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A KINETIC STUDY OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE PHOSPHOMONOESTERASE IN WHEAT LEAVES

A Thesis

Presented to

the Faculty of Graduate Studies and Research

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Chee-kok Chin

August, 1969

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ABSTRACT

An acid phosphomonoesterase which hydrolysed nicotinamide adenine dinucleotide phosphate was isolated and purified ca. 50-fold from wheat leaves (Triticum aestivum L. var. Selkirk). The enzyme was not specific but hydrolysed nicotinamide adenine dinucleotide phosphate preferentially. It had optimum activity at pH 5.9 and energy of activation of 29,000 cal/mole. It was activated by ethylenediaminotetraacetic acid (EDTA). Kinetic studies showed that the reaction mechanism to be Ping Pong Bi Bi (Cleland, Biochim. Biophys. Acta 67: 104, 1963). K_{NADP} was calculated as 1.4×10^{-4}M. Nicotinamide adenine dinucleotide phosphate at concentrations above 5×10^{-4}M showed inhibition. The product orthophosphate was a competitive inhibitor with $K_{i(Pi)} = 1.7 \times 10^{-4}$M. No enzyme activity was found in the chloroplasts.

Treatment with kinins increased the enzyme level ca. 20% whereas nicotinic acid and NAD treatment decreased the enzyme level ca. 20%.

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ABBREVIATIONS

AMP	- adenosine monophosphate
ADP	- adenosine diphosphate
ATP	- adenosine triphosphate
NMN	- nicotinamide mononucleotide
NAD	- nicotinamide adenine dinucleotide
NADP	- nicotinamide adenine dinucleotide phosphate
EDTA	- ethylenediaminotetraacetic acid
MES	- 2-(N-morpholine) ethane sulphonic acid
HEPES	- N-2-hydroxyethypiperazine-N'-2-ethane sulphonic acid
Pi	- inorganic orthophosphate
Tris	- Tris (hydroxymethyl) aminomethane
Acid phosphatase	- orthophosphoric monoester phosphohydrolase (E.C. No. 3.1.3.2)

All the abbreviations of the kinetic constants are after Cleland (1963a).

INTRODUCTION

Pyridine nucleotide coenzymes NAD and NADP are important hydrogen carriers in living cells. Though these two coenzymes have similar structure and perform a similar function, in most cases they have different specificity. For example, the important photosynthesis enzyme photosynthetic pyridine nucleotide reductase (E.C. No. 1.98.1.1) isolated by San Pietro and Lang (1958) reduces only NADP. But many other enzymes, like the dehydrogenases and oxidases found in glycolysis and in the tricarboxylic cycle require exclusively NAD as coenzyme.

Mishra and Waygood (1968) reported that wheat leaves floated on benzimidazole and kinetin, which could delay senescence, showed a marked increase in NADP content as well as in the NADP/NAD ratio.

In incorporating into the excised wheat leaves radioactive precursors of NAD such as nicotinic acid (carboxyl ^{14}C) or nicotinamide (carbonyl ^{14}C) and then floating the leaves on water or cytokinins, Waygood *et al* (1969) observed that benzimidazole treatment favoured an accumulation of radioactivity in NADP. Wheat leaves floated on water accumulated most of the radioactivity in NAD.

These studies suggest that the senescense of detached wheat leaves and the effect of cytokinins on senescense

are directly or indirectly connected to the ratio of the concentrations of NAD and NADP in wheat leaves.

NAD kinase (E.C. No. 2.7.1.23) which phosphorylates NAD to NADP (Kornberg, 1950) and the NADP-phosphatase (E.C. No.3.1.3.2) which hydrolyses NADP to NAD (Kornberg and Pricer, 1950 and Forti et al, 1962) appear to play important roles in regulating the ratio of NAD to NADP.

In this investigation the NADP-phosphatase was purified and the kinetics, specificity and the intracellar localization of the enzyme were studied.

The effect of kinetin and benzimidazole on the activity of the enzyme was tested. The changes in NADP-phosphatase levels in wheat leaves treated with benzimidazole and kinetin were also studied.

REVIEW OF LITERATURE

Three enzymes have been reported to be able to break down NADP; (1) nucleotide pyrophosphatase (Kornberg, 1950), (2) NAD(P) glycohydrolase (Zatman et al, 1953) and (3) NADP-phosphatase (Kornberg, 1950).

(1) Nucleotide pyrophosphatase

Nucleotide pyrophosphatase (E.C. No. 3.6.1.9.) was first purified by Kornberg from potato. It catalyses the reaction:

$$NADP + H_2O \rightarrow NMN + ADP \text{ (adenosine 2',5' diphosphate)}$$

This enzyme is not specific. It also catalyses the hydrolysis of the pyrophosphate link in NAD and coenzyme A (Kornberg, 1950).

(2) NAD(P) glycohydrolase

NAD(P) glycohydrolase (E.C. No.3.2.2.6.) catalyses the hydrolysis of NAD(P) with the liberation of free nicotinamide by cleavage of the nicotinamide-ribose linkage:

$$NAD(P) + H_2O \rightarrow \text{Nicotinamide} + \underset{(P)}{R\text{-}P\text{-}P\text{-}R\text{-}A}$$

This enzyme also catalyses the transfer of the adenosine diphosphate ribose from the one pyridine group to another.

$$NAD(P) + X \rightarrow \text{Nicotinamide} + \underset{(P)}{X^{+}R\text{-}P\text{-}P\text{-}R\text{-}A}$$

X may be a pyridine compound related to nicotinamide (Kaplan and Ciotti, 1956) or an imidazole (Alivasatos et al, 1962; and Kapoor and Waygood, 1964).

This enzyme was first isolated from mycelia of a Neurospora (Nason et al, 1951). Kapoor and Waygood (1964) reported the presence of this enzyme in wheat embryos.

NAD(P) glycohydrolase acts on both NAD and NADP but not on reduced forms.

(3) NADP-phosphatase

Kornberg (1950) observed that in the crude extract of potato there was a phosphatase capable of breaking down NADP to NAD. Nakamoto (1960) reported the hydrolysis of NADPH by an alkaline phosphatase. Kuo and Blumenthal (1961) purified a non-specific phosphomonoesterase from a Neurospora which showed a low phosphatase activity against NADP.

Forti et al (1962) purified a non-specific acid phosphatase (E.C. No.3.1.3.2.) from pea leaves. As this phosphatase preferentially attacked NADP but not the usual substrates of phosphatase, such as glucose-6-phosphate and p-glycerophosphate, Forti et al suggested that it was not the common acid phosphomonoesterase.

Specificity of Acid Phosphatase

Acid phosphatase has been reported to attack a wide range of substrates. Though phosphatase is generally non-

specific, more than one phosphatase has been reported to exist in one source. Roberts (1956) studied the heat stability of the phosphatase activity of wheat juice towards 16 substrates and found that there was more than one acid phosphatase. By the inhibition data he (1957 and 1963) concluded that the acid phosphatase activity of wheat leaves was caused by a group of several distinct enzymes with quite narrow substrate specificities. The existence of more than one acid phosphatase in one source has also been studied by Tsuboi et al (1957), Shinoda (1964), Igarashi and Hollander (1968) and others. Tsuboi et al (1957) have shown that the phosphatase preparation from yeast could be separated by electrophoresis analysis into at least four distinguishable compounds, each possessing closely related catalytic properties. The denaturation data from surface inactivation studies as well as thermal effects also support the evidence that this enzyme preparation consists of a number of similar, but distinct components.

The non-specificity of acid phosphatase need not be due to the existence of a heterogenous enzyme population. Non-specificity of the phosphatase has been reported for highly purified enzyme preparations (Gordon, 1968, and Igarashi and Hollander, 1968). A highly purified acid phosphatase does not hydrolyse different substrates at the same rate. It always attacks one or several substrates

preferentially (Kuo and Blumenthal, 1961, Forti et al, 1962, and Gordon, 1968).

Effect of Kinetin on the Acid Phosphatase Enzymes

Spencer (1968) studied the effect of kinetin on the growth and acid phosphatase production of nucleated and enucleated Acetabularia mediterranea. He found that though the kinetin increased the growth rate, the synthesis of acid phosphatase was only slightly affected by the presence of kinetin.

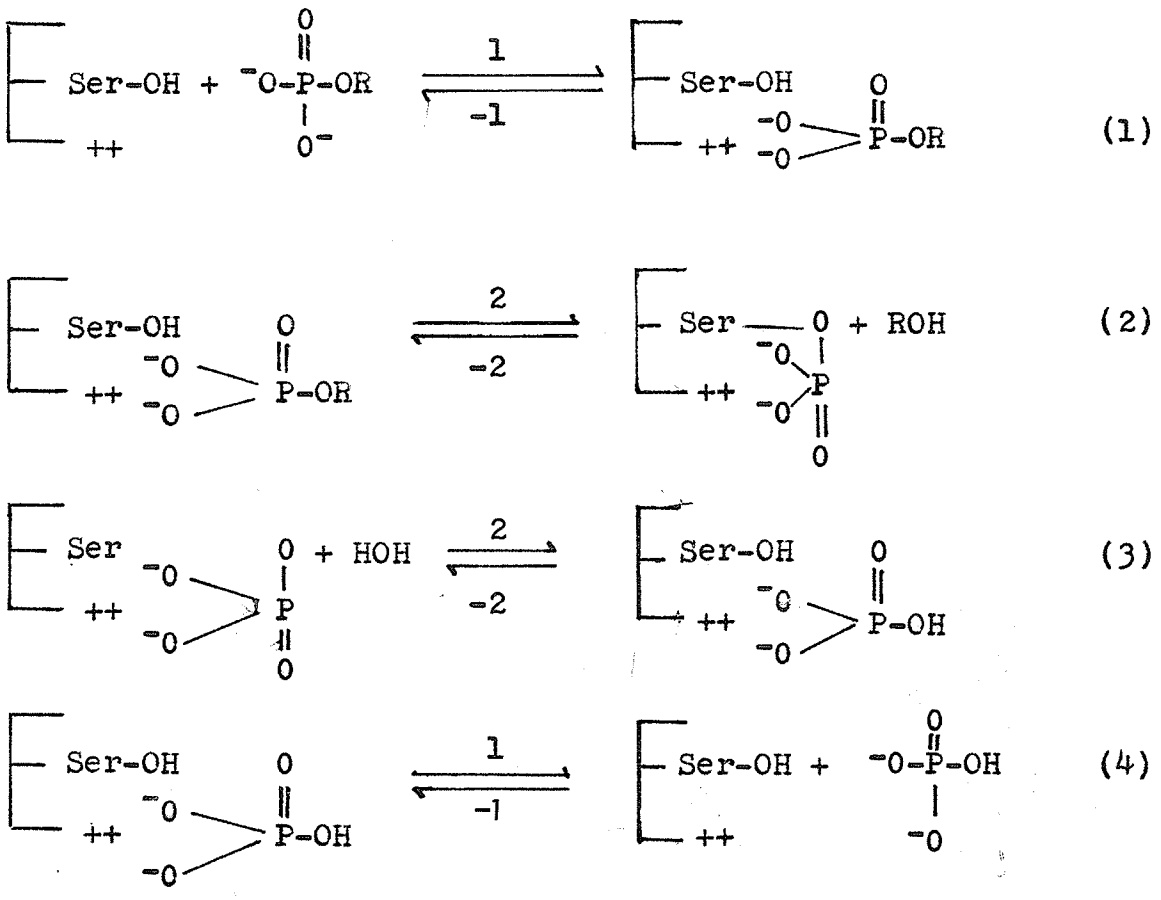
Localization of Acid Phosphatase

Spencer (1968) also studied the localization of the acid phosphatase of alga Acetabularia. He isolated the chloroplasts by the sucrose density gradient centrifugation method. He found all the phosphatase activity in the cytoplasm and no activity in the chloroplast fraction.

The Mechanism of Action of Phosphatase

Engstrom and others (Engstrom, 1961, Schwartz and Lipmann, 1961, and Greenberg and Nachmansohn, 1962) have demonstrated the formation of phosphoryl enzyme during the activity of phosphatase. They recovered radioactive serine phosphate in high yield from the protein hydrolysates of enzyme preparations that were incubated with low concentration of ^{32}P or ^{18}O labelled phosphate. Schwartz and Lipmann have suggested that both ester phosphate and

orthophosphate behave as substrates and both phosphorylate the same serine residue in the enzyme. They also have suggested that hydrolysis is just the reversal of phosphorylation when the enzyme is phosphorylated by orthophosphate. When the enzyme is phosphorylated by an ester phosphate, then in the dephosphorylation the water which ordinarily is present in high concentration will replace alcohol and this results in the hydrolysis of the phosphoryl enzyme. They have proposed the following mechanism for the action of phosphatase.



In the reaction (1) the substrate is bound to the enzyme. The enzyme is then phosphorylated at serine with the loss of the substituent alcoholic group by reaction (2). In reaction (3) the resulting phosphoryl enzyme is hydrolysed by reversal of reaction (2) to yield bound orthophosphate. This complex decomposes by reversal of reaction (1) to release free enzyme and orthophosphate as product (reaction 4). This type of enzyme mechanism is named by Cleland (1963 a) as Ping Pong Bi Bi mechanism.

MATERIALS AND METHODS

Plants

Primary leaves of 8-11 day-old wheat (*Triticum aestivum* L. var. Selkirk) which had been grown in the green house were used as a source of NADP-phosphatase.

Chemicals and Enzymes

NAD, NADP, 2'AMP, 3'AMP, 5'AMP, 2'3' cyclic AMP, 3'5' cyclic AMP, ADP, ATP, alcohol dehydrogenase, sodium isoascorbate, ethylenediamine tetraacetic acid (EDTA), kinetin and benzimidazole were purchased from Sigma Chemical Company, St. Louis, Missouri. MES, HEPES, protamine sulphate were bought from the Calbiochemical Lab., Los Angeles, California, Nicotinic acid, nicotinamide were obtained from Eastman Organic Chemicals, Rochester, New York.

Extraction and Purification of NADP-phosphatase

Crude extract: Selkirk wheat leaves were washed and homogenised with 1.5 volume of 0.05M Tris-acetate buffer, pH 7.3 containing 0.001M EDTA for five minutes at 0-5°C. All subsequent operations were carried out at this temperature. The homogenate was squeezed through four layers of cheesecloth and centrifuged for 20 minutes at 20,000 x g. The supernatant was the crude extract.

First ammonium sulphate fractionation: The crude extract was brought to 25% ammonium sulphate saturation by the addition of 14.4 grams of ammonium sulphate per 100 ml of crude extract. The suspension was stirred slowly for 2 hours. The precipitate was removed by centrifugation at 20,000 x g for 20 minutes. The supernatant was brought to 80% ammonium sulphate saturation by the addition of 39 grams of ammonium sulphate per 100 ml of supernatant fluid. The precipitate was removed by centrifugation at 20,000 x g for 20 minutes and redissolved in a volume of 0.05M Tris-acetate buffer, pH 7.3 containing 0.001M EDTA equal to one half the volume of the initial crude extract.

Protamine sulphate treatment: An equal volume of 2% protamine sulphate was added slowly with constant stirring to the first ammonium sulphate fraction. The denatured protein was removed by centrifugation at 20,000 x g for 10 minutes. The supernatant was dialysed overnight against two changes of a total of 20 volumes 0.05M Tris-acetate buffer, pH 6.0 containing 0.001M EDTA. The precipitate formed during dialysis was removed by centrifugation at 20,000 x g for 10 minutes and discarded.

Second ammonium sulphate fractionation: The supernatant fluid from the protamine sulphate treatment was brought to 45% ammonium sulphate saturation by the addition of 27.7 grams of ammonium sulphate per 100 ml of supernatant fluid.

The suspension was gently stirred for two hours and the resulting precipitate was removed by centrifugation at 20,000 x g for 20 minutes and discarded. The supernatant was brought to 65% ammonium sulphate saturation by the addition of 13.4 grams of ammonium sulphate per 100 ml of supernatant fluid and stirred for two hours. The precipitate was removed by centrifugation at 20,000 x g for 20 minutes and redissolved in a volume of 0.005M Tris-acetate buffer, pH 7.3 containing 0.001M EDTA and dialysed for 48 hours against 2 changes of the same buffer. The contents of the dialysis bag were centrifuged to remove any insoluble proteins present.

Spectrophotometric Assay of NADP Phosphatase

The NAD formed by the action of NADP-phosphatase on NADP was measured enzymatically with alcohol dehydrogenase system (Racker, 1955) in a spectrophotometer. The assay system contained Tris-acetate buffer pH 6.5, 100 umoles; EDTA 0.1 umole; ethanol, 180 umoles; alcohol dehydrogenase from baker's yeast in excess; enzyme preparation 0.1 ml; and NADP, 0.5 umole in a total volume of 3.0 ml. The reaction was started with the addition of NADP. The NAD formed was reduced by the alcohol dehydrogenase system to NADH which gave an increase in absorbance at 340 mu. Spectrophotometric measurements were made on an automatic

recording spectrophotometer (Unicam SP 800), using silica cuvettes of 1 cm light path.

Assay of NADP Phosphatase by Phosphate Liberation

In determining the phosphate liberated, the system contained Tris-acetate buffer, pH 6.0, 100 umoles; EDTA 0.1 umole; and 0.1 ml enzyme preparation in a total volume of 3.0 ml. The reaction mixture was incubated at 37°C. The reaction was started by the addition of enzyme preparation and stopped by heating at 100° for two minutes.

Orthophosphate was determined by the method of Waygood (1948) by measuring the optical densities of phosphomolybdate complex reduced by ascorbic acid at 650 mu.

Isolation of Chloroplasts

Chloroplasts were isolated by the 'laceration technique' of Mache and Waygood (1969). Solution A and B of Jensen and Bassham (1966) were used in the isolation procedure, both of which contained 0.33M sorbitol; 0.002M $NaNO_3$; 0.002M EDTA; 0.001M $MnCl_2$; and 0.001M $MgCl_2$. In addition solution A contained 0.05M MES (2-(N-morpholino) ethane sulphonic acid) adjusted with NaOH to pH 6.1; and 0.02M NaCl. Solution B contained 0.05M HEPES (N-2-hydroxyethy-piperazine-N'-2-ethane sulphonic acid) adjusted with NaOH to pH 6.7; and 0.02M NaCl. Ten grams of freshly cut leaves

were washed and placed in a glass solvent trough containing 25 ml of solution A. The leaves were gently lacerated with 5 ml, closely spaced scalpel blades attached to a rubber stopper. The suspension of chloroplasts obtained in this way was filtered through one layer of Kleenex tissue and the filtrate was centrifuged for 50 seconds at 2000 x g. The pellet was resuspended in the solution B and centrifuged on a 20%-60% sucrosed gradient for 20 minutes at 12,000 x g (IEC rotor SB 405). The chloroplasts were collected and used for enzyme activity assays.

Detached Leaf Experiments

Excised leaves in 5 gram batches were washed, dried with paper towels and floated for 3 days in trays which contained either 250 ml of distilled water; nicotinic acid (10^{-3}M); nicotinamide (10^{-3}M); NAD (10^{-3}M); benzimidazole (4.24×10^{-4}M) or kinetin (9.3×10^{-5}M). The trays were covered with Saran wrap and placed in a growth chamber at 21°C under continuous illumination at a light intensity of 1500 ft-C.

Protein Determination

Protein determinations were made by the folin phenol reagent method of Lowry et al (1951). Crystalline bovine albumin was used as a standard.

RESULTS

Purification

The purification process resulted in approximately a 50-fold increase of specific enzyme activity. The total enzyme units of the protamine sulphate fraction exceeded that of the crude extract. This was probably due to the precipitation of inhibitory substance(s) by the protamine sulphate. The over-all recovery was 58% and the over-all purification factor 56. A summary of the purification is presented in Table I.

Effect of pH

The rate of hydrolysis of NADP by NADP-phosphatase at different pH values is shown in Fig.1. The enzyme had its optimal activity at pH 5.9 and completely lost its activity above pH 8.

Effect of Temperature

The enzyme activity increased from 10°C to its optimal activity at 37°C. The enzyme activity decreased at temperature above 37°C. The temperature coefficient Q_{10} calculated is approximately 0.34. From 10°C to 30°C the Arrhenius plot of logarithm of velocity against the reciprocal of the absolute temperature shows linearity (Fig.2(b)).

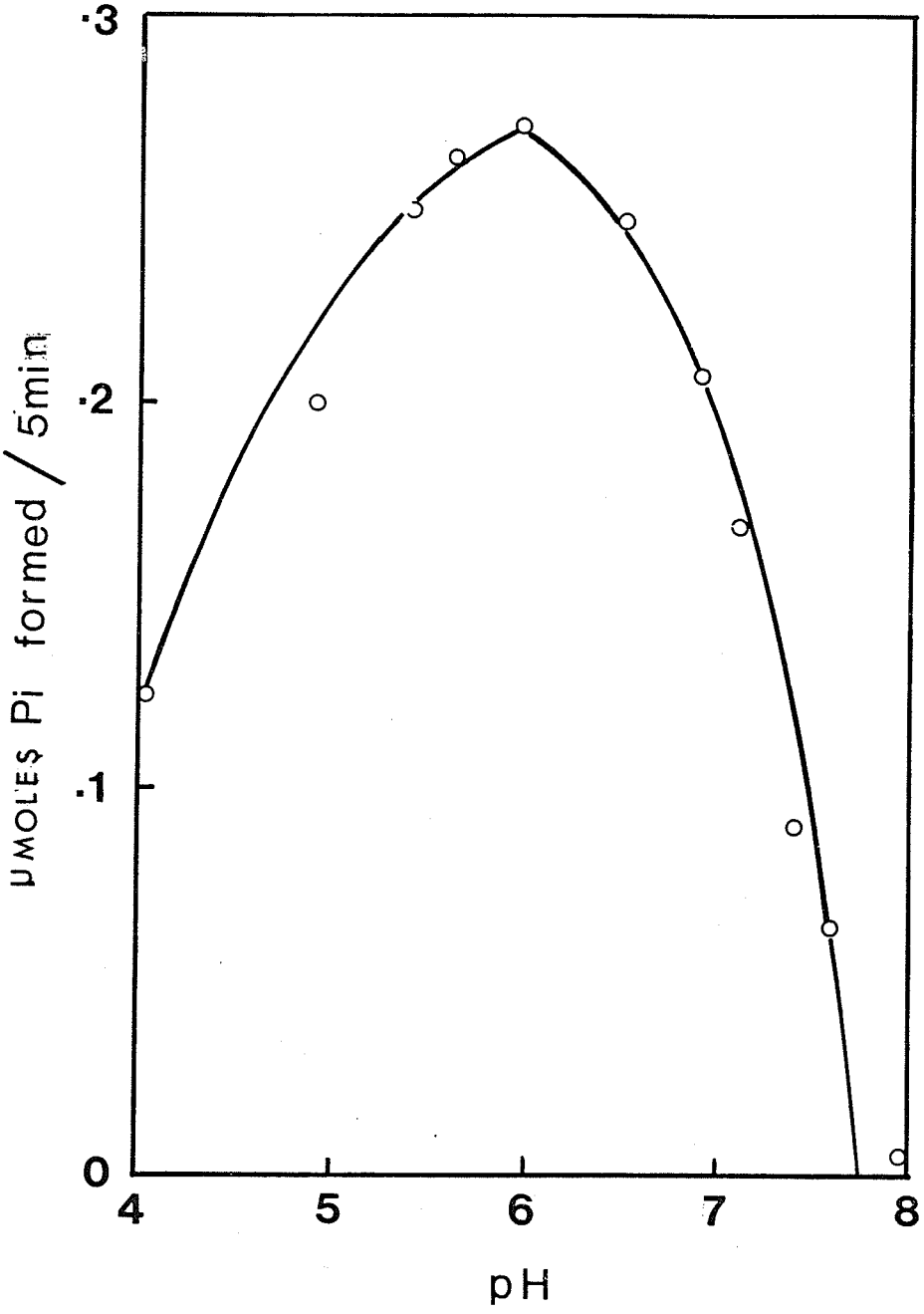
TABLE I

PURIFICATION OF NADP PHOSPHOMONOESTERASE

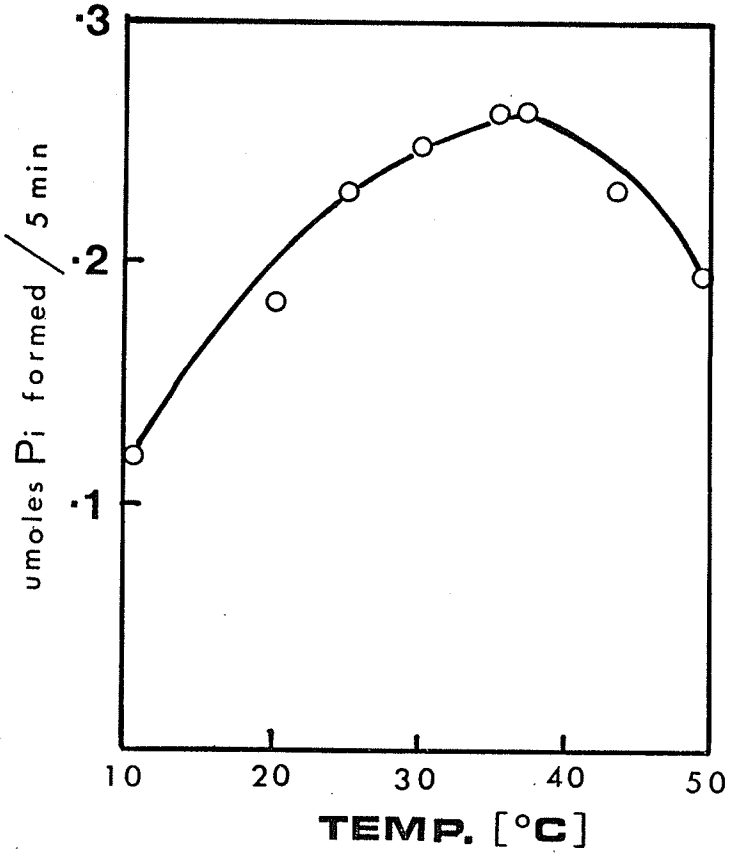
Fraction	Volume (ml)	Total enzyme units#	Total protein (mg)	Specific activity*	Recovery (%)
Crude extract	160	96	1920	0.05	100
Ammonium sulphate 25-80%	80	89	840	0.107	90
Protamine sulphate 2%	115	286	126	1.37	270
Ammonium sulphate 45-65%	20	56	18.8	2.8	58

\# One enzyme unit is defined as the amount of enzyme which catalyses the formation of 1 umole of product in 5 minutes.

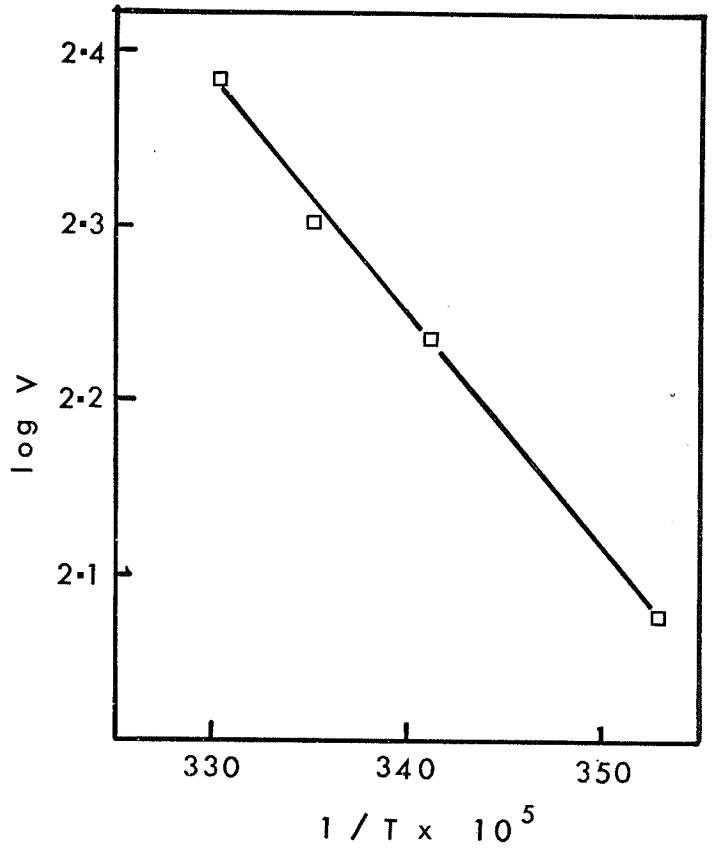
* Specific activity is expressed in units/mg of protein.


·3
·2
·1
0
4
5
6
7
8
μMOLES Pi formed / 5min
pH

[a]


·3
·2
·1
umoles Pi formed / 5 min
10
20
30
40
50
TEMP. [°C]

[b]


2·4
2·3
2·2
2·1
log V
330
340
350
1 / T x 10^5

From the Arrhenius equation;

$$- E = \frac{d(\log V)}{d\left(\frac{1}{T}\right)} \times 2.303R$$

$$E = -\text{slope} \times 2.303R$$

where T is the absolute temperature and R the gas content, the energy of activation can be calculated from Fig. 2(b).

$$E = \frac{-(2.07 - 2.38)}{\frac{353 - 330}{10^5}} \times 2.303 \times 1.986$$

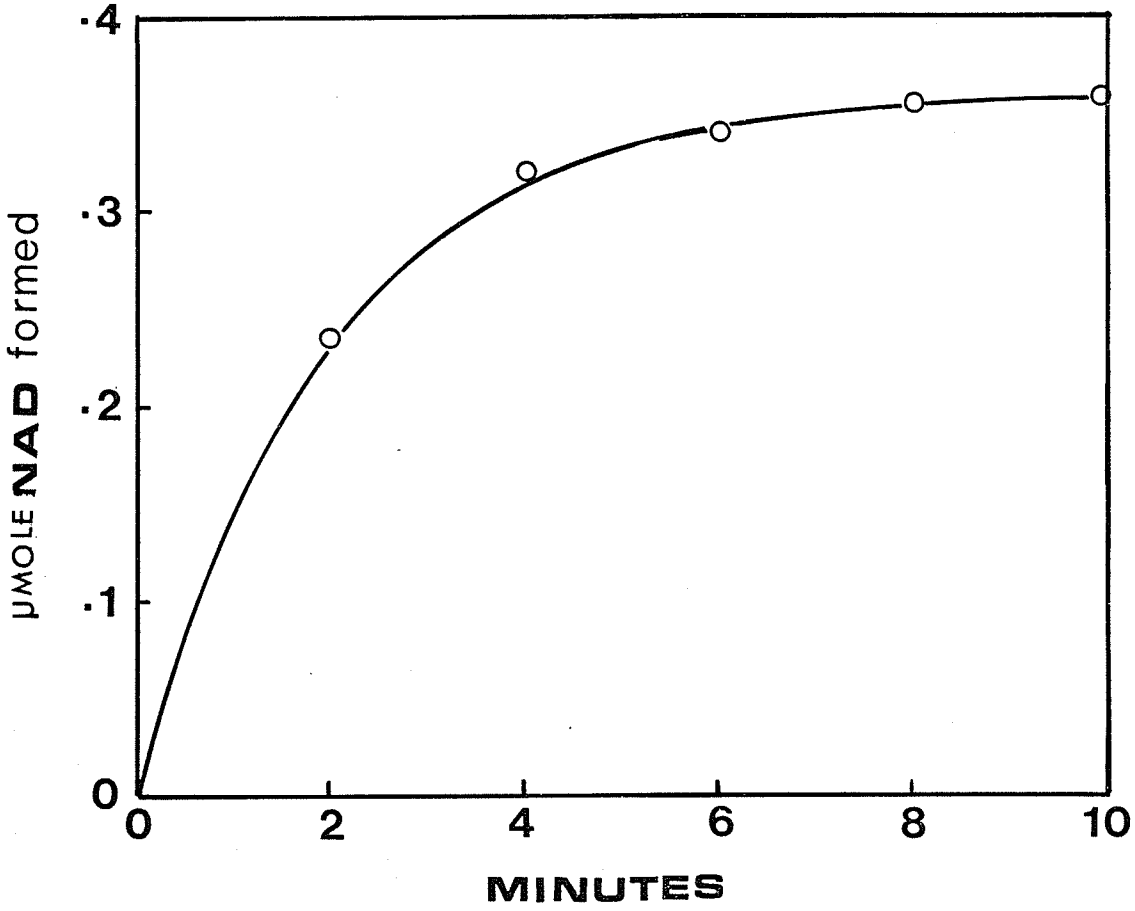
$$E = 29{,}000 \text{ cal/mole.}$$

Time Course of Hydrolysis of NADP

The time course of hydrolysis of NADP is shown in Fig.3. The figure shows that the hydrolysis began with a faster rate and after four minutes it started to approach to equilibrium. Therefore, in the kinetic studies of this investigation only the reaction rates of the first four minutes are taken as the initial velocities.

Effect of Enzyme Concentration

From the concentration of 0.025 ml to 0.1 ml of enzyme preparation, the enzyme concentration showed a linear relationship with reaction velocity (Fig.4).


·4
·3
·2
·1
0
µMOLE NAD formed
0
2
4
6
8
10
MINUTES

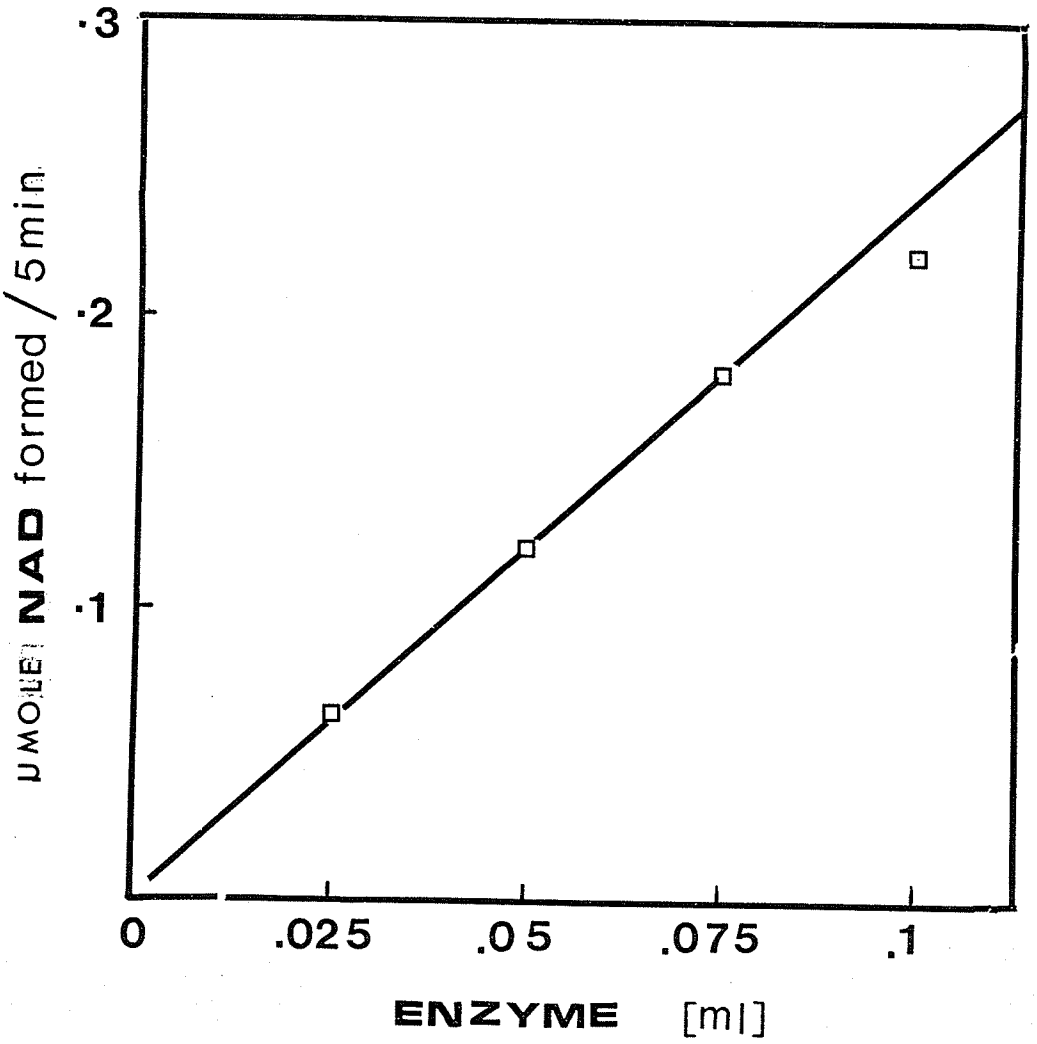

·3
·2
·1
μMOLES NAD formed / 5 min
0
.025
.05
.075
.1
ENZYME [ml]

TABLE II

EFFECT OF IONS AND OTHER COMPOUNDS ON NADP PHOSPHOMONOESTERASE*

Compounds	Concentration	% Activity
$MgCl_2$	3.33×10^{-5}M	111.5
	3.33×10^{-4}M	110
$MnCl_2$	3.33×10^{-4}M	84.6
	3.33×10^{-3}M	81
$CoCl_2$	3.33×10^{-4}M	80.7
	6.66×10^{-4}M	67.3
$FeCl_2$	3.33×10^{-4}M	87.5
	3.33×10^{-3}M	84.3
NaF	3.33×10^{-4}M	73
	3.33×10^{-3}M	48
Iodoacetic acid	3.33×10^{-6}M	75
	3.33×10^{-5}M	37.6
	3.33×10^{-4}M	26.8
EDTA	3.33×10^{-4}M	160
	3.33×10^{-3}M	173

* Tris-acetate buffer was used, final concentration 0.033M. Phosphate liberation assay.

Effect of Ions and Other Compounds

The effect of ions and other compounds with no pre-incubation on the NADP-phosphatase activity are shown in Table II.

EDTA increased the enzyme activity approximately 60% over that of the control. The magnesium ion also slightly increased the enzyme activity while all other ions tested were inhibitory.

Effect of Nicotinic Acid, Nicotinamide, Benzimidazole and Kinetin

Nicotinic acid and nicotinamide, precursors of NAD synthesis (Godavari, 1966 and Waygood *et al*, 1968), had little or no effect on the activity of the NADP-phosphatase. Similarly benzimidazole and kinetin which influence the levels of NADP in the wheat leaves (Mishra and Waygood,1968) were without effect. The results are shown in Table III.

Specificity

The partially purified NADP-phosphatase is non-specific. The enzyme was found to be very active on ATP. It was less active on either 2', 3' or 5' AMP. The 2', 3' and 3',5' cyclic AMP were attacked at 2 to 3% of the rate observed with NADP. NAD was hydrolysed at 3% of the rate of NADP. This enzyme also hydrolysed glucose-6-phosphate, a very common substrate for acid phosphatase, at 50% of the rate of NADP. The substrate specificity of NADP phosphomonesterase are shown in Table IV.

TABLE III

EFFECT OF NICOTINIC ACID, NICOTINAMIDE, BENZIMIDAZOLE, AND KINETIN ON NADP PHOSPHOMONESTERASE*

Compounds	Concentration	% Activity
Nicotinic acid	10^{-4}M	96.6
	2×10^{-4}M	93.3
	2×10^{-3}M	93.3
	4×10^{-3}M	90
Nicotinamide	10^{-4}M	95
	10^{-3}M	93.3
	2×10^{-3}M	93.3
Kinetin	3.1×10^{-6}M	100
	7.7×10^{-6}M	100
	1.5×10^{-5}M	103
	3.1×10^{-5}M	106.6
Benzimidazole	1.4×10^{-5}M	100
	7.0×10^{-5}M	93.3
	1.4×10^{-4}M	93.3

* Tris-acetate buffer (pH 6.0) was used, final concentration 0.033M. Phosphate liberation assay.

TABLE IV

SUBSTRATE SPECIFICITY OF NADP PHOSPHOMONOESTERASE*

Substrate	Activity (nmoles/5 min.)
NADP	221
NAD	6.6
ATP	233
ADP	17.6
2' AMP	77
3' AMP	55
5' AMP	77
2', 3' cyclic AMP	6.6
3', 5' cyclic AMP	4.3
Glucose-6-phosphate	114

* Reaction system contained 500 nmoles substrate and Tris-acetate buffer (pH 6.0), final concentration 0.033M. Incubation temperature was 37°.

Intracellular Localisation

No NADP phosphatase activity could be found in the highly purified chloroplasts isolated by the 'laceration technique' of Mache and Waygood (1969). Table V shows the results of intracellular localisation of NADP phosphomonoesterase.

TABLE V

INTRACELLULAR LOCALISATION OF NADP PHOSPHOMONOESTERASE*

Fraction	Total Enzyme Units**	
	Experiment 1	Experiment 2
Chloroplast sonicate	0	0
Supernatant	7.5	8

* Spectrophotometric assay.

** One enzyme unit is defined as the amount of enzyme which catalyses the formation of 1 um of product in 5 minutes.

Effect of Nicotinic Acid, Nicotinamide, Benzimidazole, Kinetin and NAD on the Levels of NADP Phosphomonesterase in Detached Wheat Leaves

Detached wheat leaves were floated on nicotinic acid (10^{-3}M), nicotinamide (10^{-3}M), NAD (10^{-3}M), benzimidazole (4.24×10^{-4}M), kinetin (9.3×10^{-5}M), and distilled water as described in the methods for three days. The treated leaves were rinsed several times with distilled water and the enzymes was extracted according to the described procedure.

TABLE VI

EFFECT OF NICOTINIC ACID, NICOTINAMIDE, NAD, BENZIMIDAZOLE AND KINETIN ON NADP PHOSPHOMONOESTERASE IN DETACHED WHEAT LEAVES*

Treatment	Protein (mg/ml)		Total enzyme units (EU)**		Specific activity (EU/mg protein)	
	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2
Immediately detached	11	10.2	2.14	2.44	2.19	2.30
Water	9.8	9.5	2.29	2.28	2.33	2.40
Nicotinic acid	9.5	9.2	1.69	2.05	1.77	2.22
Nicotinamide	9.6	9.0	3.14	2.69	3.27	2.89
NAD	10	9.1	1.44	1.72	1.44	1.89
Kinetin	10.1	9.8	3.57	3.32	3.53	3.38
Benzimidazole	10.2	9.8	2.57	3.03	2.51	3.09

* Spectrophotometric assay.

** One enzyme unit is defined as the amount of enzyme which catalyses the formation of 1 umole of NAD in 5 minutes.

The results of the effect of nicotinic acid, nicotinamide, benzimidazole, kinetin and NAD on the levels of NADP phosphomonesterase in detached wheat leaves are shown in Table VI. The levels of NADP phosphatase activity decreased in the leaves floated on NAD and nicotinic acid. The leaves floated on kinetin and nicotinamide showed an increase in NADP phosphatase activity.

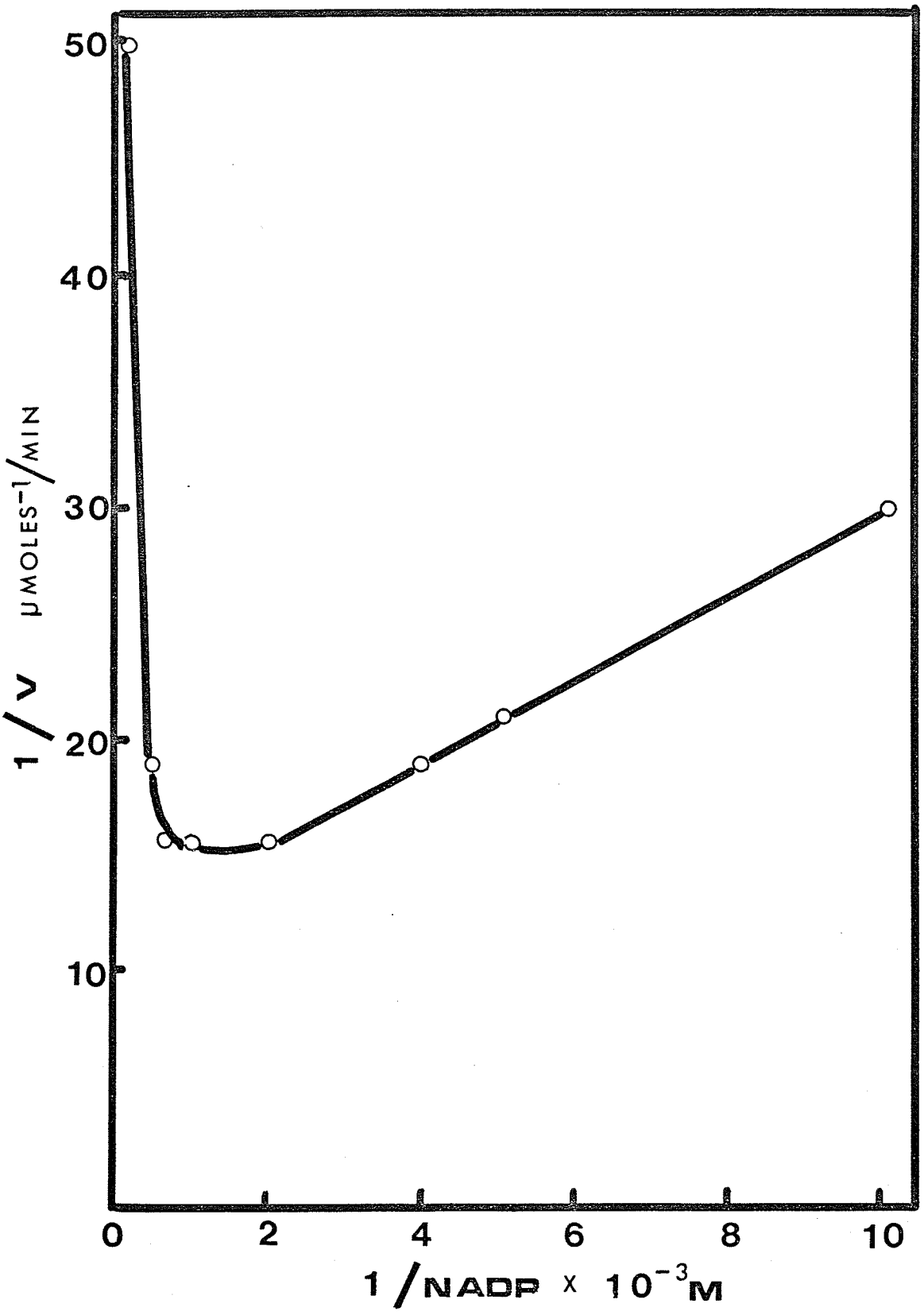
Effect of Substrate Concentration

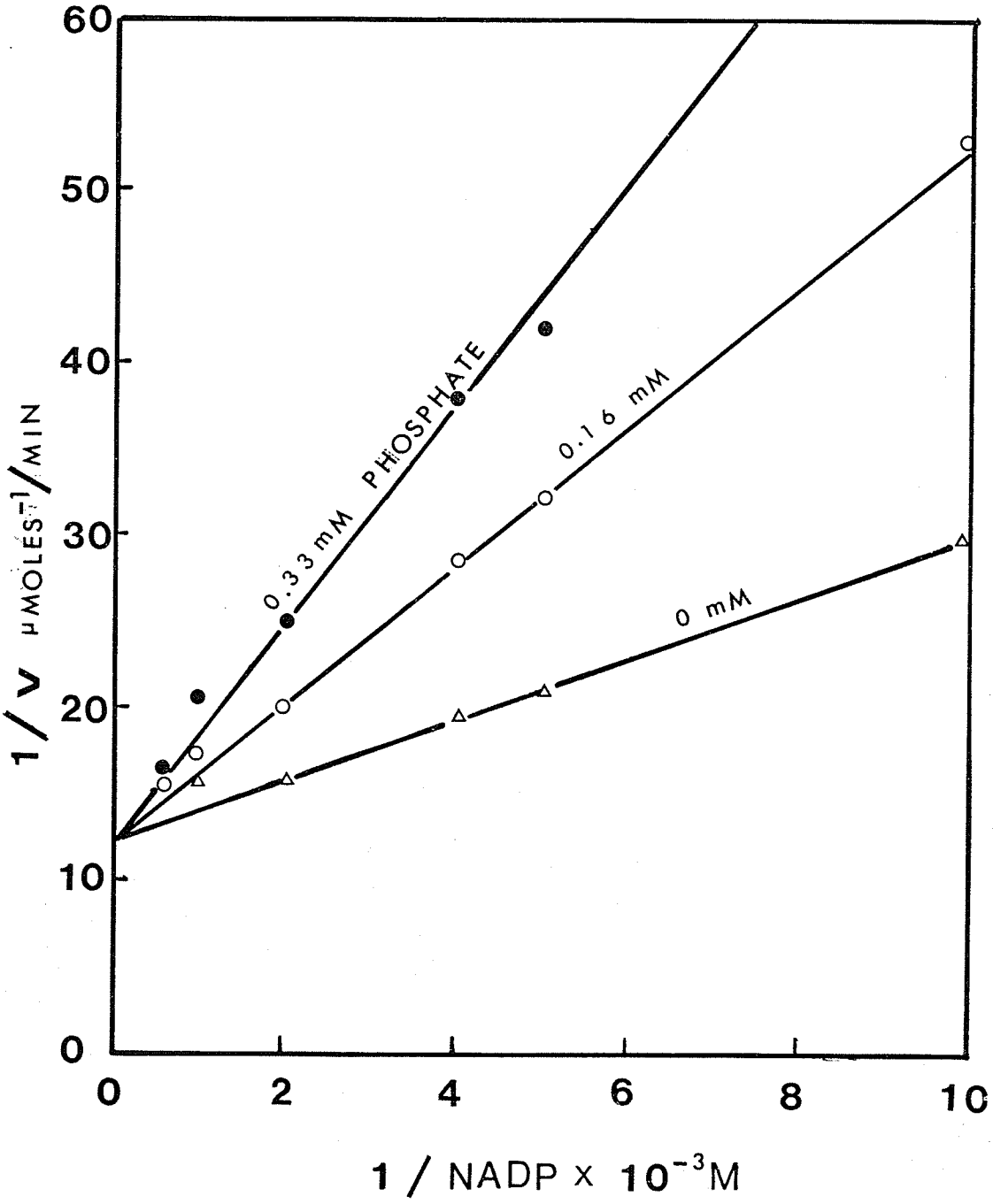
The reciprocal plot of 1/v versus 1/A with NADP as variable substrate gives a straight line at a low NADP concentration (10^{-4}M-5 x 10^{-4}M). At concentrations above 5 x 10^{-4}M the plot curves upwards showing substrate inhibition (Fig.5). The K_{NADP} calculated is 1.4 x 10^{-4}M.

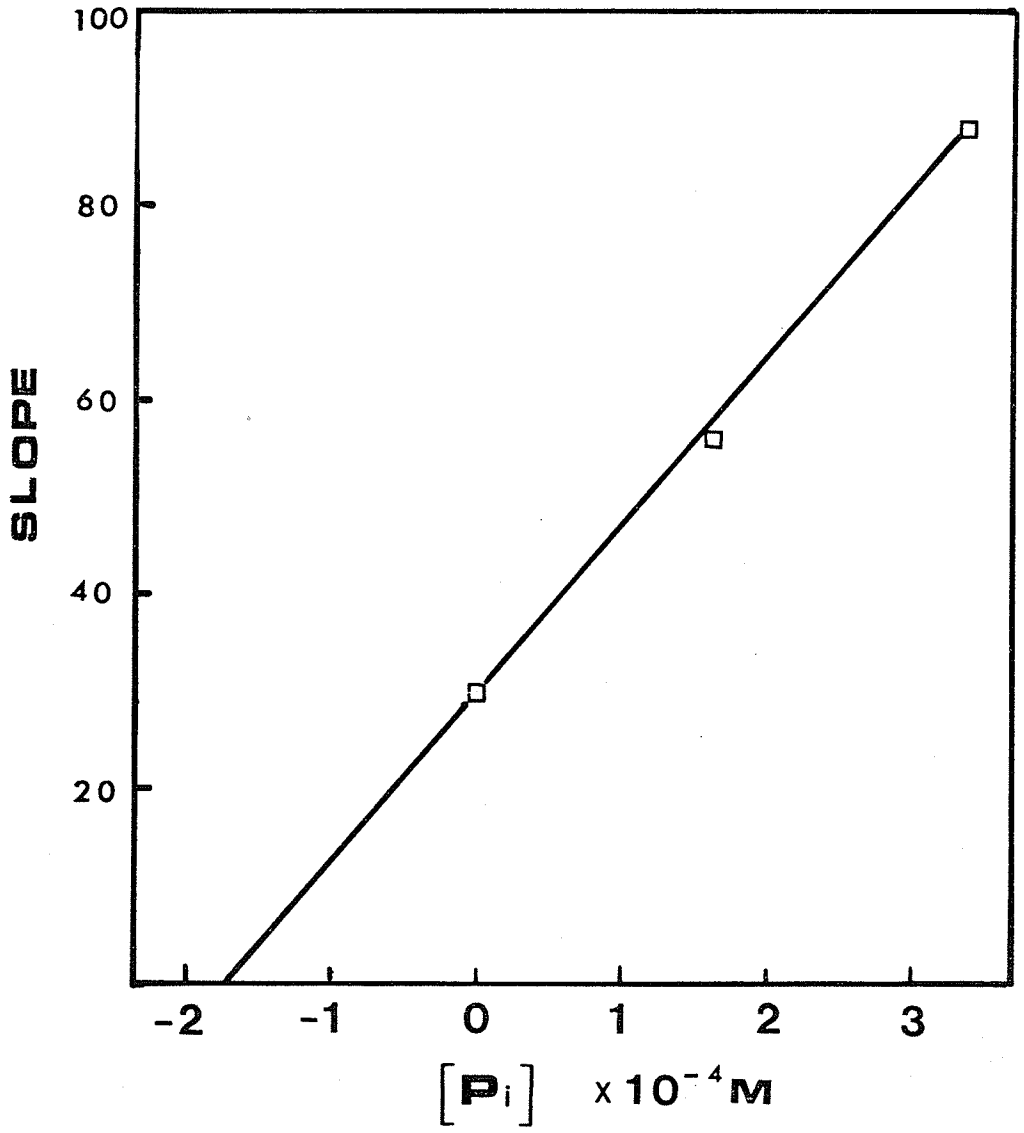
Effect of Product Concentration

The product inorganic phosphate was found to be a competitive inhibitor of the reaction (Fig.6). The $K_i(P_i)$ calculated is 1.7 x 10^{-4}M.

NAD up to 10^{-3}M did not show any inhibition.


50
40
30
20
10
1 / v μMOLES^{-1}/MIN
0
2
4
6
8
10
1 /NADP x 10^{-3}M


60
50
40
30
20
10
0
0
2
4
6
8
10
1 / v µMOLES^{-1}/MIN
1 / NADP x 10^{-3}M
0.33 mM PHOSPHATE
0.16 mM
0 mM

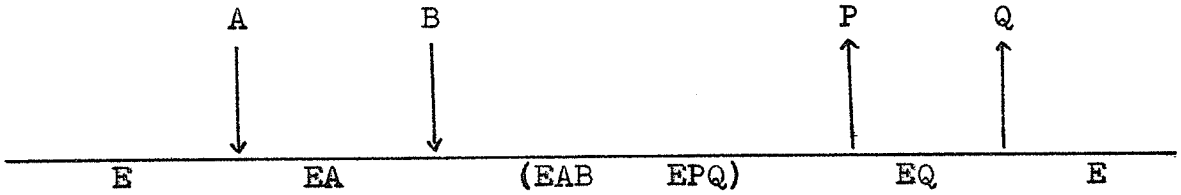

100
80
60
40
20
SLOPE
-2
-1
0
1
2
3
[Pi] x 10⁻⁴M

DISCUSSION

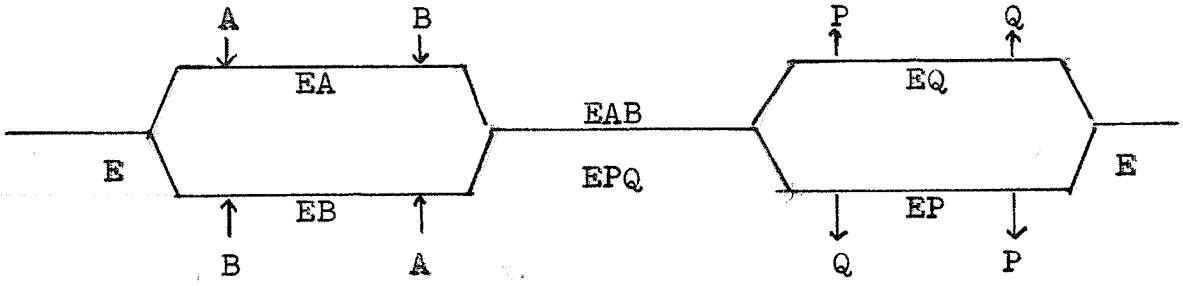
The hydrolysis of NADP catalysed by NADP-phosphatase is a two-substrate-two product reaction.

$$NADP + H_2O \underset{\text{phosphatase}}{\overset{\text{NADP}^-}{\rightleftharpoons}} NAD + Pi$$

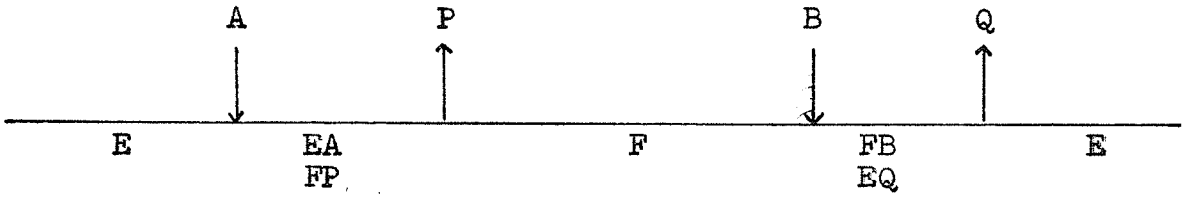
Cleland (1963a) has proposed several mechanisms which he classifies as Ordered, Random, and Ping Pong Bi Bi mechanisms for a bi reactant-bi product reaction. He defines the Ordered Bi Bi mechanism as a reaction in which the two substrates, A first then B must add to the enzyme before any products are released. It is presented graphically as:



where P and Q are the products in that order and E is the enzyme. In the Random Bi Bi mechanism the substrates do not react in obligatory order and alternate sequences exist.



In the Ping Pong Bi Bi mechanism, one product is released before the second substrate has added to the enzyme and the enzyme will exist in two stable forms between which it oscillates during the reaction:



E and F designate the two different enzyme forms.

The rate equation for the initial velocity when both products P and Q are equal to zero are the same for all three mechanisms as follow:

$$v = \frac{V_1 AB}{K_b A + K_a B + AB} \qquad (1)$$

This can be rewritten into double reciprocal form:

$$\frac{1}{v} = \frac{K_a}{V_1}\left(\frac{1}{A}\right) + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) \qquad (2)$$

where V_1 is the maximum velocity for the forward reaction and K_a and K_b are Michaelis constants for A and B respectively. When substrate B is saturating the equation (2) transforms into:

$$\frac{1}{v} = \frac{K_a}{V_1}\left(\frac{1}{A}\right) + \frac{1}{V_1} \qquad (3)$$

Figure 5 shows the reciprocal plot of 1/v versus 1/NADP with substrate B saturating since it is water involved in the hydrolysis of NADP by NADP-phosphatase. The linearity of the reciprocal plot at NADP concentrations lower than 5×10^{-4}M shows that the enzyme reaction follows rate equation (3).

This does not distinguish which of the three is the mechanism for the hydrolysis of NADP. However these three mechanisms can best be distinguished by the patterns of the product inhibition (Cleland, 1963a and 1963b).

The rate equation for Ordered Bi Bi where A and B are substrates and P and Q are products is as follow:

$$v = \frac{V_1V_2(AB - \frac{PQ}{K_{eq}})}{K_{ia}K_bV_2 + K_bV_2A + K_aV_2B + V_2AB + \frac{K_qV_1P}{K_{eq}} + \frac{K_pV_1Q}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{K_qV_1AP}{K_{ia}K_{eq}} + \frac{K_aV_2BQ}{K_{iq}} + \frac{V_2ABP}{K_{ip}} + \frac{V_1BPQ}{K_{ib}K_{eq}}} \qquad (4)$$

where K_a, K_b, K_p, and K_q are the Michaelis constants for A, B, P, and Q respectively and K_{ia}, K_{ib}, K_{ip}, and K_{iq} are the inhibition constants and K_{eq} is the equilibrium constant (Cleland, 1963a).

If B is saturating and the reaction is followed with P and not Q present equation (4) in the reciprocal form becomes:

$$\frac{1}{v} = \frac{1}{V_1} + \frac{P}{V_1 K_{ip}} + \left(\frac{K_a}{V_1 A}\right)\frac{1}{A} \qquad (5)$$

The first product P thus should give linear uncompatitive inhibition.

If the reaction is followed with B saturating in the presence of Q, but not P the rate equation is:

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1}\left(1 + \frac{Q}{K_{iq}}\right)\left(\frac{1}{A}\right) \qquad (6)$$

The second product released should thus give linear competitive inhibition.

For the NADP-phosphatase reaction the product inorganic phosphate gave competitive inhibition but there was no inhibition by the product NAD. This indicates that the NADP-phosphatase reaction does not follow the Ordered Bi Bi mechanism.

Cleland (1963a) has worked out the rate equation and the product inhibition pattern for the Random Bi Bi reaction. He has shown that if the reaction is saturated with B the products of Random Bi Bi mechanism gives noncompetitive inhibition. As none of the products of the hydrolysis of NADP gives noncompetitive inhibition the possibility of the Random Bi Bi mechanism to be the mechanism of the NADP-phosphatase action is excluded.

The rate equation for the Ping Pong Bi Bi mechanism written in kinetic constants is:

$$v = \frac{V_1V_2\ (AB - \frac{PQ}{K_{eq}})}{K_bV_2A + K_aV_2B + V_2AB + \frac{K_qV_1P}{K_{eq}} + \frac{K_pV_1Q}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{K_qV_1AP}{K_{ia}K_{eq}} + \frac{K_aV_2BQ}{K_{iq}}} \qquad (7)$$

When B is saturating in the presence of P but not Q, the equation (7) is converted to:

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1}\left(\frac{1}{A}\right) \qquad (8)$$

Thus the first product P would not give any inhibition.

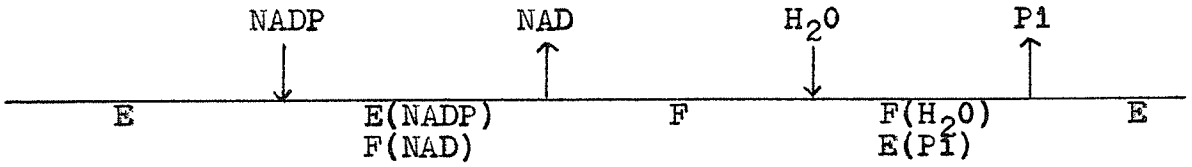
When B is saturating in the presence of Q, but not P rate equation (7) becomes:

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1}\left(1 + \frac{Q}{K_{iq}}\right)\frac{1}{A} \qquad (9)$$

Therefore the second product Q would give linear competitive inhibition with an inhibition constant equal to K_{iq}.

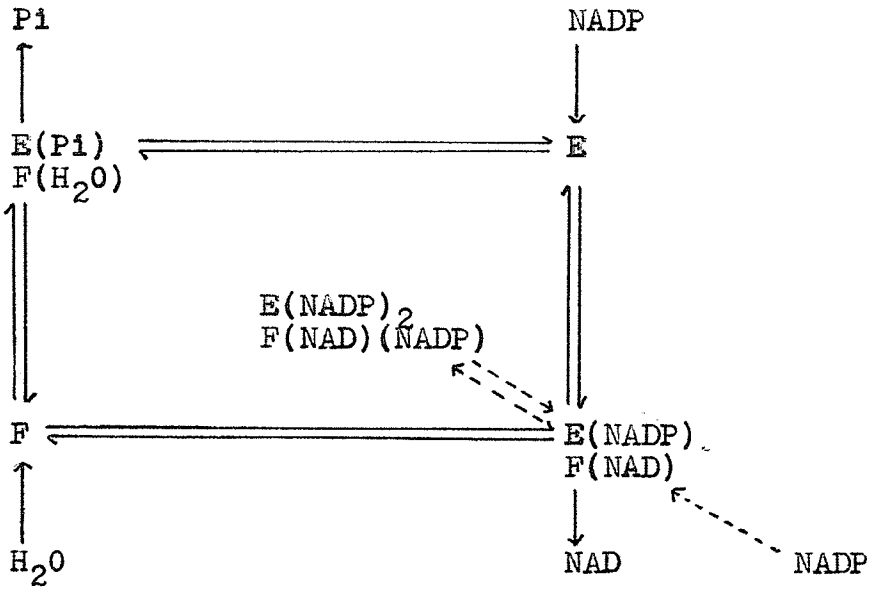
In the hydrolysis of NADP by NADP-phosphatase, the product NAD does not show inhibition but the other product inorganic orthophosphate gives linear competitive inhibition. These results thus indicate that the reaction mechanism is Ping Pong Bi Bi in which when B (H_2O) is saturating, P(NAD) will not show inhibition but Q (Pi) will be a competitive inhibitor.

Thus the mechanism of the hydrolysis of NADP can be graphically represented as below:



The Ping Pong Bi Bi mechanism for the NADP-phosphatase action obtained from the kinetic studies agrees with the mechanism proposed by Schwartz and Lipmann (1961) for the action of phosphatase. They suggest that the compound hydrolysed is first bound to the enzyme, followed by the release of an alcoholic group and the enzyme is left phosphorylated. The phosphorylated enzyme then reacts with water to liberate inorganic orthophosphate. By the nomenclature of Cleland (1963a), this type of mechanism is actually a Ping Pong Bi Bi mechanism.

As the concentration of NADP is increased to above 5×10^{-4}M NADP was found to be inhibitory indicating that NADP combines not only with free enzyme form E but perhaps also with other forms eg. with E(NADP) to form an inactive enzyme-substrate complex. This mechanism can be represented by the schematic method of King and Altman (1956) as below. Here NADP is shown to react as a substrate with enzyme form E and as an inhibitor with either E(NADP) or F(NAD) to form an inactive complex.



The rate equation of this mechanism can be written out in rate constant terms by the method of King and Altman (1956), and then transformed into kinetic constant terms by the method of Cleland (1963a). When P and Q are equal to zero and with the saturation of B, this rate equation for the initial velocity is simplified into:

$$v = \frac{V_1 A}{K_a + A + \frac{A^2}{K_i}} \qquad (10)$$

or

$$v = \frac{V_1}{1 + \frac{K_a}{A} + \frac{A}{K_i}} \qquad (11)$$

where K_i is the dissociation constant for EA_2 ($E(NADP)_2$). At high concentrations of A or NADP, the term $\frac{K_a}{A}$ in equation (11) becomes negligible and the inhibition term $\frac{A}{K_i}$

becomes dominant thus expressing the inhibition. Figure 5 fits this equation showing that the formation of the inactive complex either $E(NADP)_2$ or F(NAD)(NADP) is the mechanism of the inhibition.

However identical rate equations can be derived if the substrate A binds not with EA but with F or EQ to form other inactive complexes and consequently producing a curve similar to the one shown in Fig.5. Therefore from Fig.5 alone it is not possible to tell whether the NADP binds to E(NADP) or F or E(Pi). No further investigation was made to study the binding state of this inhibitory NADP.

The purified acid phosphatase in this study is not specific for NADP. However as it has high activity against NADP and lower activity against the common substrate of phosphatase as glucose-6-phosphate, it differs from the common phosphatase. It also differs from the phosphatase studied by Kornberg (1950), and Kuo (1961) which attacks NADP but only at very low rate. The NADP-phosphatase in this study resembles the NADP-phosphatase isolated by Forti et al (1962) from pea leaves. Both acid phosphatases hydrolyse NADP at high rates. The K_{NADP} value of the acid phosphatase from wheat leaves was 1.4×10^{-4}M in this study and the K_{NADP} value of the acid phosphatase from pea leaves purified by Forti et al was 3×10^{-4}M. Orthophosphate was found to be a competitive inhibitor of both the wheat leaf

phosphatase and pea leaf phosphatase. The $K_{iq}(K_{i(Pi)})$ was 1.7×10^{-4}M in this study.

In purification, the total NADP-phosphatase activities were increased by protamine sulphate treatment. Thus the protamine sulphate must have precipitated the natural inhibiting substance(s) in the extract.

EDTA was an activator of the NADP-phosphatase. All the ions except magnesium tested were found to be inhibitory.

Nicotinic acid, the biosynthetic precursor of NADP (Godavari, 1966) and the cytokinins benzimidazole and kinetin had little effect on the activity of NADP-phosphatase.

The excised wheat leaves floated on kinetin and benzimidazole for three days increased the specific activity of the NADP-phosphatase about 20% and the leaves floated on nicotinic acid and NAD lost about 20% of the specific activity. The results do not explain the observations of Mishra and Waygood (1968) that the leaves floated on benzimidazole and kinetin had an increase of NADP content. As the NADP-phosphatase was an active enzyme even in the crude wheat leaf extract and Mishra and Waygood (1968) reported a fairly constant NAD-NADP ratio in fresh leaves, this indicates that it must be a regulatory factor other than the enzyme level itself controlling the NAD/NADP ratio in the leaves. If so the increase of NADP-phosphatase specific activity *in vitro* will not necessarily cause the decrease of NADP

content *in vivo*. It is also possible that the main NADP pool after the leaves have been floated on the cytokinins is in the chloroplasts. Thus the change of activity of NADP-phosphatase which was not demonstrated in the chloroplasts will not affect this NADP pool.

SUMMARY

1. A procedure is described for the preparation of nicotinamide adenine dinucleotide phosphate phosphomonoesterase of wheat leaves (Triticum aestivum L. var. Selkirk), purified ca 50-fold over crude extract. Inhibitory substance(s) which can be removed by protamine sulphate treatment is found in the crude extract.
2. The pH optimum for enzyme activity is pH 5.9.
3. The enzyme has optimum activity at 37° and energy of activation of 29,000 cal/mole.
4. Ethylenediaminotetraacetic acid is an activator of the enzyme. Magnesium ion is slightly stimulatory. Iodoacetic acid > fluoride > Co^{++} > Mn^{++} > Fe^{++} are inhibitory.
5. The enzyme is nonspecific, but it hydrolyses nicotinamide adenine dinucleotide phosphate preferentially.
6. Kinetic studies show that the reaction mechanism to be Ping Pong Bi Bi (Cleland, Biochim. Biophys. Acta 67: 104, 1963).
7. K_{NADP} is calculated as 1.4×10^{-4}M. Nicotinamide adenine dinucleotide phosphate at concentrations above 5×10^{-4}M shows inhibition.
8. The product orthophosphate gives linear competitive inhibition with $K_{i(Pi)} = 1.7 \times 10^{-4}$M.

9. No enzyme activity is found in the chloroplasts.
10. Treatment with kinins (benzimidazole and kinetin) increases the enzyme level about 20%, whereas nicotinic acid and nicotinamide adenine dinucleotide treatment lowers the enzyme levels about 20%.

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