nitrogen of the asparagine residue(s) and C_1-OH group of N-acetylglucosamine.

The properties of the purified GA-AMPase resembled very closely to that of the PM-AMPase with only slight differences. For instance, the molecular weight of GA enzyme was found to be 53.7 K, the carbohydrate content was 38.46% and the optimum pH was 5.0 to 5.5. The K_m values of the two were also comparable. However, the V_{max} value of plasma membrane AMPase was two times that of Golgi-AMPase. Although, the above results were consistent, at the moment it is difficult

to say if these are significant enough to represent any modification in the enzyme during its intracellular transport.

The ER-AMPase could not be purified since it was rendered inactive during dialysis. The partially purified enzyme showed two pH optima of pH 4.5 and 6.0. The values of K_m and V_{max} were found to be 10.0 x 10^{-4} M and 232 µmol P_i/min/mg protein, respectively. The activity of the dialyzed ER-AMPase was partially restored by the addition of octylglucoside to a final concentration of 0.4%.

It is suggested that the AMPase may be used as a model surface glycoprotein for studying the intracellular transport in plant systems since it is found in ER, GA and PM.

6.1.2 Membrane-bound acid phosphatase

Besides AMPase, the membrane fractions obtained from 7-days old germinating cotyledons also contained high amount of acid phosphatase activity. However, it was observed that while the membrane fraction (12,000 - 105,000 x g pellet) from 2 days germinating cotyledons exhibited sufficiently high (40% of the maximum) acid phosphatase activity, the AMPase activity was completely absent from the membrane fraction.

The membrane fraction from 2 days old germinating cotyledons was, therefore, used for the solubilization, purification and characterization of the membrane-bound acid phosphatase. The enzyme purification was achieved by solubilization with octylglucoside followed by ion exchange chromatography on DEAE-cellulose. Three isoenzymes of acid phosphatase (APase I, APase II and APase III) were obtained from the DEAE-cellulose column at 50, 100 and 200 mM, representing 58, 11.9 and 7.1% of the total acid phosphatase activity in the crude membrane fraction. The purification folds of APase I, II and III were 69.4, 26.5 and 16.1, respectively. Of the three isoenzymes, APase I was found to be relatively pure giving a major protein band with electrophoretic mobility of 0.23 relative to bromophenol blue along with two minor bands. On SDS-PAGE the major band was splitted into two bands, one major and one minor, corresponding to molecular weights of 46.7 K and 50.1 K. Pure APase I was obtained by preparative gel electrophoresis.

The optimum pH, K_m and V_{max} values for the hydrolysis of p-nitrophenyl phosphate were found to be 4.75, 10 mM and 5.5 μmol/min, respectively. Orthophosphate inhibited the enzyme non-competitively with K_i of 34.5 mM. The energy of activation (E_a), inter-

action constant (n), free energy change (ΔG), entropy change (ΔS) and enthalpy change (ΔH), for the enzyme-p-nitrophenyl phosphate interaction were found to be 6155 Kcal/mol, 0.95, 2.77 Kcal/mol, 4.9 cal/deg and 4.25 Kcal/mol, respectively. The enzyme is a glycoprotein containing 40 $\pm$ 3% carbohydrate.

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Ref.No. RP G 009581 Amsterdam, 17 September, 1986

Studies of plant membrane 5'-nucleotidase : purification and properties of a glycoprotein adenosine 5'-monophosphatase from the plasma membr
by Sharma CB Mittal R Tanner W

Dear author,

We are pleased to inform you that the above-mentioned paper is acceptable for publication in Biochimica et Biophysica Acta. The typescript has been forwarded to the Publishers and will be included in the section devoted to General Subjects.

Please quote BBA 22393 in all future correspondence concerning this paper.

Please note that, for administrative reasons, the above order of authors is not necessarily identical to that to be published. Please check that the correct order appears on your proofs.

Yours sincerely,

Editorial Secretariat.

P.S.

We have omitted the first part of the title as it does not appear to be essential and the remaining title is sufficiently descriptive. We hope that you agree with this change.

We enclose the comments of one of the original referees. This point can be considered at proof stage.

BIOCHIMICA ET BIOPHYSICA ACTA

Editorial Secretariat
P.O. Box 1345
Amsterdam, The Netherlands

A point which I overlooked before concerns the statement on p 9, 1st sentence: "The specific activity of AMPase ....." This apparently is meant to be a definition of a unit of activity rather than a statement of the specific activity, as it does not correspond to any of the specific activities reported in the paper. In any case, it seems superfluous.

8.2

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
BANARAS HINDU UNIVERSITY

VARANASI 221005

Dated 2/9/81

International Seminar

on

Structure and Function of Enzymes

Dear Dr. --Sharma and Mittal--

Ref. Your paper entitled

"Studies on acid phosphate of plant membranes: Solubilization, partial purification and characterization of an acid phosphatase devoid of 5'-Nucleotidase activity from the Microsomal Membrane Fraction of peanut cotyledons"

1. It has been reviewed and accepted for oral/poster presentation in the above Seminar. The date and time of this presentation will be announced later. We are making arrangements for your board and lodging in the university campus. T.A. (I class rail fare both ways) will be paid to you as per U.G.C. rules if you are not getting it from any other source.
The registration fee will be payable by you/your institution (Rs. 500/-; U.S. $ 50.- for participants from outside India)
Kindly follow the enclosed guidelines for further preparation.
We look forward to seeing you at the Seminar.
Time for oral presentation 35 minutes.

X 2. It has been reviewed and not accepted for presentation.

With kind regards.

Yours sincerely,

O.P. Malhotra

(O.P. Malhotra)

RS Dubey

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8.3 SOCIETY OF BIOLOGICAL CHEMISTS (India)

53rd ANNUAL MEETING

OCTOBER 12—14, 1984

ABSTRACTS

ORGANIZED BY

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UNIVERSITY OF DELHI

AND

C.S.I.R. CENTRE FOR BIOCHEMICALS,
UNIVERSITY CAMPUS, DELHI

maximum of 8 bands appeared at a particular stage. A slow moving isozyme-band no. 9 was characteristic to only Lr isogenic lines which appeared at 48 hr stage and in succeeding germination stages. PPO revealed seven band but no characteristic isozymic pattern could be associated with Lr genes or disease resistance. Different isolines with Lr genes were characterised by combined patterns of POD and PPO. RNase1 revealed a total of 9 bands and appearence and disappearence of bands were recorded as germination advanced. No particular isozyme(s) could be clearly associated with disease resistance but one ciritical band at the 48 hr stage was present only in some isogenic lines having most potent Lr genes. RNase II and Nuclease I combined showed 7 bands at different stages. The isozymic pattern in different isogenic lines was same and did not show any relationship with leaf rust resistance.

427. Purification and characterization of constitutively expressed metallothionein in Neonatal chicken liver.

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Metallothioneins (MTs) are inducible in the liver and kidney of all animals. The high concentration MT in uninduced fetal or neonatal mammalian livers has been reported recently. We are using chicken system to study the development and differentiation related changes of MTs. MTs were purified both from $CdCl_2$ injected adult and uninduced 3 day neonatal livers of white leghorn chickens. Both yielded a homogeneous protein like MTs of identical behaviour. In 7.5% native polyacrylamide gel electrophoresis (PAGE) their migration were identical to mouse hepatic MT-I. The naturally occuring neonatal protein was *in vitro* incubated with $^{109}CdCl_2$ and run on 7.5% native PAGE. The autoradiogram revealed Cd-binding property of this protein. UV-absorption spectrum of the neonatal protein showed a peak at 254 nm which abolishes on acidification. This was another criterion of all animal MTs. Finally, this neonatal protein crossreacted with mouse MT-I antisera. This neonatal MT was absent in newly laid undeveloped eggs (both egg white and egg yolk fractions) and its level gradually diminished during maturation of the liver, ultimately disappearing from one month old chickens. This is the first evidence of a non-mammalian neonatal naturally occuring MT.

428. Interaction of colchicine and its B-ring analogues in Zn^{2+} induced polymerization of tubulin.

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At low Zn^{2++}/tubulin ratio (2.7), tubulin assembly is substoichiometrically inhibited by colchicine, whereas at high Zn^{2++}/tubulin ratio (9.3) colchicine tubulin complex itself can polymerize. At the same cation/tubulin ratio, activation energy of colchicinetubulin complex assembly is greater than that for tubulin. Binding of colchicine to tubulin decreases the affinity of the assembly. Three biologically active B-ring analogues of colchicine can also inhibit the Zn^{2++} induced assembly of tubulin, but these analogues-tubulin complexes can polymerize at less cation/tubulin ratio than that for colchicine-tubulin complex and the affinity of these analogues-tubulin complex assembly is greater than that for colchicine-tubulin complex. Thus B-ring of colchicine plays a role in the colchicine-tubulin complex.

429. Solubilization, partial purification and characterization of adenosine monophosphatase isoenzymes from a mirosomal membrane fraction of arachis hypogea cotyledons.

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The microsomal membrane fraction from 7-day-old germinating peanut cotyledons were found to contain three isoenzymes of adenosine-5-monophosphatase, AMPase I, II and III. These isoenzymes were partially purified by selective solubilization with 0.5 per cent octyl-β-D-glucopyranoside at a protein-detergent ratio of 2/3 in presence of Mg^{2++} and EDTA, followed by dialysis and chromatography on DEAE-cellulose. The specific activity of enzyme I, II and III increased about

18, 12 and 9 folds, respectively. The molecular weight of the isoenzymes are approximately 17 500, 19 500 18 600 daltons, respectively. They exhibit broad pH-optimum of 3 to 5 pH range. The partially purified enzyme fractions are highly unstable, losing total activity within 12 hours at 4°C, while at this temperture both membrane bound and the solubilized enzymes are stable for several days without any significant loss of activity. The isoenzymes show high specificity for AMP with no activity towards ATP, GTP, UTP, ADP, GDP, GMP, UMP and glucose-6-phosphate. The role of these isoenzymes in cell proliferation has been envisaged.

430. Purification and properties of a novel phytase isoenzyme from cucurbita maxima cotyledons.

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A noval phytase isoenzyme (EC 3.1.3.8) has been isolated and purified to homogeniety from germinating cotyledons of *Cucurbita maxima* by acetone extraction, $(NH_4)_2SO_4$ precipitation, gel filtration and ion exchange chromatography on DEAE and CM-cellulose. The enzyme was eluted from the CM-cellulose column by a high salt gradient (0.7 M NaCl) and has a molecular weight of 40,000 daltons. Its electrophoretic mobility relative to bromophenol blue, at pH 8.3 is 0.61. The values of pH-optimum and apparent Km for myoinositol hexaphosphate were found to be 4.4, and 6×10^{-4}M, respectively. The enzyme is strongly inhibited by Hg^{++}, Cd^{2+}, F^- and PO^{3-} ions with Ki values 15, 14, 3.5 and 14 mM, respectively. It exhibits a broad specificity towards phosphate ester substrates including ATP, and AMP. But a comparision of the kinetic data, Vmax/Km for various substrates including myoinositol poly-phosphates produced during the phytate hydrolysis, shows that the myoinositol pentaphosphate is the best substrate for the enzyme. Its important role in mobilization of phytate-phosphorous during early stage of germination is suggested.

431. Multiple molecular forms of arginase frog tissues

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Frog (*Rana tigrina*) liver and kidney extracts we chromatographed on DEAE-cellulose columns by line KCl gradient (0-0.3M) elution. The fractions showe two peaks of arginase activity suggesting the presence two forms of arginase in these tissues. The two iso enzymes of arginase from both tissues were similar molecular weights as determined by gel filtration (kidne 29,000 ± 0; liver, 28,000 ± 1200). When whole tissu extracts (18-23 mg protein/ml) were subjected to g filtration on Sephadex G-200, a single peak of arginas activity was found in both tissues corresponding molecular weights of 104,000 ± 17,000 in kidney an 82,600 ± 4,900 in liver. Dilution of both tissue extrac yielded lower molecular weight species of arginas suggesting the dissociation of the enzyme into dime (kidney, 56,000 ± 6,900, liver, 45,000 & 35,500) an probably monomers (kidney, 28,000—3,800; live 29,000 ± 1,600) at low protein concentrations. Th molecular weights of arginase isoenzymes reporte above probably represent those of dimers. Furth studies on the kinetic and immunological characteriz tion of the multiple molecular forms of arginase in fr tissues are in progress.

432. Properties of immobilized invertase fro *Aspergillus athicus*

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A fungal strain isolated from a soil sample, identifi as *Aspergillus athicus* was found to produce large quan ties of extracellular invertase when grown on whe brain. The invertase was immobilized using DEA cellulose as the matrix. Various kinetic parameters we determined using both bound and free enzyme. T free enzyme had a pH optimum at 5.7 whereas the bou enzyme showed maximum activity at pH 4.8. Althou