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1974

PARTIAL PURIFICATION AND KINETIC STUDIES OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-SPECIFIC ISOCITRATE DEHYDROGENASE OF *PHYCOMYCES BLAKESLEEANUS*

A Thesis
Presented to
the Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
of the Requirements of the Degree
Master of Science

by
Michael J. Meredith
May 1974

PARTIAL PURIFICATION AND KINETIC STUDIES OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-SPECIFIC ISOCITRATE DEHYDROGENASE OF *PHYCOMYCES BLAKESLEEANUS*

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS iv

LIST OF TABLES v

LIST OF FIGURES vi

INTRODUCTION 1

MATERIALS AND METHODS 9

Chemicals 9

Buffer systems 9

Enzyme activity 10

Protein assay 11

RESULTS 12

Purification of Enzyme 12

pH Optimum 15

Enzyme stability 24

Molecular weight 24

Enzyme kinetics 28

Substrate and co-factor specificity 28

Enzyme inhibition 28

DISCUSSION 44

SUMMARY 53

LITERATURE CITED 56

LIST OF TABLES

Page

Table 1. Partial purification of NADP$^+$-specific isocitrate dehydrogenase.......................... 18

Table 2. Determination of optimum pH for reactions of IDH.. 19

Table 3. Analysis of Variance of IDH stability as a function of temperature and reducing agents.................................. 25

Table 4. Comparison of Km values for isocitrate, Mn^{++}, and NADP$^+$ in the forward reaction............. 50

Table 5. Comparison of Km values for alpha-ketoglutarate, Mn^{++}, and NADPH in the reverse reaction.............................. 51

LIST OF FIGURES

Page

Figure 1. Typical gel filtration elution and enzyme activity profile plotted against fraction number. The solid line represents the protein elution profile (ΔAbs_{280}). The dashed line represents the enzyme activity profile (ΔAbs_{340})....................17

Figure 2. Determination of pH optimum for the forward reaction of NADP$^+$-specific isocitrate dehydrogenase....................21

Figure 3. Determination of pH optimum for the reverse reaction of NADP$^+$-specific isocitrate dehydrogenase....................23

Figure 4. Molecular weight determination of NADP$^+$-specific isocitrate dehydrogenase by gel filtration. The Ve-Vo/Vt-Vo values were plotted against $\log_{10}$ molecular weight of the standards. Ve, elution volume; Vo, void volume; Vt, bed volume. Rib, ribonuclease; Chy, chymotrypsinogen; Ova, ovalbumin; IDH, isocitrate dehydrogenase; Ald, aldolase...........27

Figure 5. Lineweaver-Burk plot for the determination of the apparent Km for threo-Ds isocitrate......30

Figure 6. Lineweaver-Burk plot for the determination of the apparent Km for Mn^{++} in the forward reaction....................32

Figure 7. Lineweaver-Burk plot for the determination of the apparent Km for Mn^{++} in the reverse reaction....................34

Figure 8. Lineweaver-Burk plot for the determination of the apparent Km for NADPH....................36

Figure 9. Lineweaver-Burk plot for the determination of the apparent Km for alpha-ketoglutarate......38

Page

Figure 10. Hill plot determination of binding order of Mn^{++}. Each line represents a separate replication....................................40

Figure 11. Hill plot determination of binding order of NADPH. Each line represents a separate replication....................................42

PARTIAL PURIFICATION AND KINETIC STUDIES OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-SPECIFIC ISOCITRATE DEHYDROGENASE OF PHYCOMYCES BLAKESLEEANUS

Michael J. Meredith 59 pages

Directed by: Martin R. Houston, David R. Hartman, and Louis B. Lockwood

Department of Biology Western Kentucky University

The nicotinamide adenine dinucleotide phosphate-specific isocitrate dehydrogenase (threo-Ds(+) isocitrate: $NADP^+$: oxidoreductase [decarboxylating]; E.C. 1.1.1.42.) of *Phycomyces blakesleeanus* was partially purified. The method used to purify the enzyme was a combination of protamine sulphate precipitation, ammonium sulphate fractionation, and ion exchange and gel filtration chromatography. The $NADP^+$-specific IDH was purified from an initial specific activity of 0.05 units/mg of protein to a final specific activity of 1.5 units/mg protein in solution.

Molecular and reaction characteristics were explored. Employing gel filtration the molecular weight of the enzyme was determined to be 88,000. The forward reaction was found to have a pH optimum of 7.5 to 8.5. The pH optimum for the reverse reaction was found to be 6.0.

Kinetic studies performed showed the apparent Km's for Mn^{++} and threo-Ds(+) isocitrate to be 2.45×10^{-4} M, and 1.57×10^{-4} M, respectively. Apparent Km's for Mn^{++} and

NADPH in the reverse reaction were found to be: Mn^{++}, 9.7×10^{-5} M, and NADPH, 1.52×10^{-4} M. Alpha-ketoglutarate did not give a linear Lineweaver-Burk plot. Hill plots for the reverse reaction showed the binding orders for Mn^{++} and NADPH to be 2 and 1, respectively.

INTRODUCTION

Isocitrate dehydrogenase (IDH), the enzyme responsible for the conversion of isocitrate to alpha-ketoglutarate, has been postulated to exist in all organisms undergoing aerobic metabolism. It has been isolated and purified to homogeneity from sources ranging from bacteria to numerous vertebrate systems (1, 4, 8, 14, 15, 34). The assumption that isocitrate dehydrogenase is a ubiquitous enzyme is well-founded, although Williams and Rainbow (37) have reported the absence of IDH in several of the _Acetobacter_ species.

Isocitrate is an intermediate in the Krebs' cycle, the major oxidative metabolic pathway in most organisms. It has also been shown to participate in a by-pass of the Krebs' cycle, the glyoxylate by-pass. Instead of being converted to alpha-ketoglutarate, isocitrate can be cleaved by isocitrate lyase to produce succinate and glyoxylate. The glyoxylate is condensed with an acetyl-Co A to yield one molecule of malate. The malate can then be decarboxylated to pyruvate, for gluconoegenesis, or re-enter the Krebs' cycle (36). This pathway does not exist in higher animals, but is well known in higher plants, bacteria and yeast (12).

The metabolic role of isocitrate dehydrogenase, in the context of these two pathways, is clearly that of both a supplier of cycle intermediates and a regulator and control point for pathway choice. It has also been suggested that IDH may be connected with the control of biosynthesis of glutamate and arginine by way of the Cori cycle (20).

Kornberg and Pricer (16) showed the existence of two separate forms of IDH differing in coenzyme requirement. One form required nicotinamide adenine dinucleotide (NAD^+) for activity, while the other required nicotinamide adenine dinucleotide phosphate ($NADP^+$). This difference in cofactor requirement has been the basis for classification of IDH function and postulation of metabolic roles.

The NAD^+-specific isocitrate dehydrogenase (threo-Ds(+) isocitrate: NAD^+: oxidoreductase [decarboxylating]; E.C. 1.1.1.41.) has been isolated from a large number of sources, thus allowing comparative studies of its metabolic roles. The enzyme isolated from higher plants, specifically from mitochondria, has been shown to have properties consistent with a role in the regulation of the Krebs' cycle. This regulatory capacity was strongly suggested by its complex kinetic behavior with regard to isocitrate (14).

The reaction using the NAD^+-specific IDH is known to yield NADH which is then used in the production of ATP by oxidative phosphorylation. This reaction has been shown to be controlled in yeast feedback from the metabolism of adenine nucleotides: AMP by allosteric activation of dehydrogenases, and ADP by stimulation of the respiratory chain (7). NAD^+-specific IDH is inhibited by ATP and NADH (5). It has been suggested that inhibition of IDH by ATP is a means by which organisms are able to reduce the activity of the Krebs' cycle when energy production exceeds demand. However, in *Thiobacillus thiooxidans*, where the Krebs' cycle functions primarily as a biosynthetic pathway, nucleotides and glycolytic intermediates still inhibit IDH (14).

The $NADP^+$-specific IDH (*threo*-Ds(+) isocitrate: $NADP^+$: oxidoreductase [decarboxylating]; E.C. 1.1.1.42.) has recently been purified from an equal variety of sources (1, 8, 24, 27, 34). However, it has not been studied to the same extent as the more common NAD^+-specific IDH.

The intracellular distribution of the $NADP^+$-specific IDH was first studied by Lowenstein (18) using mouse liver. It was found that 80-90% of the $NADP^+$-specific activity was in the soluble, extramitochondrial portion. It was his conclusion that this extramitochondrial IDH might serve as a source of NADPH for biosynthetic processes. It has since been generally accepted that the $NADP^+$-specific IDH functions in the generation of NADPH for purposes other than the production of ATP, while the NAD^+-specific form is involved with the regulation of the Krebs' cycle (35).

A large part of the information available on the $NADP^+$-specific IDH has been obtained from research on strains of *Azotobacter vinelandii*. Barrera and Jurtshuk (4) found that the IDH activity in strain O was ten to one hundred times greater than any other NAD^+- or $NADP^+$-specific dehydrogenase in the organism. This high level of activity was attributed to the N_2-fixing ability of the *Azotobacter* species. NADPH was assumed to supply a major part of the reducing equivalents needed for that series of reactions. The purified enzyme obtained by Barrera and Jurtshuk was found to have a molecular weight of 78,000, existing as a polymeric unit of monomers with molecular weights of 15-20,000. A specificity for the _threo_ isomer of D-isocitrate was found, and the apparent Km for isocitrate was found to be 3.6×10^{-5} M. The apparent Km for $NADP^+$ was 1.8×10^{-5} M. Although Mn^{++} was required for maximum activity, Mg^{++}, Co^{++}, and Cd^{++} supported partial activity.

Chung and Franzen (8) isolated the $NADP^+$-specific IDH from strain 9104 (ATCC) of _Azotobacter_ _vinelandii_. It was found to be similar in size, molecular weight of 80,000, and kinetic properties to the enzyme isolated by Barrera and Jurtshuk (4). The Km's for isocitrate and $NADP^+$ were found to be 2.3×10^{-5} M and 2.0×10^{-5} M, respectively.

By application of disc polyacrylymide electrophoresis and ion exchange chromatography, Reeves _et_ _al_. (27) partially purified two isoenzymes of $NADP^+$-specific IDH from _Escherichia coli_ grown in medium with glucose as the sole carbon source. One of these isozymes was later purified from the K-12 strain of _E_. _coli_ (28). The Km's were found to correlate with other bacterial source enzymes.

Previous investigations of $NADP^+$-specific isocitrate dehydrogenases in fungi have been confined to yeasts and several species of _Aspergillus_ and _Neurospora_. Kornberg and Pricer (16) partially purified the $NADP^+$-specific IDH from baker's yeast. Bernofsky and Utter (5) reported the sensitivity of $NADP^+$-specific IDH isolated from yeast mitochondria to deoxycholate. Treatment with the bile salt resulted in increased activity. Tsao (33) found one $NADP^+$-specific IDH band by starch gel electrophoresis in crude extracts of several strains of wild type *Neurospora crassa*.

Since *Aspergillus* is one of the principle organisms used for the industrial production of citric acid, some work has been done on the citric acid cycle enzymes of the fungus. La Nauze (17) found both NAD^+ and $NADP^+$-specific isocitrate dehydrogenases in a number of industrial strains of *Aspergillus niger*. Her work was done with regard to the accumulation of citric acid in these fungi, and little information was given beyond the report of the presence of these enzymes.

In all reported cases, $NADP^+$-specific IDH has a requirement for a divalent metal cation. Mn^{++} is the metal usually showing the highest activity. The enzyme does, however, accept other metals with a lower catalytic efficiency. Reeves *et al*. (28) showed that Mg^{++} was acceptable, but Mn^{++} was ten times more efficient. Using the enzyme purified from pig heart, Colman (11) demonstrated that Mn^{++}, Cd^{++}, Zn^{++}, Co^{++}, and Mg^{++} could be used as metal cofactors. The Km's were 1.1×10^{-5} M, 1.6×10^{-5} M, 1.39×10^{-5} M, 1.91×10^{-5} M, and 1.26×10^{-4} M, respectively. It is interesting to note that the order of affinity correlated with neither ionic radius, the Irving-Williams stability sequence, nor the crystal field stabilization energy of potential ligand-metal complexes. Cd^{++}, Co^{++}, and Zn^{++} were found to catalyze the reverse reaction. Colman (11) also demonstrated that Ca^{++} and Sr^{++} inhibited the reaction in both directions. The K_I's for the forward reaction were 5.02×10^{-4} M, and 2.79×10^{-3} M, respectively.

In the conversion of isocitrate to alpha-ketoglutarate, there are two mechanistically different reactions: an initial dehydrogenation followed by a decarboxylation. By 1957, it was realized that IDH catalyzed not one, but at least four reactions (30). These were determined to be (1) oxidative decarboxylation of isocitrate, (2) the reductive carboxylation of alpha-ketoglutarate, (3) the reduction of oxalosuccinate, and (4) the decarboxylation of oxalosuccinate.

Oxalosuccinate was postulated to be the enzyme-bound intermediate in the oxidative decarboxylation reaction. Oxalosuccinate was discovered to be the intermediate when IDH from pig heart muscle showed activity toward the beta-keto acid when substituted for isocitrate (29). It was assumed to be enzyme-bound when labeled isocitrate was added to the reaction mixture but no labeled oxalosuccinate could be detected. The reaction was run under limiting conditions, namely, treatment with iodoacetate at a pH of less than 6. Treatment of IDH in this manner resulted in a loss of dehydrogenation but not decarboxylation (10).

Early attempts to ascertain the mechanism of the forward reaction used the established oxalosuccinate intermediate as a starting point. Parallels were drawn between the decarboxylation of oxalosuccinate and the decarboxylation of acetoacetate. Acetoacetate, also a beta-keto acid, has been shown to proceed through a Schiff base formed with the 6-amino group of a lysine residue at the active site of the acetoacetate decarboxylase (35). The Schiff base intermediate was a short-lived concept, as related to the IDH reaction. Later that same year, Zalkin *et al*. (38) found that the carbonyl oxygen of alpha-ketoglutarate does not exchange with an $H_2{}^{18}O$ medium, but the product of the acetoacetate reaction does. ^{18}O was found in the carbonyl group of the product. The absence of a free beta oxygen deprives the molecule of the needed electronegativity to stabilize the Schiff base-imide intermediate (19). Zalkin also showed that when ^{14}C-labeled alpha-ketoglutarate was incubated with IDH and sodium borohydride, no radioactive protein was found. Were a Schiff base the intermediate, a catalytically inactive, labeled protein should have been detected. The oxalosuccinate intermediate has been reinforced by reports of activity in the forward reaction with $NADP^+$-specific IDH from *Pisum sativum* (24) and *Brevibacterium flavum* (25).

NAD^+-specific IDH has been shown to be irreversible; that is, it will not catalyze the conversion of alpha-ketoglutarate to isocitrate (6). $NADP^+$-specific IDH will catalyze this reaction. Since it is not directly linked to the respiratory chain, nor is it a regulatory enzyme, $NADP^+$-specific IDH is not inhibited by ATP or NADPH. It functions to maintain the concentration of NADPH and intermediate organic species when the Krebs' cycle is not active (5).

The reverse reaction of $NADP^+$-specific IDH has not been studied in great detail. Colman (10) reported the Km's for the reverse reaction of pig heart IDH to be: alpha-ketoglutarate, 1.6×10^{-4} M; NADPH, 1.3×10^{-5} M; and Mn^{++}, 9.0×10^{-7} M. The Km for Mn^{++} is approximately 100 times smaller for the reverse reaction than for the forward reaction. Omran and Dennis (24) have reported Km's for the reverse reaction in Pisum sativum to be: alpha-ketoglutarate, 1.05×10^{-3} M; NADPH, 3.5×10^{-5} M; and Mn^{++}, 1.5×10^{-4} M. The Km for Mn^{++} is again much smaller than that for the forward reaction.

The purpose of this study was to further purify and characterize the $NADP^+$-specific isocitrate dehydrogenase from Phycomyces blakesleeanus. The rationale for choice of this organism was multiple in nature. First, there are few reports in the literature on the characterization of enzyme systems from filamentous fungi. This void is true of fungi in general, with the aforementioned exceptions. Secondly, the role of IDH in the overall metabolic scheme of Phycomyces blakesleeanus is not clear. A previous study (31) detected only very small amounts of NAD^+-specific IDH. This circumstance presented the possibility of a $NADP^+$-specific IDH participating in both extra- and intramitochondrial activities.

MATERIALS AND METHODS

Chemicals

Protamine sulphate, phenazine methosulphate, nitro blue tetrazolium, asparagine, ammonium sulphate (Grade I purified), CM Sephadex A-25-120, $NADP^+$ (monopotassium salt), NADPH (trisodium salt), threo-Ds(+) isocitrate (monopotassium salt, Grade III), DL-isocitrate (Grade III) were obtained from Sigma Chemical Co. Isocitrate (trisodium and monopotassium salts), oxaloacetate, alpha-ketoglutarate (A grade), were purchased from Calbiochem. Reagent grade mono- and dibasic potassium phosphate, 2-mercaptoethanol, and thiamine were purchased from Matheson, Coleman and Bell. With the above-mentioned exception, all ion exchange and gel filtration chromatographic materials were purchased from Pharmacia Fine Chemicals, Inc. Other materials were reagent grade and obtained from various sources.

Buffer systems

Buffers used during experimentation were as follows:

Buffer A: 0.1 M potassium phosphate (pH 7.5) with 10% glycerol and 0.05% 2-mercaptoethanol (v/v).

Buffer B: Citrate-phosphate buffer (pH 5.4), 4.0 mM citrate, 8.0 mM sodium phosphate (monobasic) with 10% glycerol and 0.05% 2-mercaptoethanol (v/v).

Buffer C: 0.2 M potassium phosphate (pH 6.0) with 10% glycerol (v/v).

Buffer D: 0.5 M potassium phosphate (pH 7.5) with 10% glycerol (v/v).

Enzyme activity

Enzyme activity for the forward reaction was determined by following the reduction of $NADP^+$ by IDH at 340 nm. Preparatory assays were performed on a Bausch and Lomb Spectronic 505 Recording Spectrophotometer. Kinetic studies were done on a Cary 14 Recording Spectrophotometer. Enzyme activity for the reverse reaction was assayed by following the oxidation of NADPH at 340 nm.

The reaction mixture for assay of enzyme activity in the forward reaction contained Buffer D, 0.5 mM $MnCl_2 \cdot 4H_2O$, 0.66 mM $NADP^+$, 3.1 mM DL-isocitrate (allo free), distilled, deionized water and enzyme in a final volume of 1.0 ml. Isocitrate was used to start the reaction. One unit of enzyme activity was defined to be that amount of enzyme needed to reduce 1 μmole of $NADP^+$ (or oxidize 1 μmole of NADPH) per minute. Specific activity was expressed as the ratio of units of enzyme activity per milligram of protein in solution.

The reaction mixture for assay of activity in the reverse reaction contained Buffer C, 0.5 mM $MnCl_2 \cdot 4H_2O$, 0.5 mM NADPH, 3.0 mM alpha-ketoglutarate. Carbon dioxide was supplied by addition of a saturated solution of sodium bicarbonate to the reaction mixture. Final volume of the reaction mixture, after addition of enzyme was 1.0 ml.

Protein assay

The concentration of protein was determined by the Waddell method (23). A Bausch and Lomb Precision Spectrophotometer was used to measure absorbance.

RESULTS

Purification of Enzyme

Step I. Source of the enzyme. Stock cultures of Phycomyces blakesleeanus (+ mating type, Vanderbilt strain) were maintained on agar slants of glucose-asparagine medium with the following composition per liter: glucose, 30 gm; asparagine, 5.0 gm; KH_2PO_4, 1.5 gm; $MgSO_4 \cdot 7H_2O$, 0.5 gm, thiamine, 0.2 mg; trace elements added from a stock solution of $CaCl_2$, $FeC_6H_5O_7$ and $ZnSO_4$ with the following concentrations per ml: Ca^{++}, 80 mg; Fe^{+3}, 0.3 mg; Zn^{++}, 0.4 mg; agar 2.0%.

The glucose-asparagine medium was dispensed in 500-ml quantities into one-liter Ehrlenmeyer flasks. The medium was inoculated with a heat-shocked spore suspension (3 minutes at 50 C) and aerated by shaking at 30 oscillations per minute in a New Brunswick Scientific Company Controlled Environment Incubator Shaker. After three days, the starter culture was used to inoculate a five-gallon carboy containing 15 liters of the glucose-asparagine medium. The medium was aerated by forced air, and the mycelia were harvested after four days of growth.

The mycelia were collected by filtering the medium through cheesecloth. Fifteen liters of medium yielded about 1.0 kg of mycelia (wet weight). The mycelia were washed with cold deionized water, followed by 500 ml of Buffer A. The washed mycelia were then pressed dry by hand. All procedures after this point were carried out at 5 C.

After washing, mycelia were placed in a Waring Blender with 2.0 gm. of Alumina, 1.0 gm Glasparin, and 150 ml of Buffer A. Cell disruption was accomplished by blending at high speed for 15 seconds, four times, with a 30-second extraction period between each blending. The extract was filtered through cheesecloth. The filtrate was centrifuged in a Sorvall Superspeed Rc2-B Automatic Refrigerated Centrifuge with a Sorvall SS-34 rotor at 27,000 x g for 15 minutes. After decanting the supernatant, the pellet was discarded. The supernatant was concentrated in Amicon CH3 Concentrator to a final volume of about 80 ml. The concentrated extract was assayed for enzyme activity and protein content. Substantial loss of protein was usually seen at this step.

Step II. Protamine sulphate precipitation. Protamine sulphate (0.3 mg per mg protein in the concentrated extract) was dissolved in distilled, deionized water. The solution was added to the extract with slow stirring. The nucleoproteins were removed as a flocculent white precipitate by centrifugation for 30 minutes at 20,000 x g. IDH activity was found in the supernatant. An average increase in IDH activity of 14% was seen after the removal of the nucleoproteins.

Step III. Ammonium sulphate fractionation. The supernatant from Step II was dialyzed against the appropriate volume of cold, saturated ammonium sulphate (distilled, deionized water solution) to yield a 45% saturated extract. The ammonium sulphate solution was added slowly to the dialysis vessel over a 12-hour period. The precipitated proteins were removed by centrifugation at 34,000 x g for 30 minutes. Isocitrate dehydrogenase activity remained in the supernatant. The redissolved pellet showed no activity.

The supernatant was then dialyzed for 12 hours against the appropriate volume of ammonium sulphate to yield a 70% saturated extract. The precipitate contained the IDH activity. The precipitate was redissolved in 50 ml of Buffer A.

Step IV. Ion exchange chromatography. The redissolved precipitate from the 70% ammonium sulphate fractionation was desalted using the Amicon CH3 Concentrator. Buffer A was simultaneously replaced with Buffer B.

The desalted extract was applied to a column of Sephadex CM-25 (gel volume, 15 x 2.5 cm) which had been previously equilibrated with Buffer B. The column eluent, containing the IDH activity, was concentrated and Buffer B replaced with Buffer A.

The extract was then applied to a column packed with Sephadex CM-50 (gel volume, 15 x 2.5 cm) which had been equilibrated with Buffer D plus 0.05% 2-mercaptoethanol (v/v). The column eluent contained the enzyme. After concentration with the Amicon CH3 Concentrator, the extract was further concentrated with an Amicon Model 52 Ultrafiltration Cell using a 30,000 molecular weight membrane. The volume of the extract was about 7 ml. The extract was then further concentrated in a Collodion Bag Apparatus (Scheicher and Schuell, Inc.) with a 25,000 molecular weight collodion bag. The final volume was approximately 2 ml.

Step V. Gel filtration. Approximately 2 ml of the concentrated extract from Step IV were applied to a column of Sephadex G-150 (gel volume, 45 x 2.5 cm) previously equilibrated with Buffer A. The enzyme was eluted with Buffer A at a flow rate of 10.2 ml per hour. Fractions were collected at 20-minute intervals by an Isco Model 272 Fraction Collector. The elution profile was charted by an Isco Model UA-4 Absorbance Monitor and Type 4 Dual Beam Optical Unit. Figure 1 displays the elution profile (ΔAbs_{280}) and enzyme activity (ΔAbs_{340}) plotted against fraction number. Table 1 shows the results of a typical purification.

pH optimum

The pH optimum for the forward and reverse reaction was determined by changing the buffer component of the reaction mixture. Table 2 shows the buffers and pH ranges tested.

The pH optimum for the forward reaction in 0.1 M potassium phosphate buffer was found to be 7.4; pH 7.5 in 0.012 M citrate-phosphate buffer; and pH 9.0 in 0.1 M Tris (Figure 2). The pH optimum for the reverse reaction in 0.1 M potassium phosphate buffer was found to be 6.0; pH 5.85 in 0.012 M citrate-phosphate buffer; and pH 6.0 in 0.1 M Tris buffer (Figure 3). Sodium acetate-acetic acid buffer (0.2 M) showed no activity.

Figure 1. Typical gel filtration elution and enzyme activity profile plotted against fraction number. The solid line represents the protein elution profile (ΔAbs_{280}). The dashed line represents the enzyme activity profile (ΔAbs_{340}).

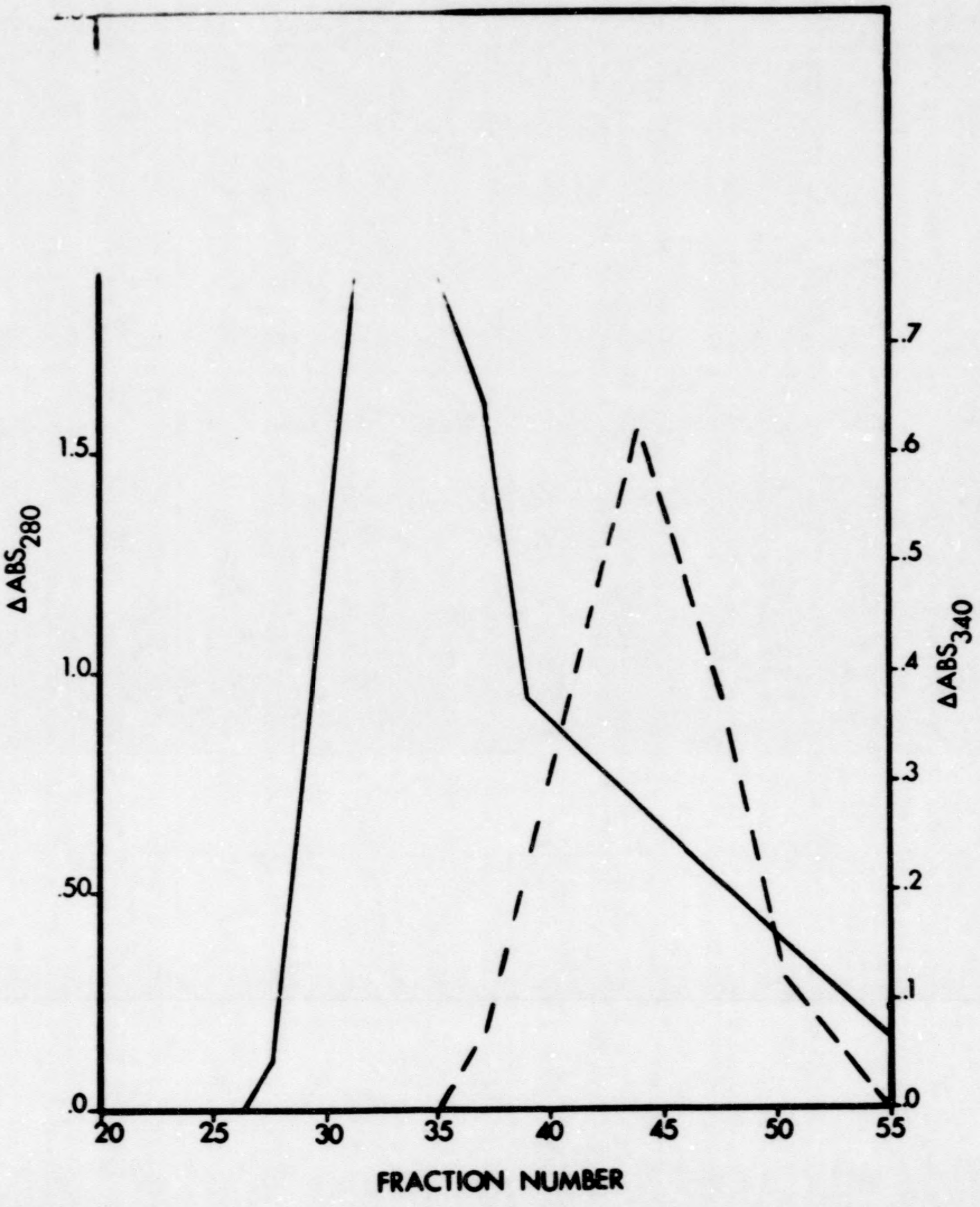

Δ ABS$_{280}$
1.5
1.0
.50
.0
.7
.6
.5
.4
.3
.2
.1
.0
Δ ABS$_{340}$
20
25
30
35
40
45
50
55
FRACTION NUMBER

TABLE 1

PARTIAL PURIFICATION OF NADP$^+$-SPECIFIC ISOCITRATE DEHYDROGENASE

STEP	VOLUME (mg)	TOTAL PROTEIN	TOTAL UNITS	SPECIFIC ACTIVITY (units/mg)	RECOVERY (%)	PURIFICATION
Crude	515	4618	315	0.07	100	0.0x
Protamine sulphate	550	3501	406	0.12	129	1.7x
0-45% Ammonium Sulphate	82	820	229	0.28	72	4.1x
45-70% Ammonium Sulphate	37	402	174	0.43	55	6.3x
Desalting	80	320	108	0.34	34	____
CM-25	64	270	85	0.31	27	____
CM-50*	48	200	54	0.27	17	____
Ultra-filtration	11	172	59	0.35	19	____
Concen-tration	2	107	50	0.47	16	6.9x
G-150 Gel Filtration*	9	15	20	1.37	6	20.2x

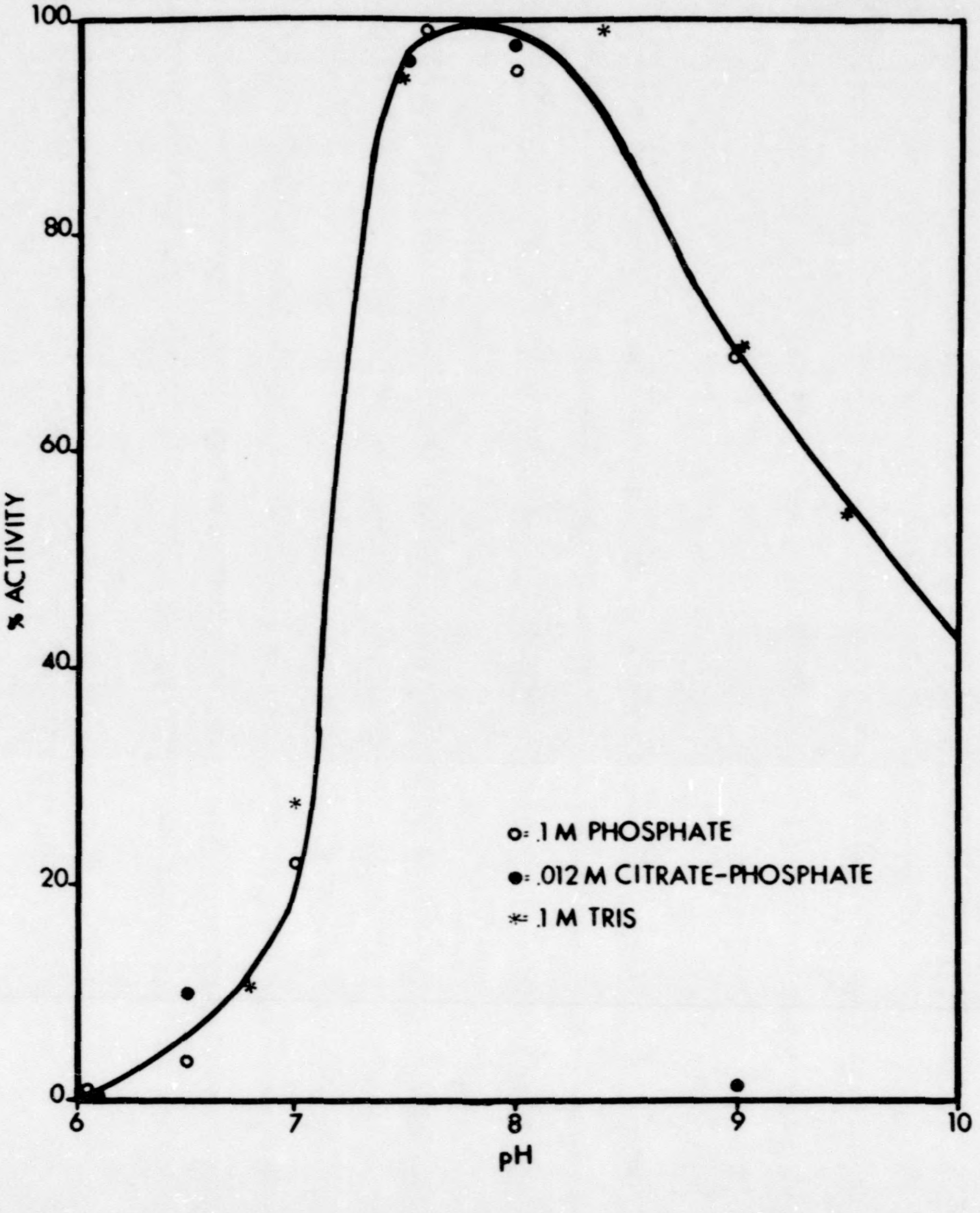
*Fractions with maximum activity were selected.

TABLE 2

DETERMINATION OF pH OPTIMUM FOR REACTIONS OF IDH

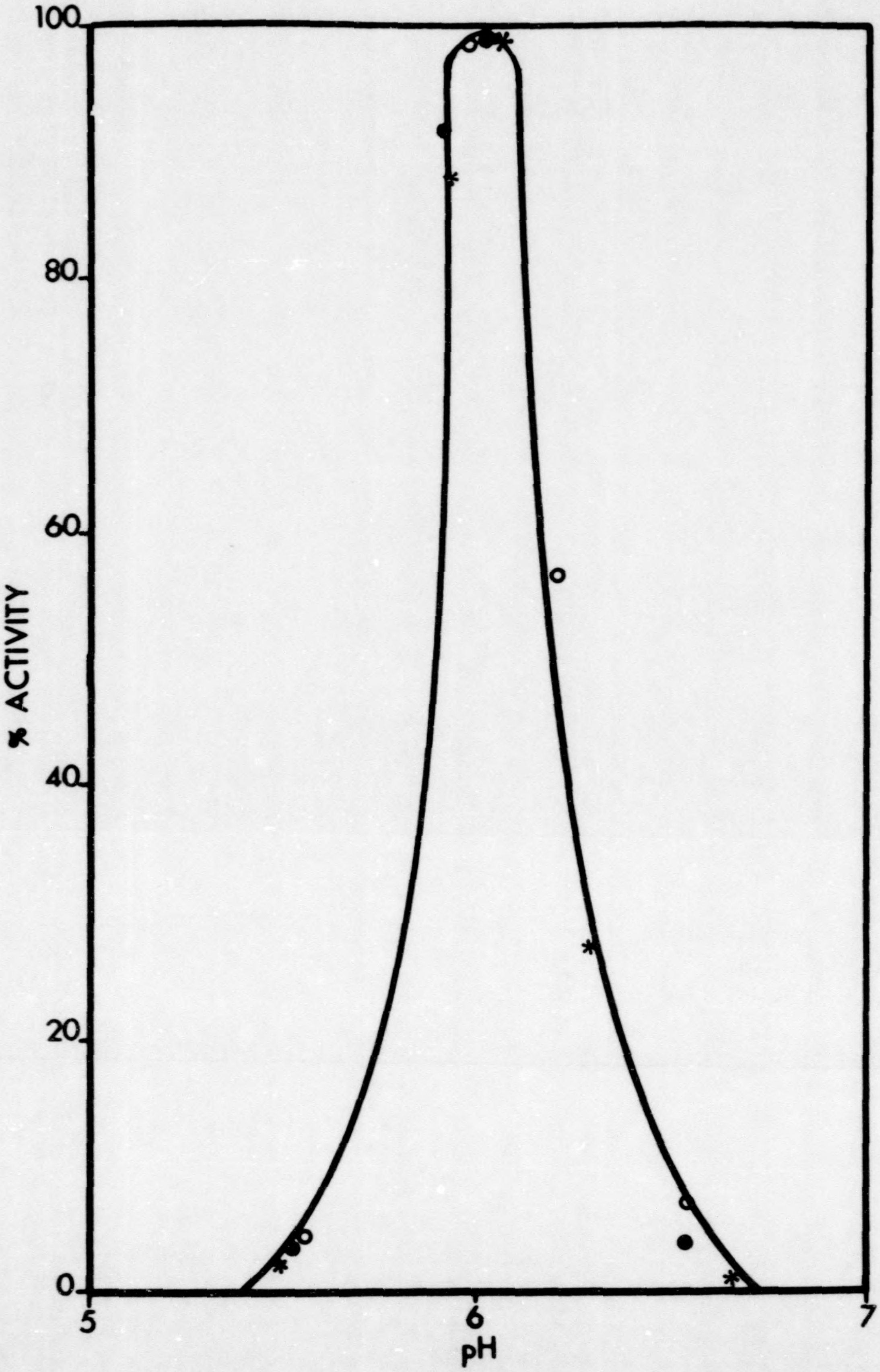
BUFFER	REACTION	pH RANGE
0.1 M Tris	forward	6.8-9.5
0.1 M Tris	reverse	4.0-8.0
0.1 M Potassium phosphate	forward	6.0-9.0
0.1 M Potassium phosphate	reverse	5.4-8.0
0.012 M Citrate-phosphate	forward	5.0-9.2
0.012 M Citrate-phosphate	reverse	5.0-8.0
0.2 M Sodium acetate-acetic acid	reverse	4.2-7.8

Figure 2. Determination of pH optimum for the forward reaction of NADP$^+$-specific isocitrate dehydrogenase.



100
80
60
40
20
0
% ACTIVITY
6
7
8
9
10
pH
○ = .1 M PHOSPHATE
● = .012 M CITRATE-PHOSPHATE
* = .1 M TRIS

Figure 3. Determination of pH optimum for the reverse reaction of NADP$^+$-specific isocitrate dehydrogenase. See Figure 2 for legend.


100
80
60
40
20
0
% ACTIVITY
5
6
7
pH

Enzyme stability

Enzyme samples, 5 units per 10 ml of Buffer A, were prepared to determine the stability of IDH as a function of temperature and reducing agents. The samples were assayed after 72 hours to determine loss of activity. The data were analyzed as a randomized block with a factorial arrangement of treatments. Table 3 presents the Analysis of Variance.

The F value for temperature was highly significiant. Linear regression analysis showed that -12 C was more conducive to retention of activity than 5 C.

F values for 2-mercaptoethanol and reduced glutathione were not significant.

The interaction of temperature and 2-mercaptoethanol was significant. Regression analysis showed 0.05% 2-mercaptoethanol (v/v) and -12 C to be most conducive to retention of activity.

Molecular weight

The molecular weight of $NADP^+$-specific isocitrate dehydrogenase from *Phycomyces blakesleeanus* was determined by gel filtration (2). A concentrated extract was applied to a calibrated column of Sephadex G-150 (gel volume, 45 x 2.5 cm) and eluted with Buffer A at a flow rate of 10.2 ml per hour. Ovalbumin, chymotrypsinogen, ribonuclease, and aldolase were used as standards for calibration of the column. The molecular weight of the enzyme was found to be 88,000 (Figure 4).

TABLE 3

ANALYSIS OF VARIANCE OF IDH STABILITY AS A FUNCTION OF TEMPERATURE AND REDUCING AGENTS

SOURCE	df	SUM OF SQUARES	MEAN SQUARED	F
Replications	2	.0004691	.0002345	3.602*
Treatments	17	.0019806	.0001165	1.789
A	(1)	.0011303	.0011303	17.363**
B	(2)	.0001646	.0000823	1.264
C	(2)	.0000439	.0000269	.4143
Interactions				
AB	(2)	.003435	.0022197	34.06**
AC	(2)	.0000917	.0000458	.7035
BC	(4)	.0006103	.0001525	2.3482
ABC	(4)	.000007	.00000017	.0215
Total	53	.004664	.0000880	

*= significance at 95% probability level

**= significance at 99% probability level

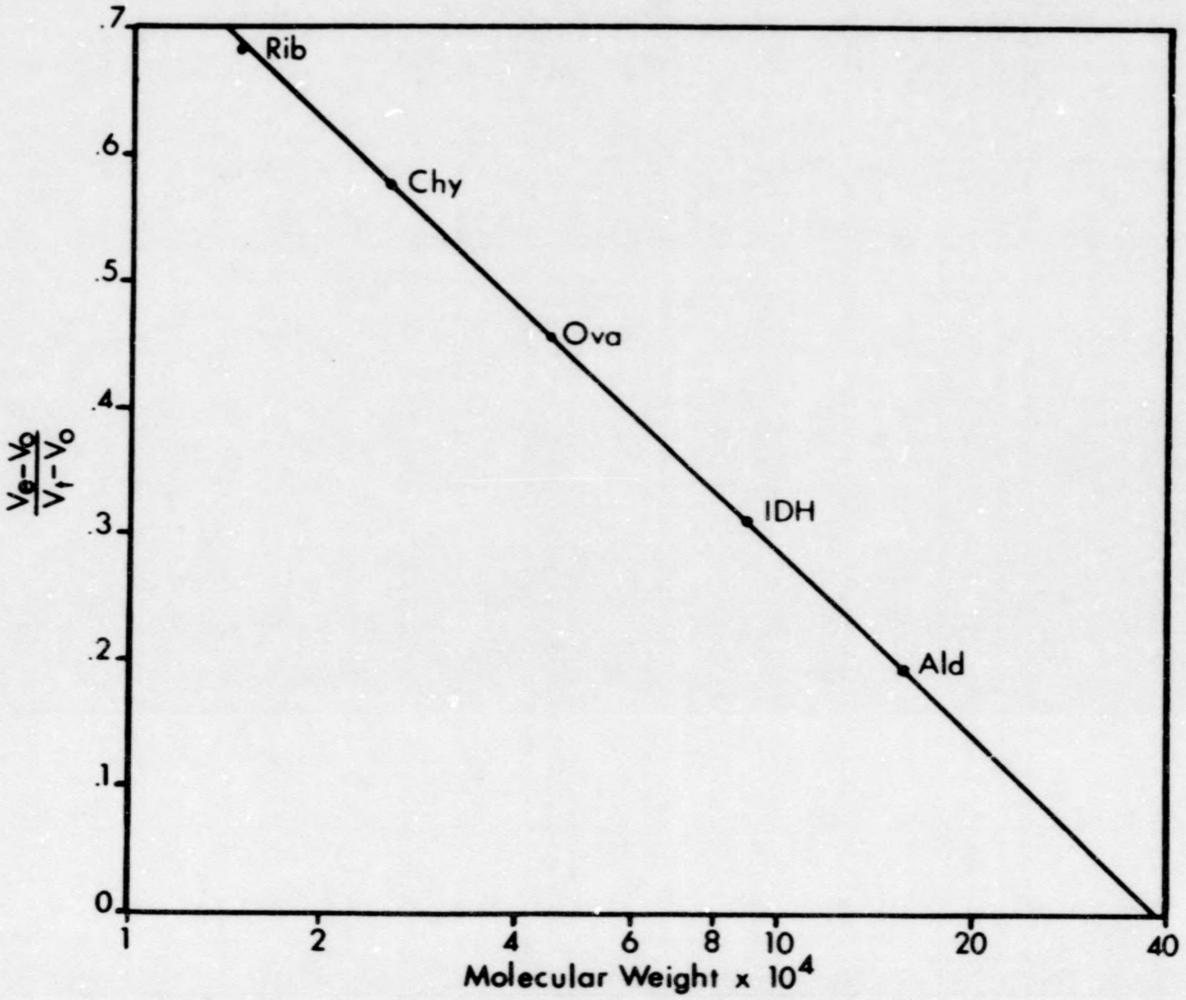
TREATMENTS

A = Temperature: -12, 5 C

B = 2-Mercaptoethanol concentration: 0%, 0.025%, 0.05%

C = Reduced glutathione concentration: 0 mM, 10mM, 20mM

Figure 4. Molecular weight determination of $NADP^+$-specific isocitrate dehydrogenase by gel filtration. The Ve-Vo/Vt-Vo values were plotted against the $\log_{10}$ molecular weight of the standards. Ve, elution volume; Vo, void volume; Vt, bed volume. Rib, ribonuclease; Chy, chymotrypsinogen; Ova, ovalbumin; IDH, isocitrate dehydrogenase; Ald, aldolase.


.7
.6
.5
.4
.3
.2
.1
0
$\frac{V_e - V_o}{V_t - V_o}$
Rib
Chy
Ova
IDH
Ald
1
2
4
6
8
10
20
40
Molecular Weight x 10^4

Enzyme kinetics

By application of the Lineweaver-Burk plot, the apparent Km's were determined for *threo*-Ds(+) isocitrate, and Mn^{++} in the forward reaction and Mn^{++} and NADPH in the reverse reaction. The Michaelis constants were found to be 1.57×10^{-4} M, 2.45×10^{-4} M, 9.70×10^{-5} M, and 1.53×10^{-4} M, respectively (Figures 5, 6, 7, and 8). Alpha-ketoglutarate was tested in the reverse reaction and was found not to follow Michaelis-Menton kinetics (Figure 9).

It was found that NADPH was not stable under the defined experimental conditions. The disappearance of NADPH occured in a concentration-independent first or pseudo-first order decay, with a rate constant of 5.8×10^{-3} minute $^{-1}$. The concentrations used for calculation of the apparent Km for NADPH were determined by statistical adjustment of the original concentration to comply with the experimental decay rate of the co-factor.

Hill plots were made for Mn^{++} and NADPH in the reverse reaction. It was found that Mn^{++} showed a binding order of 2.0 and NADPH a binding order of 1.0 (Figures 10 and 11).

Substrate and co-factor specificity

No activity was detected when NAD^+ or 3-acetyl pyridine $NADP^+$ was substituted for $NADP^+$. Substitution of citrate or alpha-ketoglutarate for isocitrate produced no detectable activity.

Enzyme inhibition

The enzyme was not inhibited by ATP, glyoxylate, or

Figure 5. Lineweaver-Burk plot for the determination of the apparent Km for threo-Ds isocitrate.

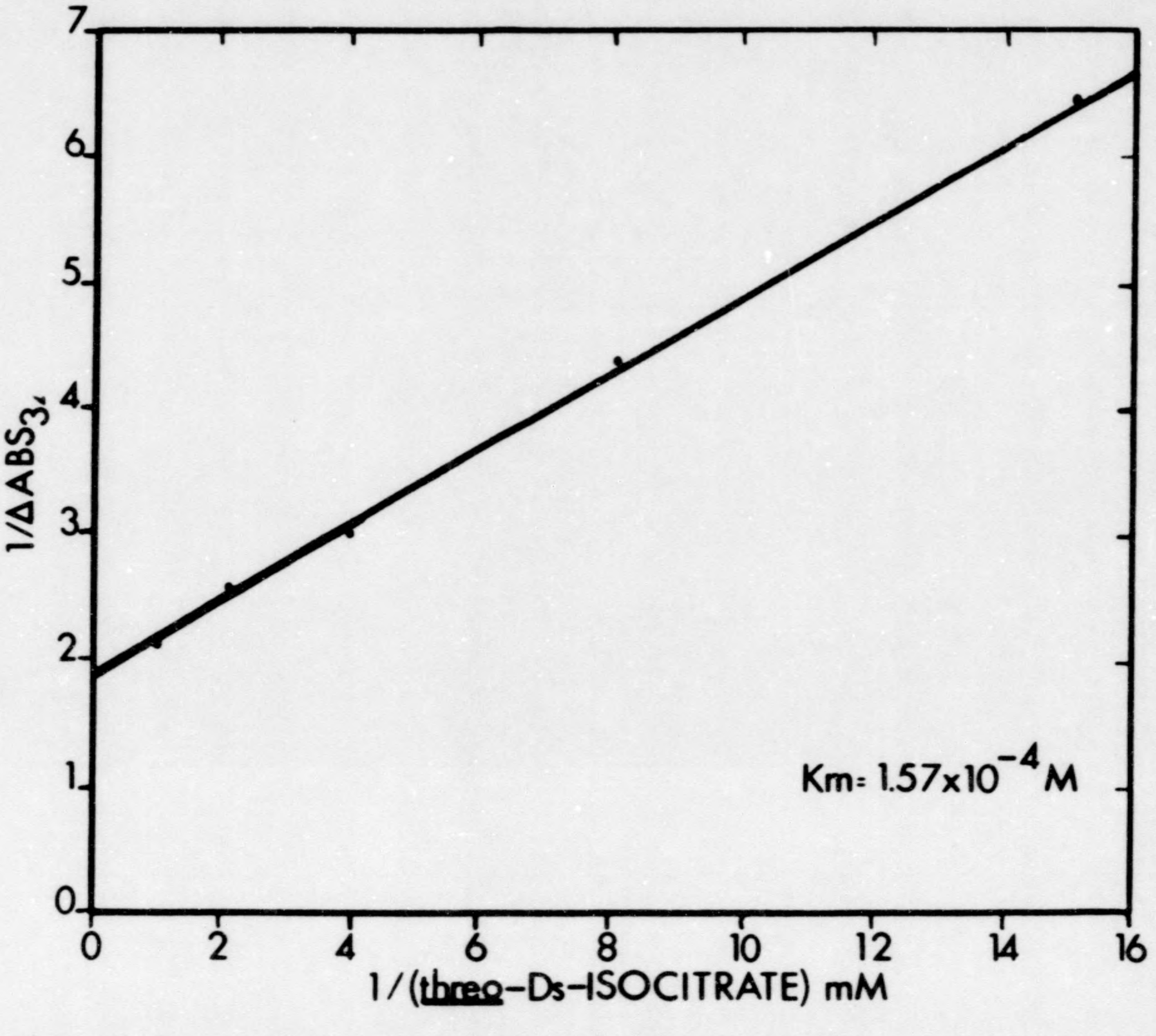

7
6
5
4
3
2
1
0
1/ΔABS340
0
2
4
6
8
10
12
14
16
1/(threo-Ds-ISOCITRATE) mM
Km= 1.57x10-4M

Figure 6. Lineweaver-Burk plot for the determination of the apparent Km for Mn^{++} in the forward reaction.

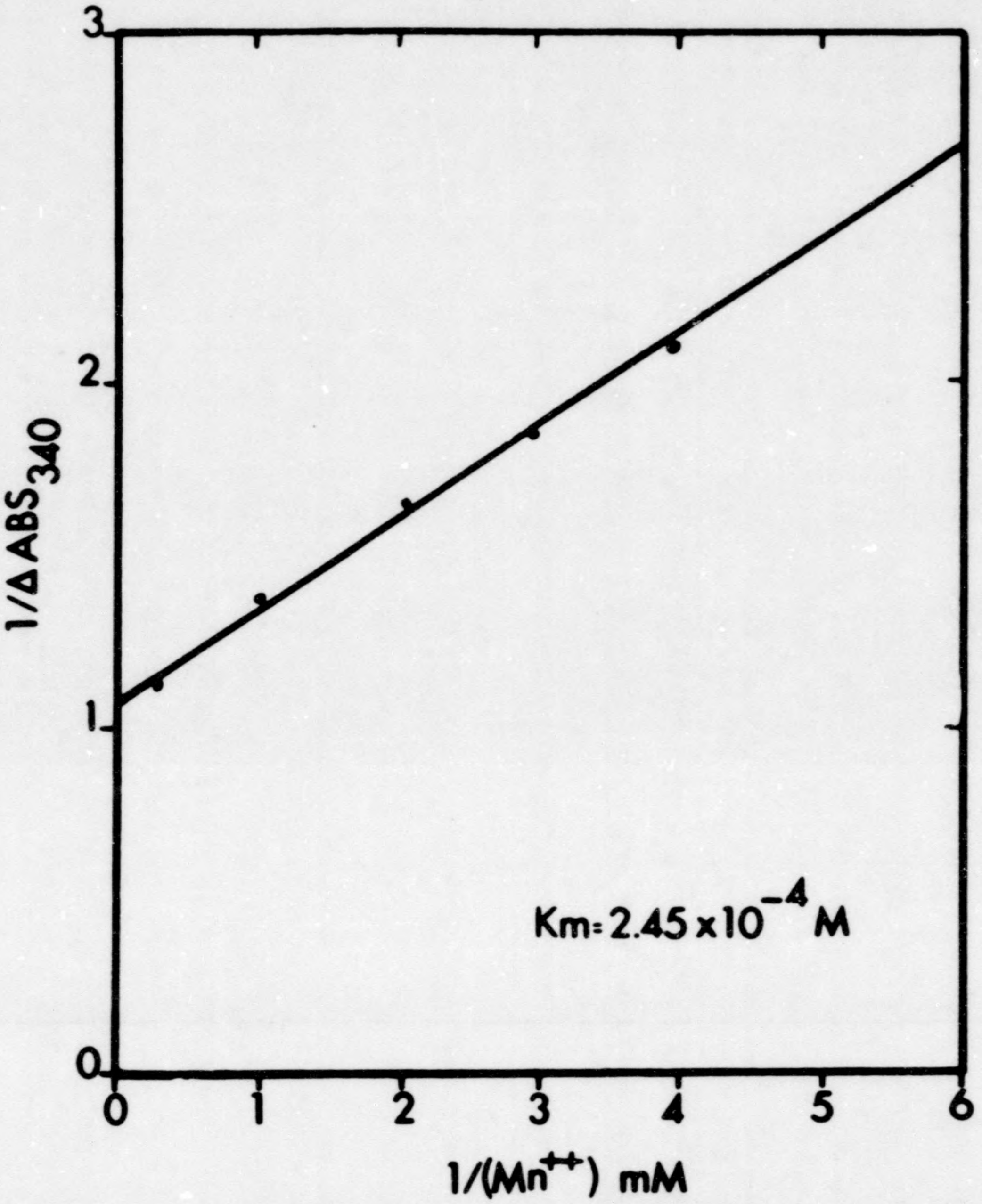

3
2
1
0
0
1
2
3
4
5
6
1/Δ ABS$_{340}$
1/(Mn^{++}) mM
Km= 2.45 x 10^{-4} M

Figure 7. Lineweaver-Burk plot for the determination of the apparent Km for Mn^{++} in the reverse reaction.

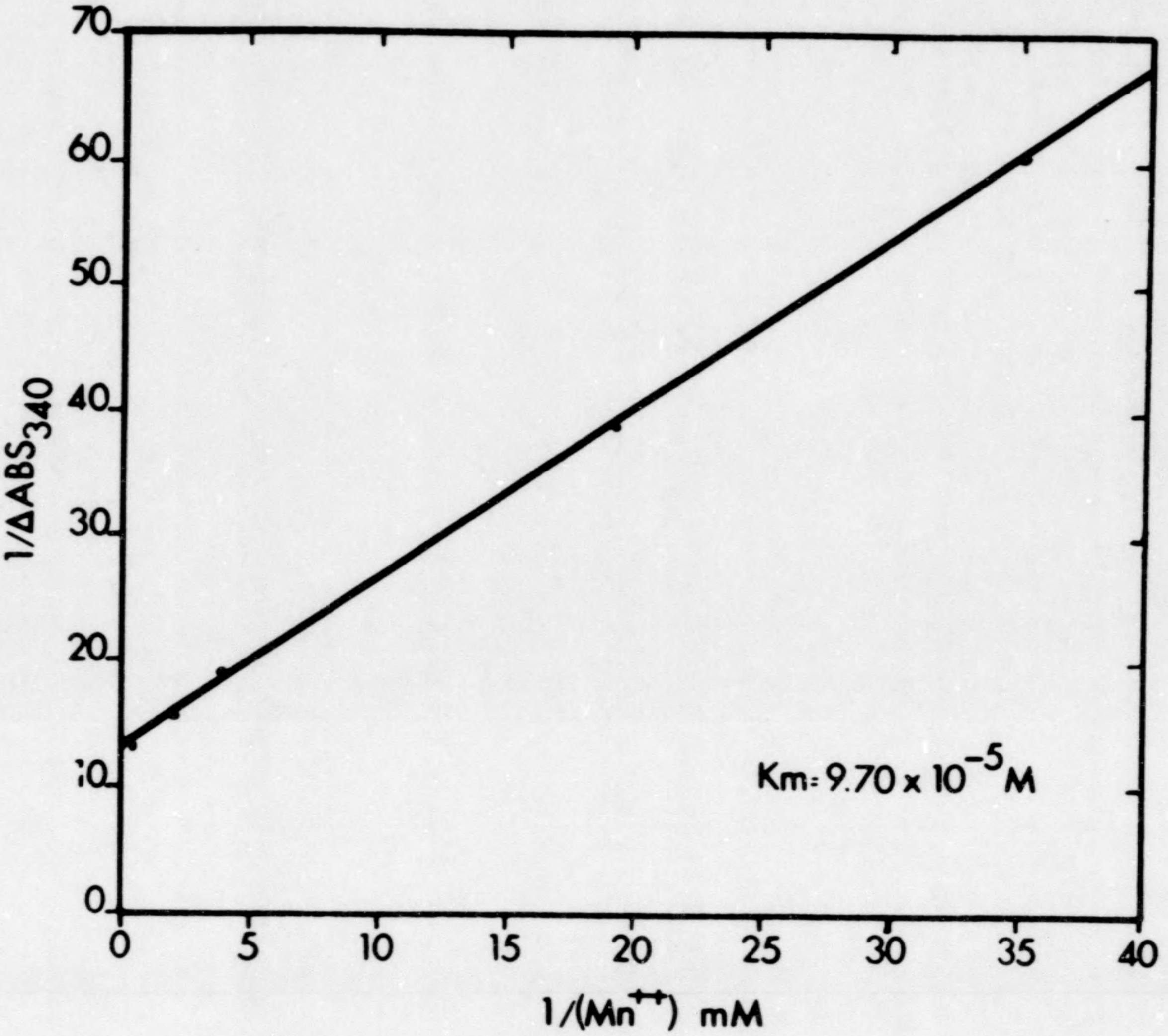

1/ΔABS340
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
1/(Mn++) mM
Km: 9.70 x 10-5M

Figure 8. Lineweaver-Burk plot for the determination of the apparent Km for NADPH.

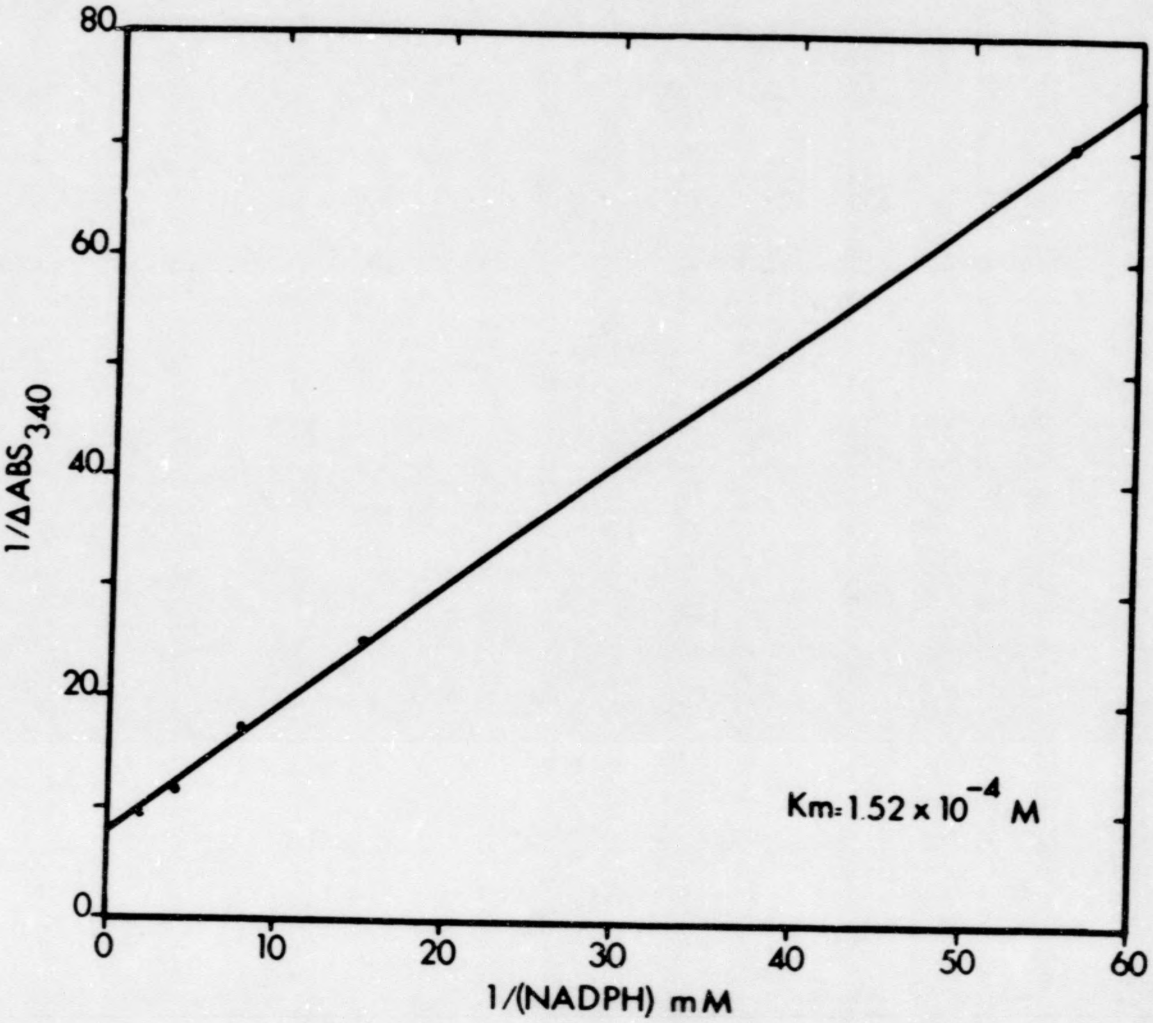

80
60
40
20
0
1/ΔABS340
0
10
20
30
40
50
60
1/(NADPH) mM
Km=1.52 x 10-4 M

Figure 9. Lineweaver-Burk plot for the determination of the apparent Km for alpha-ketoglutarate.

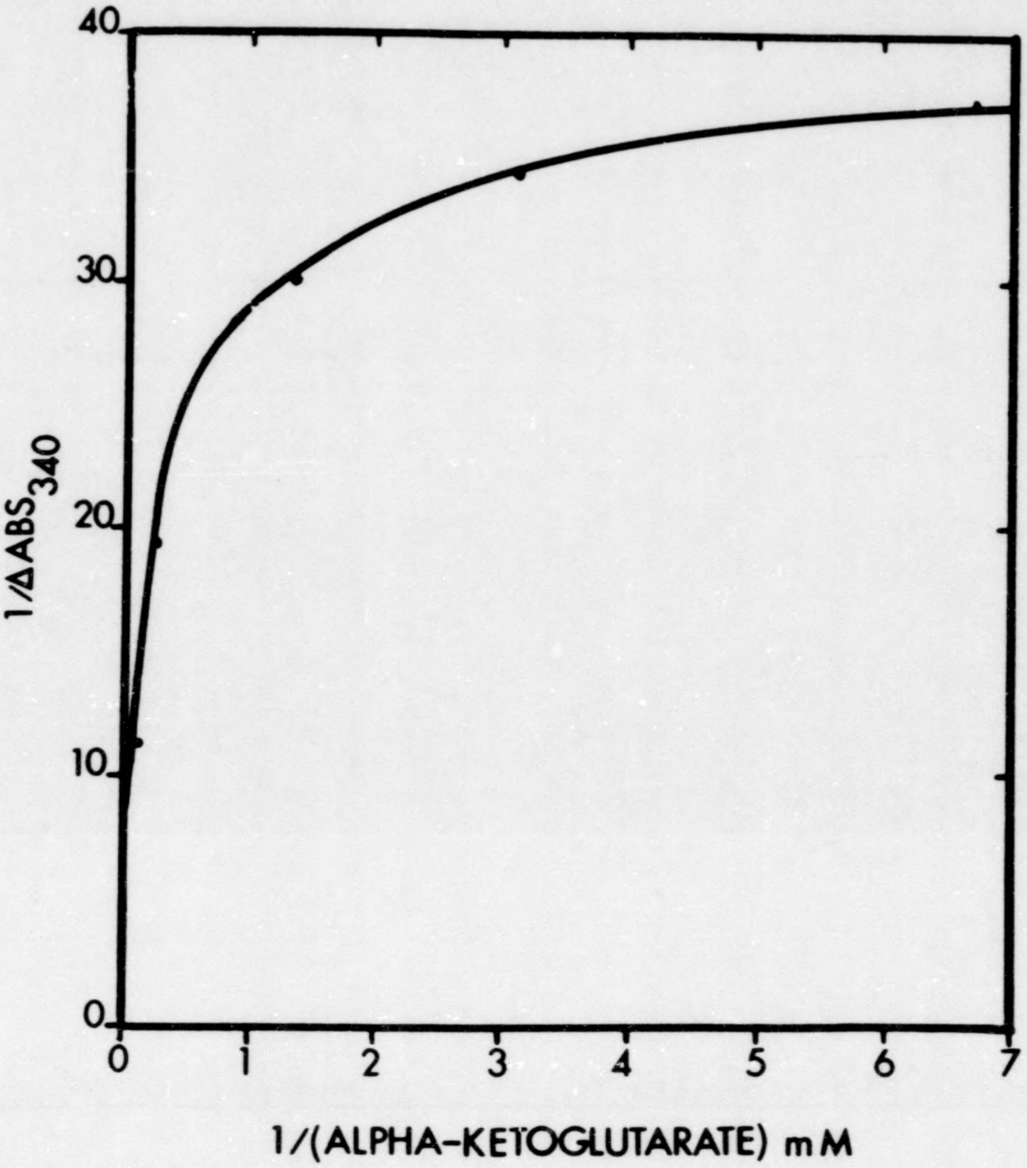

40
30
20
10
0
1/ΔABS340
0
1
2
3
4
5
6
7
1/(ALPHA-KETOGLUTARATE) mM

Figure 10. Hill plot determination of binding order of Mn^{++}. Each line represents a separate replication.

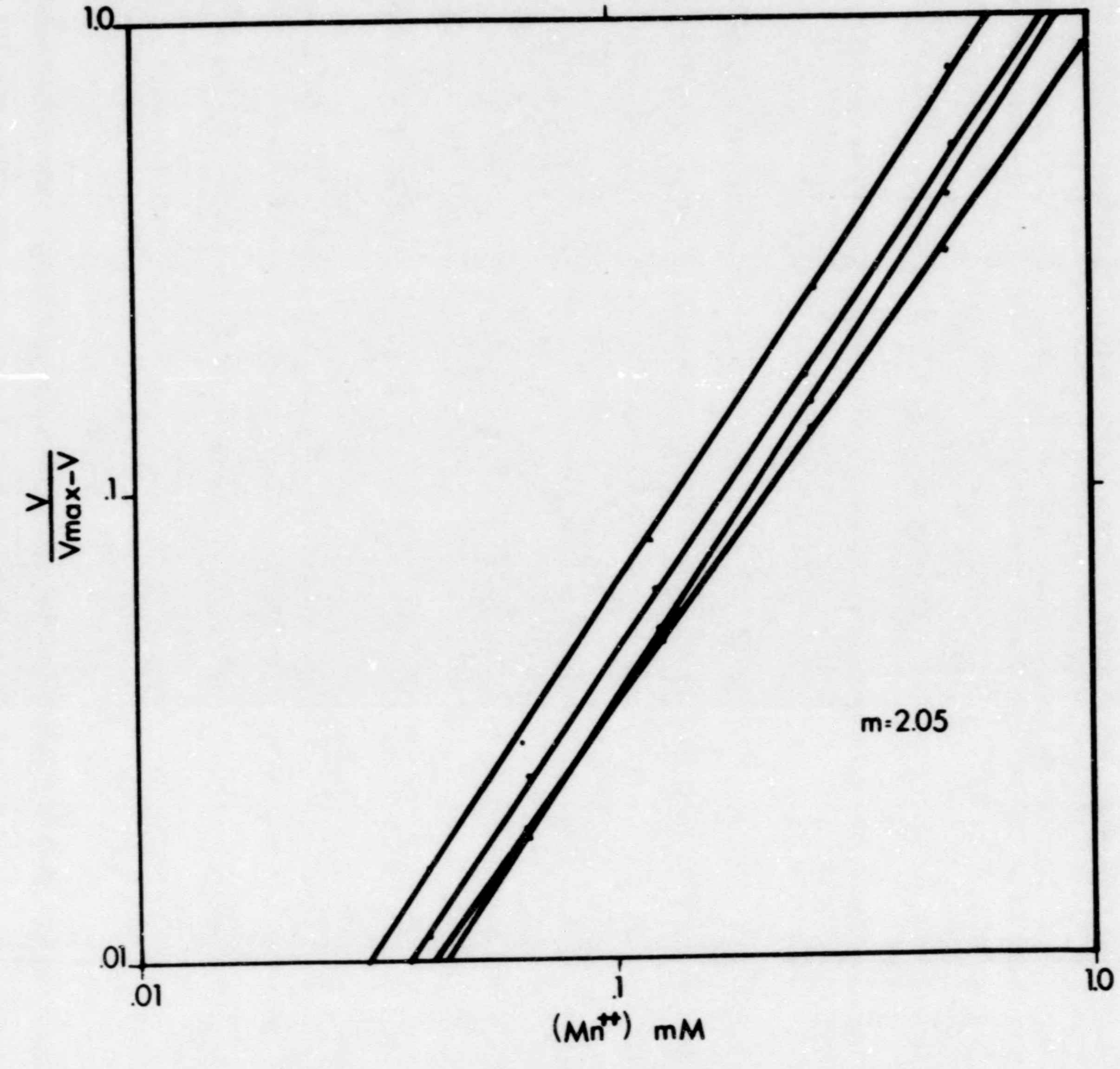
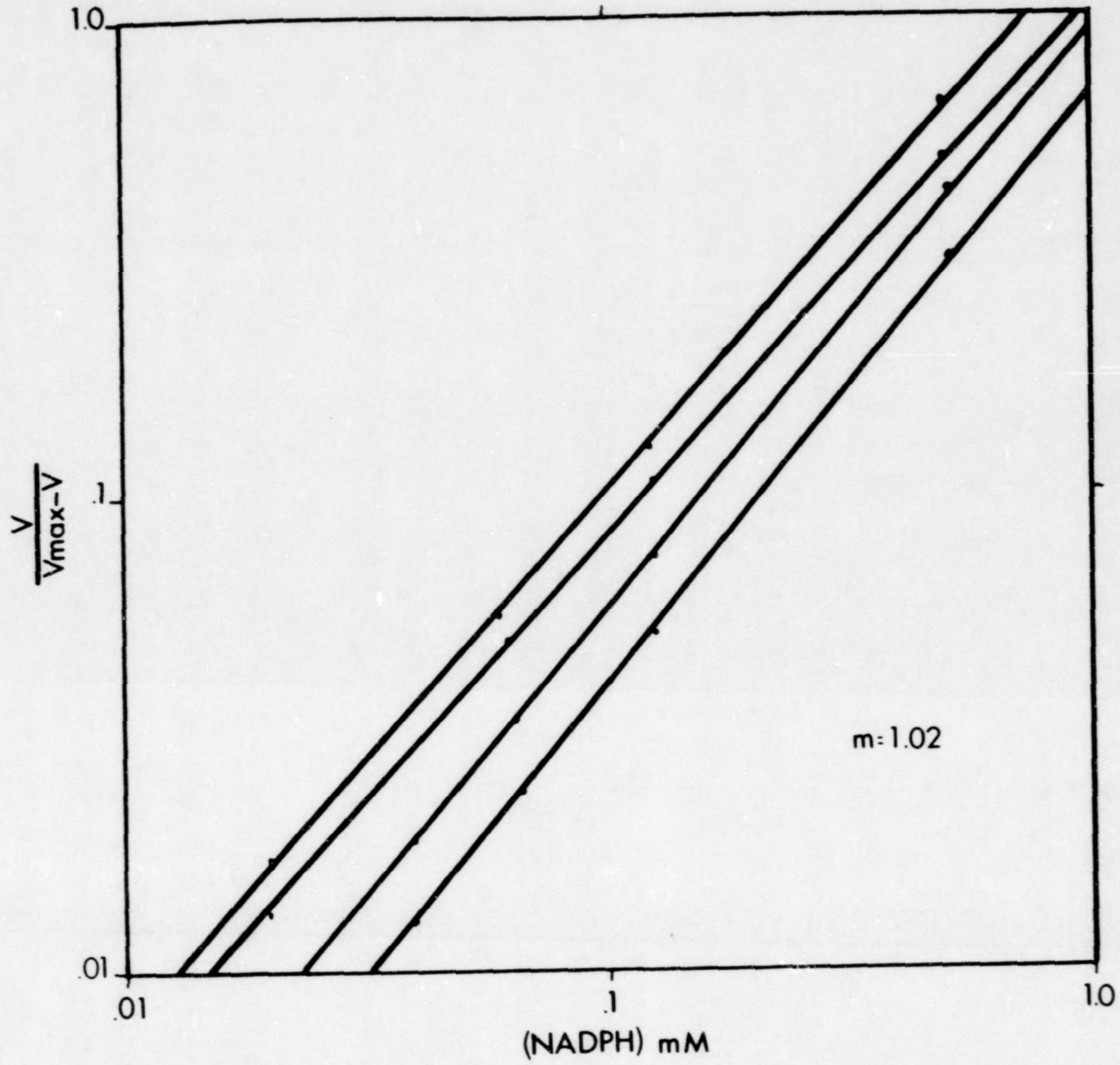

1.0
.1
.01
.01
.1
1.0
$\frac{V}{V_{max}-V}$
(Mn⁺⁺) mM
m=2.05

Figure 11. Hill plot determination of binding order of NADPH. Each line represents a separate replication.


1.0
.1
.01
$\frac{V}{V_{max}-V}$
.01
.1
1.0
(NADPH) mM
m=1.02

oxaloacetate. However, 3-indole glyoxylate inhibited the forward reaction by 24%. No inhibition was seen at elevated levels of Mn^{++}.

DISCUSSION

The preparation of NADP$^+$-specific isocitrate dehydrogenase from Phycomyces blakesleeanus resulted in an overall purification of about 20-fold. Specific activity was increased from an average of 0.05 units per mg to about 1.5 units per mg. Although this represents a 100% improvement in purification over the previous study of this enzyme (31), it is not comparable to the purity of enzymes prepared from other sources. Omran and Dennis (24) purified the NADP$^+$-specific IDH from the garden pea to a specific activity of 227, a purification of 65-fold. Chung and Franzen (8) isolated the NADP$^+$-specific IDH from Azotobacter vinelandii and purified the enzyme from a specific activity of 1.50 to a specific activity of 133, a purification of 88.6 times.

The partial purification of the NADP$^+$-specific IDH from the filamentous fungus Phycomyces blakesleeanus was accomplished by means similar to those employed in purification of other isocitrate dehydrogenases. Purification to homogeneity was not completed for a number of reasons. The first, and most obvious of these, can be seen in Table 1. IDH occurs in such low concentrations in Phycomyces blakesleeanus that initial specific activity is expressed in hundredths of a unit per milligram. Initial specific activity for other

IDH preparations ranged from 0.44 in the halophil Halobacterium salinarium (1) to 1.2 in Azotobacter vinelandii (4).

In addition to the low specific activity, the instability of the enzyme made purification difficult. All purification steps had to be carried out at less than 5 C. This temperature lability had only one precedent in the literature. Moon and Hochachka (21) reported that the NAD$^+$-specific IDH from rainbow trout liver required cold preparation. The enzymes purified by Chung and Franzen (8), Kornberg and Pricer (16), and Reeves et al. (28) from Escherichia coli was deactivated at temperatures below 23 C.

The instability of the enzyme increased with additional purification steps. After removal of low molecular weight proteins, 50,000 molecular weight or less, crude extracts could be stored at 3 C for two days with no significant loss of activity. After Step III (see Table 1), storage at 3 C for 24 hours resulted in complete loss of activity. The continuation of this stepwise loss of activity was related to the decrease in total protein. It is reasonable to speculate that the enzyme might have been protected by a coat of smaller proteins. Removal of these proteins would make the enzyme increasingly vulnerable to denaturation. Loss of activity was retarded by increasing the percentage of glycerol in the buffer. After gel filtration, the enzyme was stored in 75% glycerol (v/v) at -12 C. This treatment resulted in retention for up to five days of 90% of the activity present after gel filtration.

Addition of sulfhydral reducing agents increased enzyme stability. This increase in stability indicated the presence of sulfhydral amino acid residues at either the active site or at some point essential to the tertiary structure of the protein. These sulfhydral residues could be oxidized to form disulfide or carbomercaptan bridges (9). The reducing agents, such as 2-mercaptoethanol, reduce these sulfur links by replacing sulfur atoms with sulfhydral groups. Addition of these reducing agents either reactivates or prevents the denaturation of the enzyme. Because excess 2-mercaptoethanol did not completely stabilize the enzyme, it must be assumed that other modes of deactivation were present.

IDH was found not to bind to anionic or cationic exchangers. This lack of binding could not be altered by variation in type of buffer, pH, or ionic strength of the buffer. DEAE Sephadex removed no protein from the extracts. This inability to remove protein could have been due to removal of negatively charged proteins by NH_4^+ in the ammonium sulphate fractionation. CM Sephadex equilibrated at pH 5.4 and 7.5 removed two non-enzymic protein fractions.

Using Sephadex G-150, the molecular weight of IDH was determined according to the method of Andrews (2). Repeated determinations showed the molecular weight to be approximately 88,000. Other $NADP^+$-specific isocitrate dehydrogenases reported have had lower molecular weights: pig heart, 64,000 (22); *Azotobacter vinelandii*, 80,000 (8) and 78,000 (4).

However, Aitkens (1) demonstrated that the molecular weight of IDH from *Halobacterium salinarium* was dependent on the concentration of NaCl. At low salt concentrations, the molecular weight of IDH was 140,000, and the enzyme was composed of two catalytically active monomers of molecular weight 70,000. Aggregation of the 70,000 molecular weight monomers continued up to 4.0 M NaCl where the composite molecular weight was 251,000.

IDH from *Phycomyces blakesleeanus* was found to be specific in its requirements for substrate and co-factors, with the exception of the metal co-factor (13). Citrate was not an acceptable substitute for *threo*-Ds(+) isocitrate in the forward reaction. Since the *threo* isomer pair would be the favored configuration under physiological conditions, this specificity is not surprising (19). NAD^+ and 3-acetyl pyridine $NADP^+$ were not active as co-factors in place of $NADP^+$. Lowenstein (18) reported that the $NADP^+$-specific IDH from rat liver exhibited some activity toward these $NADP^+$ substitutes and also citrate.

$NADP^+$-specific isocitrate dehydrogenases are not generally considered to be regulatory enzymes. ATP does not inhibit the forward reaction in *Phycomyces blakesleeanus*, therefore, regulation of ATP-producing pathways is not accomplished by feedback inhibition of this IDH. Glyoxylate and oxaloacetic acid were found not to be inhibitory. However, $NADP^+$-specific IDH from *Azotobacter vinelandii* was inhibited strongly by both (4). The $NADP^+$-specific IDH from *Pisum sativum* was inhibited only slightly by glyoxylate and oxaloacetic acid. This was attributed to the fact that the glyoxylate by-pass in higher plants is located only in areas of high fatty acid breakdown. Since the plant samples were not taken from such an area, the inhibition was given little physiological significance by Omran and Dennis (24). Inhibition of $NADP^+$-specific IDH from pig heart, where the glyoxylate by-pass does not function, has been seen (32). These seemingly conflicting reports obscure the significance of a lack of inhibition by glyoxylate and oxaloacetic acid in a cell with no NAD^+-specific IDH and a non-regulatory $NADP^+$-specific IDH.

Like all other reported isocitrate dehydrogenases, the $NADP^+$-specific IDH from Phycomyces blakesleeanus had a divalent metal cation dependence. Mn^{++} was the only metal used in this study. The full spectrum of metal usage by this enzyme was explored by Dedhia (13). He demonstrated the ability of this $NADP^+$-specific IDH to utilize numerous metals, including mercury, which is usually inhibitory to enzyme-catalyzed reactions.

The basic kinetic parameters measured included Km's for the forward and reverse reaction. The forward reaction Km's were very similar to those reported for enzymes from various sources, as well as those previously reported for IDH from Phycomyces blakesleeanus (13, 31). Km's found for substrate and cofactors of the reverse reaction were similar to those for the enzyme from Pisum sativum. In those studies of the reverse reaction reported by Omran and Dennis (24), and

Colman (11), the Km's for Mn^{++} were consistently smaller than that for the forward reaction. The IDH from _P. blakesleeanus_ showed a similar disparity. The Km for Mn^{++} in the reverse reaction was three-fold smaller than in the forward reaction. Compared to a nine-fold difference in _P. sativum_ and a one hundred-fold difference in pig heart, the variation in _P. blakesleeanus_ was not significant. Tables 4 and 5 present a comparison of Km's established for the forward and reverse reactions of IDH's from various sources.

The complex kinetics obtained with alpha-ketoglutarate had no precedent in the literature. However, nonlinear Lineweaver-Burk plots have been reported for substrates of IDH in the forward reaction. Bernofsky and Utter (5) found that as the concentration of isocitrate approached high levels, a downward deflection of the plot was seen. This phenomenon was attributed to a secondary effect of the mitochondrion itself. This type of response was also seen with the regulatory NAD^+-specific IDH from bean seedlings (14). Both cases appear consistent with the regulatory nature of the enzymes involved. The construction of parallels between the reactions of these two enzymes and the IDH from _Phycomyces blakesleeanus_ would necessitate the acceptance of a monodirectional regulatory enzyme concept. Although no available data precludes this idea, the purpose of a regulatory point in the reversed Krebs' cycle is not clear. The regulatory capacity could be related to isocitrate's participation in the glyoxylate by-pass.

TABLE 4

COMPARISON OF Km VALUES FOR ISOCITRATE, Mn^{++}, AND $NADP^{+}$ IN THE FORWARD REACTION

SOURCE OF IDH	Km			REFERENCE
	ISOCITRATE	$NADP^{+}$	Mn^{++}	
Phycomyces blakesleeanus	1.57×10^{-4} M	2.6×10^{-4} M	2.45×10^{-4} M	Present Study
Azotobacter vinelandii	3.6×10^{-5} M	1.8×10^{-5} M	———	(4)
Pig heart	2.6×10^{-6} M	———	1.1×10^{-5}	(11)
Escherichia coli	1.56×10^{-5} M	3.7×10^{-5} M	1.29×10^{-5}	(28)
Thiobacillus thiooxidans	1.2×10^{-5} M	2.9×10^{-4} M	———	(15)
Pisum sativum	6.0×10^{-5} M	2.7×10^{-5} M	1.7×10^{-5} M	(24)
Pseudomonas fluorescens	1.5×10^{-5} M	1.7×10^{-5} M	———	(15)

TABLE 5

COMPARISON OF Km VALUES FOR ALPHA-KETOGLUTARATE, Mn^{++}, AND NADPH IN THE REVERSE REACTION

	Km			
SOURCE OF IDH	ALPHA-KETOGLUTARATE	NADPH	Mn^{++}	REFERENCE
Phycomyces blakesleeanus	____	1.52×10^{-4} M	9.7×10^{-5} M	Present Study
Pig heart	1.6×10^{-4} M	1.3×10^{-5} M	9.0×10^{-7}	(11)
Pisum sativum	1.0×10^{-3} M	3.5×10^{-5} M	1.5×10^{-5} M	(24)

Substantial proof, both theoretical and empirical, has been presented showing the actual substrate of IDH in the forward reaction to be an isocitrate-Mn^{++} complex (11, 34). This complex is a viable possibility for the reverse reaction. Incorporation of CO_2 is a thermodynamically unfavorable reaction. The inductive effect of the Mn^{++} on alpha-ketoglutarate, by way of chelation through the carbonyl or alpha-keto oxygens, would aid in stabilization of a carbanionic non-carboxylated carbon, making nucleophilic attack upon carbon dioxide more favorable.

Comparison of Hill plots between the IDH from *Phycomyces blakesleeanus* and IDH's from other sources could not be made for components of the reverse reaction. However, binding orders for the forward reaction have been well-studied. Dedhia (13) found the binding orders of both Mn^{++} and $NADP^+$ in the forward reaction to be 1. Atkinson (3) found the orders of Mn^{++} and NAD^+ to be 2. The binding orders in the reverse reaction were found in this study to be Mn^{++}, 2, and NADPH, 1. This fluctuation in Mn^{++} binding order would seem to indicate a difference in reaction mechanism not only among enzymes from different sources, but also between the forward and reverse reactions of the same enzyme.

SUMMARY

NADP$^+$-specific isocitrate dehydrogenase (threo-Ds(+) isocitrate: NADP$^+$: oxidoreductase [decarboxylating] E. C. 1.1.1.42) from *Phycomyces blakesleeanus* (+ mating type, Vanderbilt strain) was isolated from a cell-free extract. Although well described from vertebrates, bacteria, and yeast, IDH had not been characterized from a filamentous fungus.

Partial purification was achieved by a combination of protamine sulphate precipitation, ammonium sulphate fractionation, ion exchange chromatography, and gel filtration. The overall recovery rate was about 20%. Overall purification was approximately 20-fold with specific activity being increased from about 0.05 units/mg protein to about 1.5 units/mg protein. The molecular weight, as determined by gel filtration, was found to be 88,000.

Stability of the enzyme was found to be affected by temperature and concentration of sulfhydral reducing agents. The greatest stability was attained when the enzyme was stored at -12 C in 0.1 M potassium phosphate buffer (pH 7.5) with 10% glycerol and 0.05% 2-mercaptoethanol (v/v). The enzyme showed the greatest rate of deactivation in those preparative steps following the first ammonium sulphate precipitation. IDH preparations were highly unstable at 25 C.

Ten minutes at room temperature were usually sufficient to cause complete loss of activity.

A distinct pH optimum was seen for both the forward and reverse reactions. The reverse reaction exhibited a sharp pH peak at 6.0. The forward reaction was maximally active between pH 7.5 and 8.5.

Basic kinetic studies were performed. The apparent Km's for the forward reaction were: threo-Ds(+) isocitrate, 1.57 x 10^{-4} M; and Mn^{++}, 2.45 x 10^{-4} M. The Km for $NADP^+$ had previously been determined to be 2.6 x 10^{-4} M. Michaelis constants for Mn^{++}, and NADPH were calculated for the reverse reaction and were found to be 9.7 x 10^{-5} M, and 1.52 x 10^{-4} M, respectively. Alpha-ketoglutarate did not yield a linear Lineweaver-Burk plot. Hill plots were made for Mn^{++} and NADPH in the reverse reaction, and binding orders of 2 and 1 were found, respectively.

The $NADP^+$-specific IDH from Phycomyces blakesleeanus was not inhibited by 0.001 M concentrations of glyoxylate, oxaloacetate, or ATP. However, 3-indole glyoxylate resulted in a 25% inhibition. Addition of excess Mn^{++} relieved the inhibition. No activity was detected when 3-acetyl pyridine $NADP^+$ or NAD^+ was substituted for $NADP^+$.

Compared to other $NADP^+$-specific isocitrate dehydrogenases, the IDH from Phycomyces blakesleeanus possessed many similar characteristics. Its molecular weight was similar to those reported for other isocitrate dehydrogenases, and it had the same type and magnitude of metal requirement.

Kinetic properties differed slightly. Km's in the forward reaction were a power of ten greater than those reported for *Azotobacter* and *Aspergillus* species. Reverse reaction kinetics were comparable to those reported for *Pisum sativum* with the exception of the non-linear kinetics of alpha-ketoglutarate.

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