A KINETIC STUDY OF NICOTINAMIDE ADENINE DINUCLEOTIDE TRANSHYDROGENASE FROM 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

Chyan-Erl Jou

September 1968

ACKNOWLEDGMENTS

The author is deeply indebted to Dr. E. R. Waygood for his ever available guidance and constant encouragement throughout this investigation.

Acknowledgment is also given to the National Research Council for financial assistance.

ABSTRACT

NAD transhydrogenase which catalyzes the oxidation of NADPH by NAD was demonstrated in extracts of leaves from Triticum aestivum L. var. Selkirk. The reverse reaction could not be demonstrated even in the presence of nucleotide mono- and tri-phosphates. The enzyme was shown to be localized in chloroplasts. It has a molecular weight of 37,000 ± 5,000, optimum pH 7.7, and energy of activation 3,500 cal/mole. Kinetic studies revealed the reaction mechanism to be Ping-Pong (Cleland, Biochim. Biophys. Acta 67:104, 1963) with K_m values of 1.33×10^{-6} M and 5.0×10^{-3} M for NADPH and NAD respectively. NADPH at concentrations > 0.016 mM was a non-competitive inhibitor with a $K_i = 2.8 \times 10^{-5}$ M. 2'-AMP was a competitive inhibitor. Treatment of leaves with kinins increased the specific activity of the enzyme 2-3 fold in contrast to an almost complete loss of enzyme activity in extracts from leaves floated on water.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Abbreviations--ADP, ATP, the 5-di- and triphosphates of adenosine; 2', 3', or 5'-AMP, the 2', 3', or 5'-monophosphate of adenosine; $AgNO_3$, silver nitrate; $CaCl_2$, calcium chloride; $CoCl_2$, cobalt chloride; CTP, cytosine triphosphate; GTP, guanosine triphosphate; p-HMB, p-hydroxymercuribenzoate; ITP, inosine triphosphate; K_i, the inhibitor constant; K_m, the Michaelis constant; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP; NaCl, sodium chloride; NaOH, sodium hydroxide; NMN, nicotinamide mononucleotide; O.D., optical density; UTP, uridine triphosphate; μM, micromolar.

INTRODUCTION

Transhydrogenase ($NADPH_2$: NAD oxidoreductase; E.C.1.6.1.1.) catalyzes the reaction:

$$NADPH + NAD^+ \longrightarrow NADP^+ + NADH$$

The enzyme was first demonstrated by Colowick et al. (1952) in Pseudomonas fluorescens. Transhydrogenase has since been found in spinach leaves and bean leaves by Keister et al. (1960, 1962).

Mishra and Waygood (1968) have postulated that transhydrogenase operating in the reverse direction is an important partial reaction in the following overall reaction which appears to occur in wheat leaves:

$$NAD^+ + NADH + 2ATP + X + H^+ + e \underset{\text{Dark}}{\overset{\text{Light}}{<\!\!\longrightarrow}} NADP^+ + NADPH + 2ADP + XH$$

The other systems involved are NAD kinase, photosynthetic electron transfer and one of several biosynthetic reactions in which X is the oxidant.

The objective of the present study was to determine the intracellular localization of transhydrogenase in wheat leaves, and whether the reaction catalyzed by the enzyme was freely reversible. A detailed kinetic study of transhydrogenase was also undertaken to determine, if possible, its mechanism of action.

In addition the effect of benzimidazole and kinetin treatment of wheat leaves was tested to determine their effect on transhydrogenase in situ. This was undertaken since Mishra and Waygood (1968) and Godavari (1966) have shown that these kinins have profound effect on nicotinamide nucleotide metabolism in wheat leaves.

Other enzymes e.g. diaphorase, menadione reductase and cytochrome c reductase have been included in this study to determine the relative efficiency of NADH and NADPH as hydrogen donors.

LITERATURE REVIEW

1. HISTORICAL BACKGROUND

Two kinds of transhydrogenase have been described, one is energy-independent and the other energy-dependent.

Energy-independent transhydrogenase--This enzyme, first isolated from Pseudomonas fluorescens in 1952 (Colowick et al.), catalyzes the transfer of hydrogen from NADPH to NAD as well as certain analogues of NAD. The occurrence and properties of the enzyme have been demonstrated in microorganisms (Robinson and Mills, 1961; Keister and Hemmes, 1966; Orlando et al., 1966), in plants (Keister et al., 1960) and also tissues of higher animals (Kaplan et al., 1953; Humphrey, 1957; Stein et al., 1959; Spiro and Ball, 1961; Kaufman et al., 1961; Vignais and Vignais, 1961; Purvis, 1961; Horie et al., 1966). The reactions catalyzed by transhydrogenase are listed below:

$$NADPH + NAD^+ \longrightarrow NADP^+ + NADH \quad (1)$$

$$NADH + \text{Deamino-}NAD^+ \longrightarrow \text{Deamino-NADH} + NAD^+ \quad (2)$$

$$NADPH + \text{Deamino-}NAD^+ \longrightarrow \text{Deamino-NADH} + NADP^+ \quad (3)$$

$$NADPH + \text{3-Acetylpyridine-}NADP^+ \longrightarrow \text{3-Acetylpyridine-NADPH} + NADP^+$$

$$NADPH + \text{3-Acetylpyridine-}NAD^+ \longrightarrow \text{3-Acetylpyridine-NADH} + NADP^+$$

$$NADH + \text{3-Acetylpyridine-}NAD^+ \longrightarrow \text{3-Acetylpyridine-NADH} + NAD^+$$

$$NADH + \text{Thio-}NAD^+ \longrightarrow \text{Thio-NADH} + NAD^+$$

$$NADH + NAD^+ \longrightarrow NADH + NAD^+$$

$NADH + NAD\text{-}X \longrightarrow NADH\text{-}X + NAD^+$

$NADH + NMN \longrightarrow NMNH + NAD^+$

$NADPH + NMN \longrightarrow NMNH + NADP^+$

Transhydrogenase from different sources has different catalytic activities for the above reactions. For example, transhydrogenase from Pseudomonas readily catalyzes reactions 2 and 3, but the enzyme from beef heart showed no detectable catalysis of reaction 3 although it did catalyze reaction 2 (Kaplan et al., 1953).

Energy-dependent transhydrogenase--This enzyme was described first in submitochondrial particles by Danielson and Ernster (1962). The enzyme catalyzes the reduction of NADP by NADH. The energy for the reaction could be supplied by exogenous ATP or light (Orlando et al., 1966; Keister et al., 1967); ATP and Mg^{++} (Andreoli et al., 1964) or by the aerobic oxidation of NADH or succinate. Fumarate and malate could not drive the reaction, ruling out the possibility of direct reduction of $NADP^+$ by the products of succinate oxidation (Asana et al., 1967). Light, rather than the oxidation of a substrate, is used to generate energy for the transhydrogenase from chromatophores of Rhodospirillum rubrum (Keister et al., 1967). Studies with uncouplers and inhibitors of oxidative phosphorylation indicated that the reaction was mediated by a high energy intermediate (X-I) which was generated from ATP or by the aerobic oxidation of substrates

by the respiratory chain. The results reported by Keister and Yike (1967) demonstrated that succinate-linked NAD^+ reduction by Rhodospirillum chromatophores can be driven by ATP and that the reaction is probably mediated by a high-energy intermediate of phosphorylation according to the following equations:

$$\text{Succinate} + NAD^+ + X\text{-}I \longrightarrow \text{Fumarate} + NADH + X + I$$

and this reaction can be coupled to the energy-linked transhydrogenase,

$$NADH + NADP^+ + I\text{-}X \longrightarrow NAD^+ + NADPH + X + I$$

The reaction proceeds more readily in transferring hydrogen from NADPH to NAD than in the reverse direction of NADH with NADP (Kaplan et al., 1953; Stein et al., 1959; Lee and Ernster, 1964). In contrast to this observation, Horie and Chefurka (1966) isolated transhydrogenase from thoracic muscle and gut mitochondria of the cockroach and showed the rate of reduction of NADP by NADH to be faster than the reaction rate in the reverse direction. In speculating on the possible reason for this situation, they postulated that this might result from either high endogenous levels of 2'-AMP in the roach gut mitochondria, since Kaplan pointed out that NADH-NADP reaction could be activated by 2'-AMP in the presence of phosphate. In addition there are different K_m values for the reduced and oxidized pyridine nucleotide participating in the reaction. In the same investigation, the very active NADH-NAD reaction in the gut and thoracic

muscle mitochondria suggests that it has some physiological significance, perhaps as a link between the pool of free NADH and bound NAD of the mitochondrial respiratory chain.

The reverse of reaction 1, reduction of NADP by NADH, is catalyzed by both an energy-independent and an energy-dependent transhydrogenase (Danielson and Ernster 1963; Estabrook et al., 1963; Ernster and Lee, 1964; Asano et al., 1967; Orlando et al., 1966; Keister, 1966). However, the energy-dependent reduction of NADP by NADH is more rapid than the energy-independent reaction (Lee and Ernster, 1964).

II. RELATIONSHIP BETWEEN ENERGY-INDEPENDENT AND ENERGY-DEPENDENT TRANSHYDROGENASE

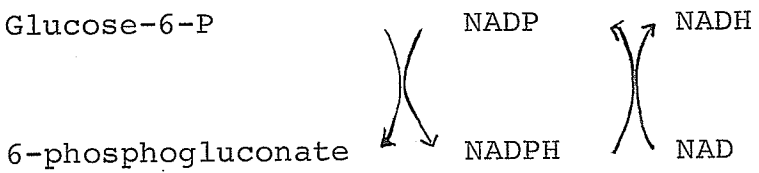
Danielson and Ernster (1963) concluded that the energy-dependent transhydrogenase reaction, involving a reduction of NADP by NADH, catalyzed by sub-mitochondrial preparations from both rat liver and beef heart is different from the transhydrogenase reaction between NADPH and NAD described by Kaplan (1953). In contrast to this conclusion the study of Hommes and Estabrook (1963) pointed to the similarity of the transhydrogenase of Kaplan to the transhydrogenase reaction mediating the energy-dependent reduction of NADP on the basis of the similar inhibition by triiodothyronine as well as the unusually high temperature coefficient for both types of reactions. Similar properties for the energy-dependent

succinate reduction of NAD suggests the activation of NAD or NADH which can then participate in transhydrogenase reaction.

The sonic supernatant from beef heart mitochondria (Kawasaki et al., 1964) isolated by sucrose density gradient showed that the energy-independent (reduction of NAD by NADPH) and ATP-dependent transhydrogenase (reduction of NADP by NADH) activities were found together in the same peak. This might be due to the involvement of energy-independent transhydrogenase in ATP-dependent NADP reduction by NADH.

III. ROLE OF TRANSHYDROGENASE IN LIVING SYSTEMS

The rate of glucose-6-P oxidation by the hexose monophosphate shunt is regulated by the availability of NADP (McGuire and Pesch, 1962) and the NADPH formed by the oxidation of glucose-6-P to 6-phosphogluconate is reoxidized by transhydrogenase which can be activated by several compounds, i.e. epinephrine, serotonin, estradiol and phenylacetaldehyde.



Pyridine nucleotide transhydrogenase not only controls glucose oxidation, but also it has been suggested that it plays a role in the utilization of fructose in mammalian brain (Stewart et al., 1964). It is known that fructose alone cannot support respiration in the brain (Harpur and Quastel, 1949)

and brain hexokinase has a low affinity for fructose (Sols and Crane, 1954). It has been shown (King and Mann, 1959 and Van Heyningen, 1959) that fructose is converted to lactate very slowly in nerve. Stewart and Passonneau (1964) suggested that possibly the role of fructose in nerves is to mediate transhydrogenation of NAD and NADP with the aid of aldose reductase and sorbital dehydrogenase as has been suggested for lens, placenta and seminal vesicles.

In mitochondria, electron transfer from NADPH to cytochrome c is catalyzed either directly by NADPH cytochrome c reductase or indirectly by transhydrogenase and NADH cytochrome c reductase (Vignais and Vignais, 1957). Studies by them suggested that the differences in oxidative phosphorylation rate in different tissues were related to their transhydrogenase activity. For example, in brain mitochondria in which the transhydrogenase via NADPH cytochrome c reductase has the maximal value for the phosphorylation quotient (P:O ratio) of 1. But in kidney or liver preparations the efficiency of the associated phosphorylation was twice as great as with brain mitochondria. It is clear that transhydrogenase plays a fundamental role in the regulation of the efficiency of phosphorylation.

Phosphorylation coupled to NADPH oxidation appears to require the initial transfer of hydrogen to NAD (Kaplan et al., 1956; Kielley et al., 1958). NADH oxidation through the

cytochrome c system is rapid and leads to phosphorylation. These separate pathways for the oxidation of NADH and NADPH are known to exist in animal tissues (Kaplan et al., 1956). The following scheme illustrates the role of pyridine nucleotide transhydrogenase in linking NADP and NAD systems:

$$\text{Substrate} \longrightarrow \text{NADH} \xrightarrow[-P]{\text{Fast}} 0$$

$$\text{Substrate} \longrightarrow \text{NADPH} \xrightarrow[-P]{\text{Slow}} 0$$

Transhydrogenase has been postulated to serve as a regulatory mechanism for the conservation of oxidative energy (Kaplan, 1955). The shifting of electrons from NADH to the NADP system would result in a lowering of phosphorylation; a shift in the direction of the NAD system would increase the level of phosphorylation.

IV. REGULATION OF TRANSHYDROGENASE BY 2'-AMP

2'-AMP apparently influences reaction 1 in Pseudomonas (Kaplan et al., 1953), promoting the completion of the oxidation of NADPH by NAD in the presence of phosphate. Reaction 1 is almost irreversible when catalyzed by bacterial enzymes except in the presence of 2'-AMP or its derivatives. Kaplan et al. (1953) concluded that it acted by reversing the inhibition caused by NADP. The stimulation of the forward reaction 1 by 2'-AMP may be due to this nucleotide causing a dissociation of a part of the NADP from the protein and

thereby making available a more active enzyme. The reverse of reaction 1 proceeded at only a very slight rate with the transhydrogenase from Chromatium in the presence of 2'-AMP (Keister and Hemmes, 1966). It should be noted that the animal tissue transhydrogenases are unaffected by 2'-AMP. Reaction 2 was very markedly stimulated by 2'-AMP in bacterial transhydrogenase (Kaplan et al., 1953).

Orlando et al., (1966) demonstrated the reduction of NADP by NADH either in the light or dark condition. They found that the presence of ADP and inorganic phosphate (P_i) slightly inhibited the NADP reduction in light and markedly enhanced this reaction in darkness. Somewhat greater inhibition in light was produced by ADP without P_i and very slight inhibition was produced by P_i alone. Both ADP and P_i separately enhanced the reaction in darkness though to a lesser extent than when both were present.

The working hypothesis suggested by Kaplan (1953) was that there may be three active sites involved in Pseudomonas transhydrogenase reaction. The first and second sites would be the points of attachments of the oxidized and reduced pyridine nucleotides; the third site would be the site of attachment for the activation of the enzyme. The first and second sites would be quite unspecific, since all the pyridine nucleotides appear to react with the Pseudomonas enzyme. However, the third site would be specific for the

phosphoadenosine derivatives, yielding an active complex with all derivatives except NADP.

V. PHOTOINDUCTION OF TRANSHYDROGENASE REACTION

Lazzarini and Woodruff (1964) suggested that the synthesis of transhydrogenase in Euglena is associated with the second phase of chloroplast maturation. In Euglena and bean leaf substantial levels of transhydrogenase were found in completely etiolated cells and this level increases 3 to 4-fold upon exposure to continuous illumination. The kinetics of this photoinduction of chlorophyll showed that both processes exhibit a 14-hour lag before the fully induced rates are attained. Preillumination by red light eliminated the lag during subsequent photoinduction.

MATERIALS AND METHODS

MATERIALS

Plants--Primary leaves of wheat (Triticum aestivum L. var. Selkirk) were grown in the greenhouse and harvested after 9 to 11 days for the preparation of transhydrogenase. To study the effect of benzimidazole and kinetin, five gram batches of 9-11 day-old leaves were harvested, washed, dried between paper towels and floated for 3 days on either 500 ml of benzimidazole (50 mg/litre), 500 ml of kinetin (5 mg/litre) or 500 ml of deionized water contained in separate glass trays. The trays were covered with Saran Wrap and immediately transferred to a growth chamber under conditions of continuous illumination at a light intensity of 3000 ft-c at 24°.

Chemicals and enzymes--NADP, NAD, NADPH, NADH, acetylpyridine-NAD, deamino-NAD, deamino-NADP, ATP, ADP, 2'-AMP, 3'-AMP, 5'-AMP, ITP, UTP, GTP, p-HMB, lactic dehydrogenase, malic dehydrogenase, sodium DL-isocitrate, Na isoascorbate, ethylenediamine tetraacetic acid (EDTA), and kinetin were obtained from Sigma Chemical Company, St. Louis, Missouri. Isocitrate dehydrogenase, protamine sulfate, HEPES and MES buffers, and Cleland's reagent (dithiothreitol) were purchased from CalBiochemical Lab., Los Angeles, California. Benzimidazole,

nicotinic acid, trigonelline were from Eastman Organic Chemicals, Rochester, New York. Acetone was from J. T. Baker Chemical Company, Phillisburg, N. J.

METHODS

Extraction and purification of transhydrogenase

From whole leaves--A modified procedure described by Keister and Stolzenbach (1960) was used in the purification of transhydrogenase from wheat leaves.

Step 1. Crude homogenate: Leaves were washed, cut, and ground into sections in a Waring blendor. The homogenate was filtered through 4-layers of cheesecloth and sufficient 0.5 M HEPES buffer containing 0.005 M Cleland's reagent, pH 7.8, was added to the dark green filtrate to give a final concentration of 0.05 M HEPES and 0.0005 M Cleland's reagent.

Step 2. Acetone fractionation: Acetone, precooled to -20°, was added to the crude extract until a 40% concentration was obtained. This mixture was then stirred for 10 minutes while the temperature was maintained at -10°. The precipitate obtained after centrifugation at 15,000 rpm* for 10 minutes (-10°) was discarded. The acetone concentration in the supernatant fluid was then raised to 70% and the resulting precipitate which contained all of the transhydrogenase activity was centrifuged as before and resuspended in 0.05 M HEPES buffer and 0.0005 M Cleland's reagent, pH 7.8. This

*Sorvall Centrifuge Model RC2-B with SS-34 Head (r=4.25 inches).

was again centrifuged at 13,000 rpm for 5 minutes to remove undissolved materials.

Step 3. Treatment with protamine sulfate and heat: One-fifth volume of 2% protamine sulfate adjusted to pH 6.5 was stirred with the enzyme preparation for 15 minutes. The mixture was heated to 52-54° for 15 minutes with gentle agitation. The mixture was rapidly cooled in an ice bath and the denatured protein was removed by centrifugation at 15,000 rpm for 10 minutes. Protamine sulfate was found to protect the enzyme during heating. The supernatant was dialyzed against 0.005 M HEPES and 0.0005 M Cleland's reagent for 2 hours.

From chloroplasts--The method of isolating chloroplasts described by Jensen et al. (1966) was used in this procedure. Each of two solutions (A and B) used in the isolation contained the following: 0.33 M sorbital; 0.002 M $NaNO_3$; 0.002 M EDTA; 0.002 M Na isoascorbate; 0.001 M $MnCl_2$; 0.002 M NaCl; 0.0005 M K_2HPO_4. Solution A also contained 0.05 M MES [2-(N-morpholino) ethanesulfonic acid], adjusted with NaOH to pH 6.1. Solution B contained 0.05 M HEPES (N-2-hydroxyethypiperazine-N'-2-ethanesulfonic acid), adjusted with NaOH to pH 6.7.

Five grams of freshly cut leaves were washed and chilled. The leaf blades and 15 ml chilled solution A were homogenized in a Waring blendor for 5 seconds. The slurry was pressed through 6 layers of cheesecloth, and the resulting juice was

centrifuged for 50 seconds at 2000 x g. The precipitate was resuspended in 2.5 ml solution B at 0°. This chloroplast fraction was used for enzyme assays.

Assay of transhydrogenase--The standard assay system for the forward reaction 1 was similar to that described by Colowick et al. (1952) but included 16 mM HEPES buffer at pH 7.8, 10 µmoles of $MgCl_2$, 10 µmoles of sodium isocitrate, an excess of NADP linked isocitrate dehydrogenase, 0.05 µmoles of NADP, 4 µmoles of NAD, in a total volume of 3.0 ml. The reaction was started by the addition of NADP which is reduced to NADPH by isocitrate dehydrogenase system. Once the reduction of NADP is complete the transhydrogenase reaction was started by the addition of NAD. NADPH is then alternately oxidized and reduced to provide a continuous supply of substrate. One unit of transhydrogenase is defined as that amount of enzyme which will cause an O.D. change of 0.01 per minute at 340 mµ. Spectrophotometric measurements were made with a Zeiss PMQ II spectrophotometer.

The assay system for the reverse of reaction 1 was similar to that described by Danielson et al. (1963) and included 16 mM HEPES buffer at pH 7.8, 10 µmoles of $MgCl_2$, 0.1 ml of 95% ethanol, an excess of alcohol dehydrogenase, 0.05 µmoles of NAD, 0.2 µmoles of NADP, 4 µmoles of ATP (pH 6.5), in a total volume of 3.0 ml. When the reduction of NAD by the alcohol dehydrogenase system was completed, 0.2

μmoles of NADP was added. One unit of transhydrogenase is defined as above.

When acetylpyridine-NAD was used as the acceptor, NADPH was used in substrate quantities rather than being generated continuously. In this assay,the reaction mixture contained 0.3 μmoles of NADPH, 0.3 μmoles of acetylpyridine-NAD, and 16 mM HEPES buffer, pH 7.8, in a total volume of 3.0 ml. The reaction was started by the addition of enzyme and the increase in optical density at 375 mμ served as a direct measure of enzymatic activity.

When deamino-NADP and deamino-NAD were used as hydrogen acceptors, the enzyme assay system was the same for the forward reaction 1. The concentration of deamino-NADP and deamino-NAD used was 0.3 μmoles.

Assay of diaphorase--Diaphorase was assayed by a modified procedure of Avron and Jagendorf (1956) using trichloro-indophenol as the electron acceptor. The system contained 16 mM HEPES buffer at pH 7.8, 0.2 μmoles of NADP or NAD, 10 μmoles of $MgCl_2$, 10 μmoles of sodium isocitrate or 0.1 ml of 95% ethanol, excess of isocitrate dehydrogenase or alcohol dehydrogenase, and sufficient trichloroindophenol dye (about 0.24 μmoles) in a total volume of 3.0 ml to give an optical density of approximately 1.5 at 620 mμ. The reaction was started by the addition of the enzyme and the activity was measured for one minute. One unit of enzyme activity is defined

as the amount of enzyme which produces an O.D. change of 0.01 per minute when measured in a 1.0-cm light path at 620 mμ.

Assay of menadione reductase--Menadione reductase was assayed by a modified procedure of Wosilait and Nason (1954), in a system containing 16 mM HEPES buffer at pH 7.8, 0.35 μmoles of NADPH or NADH, 0.6 μmoles of menadione in a final volume of 3.0 ml. The reaction was started by the addition of enzyme and the decrease in optical density at 340 mμ was measured for one minute. One enzyme unit is defined as that amount which causes a decrease in O.D. of 0.01 per minute at 340 mμ.

Assay of cytochrome c reductase--Cytochrome c reductase was assayed according to the method of Marre and Servettaz (1958). The reaction mixture contained 0.2 μmoles of NADPH or NADH, 0.3 μmoles of flavin mononucleotide, 0.2 μmoles of cytochrome c and 16 mM HEPES buffer, pH 7.8, in a final volume of 3.0 ml. After addition of the enzyme, the increase in optical density at 550 mμ was measured for one minute. One unit of enzyme is defined as that amount which produces an optical density change of 0.01 per minute when measured in a 1.0-cm light path at 550 mμ.

Molecular weight determination--Molecular weight determination of transhydrogenase was carried out by the sucrose density-gradient method (Martin et al., 1961) with a 4-20% sucrose gradient in 0.05 M HEPES buffer, pH 7.8. A sample solution

(0.2 ml) containing transhydrogenase and either malic dehydrogenase or lactic dehydrogenase as markers was carefully layered on the top of gradients and centrifuged at 35,000 rpm for 18 hours at 0° in Model L Spinco. Malic dehydrogenase and lactic dehydrogenase activities were assayed by the methods of Ochoa (1955) and Kornberg (1955), respectively. Transhydrogenase was measured as previously described.

Protein determination--Protein was determined by the phenol method of Lowery et al. (1951).

RESULTS

Purification--The purification procedures for whole leaves resulted in increased enzyme specific activity of approximately 300-fold (Table I). Activity was very low in crude extracts due to a heat stable and dialyzable inhibitor in the crude extracts.

TABLE I

SUMMARY OF ENZYME PURIFICATION FOR WHOLE LEAVES

Fraction	Total volume (ml)	Total protein (mg)	Total enzyme units	Specific activity (units/mg)
1. Crude extract	94.5	1058.4	567	0.53
2. Extract of acetone precipitate	9.4	23.5	3713	158
3. Protamine sulfate and heat step	10.4	20.8	3608.8	178.3

Intracellular localization--Enzyme activity was found to be localized in chloroplasts (Table II) isolated by the method of Jensen et al. (1966). Enzyme activity in chloroplasts progressed linearily with time.

TABLE II

INTRACELLULAR LOCALIZATION OF TRANSHYDROGENASE

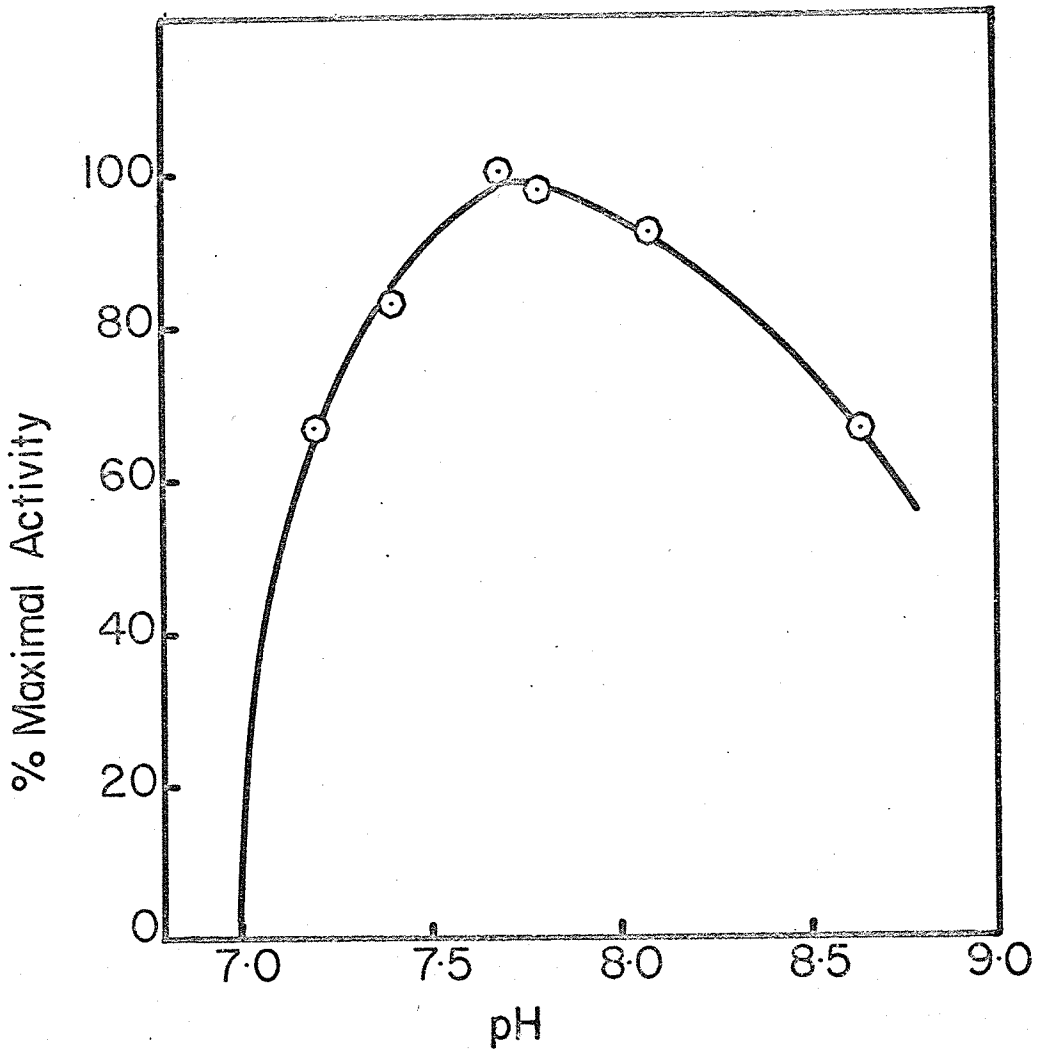
	Enzyme units		
	Exp. 1	Exp. 2	Exp. 3
Chloroplast	13	9.5	12
Supernatant	0	2.7	0

Reverse reaction--The reverse of reaction 1 (assayed as described in "Methods") could not be demonstrated either in extracts from whole leaves or chloroplasts even in the presence of ATP or 2'-AMP.

Effect of pH--The rate of reduction of NAD by NADPH at different pH values is shown in Fig. 1. Since the isocitrate dehydrogenase reaction was in excess at each pH value with the amount of pig heart enzyme used, the observed values represent the effect of pH on the rate of the transhydrogenase reaction. It is seen that the activity is optimal at about pH 7.7 - 7.8 which is lower than the pH optimum of 8.8 found for the spinach enzyme (Keister et al., 1960) and higher than the value of 6.2 to 6.3 obtained for the beef heart enzyme (Kaplan et al., 1953) and rat liver heart enzyme (Humphrey, 1957).

Effect of temperature--As the temperature was increased from

FIGURE 1. Effect of pH on the activity of transhydrogenase.


% Maximal Activity
100
80
60
40
20
0
7·0
7·5
8·0
8·5
9·0
pH

18.9° enzyme activity increased to a maximum at 32° followed by a rapid decrease to zero at 40° (Fig. 2(a)), enzyme activity being measured as the change in optical density at 340 mμ in 3 minutes.

The energy of activation was calculated using the Arrhenius equation:

$$\log \frac{k_2}{k_1} = \frac{E(T_2 - T_1)}{RT_1T_2}$$

where k_1 and k_2 are the rates at two temperatures and equivalent to v_1 and v_2, T_1 and T_2 are the absolute temperatures, E is the energy of activation, and R is the gas constant.

The Arrhenius plot of logarithm of velocity as a function of the reciprocal of the absolute temperature showed linearity between 22° to 32° and is shown in Fig. 2(b). Here, $T_1 = 295°k$, $v_1 = 255$, $T_2 = 305°k$, $v_2 = 303$,

$$\log \frac{303}{255} = \frac{E}{4.6} \times \frac{(305-295)}{295 \times 305}$$

$$E = 3{,}097 \text{ cal/mole}$$

Or, the energy of activation can be calculated from the slope:

$$\text{Slope} = -\frac{\Delta E}{4.6}$$

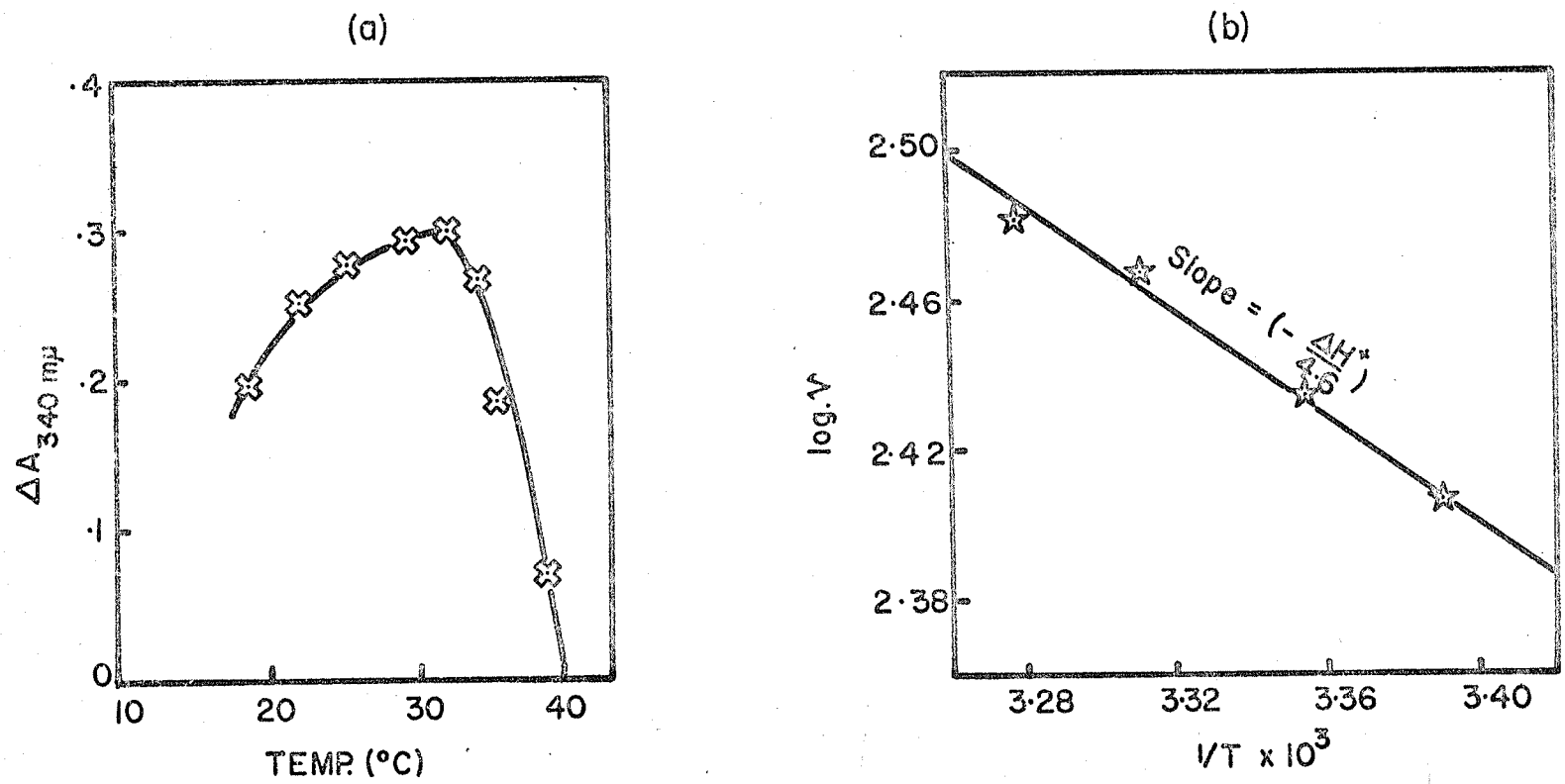
$$\frac{2.394 - 2.472}{\left(\frac{3.40 - 3.30}{10^3}\right)} = -\frac{\Delta E}{4.6}$$

$$\Delta E = 3{,}590 \text{ cal/mole}$$

Effect of enzyme concentration--There was a linear relationship

FIGURE 2(a). Effect of temperature on the activity of transhydrogenase.

2(b). Arrhenius plot of log velocity against 1/T.


(a)
.4
.3
.2
.1
0
$\Delta A_{340\ m\mu}$
10
20
30
40
TEMP. (°C)
(b)
2·50
2·46
2·42
2·38
log. v
Slope = $(-\frac{\Delta H^{\ddagger}}{4.6})$
3·28
3·32
3·36
3·40
$1/T \times 10^3$

between the reaction velocity and enzyme concentration up to 0.1 ml of enzyme preparation containing 0.25 mg protein (Fig. 3).

Effect of substrate concentration--All initial velocity data were plotted in the double reciprocal form (1/v versus 1/S) according to Lineweaver and Burk (1934). The nomenclature of reaction mechanisms and definition of kinetic constants are those proposed by Cleland (1963).

(A). NAD as variable substrate--The progress of the enzyme reaction is shown in Fig. 3 at different concentrations of NAD. This graph shows the initial reduction of NADP by the isocitrate dehydrogenase system with an increase in optical density of ca. 0.1. The reaction is linear at all concentrations of NAD during the 10 minute period.

Double reciprocal plots with NAD as the variable substrate at fixed concentration of NADPH are shown in Figs. 4 and 5. At low concentrations of NADPH (0.33 - 1 μM) the rates increased with increasing NADPH concentration as the parallel plots indicate (Fig. 4). The K_{app} for NAD changes from 10^{-4} M at 0.33 μM NADPH to 10^{-5} M at 1 μM indicating that NADPH influences the binding of NAD to the enzyme.

It is shown later that when NADPH is the variable substrate a similar parallel pattern of the double reciprocal plots emerge. Data of this type indicate that the reaction mechanism is Ping Pong Bi Bi.

FIGURE 3. Time course of transhydrogenase reaction and velocity against enzyme concentration.

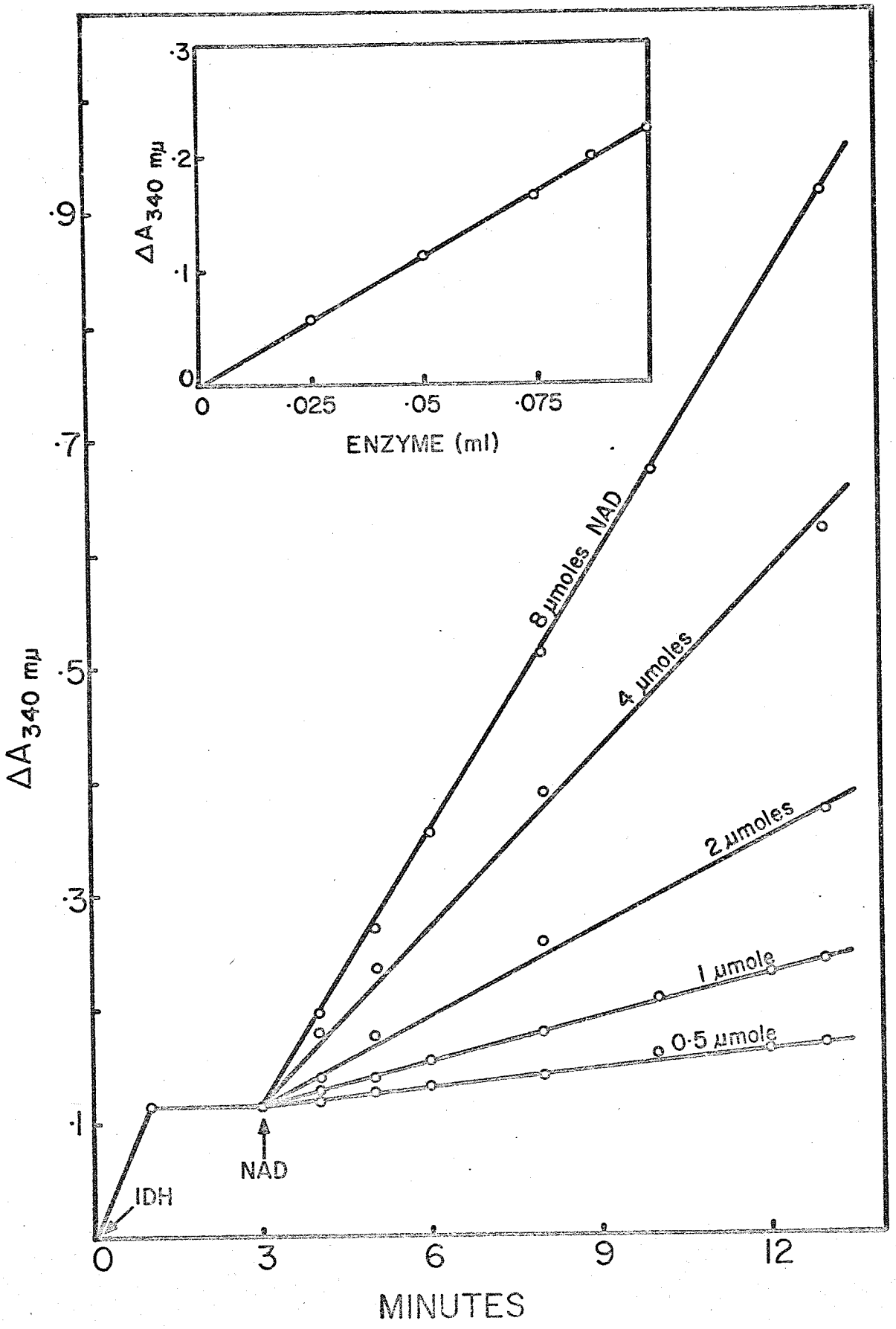

ΔA340 mμ
·9
·7
·5
·3
·1
0
3
6
9
12
MINUTES
IDH
NAD
8 μmoles NAD
4 μmoles
2 μmoles
1 μmole
0·5 μmole
ΔA340 mμ
·3
·2
·1
0
0
·025
·05
·075
ENZYME (ml)

FIGURE 4. Initial velocity pattern with NAD as variable substrate at three fixed concentrations of NADPH (0.33 μM, 0.67 μM, and 1 μM).

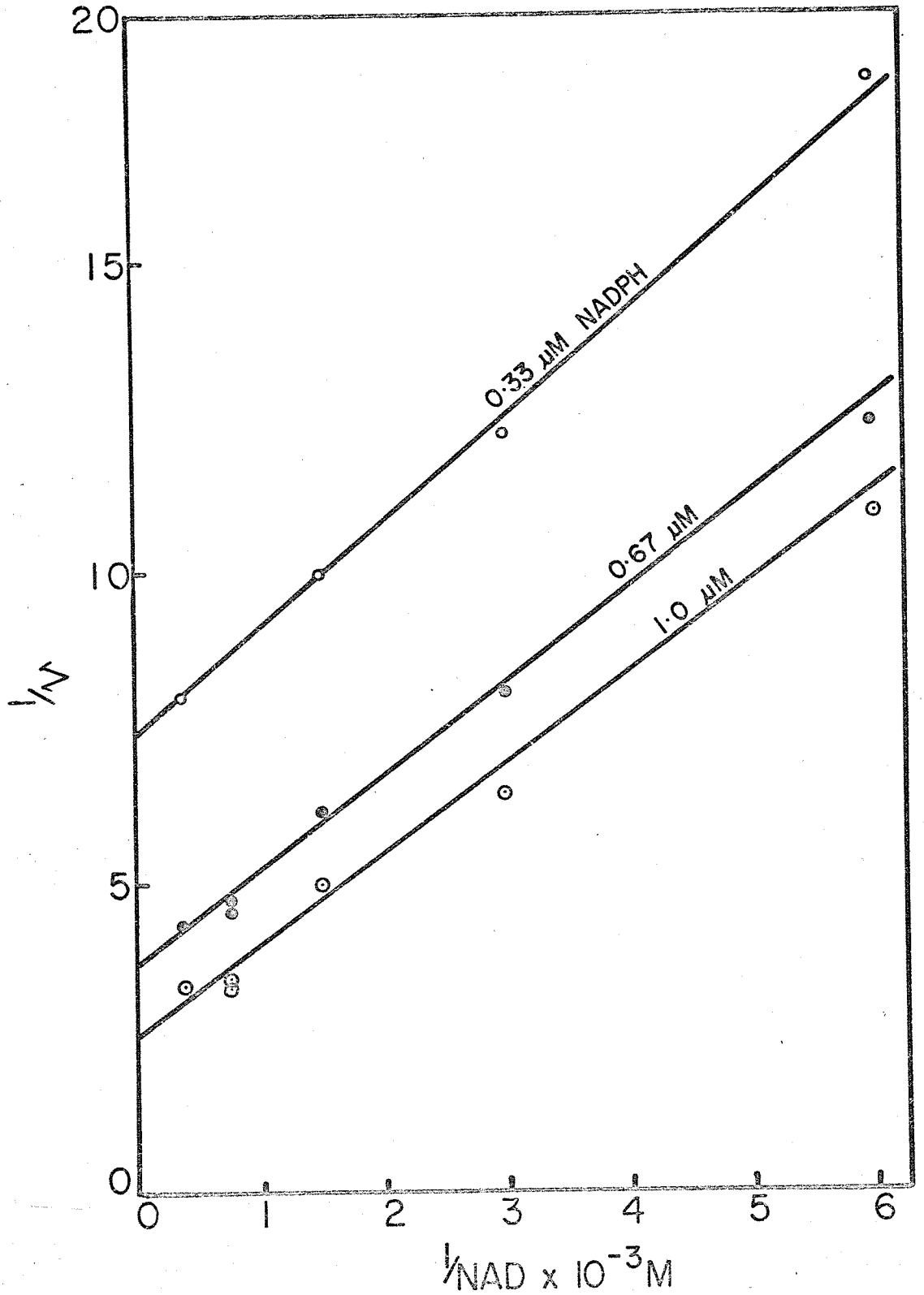
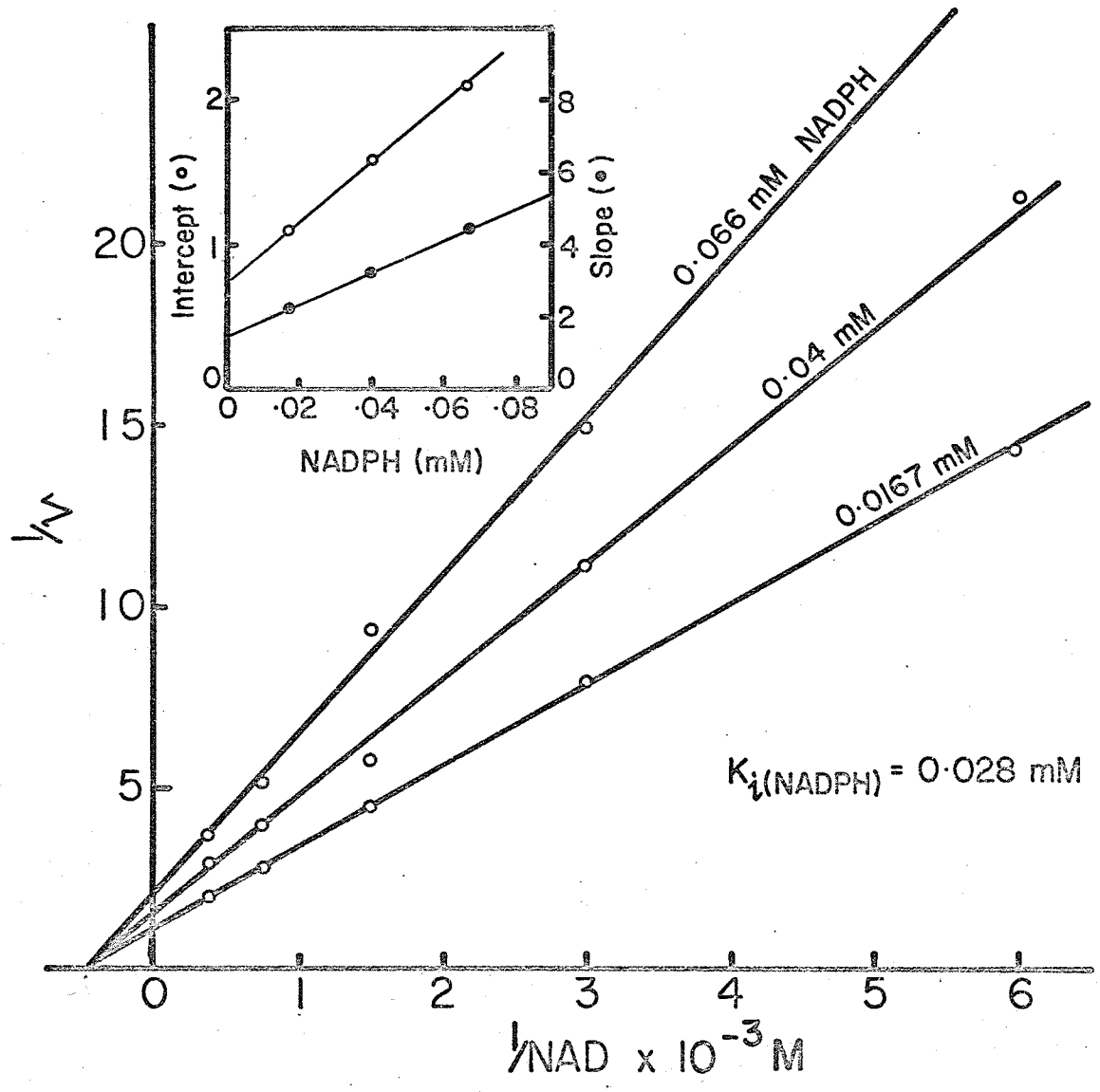
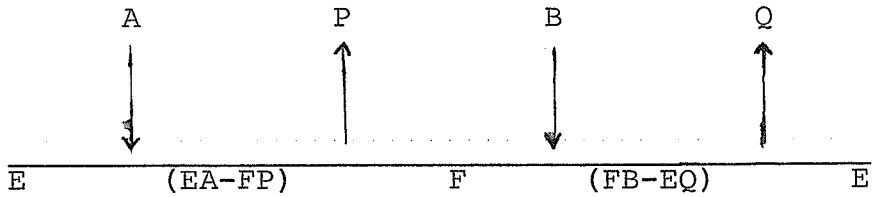

20
15
10
5
0
1/V
0.33 μM NADPH
0.67 μM
1.0 μM
0
1
2
3
4
5
6
1/NAD x 10⁻³M

FIGURE 5. Reciprocal plots with NAD as variable substrate at three fixed concentrations of NADPH showing non-competitive inhibition.

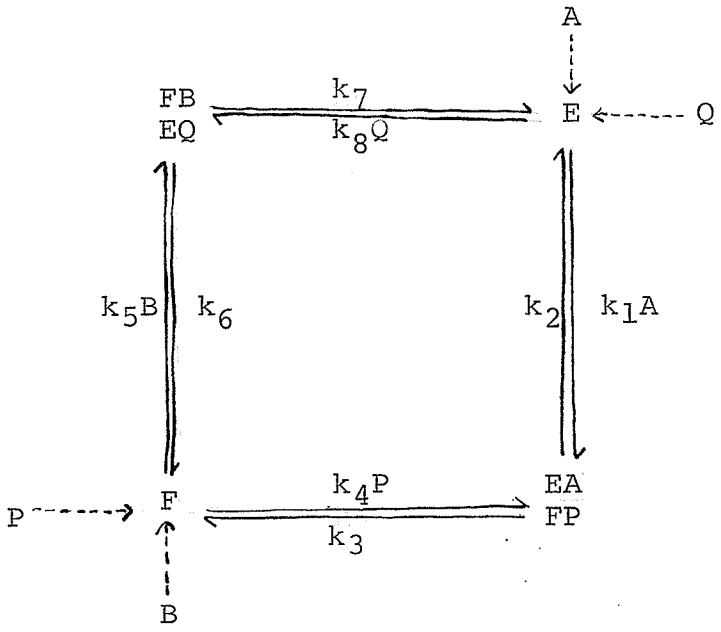

0·066 mM NADPH
0·04 mM
0·0167 mM
$K_{i(NADPH)}$ = 0·028 mM
1/V
1/NAD x 10^{-3} M
Intercept (•)
Slope (•)
NADPH (mM)
0
·02
·04
·06
·08
0
1
2
3
4
5
6
0
5
10
15
20
0
2
4
6
8

In such a reaction the enzyme oscillates between two stable forms (E and F) as in Cleland's representation:



where A and B are substrates and P and Q are products.

or by the schematic method of King and Altman (1956):



The initial velocity rate equation in terms of kinetic constants when P and Q are zero is:

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

which in the double reciprocal form is:

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

where V_1 is the maximum velocity for the forward direction, k_1 ..... k_8 are rate constants and K_a and K_b are the Michaelis constants for A and B respectively.

At higher concentrations (> 16 μM) NADPH becomes a non-competitive inhibitor as shown by the intercept of the reciprocal plots at the left of the ordinate when 3 fixed concentrations of NADPH were used (Fig. 5). In non-competitive inhibition, the double reciprocal plot of 1/v versus 1/S, the slope and intercept are both altered by factors $(1 + I/K_i)$, where I is the concentration of the inhibitor and K_i is the inhibition constant. K_i can be evaluated from the replot of slopes or intercepts versus I as follows:

from equation II when slope is plotted against I:

$$\text{Slope} = \frac{d(\frac{1}{v})}{d(\frac{1}{A})} = \frac{K_a}{V_1}\left(1 + \frac{I}{K_i}\right)_{\text{slope}}$$

when slope = 0:

$$1 + \frac{I}{K_i}_{\text{slope}} = 0 \qquad \therefore \; I = -K_{i_{\text{slope}}}$$

similarly from equation II when intercept is plotted against I,

$$\text{Intercept} = \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right)\left(1 + \frac{I}{K_i}_{\text{intercept}}\right)$$

when intercept = 0,

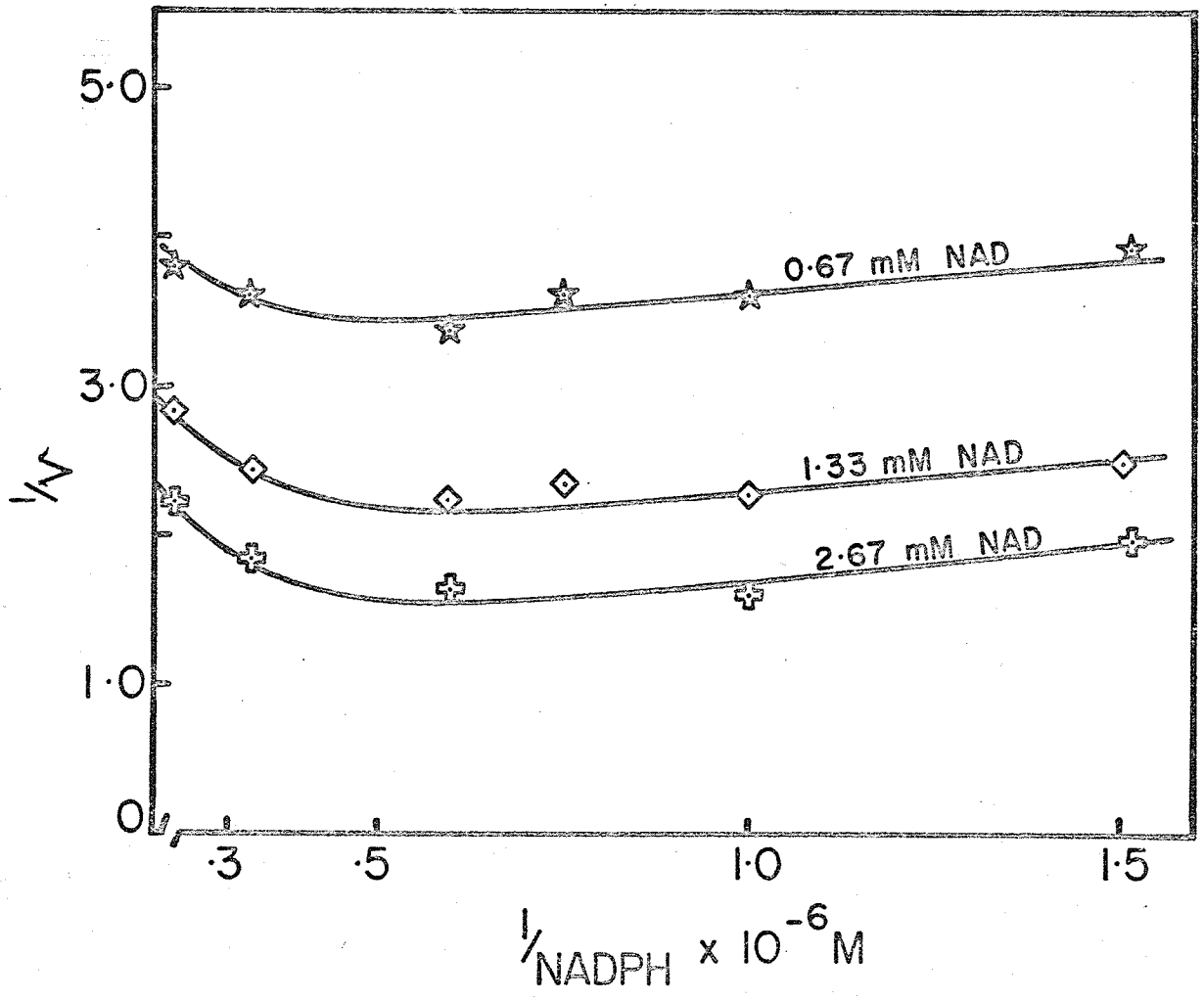
$$1 + \frac{I}{K_i}_{\text{intercept}} = 0 \qquad \therefore \; I = -K_{i_{\text{intercept}}}$$

Extrapolation of the plots to the I axis gives a K_i value of 2.8×10^{-5} M for NADPH in both cases. The $K_{i\,(NADPH)}$ evaluated from the replot of intercept is identical to the value obtained from the replot of slope. This indicated that NADPH has the same affinity for binding to E and F.

(B). NADPH as variable substrate--When NADPH was varied at 3 fixed concentrations of NAD differing in concentration in the order of 1:1000, respectively, the double reciprocal plots showed a parallel pattern (Fig. 6). The plots curve upwards at the highest NADPH concentration indicating inhibition by this substrate as would be expected from the data of Fig. 5.

The parallel patterns of the double reciprocal plots emerge when either NAD or NADPH is variable substrate (Figs. 4 and 6) which indicates the reaction mechanism is Ping Pong with rate equations as in equations I and II. In the Ping Pong mechanism, the apparent $V = \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right)$ is the same as in Ordered Bi Bi mechanism, but the K_{app} = Slope/Intercept = $\frac{K_a}{\left(1+\frac{K_b}{B}\right)}$ is different and the apparent K, since it is a linear function of B, must always increase with increase to the limiting constant K_a. This is obtained when $B > K_b$ or when B is saturating. Whereas the plot of $1/K_{app}$ against 1/B is a hyperbola in Ordered Bi Bi mechanisms, in Ping Pong

FIGURE 6. Initial velocity pattern with NADPH as variable substrate at three fixed concentrations of NAD (o.67 mM, 1.33 mM, and 2.67 mM).


5·0
3·0
1·0
0
1/v
0·67 mM NAD
1·33 mM NAD
2·67 mM NAD
·3
·5
1·0
1·5
1/NADPH x 10⁻⁶ M

mechanisms it is a straight line.

The ratio of K_{app}/V is a constant and equals $K_a V_1$ in the Ping Pong Bi Bi mechanism but it is a linear function of 1/B in the Ordered Bi Bi mechanism. Since there is no reversibility between A and B if P = 0, a plot of 1/v against 1/A at constant, non-saturating levels of B will give parallel lines and not the intersecting pattern as observed in Ordered Bi Bi.

These relationships serve to distinguish Ping Pong from other mechanisms. Also it is not necessary, in the case of Ping Pong mechanisms, to plot the intercepts against 1/A or 1/B to evaluate K_a and K_b as is done with Ordered Bi Bi. The reciprocal form of the rate equation II shows that if the ratio of A/B is held constant, e.g. A/B = x then the equation II becomes:

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

and if the concentrations of A/B are varied together in constant ratio and 1/v plotted against 1/A or 1/B, V_1 can be determined directly from the intercept. Since a plot of 1/v against 1/A or 1/B gives a non-intersecting pattern in which the slopes are K_b/V_1 and K_a/V_1 respectively, (equation II), K_a and K_b can be evaluated from the two plots. This cannot be done with the Ordered Bi Bi mechanisms.

To explore whether equation III holds, the enzyme was assayed under conditions where the two substrates were varied

at constant ratio of 1:1000 (mole:mole) and the reaction rates measured. The curve obtained when velocity versus concentration was plotted in the double reciprocal form is a straight line (Fig. 7). The V_{max} directly obtained from the intercept of the constant ratio plot was 3.3. The K_m with respect to NAD was 5.0×10^{-3} M and with respect to NADPH was 1.33×10^{-6} M.

Nucleotide specificity--Table III shows the nucleotide specificity of the wheat leaf enzyme. This is similar to that reported earlier by Keister et al. (1960) for spinach leaf transhydrogenase. With NADPH as a hydrogen donor, the enzyme catalyzes the reduction of NAD, deamino-NADP, deamino-NAD and acetylpyridine-NAD. It is seen that the hydrogen transfer reaction is greatest when deamino-NADP is the acceptor; whereas, it is lowest with either deamino-NAD or acetylpyridine-NAD. With NADH as a reductant, deamino-NAD readily served as a hydrogen acceptor but not the NADP analogue or acetylpyridine-NAD. Therefore, wheat leave transhydrogenase may be regarded as NADP specific enzyme.

Effect of ions--Table IV shows the effect of ions on the activity of transhydrogenase. Of the divalent cations tested, only Mg and Mn activated the reaction, Mg being the most effective. All other cations tested were inhibitory.

Effect of inhibitors

FIGURE 7. Reciprocal plots for the enzyme with the concentrations of both substrates varied in constant ratio (see text).

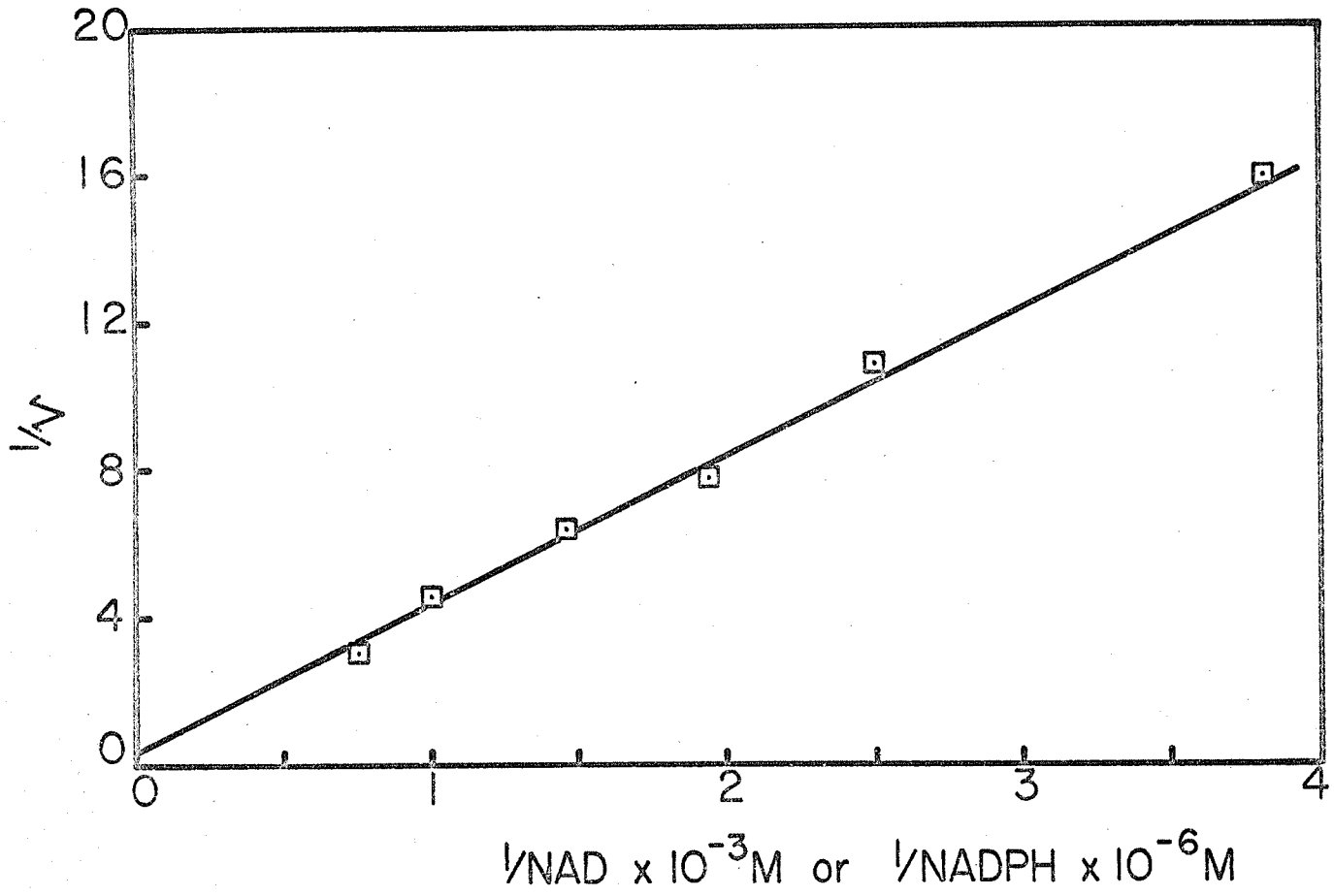

20
16
12
8
4
0
1/v
0
1
2
3
4
1/NAD x 10^-3 M or 1/NADPH x 10^-6 M

TABLE III

THE PYRIDINE NUCLEOTIDE SPECIFICITY OF TRANSHYDROGENASE

Hydrogen donor	Hydrogen acceptor	Activity (enzyme units)
NADPH	NAD	86
NADPH	Deamino-NADP	91
NADPH	Deamino-NAD	14
NADPH	Acetylpyridine-NAD	15
NADH	NADP	0
NADH	Deamino-NADP	14
NADH	Deamino-NAD	540
NADH	Acetylpyridine-NAD	0

TABLE IV

EFFECT OF IONS

Ion	Concentration	% Inhibition	% Activation
$MgCl_2$	1.6×10^{-3} M		20.6
$CoCl_2$	1.0×10^{-4} M	16.3	
$MnCl_2$	1.0×10^{-4} M		18.7
$CaCl_2$	1.0×10^{-4} M	44.8	
KCN	3.3×10^{-5} M	16.9	
$AgNO_3$	1.0×10^{-4} M	55.6	

(A). 2'-AMP--2'-AMP has been found to be a competitive inhibitor of many NADP-linked enzymes (Neufeld et al., 1955). In this study 2'-AMP was also found to be a competitive inhibitor with respect to NADPH as well as to NAD. The results are shown in Figs. 8 and 9 in the double reciprocal form. The K_i was calculated by replotting the concentration of 2'-AMP against the slope. The K_i for NADPH is 1.2×10^{-4} M and for NAD is 8.5×10^{-4} M.

(B). Other inhibitors--As can be seen from Table V, neither 3'-AMP nor 5'-AMP exhibited any inhibitory effect, nor did dinitrophenol. All other compounds tested were inhibitory to some degree, p-HMP being the most effective as it gave 100% inhibition at the test concentration.

Effect of nicotinic acid, nicotinamide, trigonelline and 4-amino-3,5,6-trichloro-picolinic acid--Nicotinic acid and nicotinamide are intermediates in the biosynthesis of NAD in wheat leaves (Godavari, 1966). Trigonelline is a methyl derivative of nicotinic acid also found in wheat leaves and 4-amino-3,5,6-trichloro-picolinic acid is a herbicide. The concentration of these compounds used in the assay system was 1.0×10^{-4} M. Nicotinic acid inhibited the reaction by 7.6%. The other compounds increased the rate of reaction by 16.8, 15, and 21.9% respectively.

Molecular weight determination--Schachman (1959) calculated

FIGURE 8. Competitive inhibition by 2'-AMP with NADPH as variable substrate.

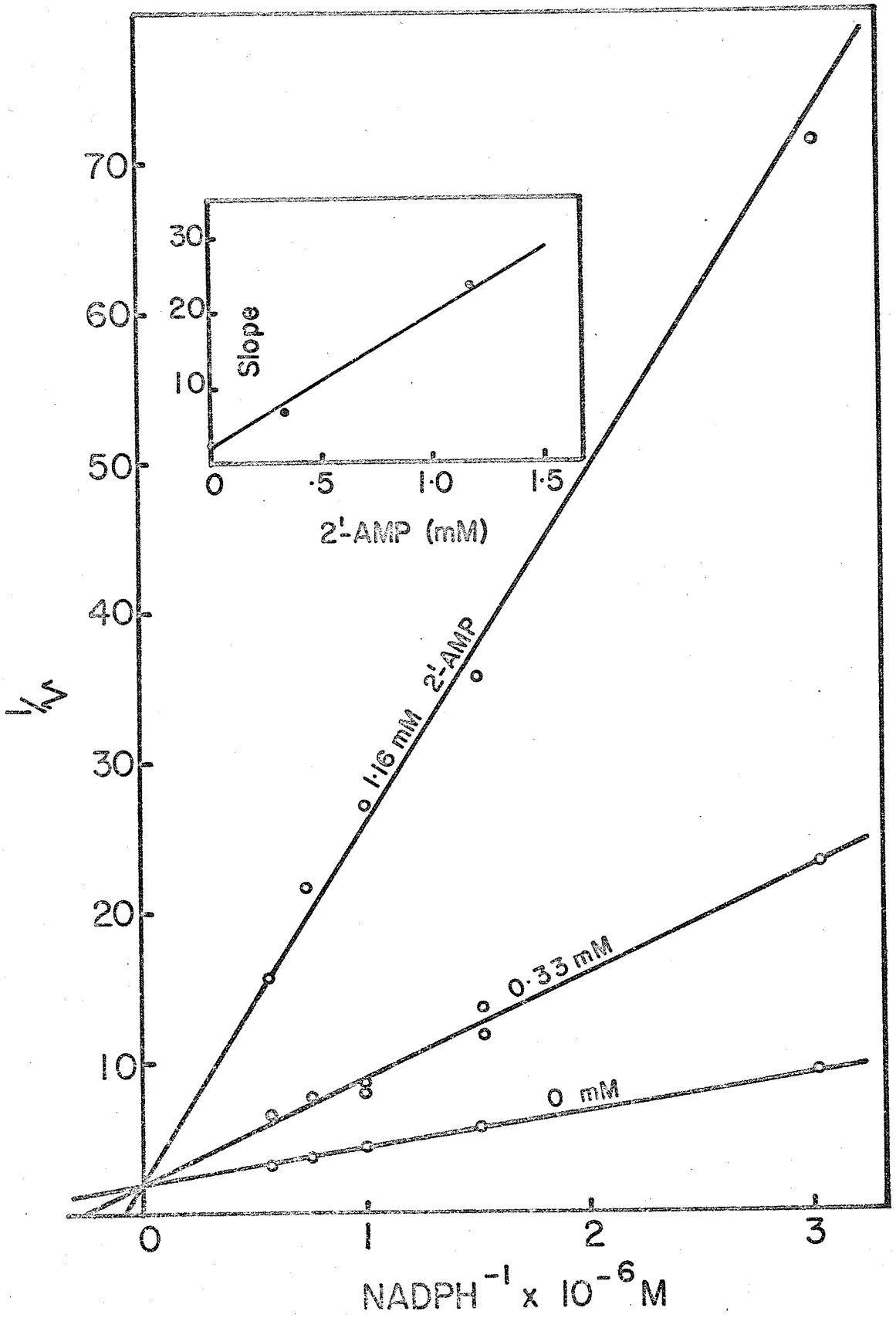

70
60
50
40
30
20
10
1/v
30
20
10
Slope
0
·5
1·0
1·5
2'-AMP (mM)
1·16 mM 2'-AMP
0·33 mM
0 mM
0
1
2
3
NADPH⁻¹ x 10⁻⁶ M

FIGURE 9. Competitive inhibition by 2'-AMP with NAD as variable substrate.

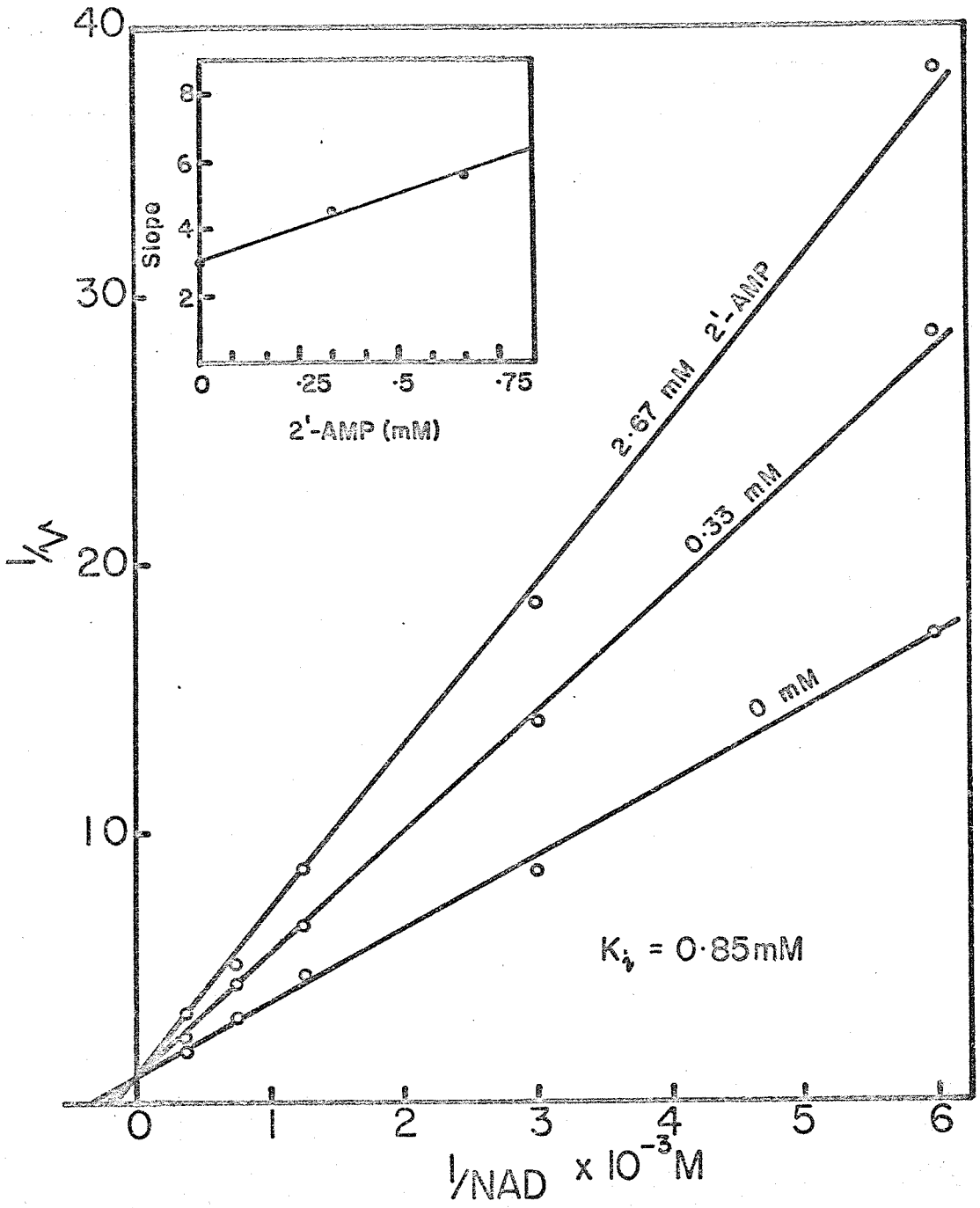

40
30
20
10
1/V
0
1
2
3
4
5
6
1/NAD x 10⁻³M
2·67 mM 2'-AMP
0·33 mM
0 mM
Ki = 0·85mM
Slope
8
6
4
2
0
·25
·5
·75
2'-AMP (mM)

TABLE V

EFFECT OF INHIBITORS ON TRANSHYDROGENASE

Compound	Concentration	% Inhibition
2'-AMP	0.33×10^{-4} M	42
3'-AMP	0.66×10^{-3} M	0
5'-AMP	0.66×10^{-3} M	0
ADP	0.66×10^{-3} M	21.8
ATP	1.0×10^{-4} M	19.7
ITP	"	35
UTP	"	56.4
GTP	"	14.2
Sodium pyrophosphate	0.66×10^{-3} M	11
Adenine	2×10^{-4} M	33.1
Dinitrophenol	2×10^{-4} M	0
p-HMP	3.3×10^{-5} M	100
Benzimidazole	1.3×10^{-5} M	13.2
Kinetin	7.6×10^{-7} M	14.7

the molecular weights of macromolecules using a sedimentation equilibrium technique and he showed that the sedimentation constants of two different macromolecules were related to their respective molecular weights according to the following equation:

$$S_1/S_2 = (MW_1/MW_2)^{2/3} \qquad (1)$$

where S is sedimentation constant and MW is molecular weight.

Martin and Ames (1961) demonstrated that molecular weights of protein mixtures could be determined by measurement of the sedimentation velocity after centrifugation in a sucrose gradient. The distance (D) a protein travelled from the meniscus was a direct measure of its sedimentation velocity and S_1/S_2 equalled D_1/D_2 thus, by substitution, equation (1) becomes:

$$D_1/D_2 = (MW_1/MW_2)^{2/3} \qquad (2)$$

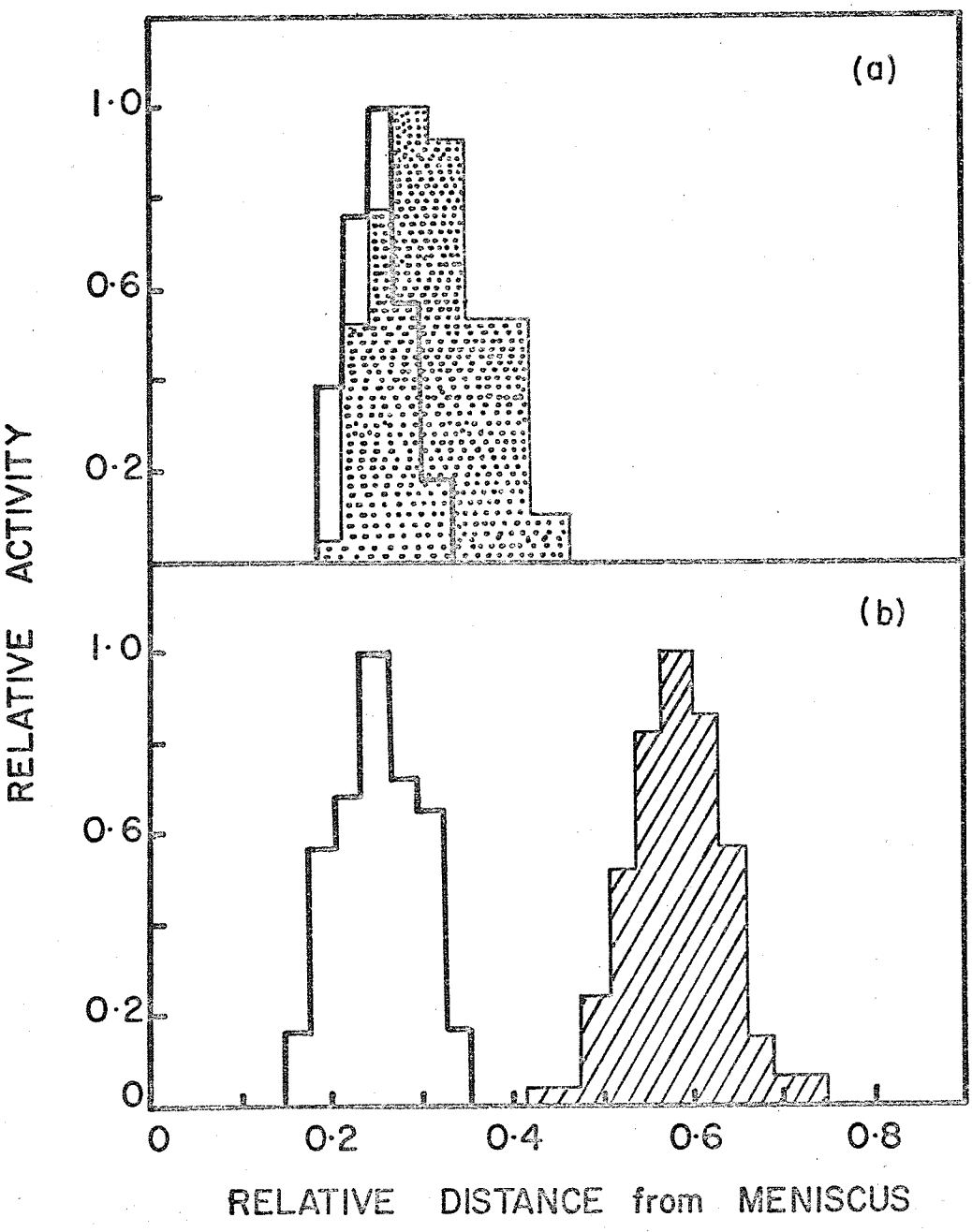
It is therefore possible to determine the molecular weight of a protein when it is combined in a mixture with a protein of known molecular weight.

Malic dehydrogenase (MW. 40,000) and lactic dehydrogenase (MW. 120,000) were used as markers. It can be seen from Fig. 10 that transhydrogenase overlapped with the malic dehydrogenase marker whereas lactic dehydrogenase which has a higher molecular weight than malic dehydrogenase was separated very well. Molecular weight of transhydrogenase calculated with malic dehydrogenase is 34,510 and with lactic

FIGURE 10. Relative distance travelled by transhydrogenase and reference proteins in a sucrose density gradient centrifugation.

(a) Transhydrogenase and malic denydrogenase.

(b) Transhydrogenase and lactic dehydrogenase.


(a)
(b)
1·0
0·6
0·2
0
RELATIVE ACTIVITY
0
0·2
0·4
0·6
0·8
RELATIVE DISTANCE from MENISCUS

dehydrogenase as marker is 40,360. The mean value of the molecular weight of transhydrogenase is therefore 37,000 ± 5,000.

Effect of benzimidazole and kinetin on the levels of transhydrogenase in chloroplasts from detached wheat leaves--Detached wheat leaves, exposed to light as described in methods, were floated for 3 days. At the end of this flotation period, enzymes were extracted from the chloroplasts according to the described procedure. When leaves were floated on water, most of the transhydrogenase activity was lost. On the other hand, in leaves floated on benzimidazole (50 mg/litre) and kinetin (5mg/litre) the levels of transhydrogenase activity were increased 2 to 3 times over that in immediately detached leaves (Table VI).

TABLE VI

EFFECT OF BENZIMIDAZOLE AND KINETIN ON TRANSHYDROGENASE LEVELS IN CHLOROPLASTS FROM DETACHED WHEAT LEAVES

Treatment	Protein (mg/ml)		Enzyme units		Specific Activity	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
1. Immediately detached	3.34	3.67	19	24	5.6	6.5
2. Water	2.61	2.73	5	0	1.9	0
3. Benzimidazole	3.24	3.20	43	59	13.2	18.4
4. Kinetin	3.30	3.51	52	57	14.8	16.2

Other enzymatic activity present in partially purified enzyme--The partially purified transhydrogenase preparations exhibit a number of other enzymatic activities, for example, diaphorase, menadione reductase and cytochrome c reductase (Table VII). It is difficult to decide whether each of these activities is associated with a single enzyme or different enzymes. However, when NADPH was used as a reductant the reaction was much faster than with NADH. This again shows that wheat leaf transhydrogenase is a NADP-specific enzyme.

TABLE VII

OTHER ENZYMATIC ACTIVITIES PRESENT IN PARTIALLY PURIFIED ENZYME

	Δ O.D. at 340 mμ in a minute
1. Diaphorase	
(1) NADPH as a reductant	1.58
(2) NADH as a reductant	0.5
2. Menadione reductase	
(1) NADPH as a reductant	0.18
(2) NADH as a reductant	0.095
3. Cytochrome c reductase	
(1) NADPH as a reductant	0.372
(2) NADH as a reductant	0.042

Enzyme stability--After dialysis against a solution containing 0.05 M Hepes buffer with 10^{-4} M Cleland's reagent added, the enzyme is stable for at least one month when stored at -15°.

Enzyme preparations can be heated to 52° - 54° without losing any activity. In contrast, in the complete assay system, activity is lost at temperatures above 40°.

DISCUSSION

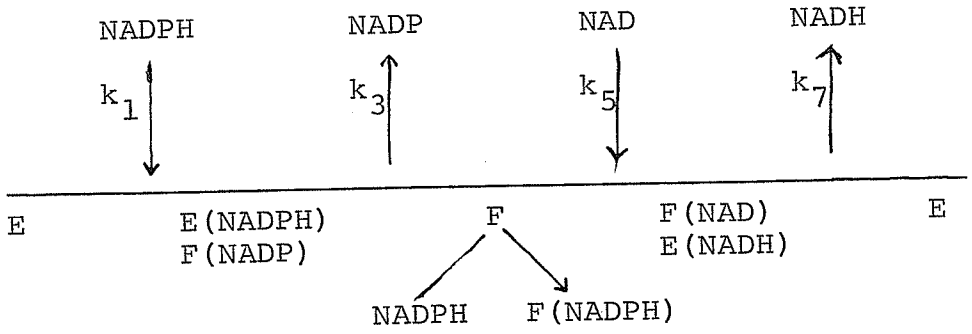
The kinetic analysis of initial velocity studies of transhydrogenase indicated that the enzyme followed a Ping Pong Bi Bi mechanism in which the two substrates are not present on the enzyme simultaneously but combine with different forms of the enzyme, in this case an oxidized form E and a reduced form F.

When one substrate was varied at several fixed concentrations of the second substrate, double reciprocal plots resulted in a family of linear parallel lines (Figs. 4 and 6) as expected from equation II. In an Ordered or a Rapid Equilibrium Random mechanism, both the slopes and intercepts would change. Accordingly, the reaction mechanism is Ping Pong Bi Bi rather than Ordered Bi Bi (Cleland, 1963).

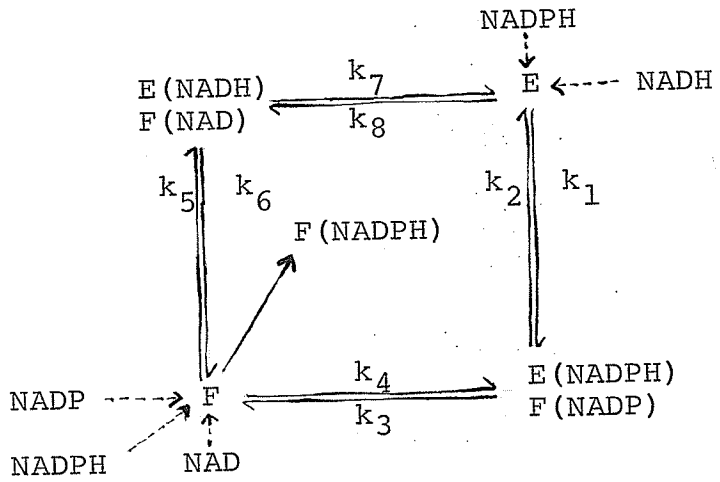
Cleland (1963) described a method to differentiate a Ping Pong Bi Bi mechanism from Ordered and Rapid Equilibrium Random Bi Bi mechanism. The rate equation for a Rapid Equilibrium Random Bi Bi mechanism is identical with that of an Ordered Bi Bi mechanism. When initial velocities are measured at various concentrations of one substrate, with a constant ratio of the two substrate concentrations, a double reciprocal plot of a Ping Pong Bi Bi reaction is the only mechanism that results in a straight-line relationship. This

is obvious from equation III if A/B is held constant. Maintaining a constant ratio of NADPH/NAD concentrations, a double reciprocal plot of transhydrogenase activity resulted in a linear relationship as shown in Fig. 7. Thus the reaction mechanism is Ping Pong Bi Bi.

At higher concentrations, NADPH was shown to be a non-competitive inhibitor of the enzyme indicating that it combined with two different forms of the enzyme, E and F or any complex of F. Neither NADH, NAD, nor NADP were inhibitory, therefore they combine with only one form of the enzyme. Since the reverse reaction with NADH and NADP as substrates does not operate then the order of substrate addition and product release is as follows:



or by the schematic method of King and Altman (1956):



where E is presumably an oxidized form of the enzyme and F is a reduced form.

In the diagrams above the substrate and non-competitive inhibitor NADPH is shown combining only with E as an enzyme-substrate complex and with F as an enzyme-inhibitor complex. However it is not possible from kinetic studies to establish the enzyme complex with which the inhibitor combines. Therefore the possibility exists that NADPH binds at an additional site on the complex F(NADP) preventing the release of NADP or on F(NAD) preventing the reduction of NAD. No further investigation was made to establish the exact binding site. However, it should be noted that in the non-competitive inhibition by NADPH the $K_{i_{slope}}$ was the same as $K_{i_{intercept}}$ (2.8×10^{-5} M). This could be interpreted as NADPH occupying the same site on enzyme form F as it does on E. If there was an additional site on enzyme for F which binds NADPH, one would expect different K_i values for slope and intercept. Also the K_m for NAD is considerably greater (5.0×10^{-3} M) than the K_m for NADPH (1.33×10^{-6} M) and the K_i for NADPH. For these reasons NADPH is shown binding to enzyme form F.

The reverse of reaction 1, that is

$$NADH + NADP^+ \longrightarrow NAD^+ + NADPH$$

was assayed using alcohol dehydrogenase for the generation of NADH. A considerable number of tests were made with the addition of 2'-AMP and ATP which have been shown in other

systems to activate this reaction (Kaplan et al., 1953; Danielson et al., 1963). However all assays were negative and it was concluded that reaction 1 was irreversible.

Further investigations on the reversibility of this reaction were based on the reaction mechanism as described previously. For example, the following partial reactions should occur:

$$NADPH + E \longrightarrow NADP^{+} + F$$

$$NADP^{+} + F + NADH \longrightarrow NAD^{+} + NADPH + E$$

In the first reaction NADPH would combine with E and become oxidized with the release of F. Under aerobic condition there was some oxidation of NADPH as measured by the absorbancy at 340 mμ but none under anaerobic conditions. Therefore, if the mechanism is correct, it would indicate that NADPH cannot be oxidized and NADP released unless the acceptor, NAD, is present on presumably an adjacent site.

If the first reaction takes place, then the product, NADP, in the presence of F, should be reduced by NADH (second reaction) and the formation of NADPH could be measured by the NADPH specific glutathione reductase system according to the reaction:

$$NADPH + G\text{-}S\text{-}S\text{-}G \longrightarrow NADP^{+} + 2\ GSH$$

However, neither this nor any method listed demonstrated the operation of these partial reactions. It is concluded that <u>in vitro</u> the powerful inhibitory effect of NADPH prevents the

reverse reaction. This may not be so in vivo where NADPH is rapidly oxidized and is in low concentration in the chloroplasts (Mishra and Waygood, 1968).

It has been shown in these studies that certain divalent metals and other compounds are potent inhibitors or activators of wheat leaf transhydrogenase. Notable amongst the inhibitors are 2'-AMP, p-HMP, Ca^{++} and Ag^{+}, while Mg^{++} and Mn^{++} are activators. The inhibition by 2'-AMP was of particular interest, since 2'-AMP has different effects on the transhydrogenases from various sources. The results show that 2'-AMP competitively inhibited the wheat leaf transhydrogenase with respect to both substrates, NADPH and NAD.

The results presented in this study indicate that the properties of transhydrogenase are similar to those reported by Keister and Stolzenbach (1960) for the same enzyme. Firstly, K_m values of both substrates are quite similar. The K_{NADPH} for wheat leaf enzyme was 1.33×10^{-6} M and for spinach leaf enzyme it was 2.3×10^{-6} M. However it was different from that found in insect enzyme (Horie et al., 1966) and in Pseudomonas enzyme (Kaplan et al, 1953); the K_{NADPH} being 1.2×10^{-5} M and 5×10^{-5} M, respectively. Likewise, K_{NAD} for wheat leaf enzyme was 5.0×10^{-3} M and for spinach leaf enzyme was 1.8×10^{-3} M, but 3.0×10^{-4} M for insect enzyme and 0.7×10^{-4} M for the bacterial enzyme. Secondly, 2'-AMP was shown to be a competitive inhibitor for the enzyme

isolated from both wheat and spinach leaves. Thirdly, it had the same catalytic specificity for pyridine nucleotides. Fourthly, other enzyme activities, i.e. diaphorase, menadione reductase and cytochrome c reductase, were present in both enzyme preparations.

Hommes and Estabrook (1963) showed the unusually high energy of activation for both energy-dependent and energy-independent reactions (> 20,000 cal/mole). In contrast to this observation, the activation energy of wheat leaf enzyme was quite low (3,500 cal/mole).

These studies show that benzimidazole and kinetin increased the level of transhydrogenase in Selkirk wheat leaves 3 days after detachment. Mishra and Waygood (1968) have shown both benzimidazole and kinetin have a profound effect on pyridine nucleotide metabolism in wheat leaves and these authors have implicated transhydrogenase in the diurnal variation of NAD(H) and NADP(H).

SUMMARY

1. A method for the preparation of transhydrogenase from wheat leaves (Triticum aestivum L. var. Selkirk) has been described. A natural inhibitor was found in crude extracts. It can be removed by acetone fractionation.
2. The enzyme was determined to be localized in chloroplasts.
3. Kinetic studies revealed the reaction mechanism to be Ping Pong with K_m values of 1.33×10^{-6} M and 5.0×10^{-3} M for NADPH and NAD, respectively. NADPH at higher concentrations (> 0.016 mM) showed non-competitive inhibition with a $K_i = 2.8 \times 10^{-5}$ M.
4. The pH optimum of the enzyme was pH 7.7.
5. The enzyme was found to have an energy of activation of 3,500 cal/mole. When the temperature was increased from 18.9 to 32°, there was an increase in enzyme activity followed by a rapid decrease to zero at 40°.
6. Transhydrogenase has been shown to be inhibited by Co^{++}, Ca^{++}, Ag^{+}, CN^{-}, sodium pyrophosphate, adenine, p-HMB, 2'-AMP, ADP, ATP, ITP, UTP, and GTP. 2'-AMP is a competitive inhibitor with respect to both substrates, NADPH and NAD. Whereas, 3'-AMP and 5'-AMP had no inhibitory effect.
7. The reverse reaction could not be demonstrated even in the

presence of ATP or 2'-AMP.

8. The substrate, NADPH, was quite specific whereas the substrate, NAD, could be replaced by deamino-NADP, deamino-NAD or acetylpyridine-NAD.

9. Diaphorase, menadione reductase, and cytochrome c reductase have been found in the enzyme preparation. NADPH is preferable to NADH as hydrogen donor for these enzymatic reactions.

10. The molecular weight of transhydrogenase was 37,000 ± 5,000 as determined by sucrose density gradient centrifugation.

11. Treatment of leaves with kinins (benzimidazole and kinetin) increased the specific activity of the enzyme 2-3 fold in contrast to an almost complete loss of enzyme activity in extracts from leaves floated on water.

BIBLIOGRAPHY

Andreoli, T. E., R. L. Pharo and D. R. Sanadi. 1964. On the mechanism of oxidative phosphorylation. VIII. Further evidence for distinct transhydrogenase reactions in submitochondrial particles. Biochim. Biophys. Acta 90: 16-23.

Asano, A., K. Imai and R. Sato. 1967. Oxidative phosphorylation in Micrococcus denitrificans. II. The properties of pyridine nucleotide transhydrogenase. Biochim. Biophys. Acta 143:477-486.

Avron, M. and A. T. Jagendorf. 1956. A TPNH diaphorase from chloroplasts. Arch. Biochem. Biophys. 65:475.

Ball, E. G. and O. Cooper. 1957. The oxidation of reduced triphosphopyridine nucleotide as mediated by the transhydrogenase reaction and its inhibition by thyroxine. Proc. Nat. Acad. Sci. U. S. 43:357-364.

Cleland, W. W. 1963. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. II. Inhibition: Nomenclature and theory. III. Prediction of initial velocity and inhibition patterns. Biochim. Biophys. Acta 67:104-188.

Colowick, S. P., N. O. Kaplan, E. F. Neufeld and M. M. Ciotti. 1952. Pyridine nucleotide transhydrogenase. I. Indirect evidence for the reaction and purification of the enzyme. J. Biol. Chem. 195:95-105.

Danielson, L. and L. Ernster. 1963. Energy dependence of pyridine nucleotide-linked dismutations in rat liver mitochondria. Biochem. Biophys. Res. Comm. 10:85-90.

_____ and _____. 1963. Demonstration of a mitochondrial energy-dependent pyridine nucleotide transhydrogenase reaction. Biochem. Biophys. Res. Comm. 10:91-96.

_____ and _____. 1963. Energy-dependent reduction of $\overline{TPN}^+$ by $\overline{DPNH}$ with submitochondrial particles. Acta Chem. Scand. 17:883.

Godavari, H. R. 1966. Nicotinamide adenine dinucleotide metabolism in higher plants. Ph. D. thesis, University of Manitoba, Winnipeg, Canada.

Harpur, R. P. and J. H. Quastel. 1949. Relations between acetylcholine synthesis and metabolism of carbohydrates and d-glucosamine in the central nervous system. Nature 164:779-782.

Hommes, F. and R. W. Estabrook. 1963. The role of transhydrogenase in the energy-linked reduction of TPN. Biochem. Biophys. Res. Comm. 11:1-6.

Horie, Y. and W. Chefurka. 1966. The distribution and properties of transhydrogenase in insect tissues. Comp. Biochem. Physiol. 17:1-18.

Humphrey, G. F. 1957. The distribution and properties of transhydrogenase from animal tissues. Biochem. J. 65: 546-550.

Jensen, R. G. and J. A. Bassham. 1966. Photosynthesis by isolated chloroplasts. Proc. Nat. Acad. Sci. U. S. 56:1095-1101.

Kaplan, N. O. 1955. Symposium on electron transport in the metabolism of micro-organisms. Bacteriol. Rev. 19:235-239.

_____, S. P. Colowick and E. F. Neufeld. 1952. Pyridine nucleotide transhydrogenase. II. Direct evidence for and mechanism of the transhydrogenase reaction. J. Biol. Chem. 195:107-119.

_____, _____ and _____. 1953. Pyridine nucleotide transhydrogenase. III. Animal tissue transhydrogenases. J. Biol. Chem. 205:1-15.

_____, _____, _____ and M. M. Ciotti. 1953. Pyridine nucleotide transhydrogenase. IV. Effect of adenylic acid a on the bacterial transhydrogenases. J. Biol. Chem. 205:17-29.

_____, M. N. Swartz, M. E. Frech and M. M. Ciotti. 1956. Phosphorylative and nonphosphorylative pathways of electron transfer in rat liver mitochondria. Proc. Nat. Acad. Sci. U. S. 42:481-487.

Kaufman, B. and N. O. Kaplan. 1961. Pyridine nucleotide transhydrogenase. VIII. Properties of transhydrogenase reactions of an enzyme complex isolated from beef heart. J. Biol. Chem. 236:2133-2139.

Kawasaki, T. and K. Satoh. 1964. The involvement of TD transhydrogenase in ATP-linked TPN reduction by DPNH. Federation Proc. 23:432.

Keister, D. L. and R. B. Hemmes. 1966. Pyridine nucleotide transhydrogenase from Chromatium. J. Biol. Chem. 241: 2820-2825.

_____, A. Jagendorf and A. San Pietro. 1962. Development of bean-leaf transhydrogenase in etiolated leaves. Biochim. Biophys. Acta 62:332-337.

_____, A. San Pietro and F. E. Stolzenbach. 1960. Pyridine nucleotide transhydrogenase from spinach. I. Purification and properties. J. Biol. Chem. 235:2989-2996.

_____ and N. J. Yike. 1966. Studies on an energy-linked pyridine nucleotide transhydrogenase in photosynthetic bacteria. I. Demonstration of the reaction in Rhodospirillum rubrum. Biochem. Biophys. Res. Comm. 24:519-525.

_____ and _____. 1967. Energy-linked reactions in photosynthetic bacteria. I. Succinate-linked ATP-driven NAD^+ reduction by Rhodospirillum rubrum chromatophores. Arch. Biochem. Biophys. 121:415-422.

Kielly, W. W. and J. R. Bronk. 1958. Oxidative phosphorylation in mitochondrial fragments obtained by sonic vibration. J. Biol. Chem. 230:521-533.

King, E. L. and C. Altman. 1956. A schematic method of deriving the rate laws for enzyme-catalyzed reactions. J. Phys. Chem. 60:1375-1378.

King, T. E. and T. Mann. 1959. Sorbitol metabolism in Spermatozoa. Proc. Roy. Soc. B. 151:226-243.

Kornberg, A. 1955. Lactic dehydrogenase of muscle. In Methods in Enzymology. Vol. I. pp. 441-443. Academic Press, New York.

Lazzarini, R. A. and M. Woodruff. 1964. The photoinduction of transhydrogenase in Euglena. Biochim. Biophys. Acta 79:412-415.

Lee, C. and L. Ernster. 1964. Equilibrium studies of the energy-dependent and non-energy-dependent pyridine nucleotide transhydrogenase reactions. Biochim. Biophys. Acta 81:187-190.

Lineweaver, H. and D. Burk. 1934. Determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56:658-666.

Lowery, N. O., N. J. Rosenbrough, A. L. Farr and R. J. Randall. 1961. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Marre, E. and O. Servfttaz. 1958. TPNH-cytochrome c reductase of chloroplasts and its role in photosynthetic phosphorylation. Arch. Biochem. Biophys. 75:309-323.

Martin, R. G. and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: Application to protein mixtures. J. Biol. Chem. 236:1372-1379.

Mishra, D. and E. R. Waygood. 1968. Effect of benzimidazole and kinetin on the nicotinamide nucleotide content of senescing wheat leaves. Canad. J. Biochem. 46:167-178.

McGuire, J. and L. Pesch. 1962. Control of glucose oxidation in anterior pituitary by hormonally sensitive pyridine nucleotide transhydrogenase. Proc. Nat. Acad. Sci. U. S. 48:2157-2163.

Neufeld, E. F., N. O. Kaplan and S. P. Colowick. 1955. Effect of adenine nucleotides on reactions involving triphosphopyridine nucleotide. Biochim. Biophys. Acta 17:525-535.

Ochoa, S. 1955. Malic dehydrogenase from pig heart. In Methods in Enzymology. Vol. I, pp. 735-739.

Orlando, J. A., D. Sabo and C. Curnyn. 1966. Photoreduction of pyridine nucleotide by subcellular preparations from Rhodoseudomonas spheroides. Plant Physiol. 41:937-945.

Purvis, J. L. 1961. Pathway of oxidation of isocitrate by mitochondria and the role of the transhydrogenase reaction. Biochim. Biophys. Acta 52:148-155.

Robinson, D. A. and R. C. Mills. 1961. Oxidation of reduced pyridine nucleotides in Pasteurella tularensis. I. Distribution of diaphorases and transhydrogenases. Biochim. Biophys. Acta 48:77-84.

San Pietro, A., N. O. Kaplan and S. P. Colowick. 1955. Pyridine nucleotide transhydrogenase. VI. Mechanism and sterospecificity of the reaction in Pseudomonas fluorescens. J. Biol. Chem. 212:941-952.

Schachman, H. K. 1959. Ultracentrifugation in biochemistry. Academic Press. New York, N. Y.

Sols, A. and R. K. Crane. 1954. Substrate specificity of brain hexokinase. J. Biol. Chem. 210:581-595.

Spiro, M. and E. G. Ball. 1961. Studies on the respiratory enzymes of the adrenal gland. I. The Cortex. II. The medulla. J. Biol. Chem. 236:225-235.

Shin, M. and D. I. Arnon. 1965. Enzymic mechanisms of pyridine nucleotide reduction in chloroplasts. J. Biol. Chem. 240:1405-1411.

Stein, A. M., N. O. Kaplan and M. M. Ciotti. 1959. Pyridine nucleotide transhydrogenase. VII. Determination of the reactions with coenzyme analogues in mammallian tissues. J. Biol. Chem. 231:521-533.

_____, B. T. Kaufman and N. O. Kaplan. 1960. On the mechanism of the transhydrogenase reaction catalyzed by a beef heart flavoprotein. Biochem. Biophys. Res. Comm. 2:354-357.

Stewart, M. A. and J. V. Passonneau. 1964. Identification of fructose in mammalian nerve. Biochem. Biophys. Res. Comm. 17:536-541.

Van Heyningen, R. 1959. Metabolism of xylose by the lens. 2. Rat lens in vivo and in vitro. Biochem. J. 73:197-207.

Vignais, P. V. and P. M. Vignais. 1961. Rôle des di-et tri-phosphopyridine nucleotides dans l'oxidation mitochondriale de l'isocitrate. Biochim. Biophys. Acta 47:515-528.

_____ and _____. 1957. Oxidation of reduced triphosphopyridine nucleotide and associated phosphorylation. J. Biol. Chem. 229:265-278.

Wosilait, W. D. and A. Nason. 1954. Pyridine nucleotide-quinone reductase. I. Purification and properties of the enzyme from pea seeds. J. Biol. Chem. 206:255-270.