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Dedhia,

Devji K.

1973

KINETIC PROPERTIES OF PARTIALLY PURIFIED ISOCITRATE DEHYDROGENASE

A Thesis

Presented to

the Faculty of the Department of Chemistry

Western Kentucky University

Bowling Green, Kentucky

In Partial Fulfillment

of the Requirement for the Degree

Master of Science

by

Devji K. Dedhia

May 1973

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KINETIC PROPERTIES OF PARTIALLY PURIFIED

ISOCITRATE DEHYDROGENASE

APPROVED August 2, 1973:
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Devji K. Dedhia

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STATEMENT OF THE PROBLEM

Within the past few years kinetic studies of $NADP^+$-specific isocitrate dehydrogenase have been conducted extensively following isolation from bacteria, yeast, and vertebrates. However, the kinetic properties of $NADP^+$-specific isocitrate dehydrogenase have not been adequately characterized in filamentous fungi. Therefore, the present study was undertaken to examine the kinetic behavior of partially purified $NADP^+$-specific isocitrate dehydrogenase enzyme isolated from Phycomyces blakesleeanus.

During this study Michaelis constants (Km) and maximum velocities (Vmax) were determined for threo-Ds-isocitrate, $NADP^+$, and Mn^{2+} as well as the number of binding sites of these components to the enzyme. The order of reaction with respect to enzyme was also established. The effects of ionic strength, metal ions, coenzymes, coordinating ligands, and some inhibitors on the rate of reaction were also investigated. Some experiments were performed to elucidate a possible reaction mechanism for the conversion of D-isocitric acid to α-ketoglutaric acid in the presence of coenzyme, $NADP^+$, and Mn^{2+}.

INTRODUCTION

Enzymes are among the most efficient and selective catalysts of chemical reactions occurring in biological systems. The kinetic behavior of enzymes has been characterized by L. Michaelis and M. L. Menten (1913)[1] where the existence of an enzyme-substrate, (ES), complex was hypothesized as the basis for a theoretical analysis of enzymatic reactions. The overall reaction is:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E \qquad (1)$$

with the rate of reaction being equal to the rate of dissociation of the enzyme-substrate, ES, complex. Since the rate depends on the concentration of ES, it follows that the maximum rate should be attained when the enzyme is saturated. The substrate concentration at which the enzyme reaction reaches one-half the maximum velocity is defined as Km, the Michaelis constant (expressed in moles/liter), which approximates the dissociation constant of the enzyme-substrate complex.

The Michaelis-Menten equation (2) is given as:

$$v_o = \frac{Vmax[S]}{Km + [S]} \qquad (2)$$

where v_o = initial velocity, Vmax = maximum velocity, [S] = substrate concentration, and Km = Michaelis constant. Rearrangement of equation 2 gives equation 3:

$$Km = [S]\left(\frac{Vmax}{v_o} - 1\right) \qquad (3)$$

The maximum velocity and Michaelis constant can be obtained from the sigmoid curve shown in Figure 4. In practice there are some disadvantages in measuring the Michaelis constant by the above method. At extremely high concentrations of substrate, anomalies are present and it is often difficult to determine when the maximum velocity is reached, i.e., the rate continues to increase by very small increments as the concentration of substrate is increased.

This difficulty was resolved by H. Lineweaver and D. Burk[2] by taking the reciprocal of both terms in equation 2:

$$\frac{1}{v_o} = \frac{Km}{Vmax} \cdot \frac{1}{[S]} + \frac{1}{Vmax} \qquad (4)$$

This represents the equation of a straight line obtained from a plot of $1/v_o$ vs $1/[S]$, which gives a slope of Km/Vmax and an intercept of 1/Vmax.

The Michaelis-Menten equation for an enzyme having 'n' interacting sites for the binding of a substrate is given by equation 5:

$$v_o = \frac{Vmax[S]^n}{Km + [S]^n} \qquad (5)$$

which can be rearranged to equation 6, or better known as the Hill equation:[3]

$$\log\left(\frac{v_o}{Vmax - v_o}\right) = n \cdot \log(S) - \log K_{eq} \qquad (6)$$

where n = number of binding sites on the enzyme molecule and

$$K_{eq} \equiv \frac{[E][S]^n}{[ES_n]}$$

with (S), v_o, and Vmax having their usual meaning. A straight line is obtained from a plot of $\log (v_o/Vmax - v_o)$ vs $\log (S)$ with a slope of 'n'. The value of 'n' does not necessarily indicate the order of reac-

tion nor does it indicate that binding with substrate should occur during the rate determining step. Atkinson et al.[3] have suggested that the enzyme molecule binds with 'n' molecules of substrate before the rate determining step forming a complex which dissociates to give products during the rate determining step.

The Michaelis-Menten equation (2) can also be expressed as:

$$v_o = \frac{k_3 e_o^n (S)}{Km + (S)} \qquad (7)$$

where n = number of enzyme molecules, e_o = initial enzyme concentration, k_3 = rate constant, and v_o, Km, and S have their usual meanings. Equation 7 can be modified to give equation 8:

$$v_o = k e_o^n \qquad (8)$$

where $k = k_3(S)/Km + (S)$. Equation 8 indicates that the initial reaction rate will increase with enzyme concentration provided n is a positive integer. The logarithm of both sides in equation 8 gives the following result:

$$\log v_o = \log k + n \cdot \log e_o \qquad (9)$$

A straight line is obtained from a plot of $\log v_o$ vs $\log e_o$ yielding a slope 'n' equal to the number of enzyme molecules taking part in the reaction under consideration. Thus, n indicates the order of reaction with respect to the enzyme molecule.

It has been shown recently[4] that the use of a computer improves the accuracy of the calculations. Therefore, kinetic results have been obtained in this study by utilizing the PDP-8 computer.

$NADP^+$-specific isocitrate dehydrogenase is widely distributed in nature.[5, 6] There are marked differences in the physical and chemical properties of the enzyme isolated from different sources. Moreover,

evidence has been presented to show the existence of multiple forms of $NADP^+$-specific isocitrate dehydrogenase in several organisms.[7]

The biological breakdown of D-isocitric acid to α-ketoglutaric acid has been assumed to occur through an enzymatic dehydrogenation[8] forming an intermediate, oxalosuccinic acid, which then decarboxylates. The enzyme, isocitrate dehydrogenase, is specific for the D-form of isocitric acid as suggested by Barrera and Jurtshuk.[9] The conversion of D-isocitric acid to α-ketoglutaric acid is proposed to take place in two steps. Adler et al.[10] demonstrated that $NADP^+$ and Mn^{2+} ions are essential components of the system. Their proposed mechanism is as follows:

$$\text{D-isocitric acid} + E + NADP^+ \rightleftharpoons NADPH + H^+ + [\text{oxalosuccinic acid} - E] \quad (10)$$

$$[\text{oxalosuccinic acid} - E] \xrightleftharpoons{Mn^{2+}} \alpha\text{-ketoglutaric acid} + E + CO_2 \quad (11)$$

It has been suggested by Ochoa[8] that crude solutions of the enzyme contain two distinct forms which catalyze reactions 10 and 11. Reaction 10 is reportedly catalyzed by isocitrate dehydrogenase and occurs in the absence of Mn^{2+}. Reaction 11 is believed to be catalyzed by a specific enzyme, oxalosuccinic carboxylase,[11] and requires Mn^{2+}.

The work of Adler et al.[10, 11] shows there is a specific requirement of $NADP^+$ for the dehydrogenase reaction and Mn^{2+} for the decarboxylase reaction. However, these studies do not show conclusively that Mn^{2+} is not also required for the dehydrogenase reaction. It has been shown by Moyle[12] that the initial velocity is dependent on the Mn^{2+} ion concentration. Inhibition by EDTA in the reaction mixture was attributed to the chelation of EDTA with Mn^{2+} and not through any non-specific inactivation of the enzyme. There are two possible explanations for these findings. Either Mn^{2+} ions are required for the dehydrogenase reaction or the rate of decarboxylation limits that of dehydrogenation.

Metal ions have long been known to activate reactions catalyzed by isocitrate dehydrogenase although their mode of interaction is not fully understood.[11] Most systems of isocitrate dehydrogenase have been catalyzed by either manganese or magnesium, although it is known that other metals[5, 9] will catalyze the decarboxylation of oxalosuccinic acid in the presence of isocitrate dehydrogenase.

Nicotinamide adenine dinucleotide phosphate, $NADP^+$, generally acts as the electron acceptor but in some instances nicotinamide adenine dinucleotide, NAD^+, can also act as an electron acceptor.[13] It is well established[14] that reduction of $NADP^+$ occurs in the pyridine ring.

The reaction considered during this study is part of the Krebs cycle (also known as citric acid cycle) and constitutes the final common pathway in the degradation of food stuff and cell constituents to CO_2 and H_2O. Each complete cycle results in the conversion of one molecule of acetic acid to CO_2 and H_2O with energy liberated.

Factors other than enzyme, substrate, and metal ion concentration which influence this reaction are pH, temperature, ionic strength, inhibitors, coordinating ligands, changes in the structure of the coenzyme, etc.

The stability of certain enzymes is affected by pH and temperature as evidenced by the fact that some enzymes denature and lose activity at different pH levels and temperatures. In the case of pyridine nucleotide-linked enzymatic reactions, a change in pH could change the position of an equilibrium as can be seen by reaction 10. Most of the kinetic studies of isocitrate dehydrogenase obtained from different sources were carried out in a pH range of 7 to 8[5, 13] and in a temperature range of 20° to 30°C. In this temperature interval, there is

little denaturation and the velocity of the enzyme reaction increases with temperature. Reaction rates achieve an optimum in the temperature range of 25° to 30°C.[5, 12] However, in the case of isocitrate dehydrogenase isolated from A. vinelandii, the temperature was 37°C.[9]

At the present time there are a number of factors which are responsible for the change in activity with variation in ionic strength. It is possible that at high concentrations of electrolyte, i.e., at high ionic strength, anion inhibition as suggested by Cennamo et al.[13] may occur. Inhibition may also occur due to the affinity of an inhibitor toward one of the active sites present in the enzyme molecule, i.e., an -SH group. Loss in activity can be recovered by addition of some reagent which contains an -SH group: e.g., cysteine, glutathione, etc. Inhibition may also occur with a substance which can combine with the substrate, coenzyme, or metal activator rendering them unavailable for the reaction.

Studies of metal catalyzed enzymatic reactions in the presence of selected polydentate ligands may provide some insight regarding the effect of coordinating properties of metals on the enzyme activity in biological reactions. For example, the reaction rate in the presence of EDTA is decreased due to the chelation of EDTA with the Mn^{2+} ion.[12] Structural alterations in the coenzymes markedly affect their capability to serve as electron acceptors for dehydrogenase enzymes and consequently affect their rate of reaction. For example, replacing the $-NH_2$ group with $-CH_3$ in $NADP^+$ strongly affects the capacity of this analogue for use as a coenzyme.[15]

In this study, the kinetic behavior of partially purified $NADP^+$-specific isocitrate dehydrogenase from Phycomyces blakesleeanus was examined. The Km and Vmax values for D-isocitrate, $NADP^+$, and Mn^{2+} as

well as their number of binding sites to the enzyme were determined. The reaction order with respect to enzyme as well as a possible mechanism for this enzymatic reaction was determined. The effects of ionic strength, polydentate ligands, different metal ions, and some inhibitors were examined. The change in activity due to structural alterations in $NADP^+$ was also measured in this kinetic study.

EXPERIMENTAL

This section of the thesis consists of 4 parts, i.e., materials, apparatus, kinetic runs, and preliminary experiments.

A. Materials

1. Enzyme

$NADP^+$-specific isocitrate dehydrogenase was isolated from Phycomyces blakesleeanus (+ mating type, Vanderbilt strain). The purification procedure[16] yielded approximately 50% recovery of the partially purified enzyme which was stored at -12°C to preserve its activity. One unit of enzyme activity is defined as one millimole of $NADP^+$ reduced per minute. An activity of 2.5 - 3.5 units/ml was maintained during the entire study by adjusting the amount of enzyme but the reaction mixture was always kept at a total volume of 1 ml for each kinetic run.

2. Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$)

This chemical was research grade and purchased from the Sigma Chemical Co. as the monosodium salt with 99% purity. The molar optical absorptivity is $6.18 \times 10^3\ M^{-1}cm^{-1}$ at $\lambda = 340$ nm for the reduced form, NADPH.

3. DL-Isocitrate

The trisodium salt of DL-isocitrate was grade A (allo free). It was purchased from Calbiochem Laboratory and was 93% pure. Kinetic measurements showed that the DL-mixture contained 41% of the D-form and 59% of the L-form.

The DL-isocitrate (monopotassium salt) was also grade A and purchased from Calbiochem Laboratory. It was 98% pure.

4. Metal Salts

Metal salts of $MnCl_2 \cdot 4H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, $CuCl_2 \cdot 2H_2O$, LiCl, $HAuCl_4 \cdot 3H_2O$ and $ZnCl_2$ were all reagent grade and purchased from Matheson-Coleman and Bell Co. while reagent grade $IrCl_3 \cdot 3H_2O$, $RuCl_3 \cdot 3H_2O$, $RhCl_3 \cdot 3H_2O$, K_2PtCl_4, and $PdCl_2$ were purchased from A. D. Mackay, Inc. Reagent grade $HgCl_2$ and AgCl were purchased from J. T. Baker Chem. Co. while reagent grade RbCl was obtained from the Fisher Scientific Company.

5. Polydentate Ligands

a. Terpyridine

Reagent grade terpyridine was purchased from G. Frederick Smith Chemical Company and was further purified[17] by sublimation in a vacuum (0.005 - 0.010 mm) at 130° - 140°C for several hours. The white crystalline sublimate was collected on a cold finger. The pK_a values of terpyridine are: $pK_1 = 4.7$ and $pK_2 = 3.3$.

b. Trien, Tetraen, and EDTA

Technical grade triethylenetetramine (trien) and tetraethylenepentamine (tetraen) were purchased from Fisher Scientific Company. Trien ($pK_1 = 3.89$, $pK_2 = 7.01$, $pK_3 = 9.36$ and $pK_4 = 9.99$)[18] was further purified[18] by vacuum distillation at 1.5 mm and the fraction of B.P. 114°C was collected. Tetraen ($pK_1 = 2.98$, $pK_2 = 4.72$, $pK_3 = 8.08$, $pK_4 = 9.10$ and $pK_5 = 9.68$)[19] was also purified[20] by distillation in a vacuum of 1.2 mm and the fraction at B.P. 148°C was collected. The tetrasodium salt of ethylenediaminetetraacetic acid (EDTA) was reagent grade and purchased from J. T. Baker Chem. Co. The pK_a values for EDTA are:[21] $pK_1 = 1.99$, $pK_2 = 2.67$, $pK_3 = 6.16$, and $pK_4 = 10.26$.

6. Phosphate Salts

The buffer consisted of KH_2PO_4 and K_2HPO_4 salts which were reagent grade and purchased from J. T. Baker Chem. Company.

7. Inhibitors

All inhibitors were research grade and were purchased from the Sigma Chemical Co.

8. 3-Acetyl $NADP^+$

This coenzyme was research grade and obtained from Sigma Chemical Company.

B. Apparatus

1. Spectral and Kinetic Measurement Assembly

The Cary Model 14 recording spectrophotometer was used to follow the rate of reaction and to obtain the spectral measurements. A thermostated sample jacket was inserted in the sample compartment of the Cary Model 14 spectrophotometer to maintain the reaction mixture at a constant temperature inside the spectral cell. A Haake Model FE constant temperature circulator was used to circulate water through the sample jacket via 1/4 inch tygon tubing. The thermostated cell jacket permits solutions in the spectral cell to be maintained within ±0.03°C at temperatures from -30°C to 55°C. A bucket of ice water served as the cold water source to the Haake circulator to prevent excessive heating during its operation. Cold water was circulated to the Haake circulator via a Vertical Centrifugal Immersion Pump manufactured by Sargent-Welch Scientific Co. (Model No. B-6). Connections to and from the pump consisted of 1/4 inch tygon tubing. Quartz semi-micro cuvettes with a 1 cm optical pathlength were used for the spectral and kinetic studies. All reaction components

were transferred to the cuvette by suitable pipets and mixed well with the semi-micro cuvette, "Add-A-Mixer," manufactured by Precision Cells Incorporated.

2. PDP-8 Computer

The PDP-8 Computer manufactured by Digital Equipment Corporation was used to obtain increased accuracy during the calculation of Michaelis constants, maximum velocities, and the number of binding sites in the case of D-isocitrate, $NADP^+$, and Mn^{2+}. The programming language used during these calculations was "FOCAL." The program selected in this study was the "Linear Least Squares Curve Fit."

3. pH Meter

The Corning Model 10 pH meter was used with calomel and glass electrodes. It was distributed by Will Scientific, Inc. and subsidiaries. The combination electrode (No. S-30070-10) manufactured by Sargent-Welch Scientific Co. was used to measure the pH of solutions when the volume was very small.

4. Sublimation Assembly

All components of the sublimator in the sublimation assembly were made of pyrex glass and purchased from Ace Glass Co. The material to be sublimated was placed in a 50 ml flask which was connected to a fraction-cutter via a condenser. This assembly allowed different fractions of material to be collected separately. The flask was connected to a vacuum pump manufactured by Central Scientific Co. when the sublimation was performed under a vacuum. The vacuum in the system was measured by Virtis's McLeod Gauges. During the sublimation of terpyridine, solid fractions were collected on a cold finger maintained via cold water circulated through an outer jacket.

5. Balance

All weight measurements were carried out on a 200 g analytical balance which has an accuracy of ±0.1 mg in the range of 100 mg - 200 g. The balance was manufactured by Sartorius-Werke AG Gottingen Co.

C. Kinetic Runs

The kinetic measurements were obtained spectrally by following the rate of reduction of $NADP^+$ at 340 nm on the Cary Model 14 spectrophotometer. The reaction mixture was maintained at 25°C by thermostating with a Haake constant temperature circulator. The phosphate buffer was prepared by mixing the appropriate amounts of K_2HPO_4 (0.5 M) and KH_2PO_4 (0.5 M) solutions to obtain a pH of 7.5 and an ionic strength of 0.405 M. This enzyme has been found to be active in the pH range of 7.2 to 8.2 with the pH optimum estimated at 7.5 under the assay conditions employed.[16] The conditions used to maintain the enzyme at complete saturation were determined previously.[16] A typical reaction mixture contained 6.6×10^{-4} M $NADP^+$ in 0.15 M phosphate buffer (0.50 ml), 3.1×10^{-3} M DL-isocitrate (0.05 ml), 5×10^{-4} M Mn^{2+} (0.10 ml), enzyme (0.05 ml), and water (0.30 ml) to give a total volume of 1.00 ml. This mixture gives a total ionic strength of 0.425 M. Ionic strength effects due to $NADP^+$ were very small. The deionized water used in this study was glass distilled over potassium permanganate before use.

1. Determination of Michaelis Constants (Km), Maximum Velocities (Vmax), and Number of Binding Sites (n).

a. threo-Ds-Isocitrate

DL-isocitrate was used as the source of substrate with only the D-form being active. Consequently, all the substrate concentrations refer to the D-form only. The enzyme activity used during this study

was 3.31 units/ml. Initial reaction rates were measured over a concentration range of $1.27 \times 10^{-3} - 6.45 \times 10^{-5}$ M D-isocitrate with the remaining components maintained at the same concentrations described in the typical reaction mixture above.

b. Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$)

The reaction mixture contained all the components at the same concentrations described in the typical reaction mixture mentioned above with the enzyme activity maintained at 0.713 units/ml. The initial reaction rates were measured over a concentration range of $1.72 \times 10^{-4} - 5.00 \times 10^{-4}$ M $NADP^+$.

c. Mn^{2+}

The typical reaction mixture described above was used with the Mn^{2+} concentration varied in a range of $5 \times 10^{-4} - 5 \times 10^{-6}$ M. The corresponding initial reaction rates were recorded at an enzyme activity of 0.83 units/ml.

The values of Km and Vmax for threo-Ds-isocitrate, $NADP^+$, and Mn^{2+} were obtained by Lineweaver-Burk plots (Figures 1, 2, and 3). The number of binding sites for threo-Ds-isocitrate, $NADP^+$, and Mn^{2+} with respect to enzyme were determined by Hill plots (Figures 5, 6, and 7).

2. Enzyme Reaction Order

The reaction mixture contained all the components in the typical reaction mixture except different volumes of enzyme (activity = 0.71 units/ml) were used to vary the enzyme concentration. The initial reaction rates were measured at different volumes of enzyme (Vv) in the range of 0.05 - 0.30 ml. The value of the slope 'n' was obtained from a plot of log v_o vs log Vv (Figure 8).

3. Effect of Ionic Strength

The reaction rates were measured at different ionic strengths in the range of 0.03 - 0.73 M. The ionic strength conditions were achieved by using the appropriate amounts of KH_2PO_4 and K_2HPO_4 salts while keeping the pH at 7.5. The effect of ionic strength on the enzymatic reaction rate is shown in Figure 9. The enzyme activity during these runs was 3.85 units/ml.

4. Polydentate Ligands

a. Terpyridine (Terpy)

The reaction mixture with an enzyme activity of 0.93 units/ml contained all the components at the same concentration described in the typical reaction mixture except for the Mn^{2+} concentration. Terpyridine (10^{-4} M, 2×10^{-4} M, and 3×10^{-4} M) was added to solutions containing 10^{-4} M Mn^{2+} so that 1:1, 1:2, and 1:3 ratios of Mn^{2+} to terpy could be obtained. The corresponding reaction rates were measured. In the fourth run an excess amount of solid terpyridine was added to the reaction mixture and the reaction rate was measured. Results of these studies are reported in Table II.

b. Triethylenetetramine (Trien), Tetraethylenepentamine (Tetraen), and Ethylenediaminetetraacetic Acid (EDTA)

Trien, tetraen, and EDTA each at a concentration of 5×10^{-4} M was added to a typical reaction mixture in which the Mn^{2+} concentration was 5×10^{-4} M and the corresponding reaction rates were measured at pH 9.5 and 10.5. The results are reported in Table III. The enzyme activity was 2.33 units/ml in this study.

5. Activities in the Presence of Different Components

a. Metal Ions

The reaction rate of the typical reaction mixture was measured in the presence of several metal ions which replaced Mn^{2+} each at a concentration of 5×10^{-4} M. The enzyme activity during these studies was 1.14 units/ml. The relative activity associated with each metal ion compared to Mn^{2+} is reported in Table V.

b. Inhibitors

A few inhibitors which are specific for the sulfhydryl group were added to the reaction mixture at a concentration of 5×10^{-4} M. The reaction rate was measured in each run and compared with the rate in the absence of inhibitors. The enzyme activity for these runs was 3.6 units/ml. The results are reported in Table VI.

c. 3-Acetyl $NADP^+$

The reaction rate was followed at 357 nm when $NADP^+$ was replaced by 3-acetyl $NADP^+$ at the same concentration. The kinetic results of these two coenzymes obtained in the typical reaction mixture are compared in Table VII. The enzyme activity during these runs was 3.6 units/ml.

D. Preliminary Experiments

1. Enzyme Activity in the Presence of Organic Solvents

Most of the studies concerning enzymatic reactions are reportedly carried out in the presence of water as the solvent. Some effort was made to examine the effects of a few common organic solvents on enzymatic reactions. Organic solvents selected for study were ethanol, methanol, carbon tetrachloride, benzene, and carbon disulphide. All attempts were unsuccessful. In the presence of ethanol and methanol no kinetic results

could be obtained due to the precipitation of $NADP^+$. Carbon tetrachloride, benzene, and carbon disulphide were not miscible with water.

2. Displacement of Terpyridine by Isocitrate in the Manganous Bis Terpyridine Complex

Preliminary spectral measurements of solutions containing $Mn(terpy)_2^{2+}$ and isocitrate indicated the presence of a Mn^{2+} complex containing both terpyridine and isocitrate. This is supported by the fact that the observed absorption of the $Mn(terpy)_2^{2+}$ complex with its absorption maximum at 400 nm was lower than the theoretical value in the presence of isocitrate. This result suggests that terpyridine is being displaced by isocitrate in the $Mn(terpy)_2^{2+}$ complex. The difference in absorption between the observed and theoretical value increases when the ratio of isocitrate to $Mn(terpy)_2^{2+}$ is elevated suggesting that more terpyridine is being displaced at the higher concentration of isocitrate. It should be pointed out that there is essentially no absorption by the isocitrate complex of Mn^{2+} at 400 nm.

RESULTS AND DISCUSSION

The rate of conversion of the substrate, D-isocitric acid, to the product, α-ketoglutaric acid, in the presence of $NADP^+$ and Mn^{2+} was slow under the conditions employed and consequently could be measured spectrophotometrically on the Cary Model 14. The partially purified isocitrate dehydrogenase used in this study was not active in the presence of coenzyme NAD^+.[16]

The kinetic results presented in this section of the thesis include Km values (Michaelis constants), maximum velocities (Vmax), and the number of binding sites for isocitrate, $NADP^+$, and Mn^{2+} respectively. The reaction order with respect to the enzyme was determined and a mechanism for the overall reaction has been proposed. The effects of ionic strength, polydentate ligands, metal ions, inhibitors, and 3-acetyl $NADP^+$ were also studied.

A. Determination of Michaelis Constants and Maximum Velocities

Lineweaver-Burk plots showing the values of the Michaelis constants (Km) and maximum velocities (Vmax) for threo-Ds-isocitrate, $NADP^+$, and Mn^{2+} (Figures 1, 2, and 3) were found to be linear which is consistent with Michaelis-Menten kinetics. However, there are a number of examples where this enzyme obtained from different sources does not obey Michaelis-Menten kinetics.[22-24]

The Michaelis constant (Km) for threo-Ds-isocitrate was also obtained from a plot of rate vs substrate concentration. The Km value obtained by

Figure 1. Lineweaver-Burk Plot for the Determination of Km and Vmax for threo-Ds-Isocitrate

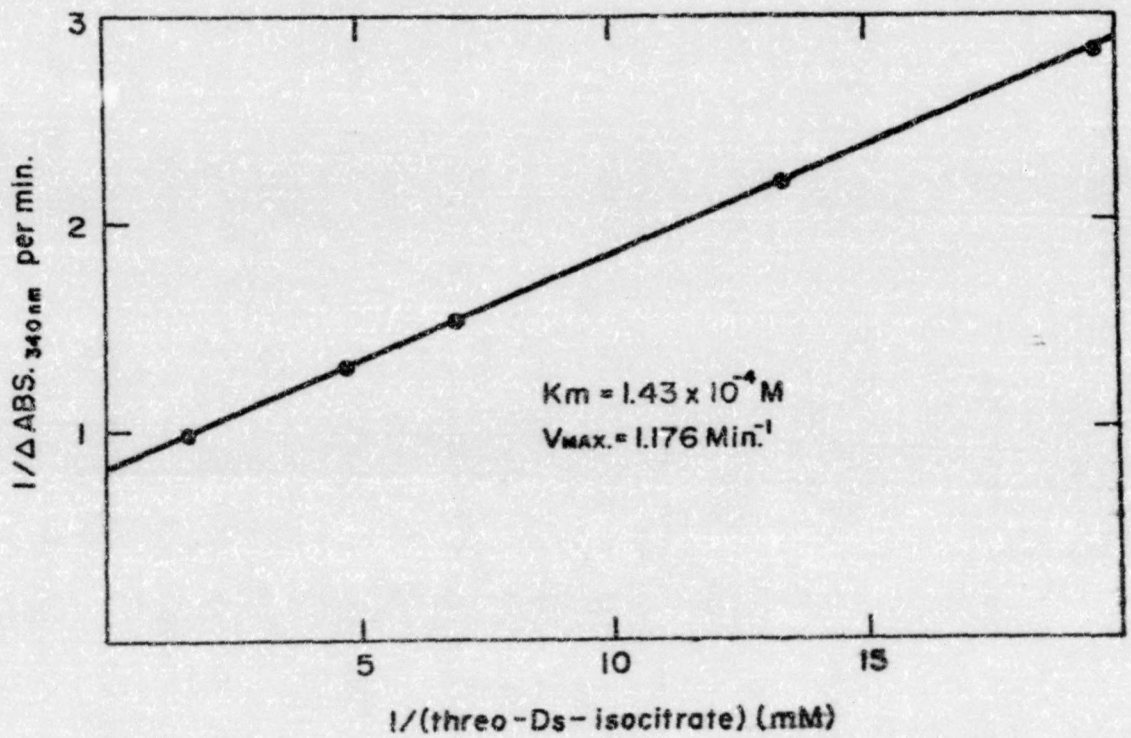

3
2
1
1/ΔABS. 340nm per min.
Km = 1.43 x 10⁻⁴ M
VMAX. = 1.176 Min.⁻¹
5
10
15
1/(threo-Ds-isocitrate) (mM)

Figure 2. Lineweaver-Burk Plot for the Determination of Km and Vmax for $NADP^+$

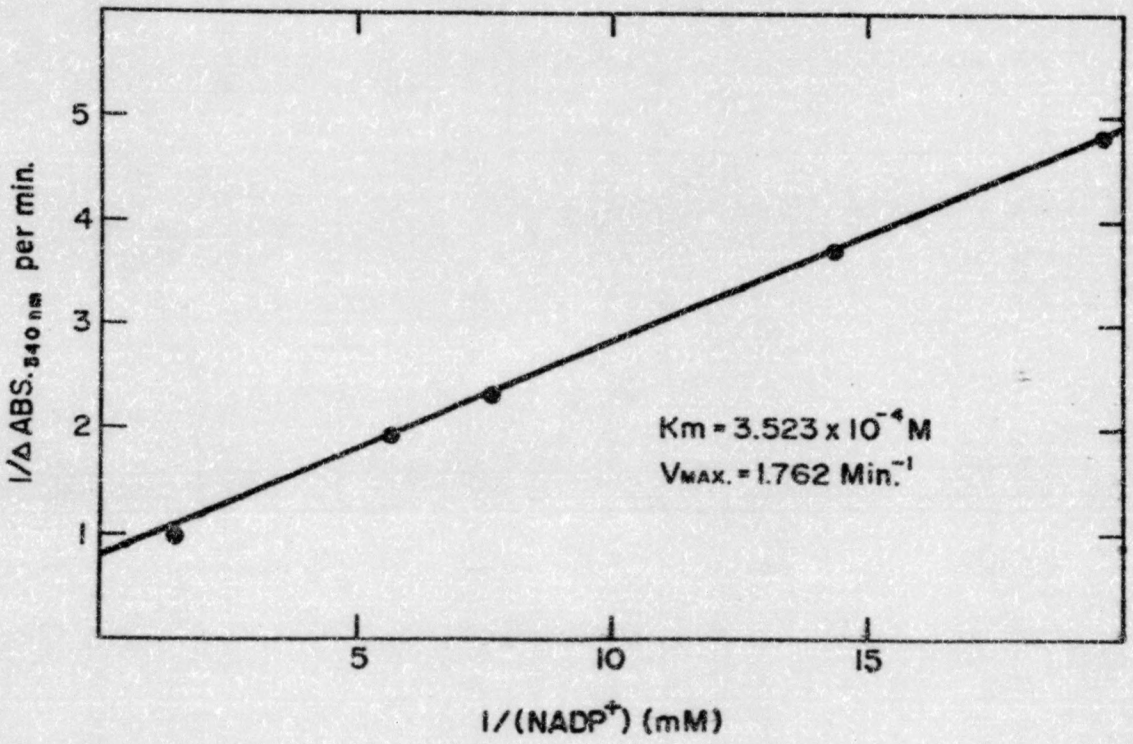
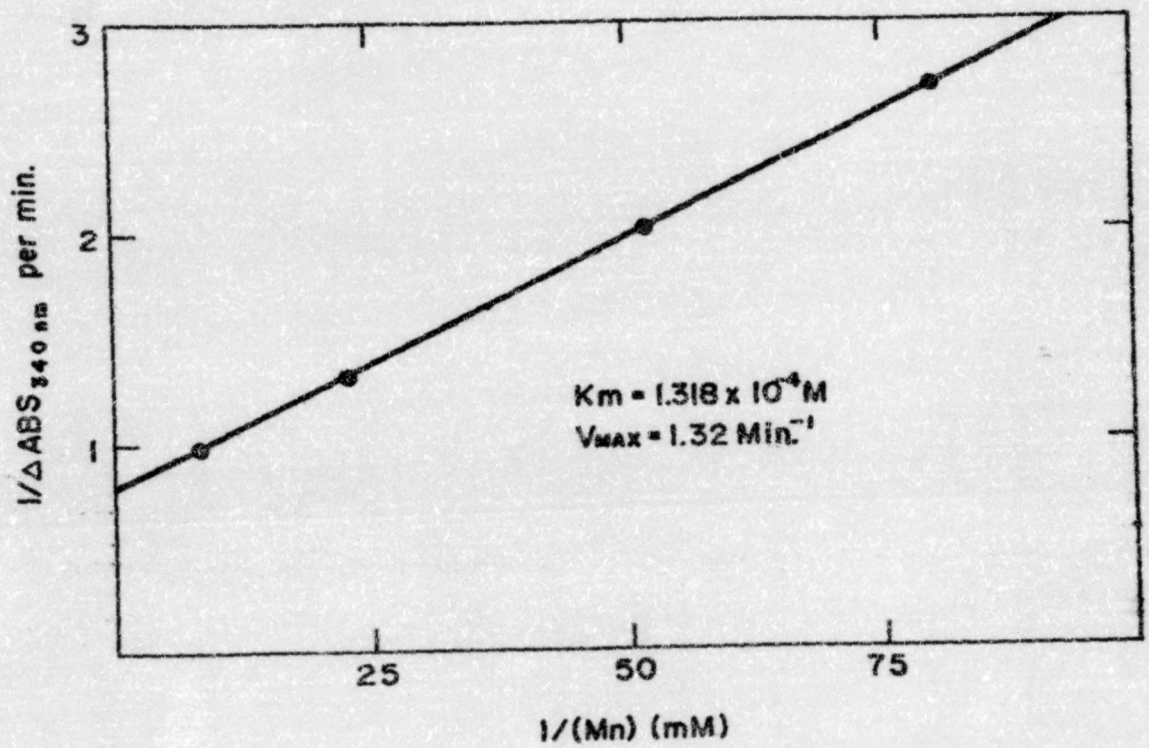

1/Δ ABS.340 nm per min.
1
2
3
4
5
5
10
15
1/(NADP+) (mM)
Km = 3.523 x 10-4 M
VMAX. = 1.762 Min.-1

Figure 3. Lineweaver-Burk Plot for the Determination of Km and Vmax for Mn^{2+}


3
2
1
$1/\Delta ABS_{340\,nm}$ per min.
$Km = 1.318 \times 10^{-4} M$
$V_{MAX} = 1.32\ Min.^{-1}$
25
50
75
1/(Mn) (mM)

this method is shown in Figure 4 and is in close agreement with the result obtained by the Lineweaver-Burk plot. However, there are some disadvantages in determining the Michaelis constant and maximum velocity from this type of plot as discussed previously.

The Km values obtained for threo-Ds-isocitrate, $NADP^+$, and Mn^{2+} are 1.43×10^{-4} M, 3.52×10^{-4} M and 1.32×10^{-4} M respectively as shown in Table I. The corresponding Km values obtained for the same enzyme isolated from different sources are also included in the table for comparison. However, it is very difficult to evaluate these results since the experiments in each instance were performed under slightly different conditions, e.g., enzyme purity, ionic strength, pH, metal ion concentration, etc. Marked differences in physical and chemical properties of the enzyme obtained from different sources would also contribute to the variation in the Km values. Consequently, it is difficult to determine whether the differences in the Km values reflect different kinetic behavior of the enzyme isolated from different sources.

B. Binding Sites of Reaction Components to Enzyme Molecule

The number of binding sites of threo-Ds-isocitrate, $NADP^+$, and Mn^{2+} to the enzyme molecule was determined via the Hill equation:[3]

$$\log \left(\frac{v_o}{Vmax - v_o}\right) = n \cdot \log(S) - \log K_{eq} \qquad (12)$$

where the definition of each term is described in the introduction section. The straight line obtained from a plot of $\log (v_o/Vmax-v_o)$ vs $\log (S)$, yields a value of the slope 'n', the number of binding sites on the enzyme molecule with a given species and (S) corresponds to the concentration of threo-Ds-isocitrate, $NADP^+$, or Mn^{2+}. The number of binding sites for threo-Ds-isocitrate, $NADP^+$, and Mn^{2+} obtained from

Figure 4. Alternate Method for the Determination of Km and Vmax for threo-Ds-Isocitrate

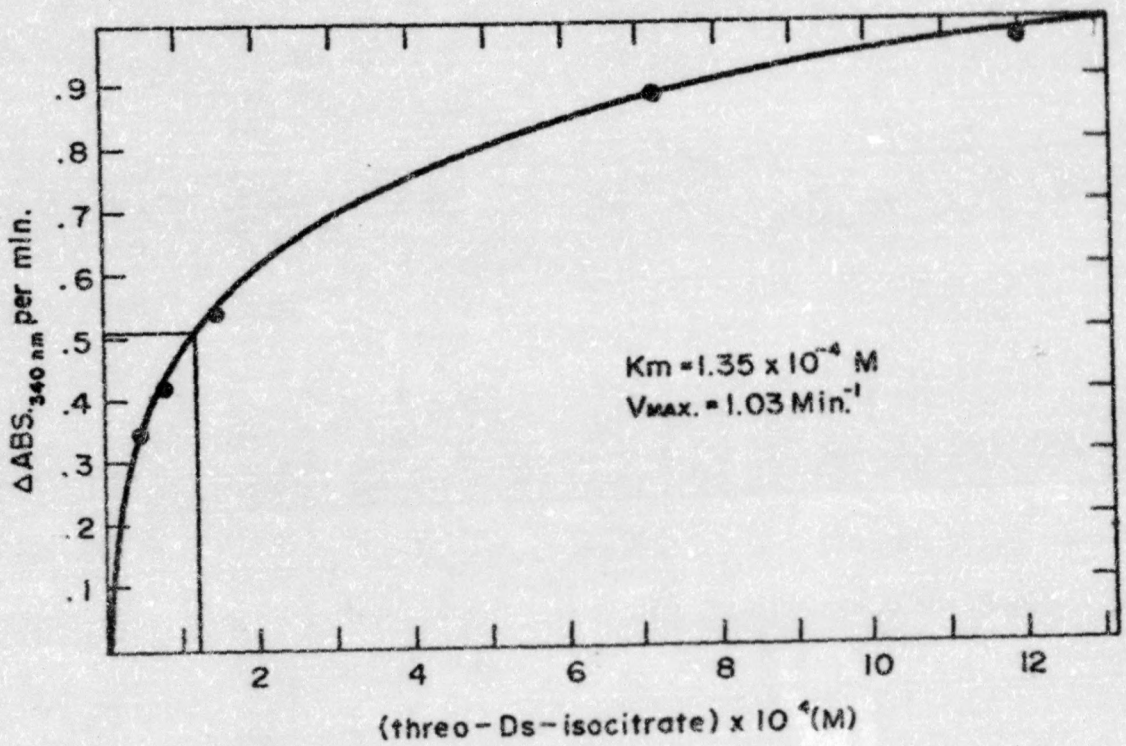
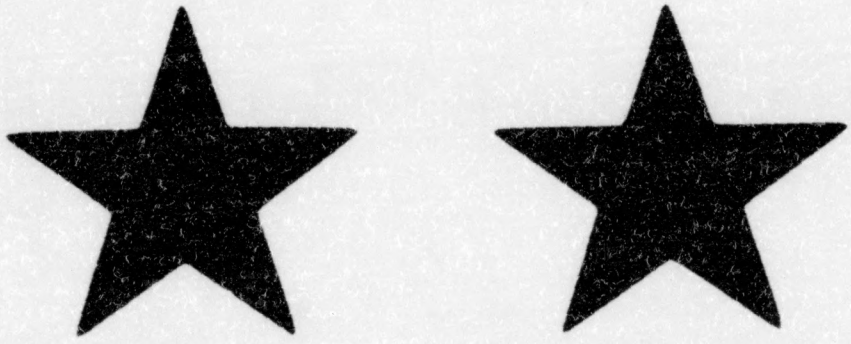

ΔABS.340 nm per min.
.9
.8
.7
.6
.5
.4
.3
.2
.1
2
4
6
8
10
12
Km = 1.35 x 10⁻⁴ M
VMAX. = 1.03 Min.⁻¹
(threo-Ds-isocitrate) x 10⁻⁴(M)

TABLE I.

COMPARISON OF Km VALUES FOR threo-Ds-ISOCITRATE, $NADP^+$, AND Mn^{2+} FROM DIFFERENT SYSTEMS

Source of Isocitrate Dehydrogenase	Km			References
	Ds-Isocitrate	$NADP^+$	Mn^{2+}	
Phycomyces blakesleeanus	1.43×10^{-4} M	3.52×10^{-4} M	1.32×10^{-4} M	Present Study
Azotobacter vinelandii	3.60×10^{-5} M	1.80×10^{-5} M	---	9
Escherchia coli	1.56×10^{-5} M	3.70×10^{-5} M	1.29×10^{-5} M	25
Pig heart	2.60×10^{-6} M	---	---	12
Thiobacillus novellus	8.30×10^{-6} M	1.30×10^{-5} M	3.40×10^{-6} M	26
Salmonella typhimurium	1.90×10^{-6} M	1.15×10^{-5} M	1.89×10^{-6} M	5
Azotobacter vinelandii	2.00×10^{-5} M	2.30×10^{-5} M	---	27
Pseudomonas flourescenes	1.50×10^{-5} M	1.80×10^{-5} M	---	28
Euglena gracilis var. bacillueris	3.00×10^{-5} M	---	---	29

CORRECTION



PRECEDING IMAGE HAS BEEN REFILMED TO ASSURE LEGIBILITY OR TO CORRECT A POSSIBLE ERROR

TABLE I.

COMPARISON OF Km VALUES FOR threo-Ds-ISOCITRATE, $NADP^+$, AND Mn^{2+} FROM DIFFERENT SYSTEMS

Source of Isocitrate Dehydrogenase	Km			References
	Ds-Isocitrate	$NADP^+$	Mn^{2+}	
Phycomyces blakesleeanus	1.43×10^{-4} M	3.52×10^{-4} M	1.32×10^{-4} M	Present Study
Azotobacter vinelandii	3.60×10^{-5} M	1.80×10^{-5} M	---	9
Escherchia coli	1.56×10^{-5} M	3.70×10^{-5} M	1.29×10^{-5} M	25
Pig heart	2.60×10^{-6} M	---	---	12
Thiobacillus novellus	8.30×10^{-6} M	1.30×10^{-5} M	3.40×10^{-6} M	26
Salmonella typhimurium	1.90×10^{-6} M	1.15×10^{-5} M	1.89×10^{-6} M	5
Azotobacter vinelandii	2.00×10^{-5} M	2.30×10^{-5} M	---	27
Pseudomonas flourescenes	1.50×10^{-5} M	1.80×10^{-5} M	---	28
Euglena gracilis var. bacillueris	3.00×10^{-5} M	---	---	29

these plots are listed in Figures 5, 6, and 7 respectively. The values of 'n' are 2, 1, and 1 for threo-Ds-isocitrate, $NADP^+$, and Mn^{2+} respectively which suggests that two molecules of threo-Ds-isocitrate, one molecule of $NADP^+$, and one molecule of Mn^{2+} can bind with the enzyme molecule. However, these values of 'n' do not necessarily indicate the overall order of the reaction. For example, the reaction:

$$E + 2SH + M + N^+ \longrightarrow [ES_2MN]^- + 2H^+ \qquad (13)$$

does not necessarily take place during the rate determining step. In the above reaction SH, N^+, and M represent threo-Ds-isocitrate, $NADP^+$, and Mn^{2+} respectively. It has been previously shown[3] that isocitrate dehydrogenase isolated from baker's yeast can bind with 4 molecules of threo-Ds-isocitrate, 2 molecules of AMP, 2 molecules of DPN^+, and 2 molecules of Mg^{2+} and that this complex is present in the rate determining step. The rate law was given by the equation:

$$v_o = k[ES_4A_2D_2M_2] \qquad (14)$$

where v_o is the initial reaction velocity, k is the rate constant, A, D, and M represent AMP, DPN^+, and Mg^{2+} respectively. It was proposed that the above complex forms in a series of steps none of which are necessarily rate determining.

For most complex enzymatic reactions the overall reaction order is unknown because it is difficult to determine the reaction order of each component involved. Additional complications arise when a multistep mechanism is operating and the reaction mechanism is unknown.

The sigmoid curve shown in Figure 4 supports the fact that more than one molecule of substrate binds with the enzyme examined here. If only one molecule of substrate was bound to the enzyme, a straight line

Figure 5. Determination of Binding Sites for threo-Ds-Isocitrate to Enzyme

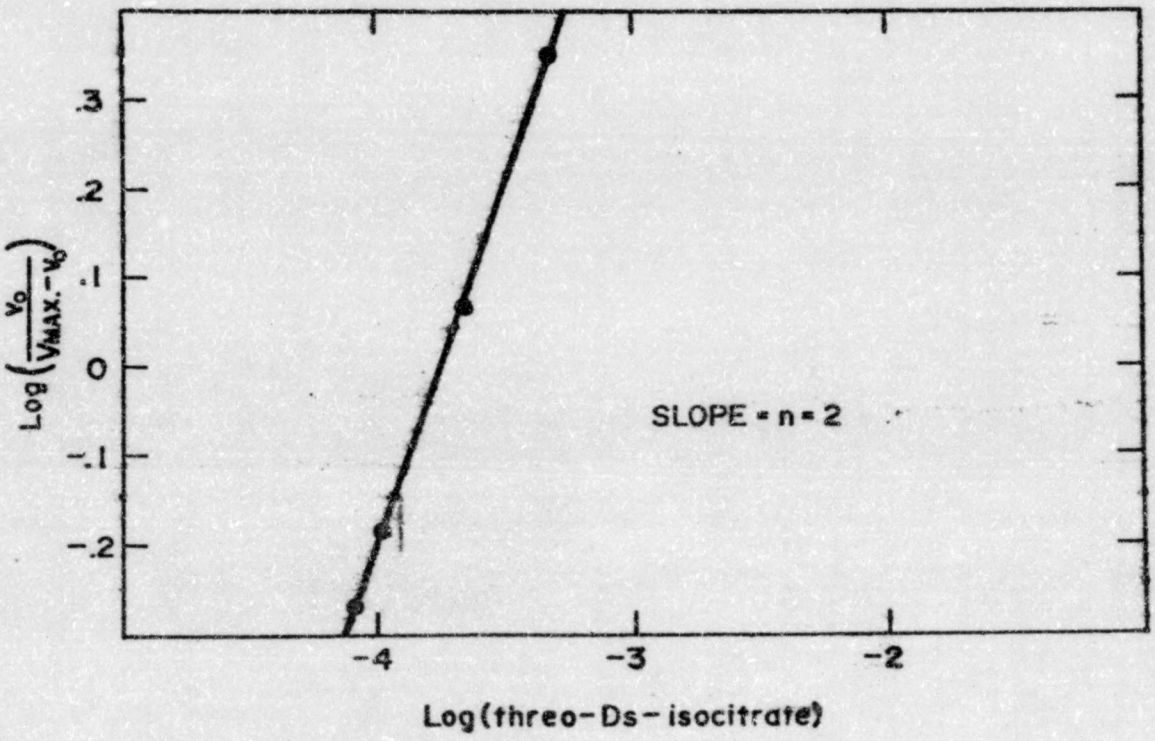

Log $\left(\frac{V_0}{V_{MAX.}-V_0}\right)$
.3
.2
.1
0
-.1
-.2
SLOPE = n = 2
-4
-3
-2
Log (threo-Ds-isocitrate)

Figure 6. Determination of Binding Sites for $NADP^+$ to Enzyme

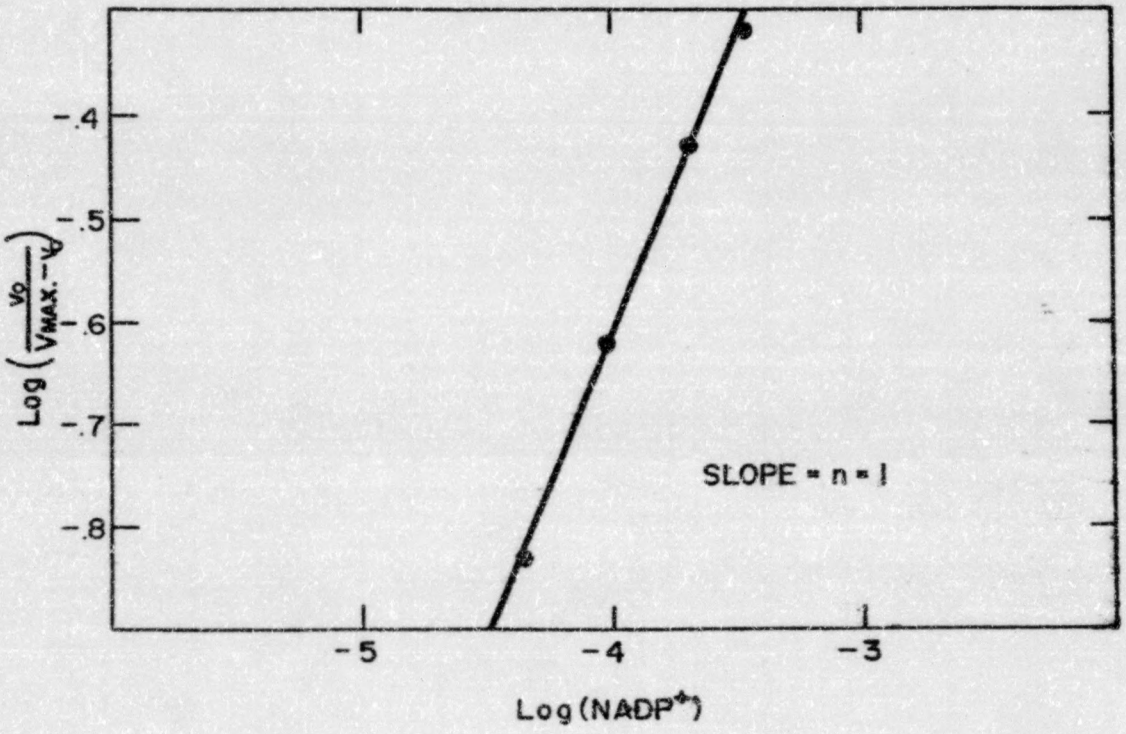
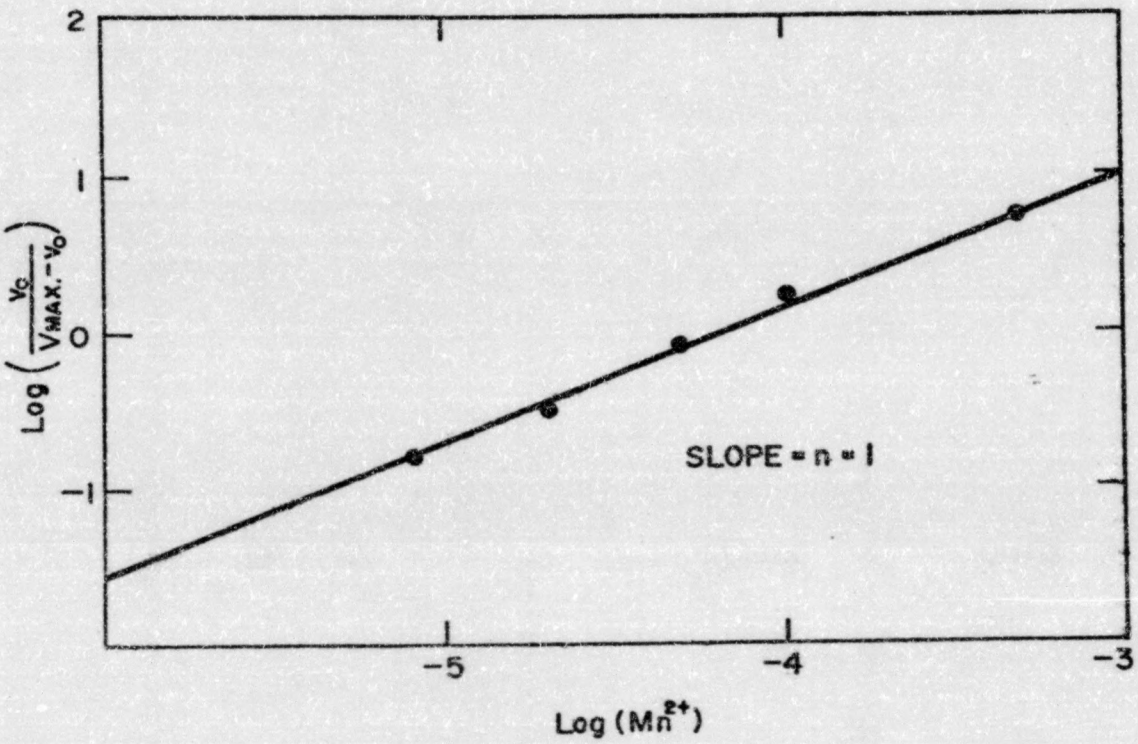

-.4
-.5
-.6
-.7
-.8
Log $\left(\frac{V_0}{V_{MAX.}-V_0}\right)$
SLOPE = n = 1
-5
-4
-3
Log (NADP$^+$)

Figure 7. Determination of Binding Sites for Mn^{2+} to Enzyme


2
1
0
-1
Log $\left(\frac{V_o}{V_{MAX.}-V_o}\right)$
SLOPE = n = 1
-5
-4
-3
Log (Mn^{2+})

would have been obtained. Results obtained from the Hill plots suggest that two molecules of threo-Ds-isocitrate, one molecule of $NADP^+$, and one molecule of Mn^{2+} bind to the enzyme and form a complex which then reacts during the rate determining step. If this is so, the rate law would be:

$$v_o = k[ES_2MN]^- \qquad (15)$$

The above rate law is not supported by the stoichiometry of the enzyme reaction which shows that one mole of threo-Ds-isocitrate yields one mole of NADPH from kinetic measurements. Nevertheless, this could be explained by the fact that one of the two molecules of isocitrate binds to the enzyme less effectively and hence does not form the product. However, based on the stoichiometry of the enzyme reaction alone, the rate law would be:

$$v_o = k[ESMN] \qquad (16)$$

This point will be reconsidered later when the reaction mechanism is discussed.

C. Enzyme Reaction Order

According to Michaelis-Menten theory the initial velocity of the reaction:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E \qquad (17)$$

can be given by the equation:

$$v_o = ke_o^n \qquad (18)$$

where the definition of each term is given in the introduction section.

In the case of the partially purified enzyme, the enzyme concentration (e_o) is unknown. However, the enzyme volume (Vv) which is

directly proportional to the enzyme concentration was used instead:

$$e_o \propto Vv \tag{19}$$

$$e_o = RVv \tag{20}$$

$$(e_o)^n = (RVv)^n \tag{21}$$

where R is a proportionality constant. By substituting the value of $(e_o)^n$ in equation 18, we obtain:

$$v_o = k(RVv)^n \tag{22}$$

$$v_o = kR^n(Vv)^n \tag{23}$$

$$v_o = k'(Vv)^n \tag{24}$$

where $k' = kR^n$. The logarithm of both sides of equation 24 yields:

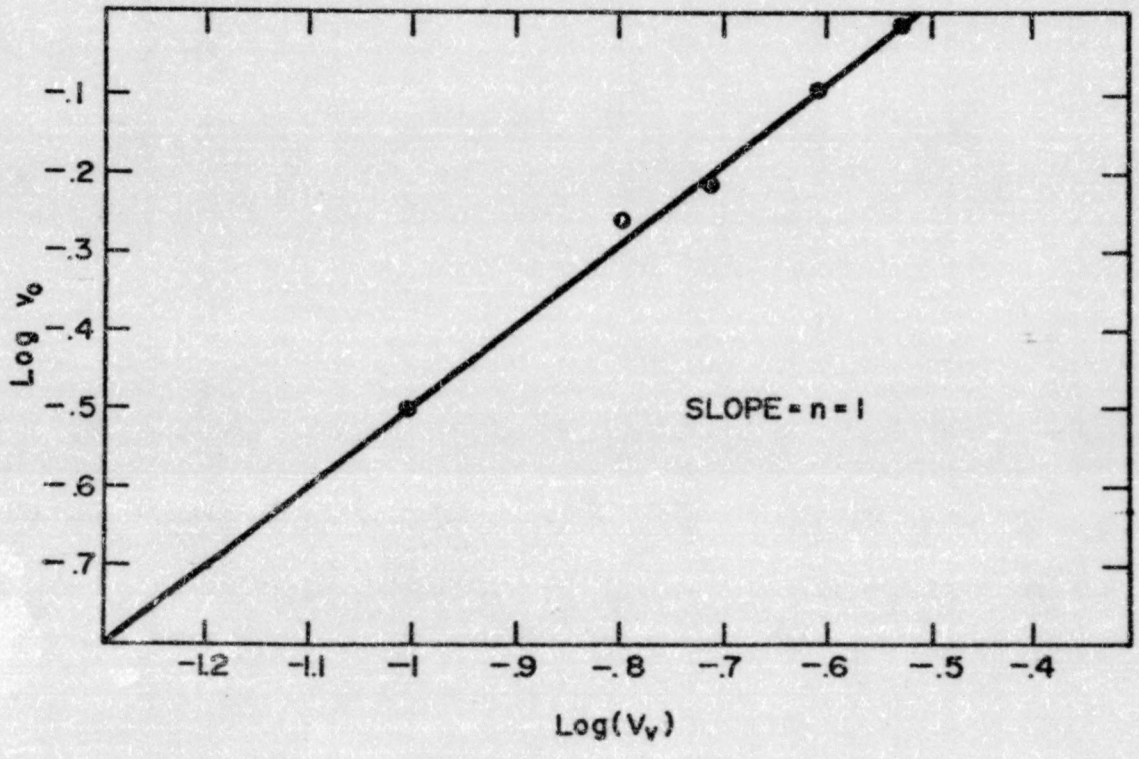
$$\log v_o = \log k' + n \cdot \log (Vv) \tag{25}$$

Therefore, the order of reaction with respect to the partially purified enzyme could be obtained with the enzyme concentration unknown. The value of the slope obtained from a plot of $\log v_o$ vs $\log (Vv)$ was unity (Figure 8) indicating that the reaction under consideration is first order with respect to enzyme. Several studies of other systems have also shown this enzymatic reaction to be first order with respect to enzyme.[3] The value of the intercept ($\log k'$) obtained from this plot has no significance since the true concentration of the enzyme was unknown.

D. Ionic Strength Effects

Potassium phosphate buffer was used during this study to maintain a pH of 7.5 in the reaction mixture. The ionic strength of the solution was varied by using different concentrations of phosphate salts[30] in

Figure 8. Determination of Enzyme Reaction Order


-.1
-.2
-.3
-.4
-.5
-.6
-.7
Log v_o
SLOPE = n = 1
-1.2
-1.1
-1
-.9
-.8
-.7
-.6
-.5
-.4
Log(V_v)

the buffer and then measuring the corresponding rate of reaction. A plot of the reaction rate vs the ionic strength is shown in Figure 9.

The rate of the observed reaction reached a maximum between an ionic strength of 0.07 M to 0.13 M. The rate decreased sharply when the ionic strength was decreased below 0.07 M but decreased slowly as the ionic strength was increased beyond 0.13 M.

It is very difficult to explain the behavior of ionic strength on an enzymatic reaction since a number of factors could be responsible. Some of these factors are outlined below.

1. The equilibrium constant associated with the complex, ES, in the reaction:

$$E + S \rightleftharpoons ES \xrightarrow{k} P + E \qquad (26)$$

is given by

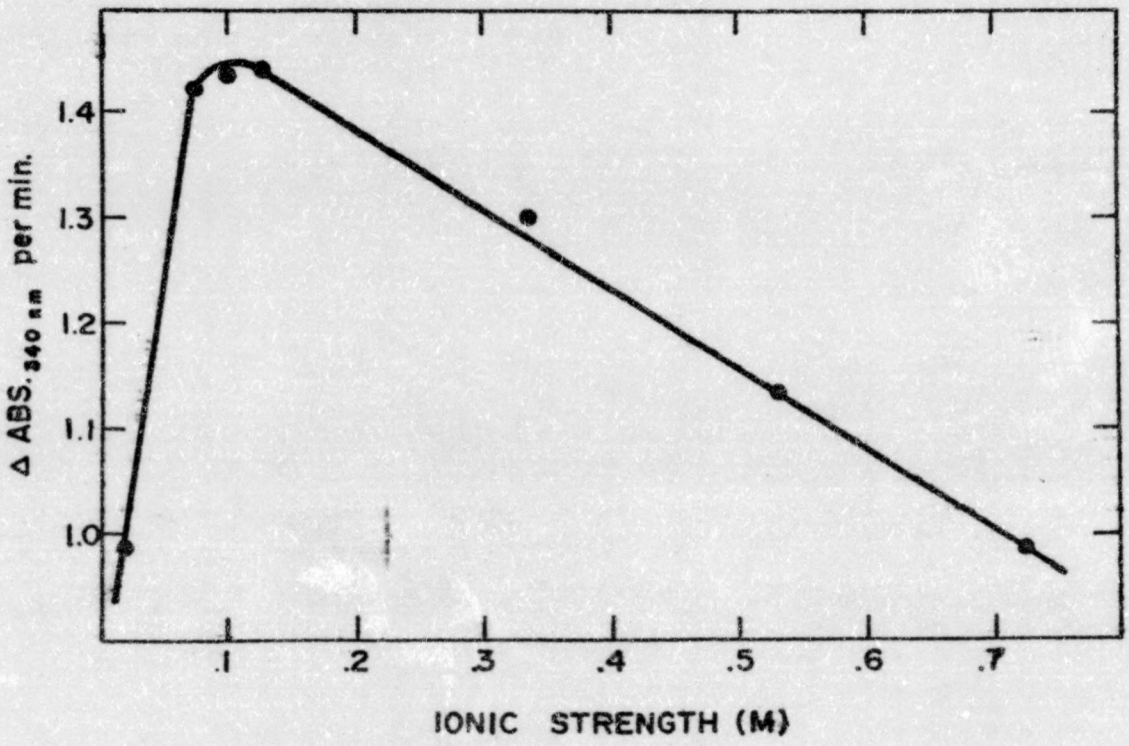
$$K_{eq} = \frac{[ES]}{[E][S]} \qquad (27)$$

According to Bronsted[31] and Bjerrum[32] K_{eq} can be expressed more accurately as:

$$K_{eq} = \frac{a_{ES}}{a_E \cdot a_S} = \frac{f_{ES}}{f_E \cdot f_S} \cdot \frac{[ES]}{[E][S]} \qquad (28)$$

where the a's are the respective activities and f's are the corresponding activity coefficients. The activity coefficients f_{ES}, f_E, and f_S are dependent on the ionic strength. Any variation in ionic strength changes the value of the activity coefficients and thus affects the value of K_{eq}. Since K_{eq} controls the concentration of ES which in turn affects the rate of reaction, ionic strength can therefore influence an enzymatic reaction rate.

Figure 9. Ionic Strength Effects on the Reaction Rate


Δ ABS.340 nm per min.
1.0
1.1
1.2
1.3
1.4
.1
.2
.3
.4
.5
.6
.7
IONIC STRENGTH (M)

2. The ionization constants of protein groups vary with ionic strength.[33] The acid dissociation constants of an enzyme generally increase with ionic strength thus favoring binding with substrate. This may possibly account for the increase in reaction rate with ionic strength in the range of 0.03 - 0.07 M.

3. As pointed out above (in 2), the reaction rate can increase with an increase in ionic strength. However, a decrease in the reaction rate occurs after reaching a maximum value at an ionic strength of 0.13 M. This could result from anion inhibition due to the increased concentration of the phosphate anion as demonstrated in other systems.[13] Inhibition by phosphate, acetate, bicarbonate, and other buffer anions frequently results from their competition with a negatively charged substrate for a cationic site on the enzyme.[33] The anion may even combine with a metal ion incorporated in the enzyme reaction.

Factors which tend to stabilize the ES complex will enhance the rate of reaction. Ionic strength being one of these factors will therefore affect the rate of reaction.[33] Moreover, ionic strength can also influence the stability of the metal-substrate complex[34] and the structure of the enzyme.[35] Both of these factors play important roles in affecting the rate of reaction.

E. Influence of Polydentate Ligands

A reduction in enzyme activity catalyzed by a metal ion may occur by the introduction of certain polydentate ligands. These polydentate ligands can coordinate with the metal ion and thus decrease its availability for the reaction under consideration. It is not always necessary for these ligands to form a more stable complex with the metal ion than the substrate, but only to compete with the substrate in order to

decrease the reaction rate. The reaction rate may also decrease if these ligands bind to the active site(s) of the enzyme molecule which is not believed to be the case here.

1. Terpyridiene (Terpy)

Activities obtained during the addition of varying amounts of terpyridine to the reaction mixture are reported in Table II. When the ratio of Mn^{2+} to terpyridine is 1:1, 73.7% of the reactivity (compared to the absence of terpyridine) remains. Spectral measurements of solutions containing a 1:1 Mn^{2+} to terpy ratio give an absorption band at 400 nm which corresponds to the absorption band of the bis (1:2) complex. The intensity of that band indicates that none of the mono species is present. This may be explained by the following set of reactions.

$$Mn(terpy)^{2+} \rightleftharpoons Mn^{2+} + terpy \qquad (29)$$

$$Mn(terpy)^{2+} + terpy \rightleftharpoons Mn(terpy)_2^{2+} \qquad (30)$$

$$\text{Overall reaction: } 2Mn(terpy)^{2+} \rightleftharpoons Mn(terpy)_2^{2+} + Mn^{2+} \qquad (31)$$

If equation 31 was the only reaction occurring in the presence of terpyridine, then a 50% loss in activity would be expected in the presence of a 1:1 Mn^{2+} to terpy ratio. (No activity is assumed to be present with the $Mn(terpy)_2^{2+}$). However, the observed activity was 73.7%. The additional reactivity (23.7%) in the presence of a 1:1 Mn^{2+} to terpy ratio may be attributed to the terpyridine in $Mn(terpy)_2^{2+}$ being displaced by isocitrate leaving 26.3% of the product mixture as $Mn(terpy)_2^{2+}$. The displacement of terpyridine in $Mn(terpy)_2^{2+}$ depends on the concentration of isocitrate, terpyridine, and the relative stability constants of $Mn(terpy)_2^{2+}$ and the Mn^{2+} complex containing isocitrate. Increasing the

TABLE II.

EFFECT OF TERPYRIDINE ON THE RATE OF REACTION

Concentration		% Activity
Mn^{2+}	Terpyridine	
1×10^{-4} M	-	100.0%
1×10^{-4} M	1×10^{-4} M	73.7%
1×10^{-4} M	2×10^{-4} M	46.7%
1×10^{-4} M	3×10^{-4} M	34.0%
1×10^{-4} M	$>> 5 \times 10^{-4}$ M	Immeasurably Slow

concentration of terpyridine would favor the formation of the bis complex which decreases the rate of reaction as observed in this study.

If the above explanation is true then the 53.3% and 66.0% loss in activity in the presence of 1:2 and 1:3 Mn^{2+} to terpy ratios corresponds to a product mixture composed of 53.3% and 66.0% of the $Mn(terpy)_2^{2+}$ complex respectively. An extremely slow reaction occurs in the presence of excess terpyridine indicating that essentially all of the Mn^{2+} species is coordinated to form the $Mn(terpy)_2^{2+}$ complex. In this complex all six sites of Mn^{2+} are unavailable for the reaction. These results suggest that when all six sites on a metal ion are involved in coordination, the metal ion ceases to function as an activator.

2. Triethylenetetramine (Trien), Tetraethylenepentamine (Tetraen), Ethylenediaminetetraacetic Acid (EDTA)

An effort was made to determine the effect of other polydentate ligands on the enzyme reactivity. There is appreciable activity when trien and tetraen, which are tetradentate and pentadentate ligands respectively, are introduced into the system as can be seen from Table III. This suggests that there is still activity associated with the Mn^{2+} ion when either one or two open sites are present. However, when six coordination sites on the Mn^{2+} ion are occupied as in the case of EDTA and the $Mn(terpy)_2^{2+}$ complex, essentially all the activity is lost.

The enzyme activity of this reaction with only one open site on the Mn^{2+} ion suggests that a linear intermediate of structure [E-S-M] or [M-E-S] is involved. If a cyclic structure such as $E\overset{M}{\underset{S}{}}$ is proposed,[36] then it would be necessary for the Mn^{2+} ion to attain a coordination number of seven. Seven coordinated Mn^{2+} complexes are plausible since this coordination number has been achieved by Mn^{2+} ion in other complexes.[37]

TABLE III.

EFFECT OF TRIEN, TETRAEN, AND EDTA ON THE RATE OF REACTION

Activity at pH = 9.5 in the presence of 5×10^{-4} M Mn^{2+}				
Polydentate Ligand	—	Trien	Tetraen	EDTA
Concentration	—	5×10^{-4} M	5×10^{-4} M	5×10^{-4} M
% Activity	100%	88.9%	70.4%	4.2%

Activity at pH = 10.5 in the presence of 5×10^{-4} M Mn^{2+}				
Polydentate Ligand	--	Trien	Tetraen	EDTA
Concentration	--	5×10^{-4} M	5×10^{-4} M	5×10^{-4} M
% Activity	100%	84.4%	62.5%	0.03%

However, if the structure of the intermediate is linear, then it would not be necessary for the Mn^{2+} ion to form a seven coordinated complex which is generally of low stability and difficult to achieve.

When the number of donor atoms increases beyond five as in the case of EDTA, nearly all of the enzyme activity is lost. The slight activity detected in the presence of EDTA may be attributed to the fact that at a pH of 9.5 (or 10.5), one site on the metal ion may still be partially available for the enzymatic reaction due to an equilibrium involving five and six coordinated manganous-EDTA complexes:

$$Mn(EDTA)^{2-} + H^{+} \rightleftharpoons Mn(EDTAH)^{-} \qquad (32)$$

This type of equilibrium is possible in the case of EDTA under the pH conditions employed in this study.[38] Since all of the polydentate ligands except terpyridine exhibit the same type of behavior, the higher activity observed at a pH of 9.5 compared to 10.5 may be due to increased protonation of the ligands thus favoring the lower coordinated structure.

F. Metal Ion Effects

There appears to be a number of parameters which influence the catalytic properties of metal ions in enzymatic reactions. Most systems of isocitrate dehydrogenase have been catalyzed using either Mn^{2+} or Mg^{2+}, although it is known that other metals will catalyze the reaction.[5, 9, 39] The relative effectiveness of different metal ions as activators on enzymatic reactions of isocitrate dehydrogenase isolated from different sources are shown in Table IV. These results indicate there are a number of parameters which are responsible for the catalytic property of metal ions.

TABLE IV.

COMPARISON OF METAL ION ACTIVATORS ON REACTIONS INVOLVING ISOCITRATE DEHYDROGENASE FROM DIFFERENT SOURCES

Source	Metal Ions		Reference
	Activator	Non-activator	
Azotobacter vinelandii	$Mn^{2+} > Mg^{2+} > Co^{2+} > Cd^{2+} > Cu^{2+} > Zn^{2+}$	Fe^{3+}, Mo^{6+}, Ni^{2+}, Ca^{2+}, Al^{3+}, Ba^{2+}, Fe^{2+}, Hg^{2+}, and Sr^{2+}.	9
Thiobacillus novellus	$Mn^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > Ni^{2+} > Hg^{2+} > Pb^{2+} > Ca^{2+}$	—	26
Pig heart	$Mn^{2+} > Cd^{2+} > Zn^{2+} > Co^{2+} > Mg^{2+}$	—	39
Salmonella typhimurium	$Mn^{2+} > Mg^{2+} > Co^{2+}$	Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}.	5
Phycomyces blakesleeanus	$Mn^{2+} > Hg^{2+} > Mg^{2+} > Pd^{2+} > Ru^{3+} > Ir^{3+} > Rh^{3+} \sim Cu^{2+} \sim Pt^{2+} \sim Zn^{2+}$.	Ag^{+}, Ag^{3+}, Li^{+}, Ca^{2+}, Rb^{+}, AsO_2^{2-}, and SbO^{+}.	Present Study

The enzyme activity obtained with a limited number of different metal ions is reported in Table V. All metal ions which activated the enzyme appreciably are members of either the first, second, or third transition metal series except Mg^{2+}, whose properties are very closely related to Mn^{2+} in many biological systems. The relative order of effectiveness of the different metal ions as activators in this enzymatic reaction is as follows: $Mn^{2+} > Hg^{2+} > Mg^{2+} > Pd^{2+} > Ru^{3+} > Ir^{3+}$. Metal ions such as Rh^{3+}, Cu^{2+}, Pt^{2+}, and Zn^{2+} showed very little activation while Ag^{+}, Au^{3+}, Li^{+}, Ca^{2+}, AsO_2^{2-}, SbO^{+}, and Rb^{+} showed no effectiveness as an activator.

Interestingly enough the Hg^{2+} ion, which generally inhibits the reactions of enzymes having -SH groups as active sites functioned as the second best activator in this reaction. Inhibition by the mercuric ion results from its affinity for the sulfur atoms of the -SH groups present on isocitrate dehydrogenase which catalyze their reactions. However, the mercuric ion has also been known to act as a catalyst for this type of enzymatic reaction.[26] The activation exhibited by the Hg^{2+} ion may be attributed to the catalysis of some other reaction which might be taking place due to impurities in the enzyme. However, other factors could also be responsible for the observed effect.

G. Inhibitor Effects

The decrease in enzyme activity with different inhibitors in the reaction mixture are shown in Table VI. It can be seen from Table VI that as the concentration of inhibitor increases from 2.5×10^{-4} M to 5.0×10^{-4} M, inhibition increases in each case. Some of the inhibitors studied have an affinity for the -SH group on the enzyme while others inhibit by reaction with $NADP^{+}$, substrate, or metal ion.

TABLE V.

METAL ION EFFECTS ON THE REACTION RATE

Metal Ion	% Activity
Mn^{2+}	100.0%
Hg^{2+}	56.3%
Mg^{2+}	50.0%
Pd^{2+}	28.1%
Ru^{3+}	25.0%
Ir^{3+}	6.3%
Rh^{3+}	Immeasurably Slow
Cu^{2+}	Immeasurably Slow
Pt^{2+}	Immeasurably Slow
Zn^{2+}	Immeasurably Slow
Ag^{+}	No Reaction
Au^{3+}	No Reaction
Li^{+}	No Reaction
Ca^{2+}	No Reaction
Rb^{+}	No Reaction
AsO_2^{-}	No Reaction
SbO^{+}	No Reaction

TABLE VI.

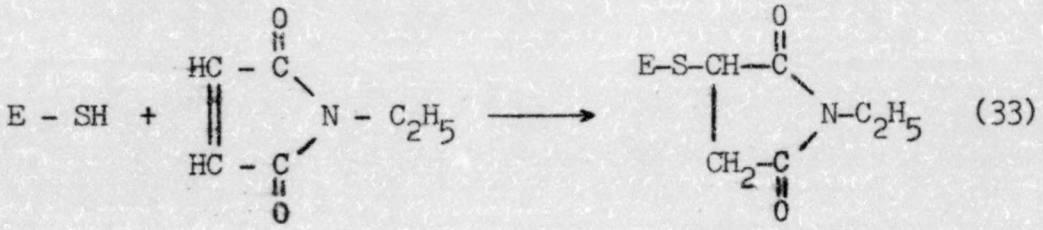
INHIBITOR EFFECTS ON THE REACTION RATE

Inhibitors	Concentration (M)	% Inhibition
3-Indol Glyoxylic acid	2.5×10^{-4} M	5.3%
	5.0×10^{-4} M	21.0%
Semicarbazide-HCl	2.5×10^{-4} M	15.8%
	5.0×10^{-4} M	26.3%
Iodoacetic Acid	2.5×10^{-4} M	29.8%
	5.0×10^{-4} M	31.6%
Quinacrine-Di-HCl	2.5×10^{-4} M	36.9%
	5.0×10^{-4} M	47.4%
p-Hydroxy Mercury Benzoate	2.5×10^{-4} M	29.0%
	5.0×10^{-4} M	42.1%
N-Ethylmaleimide	2.5×10^{-4} M	23.1%
	5.0×10^{-4} M	40.4%

The inhibition of enzymes by N-ethylmaleimide is seldom rapid based on reactions of N-ethylmaleimide with proteins.[40a] The advantages of N-ethylmaleimide as an -SH reagent may be summarized below:

(1) High probable specificity for -SH groups.

(2) Reaction with only certain -SH groups on enzymes making possible specific metabolic inhibition. The general mode of reaction is:



(33)

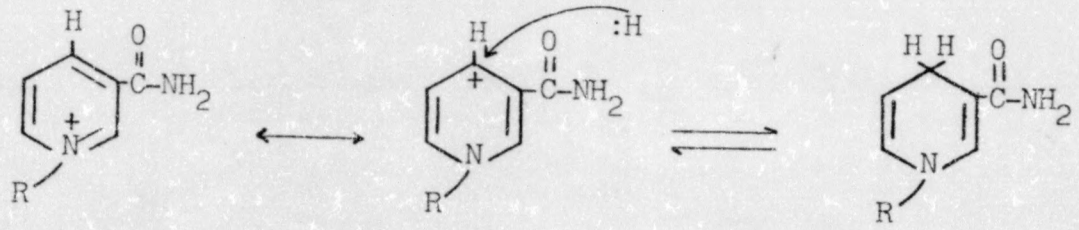
Several NAD^+- and $NADP^+$-dependent dehydrogenases are inhibited by iodoacetate present in reasonably low concentrations.[40b] Inhibition is believed to result from carboxymethylation of -SH groups on the dehydrogenases. However, it is possible that oxidation of NADPH occurs. The probable reaction between iodoacetate and the -SH group of the enzyme may be represented as:

$$E\text{-}SH + I\text{-}CH_2\text{-}COO^- \longrightarrow E\text{-}S\text{-}CH_2COO^- + H^+ + I^- \quad (34)$$

Inhibition by semicarbazide-HCl is believed to result from its interaction with either $NADP^+$ or substrate but not by reaction with the enzyme.[41] Other inhibitors examined in this study are: p-hydroxy mercury benzoate, 3-indol glyoxylic acid and quinacrine-di-HCl.

H. Enzyme Activity in the Presence of 3-Acetyl $NADP^+$

$NADP^+$ acts as an electron acceptor and receives two electrons from the hydride ion which is transferred from the substrate molecule to the pyridine ring of the $NADP^+$ molecule:[14]



(35)

(R represents the remaining part of the $NADP^+$ molecule.)

The structure of 3-acetyl $NADP^+$ is identical to that of $NADP^+$ when the $-NH_2$ group is replaced by a methyl group.

Differences in the enzyme activity (Table VII) when these coenzymes are present support the observation that the addition of hydride ion takes place on the pyridine ring of the coenzyme as found by other workers.[14] The low activity in the case of 3-acetyl $NADP^+$ compared to $NADP^+$ may be attributed to the difference in electron density on the pyridine ring. The electron density on the pyridine ring of $NADP^+$ would be lowered due to the greater electron withdrawal of the $-NH_2$ group compared to the methyl group. Consequently, the electrons of the hydride ion (from the substrate molecule) will be more readily accepted by $NADP^+$ rather than 3-acetyl $NADP^+$. Resonance effects due to a lone pair of electrons on the nitrogen atom may also be operating in the case of $NADP^+$ but this effect is expected to be weak compared to the inductive effect.

I. Reaction Mechanism

It has been postulated by several workers[10, 11] that the reaction under consideration takes place in two steps and both steps are catalyzed by the same enzyme with Mn^{2+} required only for the decarboxylation step:

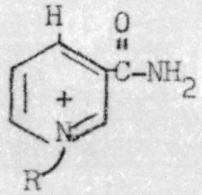
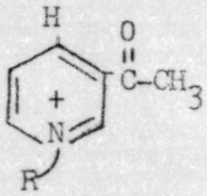
$$\text{D-isocitric acid} + E + NADP^+ \xrightarrow{RDS} [\text{E-oxalosuccinic acid}] + NADPH + H^+ \quad (36)$$

$$[\text{E-oxalosuccinic acid}] \underset{}{\overset{Mn^{2+}}{\rightleftharpoons}} \alpha\text{-ketoglutaric acid} + CO_2 + E \quad (37)$$

TABLE VII.

COMPARISON OF ENZYME ACTIVITY IN THE PRESENCE OF

3-ACETYL $NADP^+$ AND $NADP^+$

Coenzyme	Structure	% Activity
$NADP^+$		100% (λ = 340 nm)
3-Acetyl $NADP^+$		5% (λ = 357 nm)

According to this mechanism, removal of the hydride ion (to form NADPH) and the proton takes place simultaneously. If this is so, then the rate of change of pH due to the proton removal should be equal to the rate of formation of NADPH. Furthermore, the reduction of $NADP^+$ to NADPH should take place in the absence of Mn^{2+}.

The results obtained during this study are not consistent with the above mechanism. A very rapid pH change is observed when the enzyme is added to isocitrate in the absence of $NADP^+$ and buffer. The NADPH formation is much slower and is possible only in the presence of Mn^{2+} as observed on the Cary Model 14 at 340 nm. These facts are consistent with the following proposed five-step mechanism:

$$SH + E \underset{}{\overset{OH^-}{\rightleftharpoons}} ES^- + H_2O;\ K_1 \text{ (fast)} \qquad (38)$$

$$ES^- + N^+ + M \rightleftharpoons [ESNM];\ K_2 \qquad (39)$$

$$[ESNM] \overset{RDS}{\rightleftharpoons} [EOM] + NH \qquad (40)$$
(Intermediate)

$$[EOM] \rightleftharpoons \alpha\text{-K} + E + CO_2 + M \qquad (41)$$
(Intermediate) (enol)

$$\alpha\text{-K} \rightleftharpoons \alpha\text{-K} \qquad (42)$$
(enol) (keto)

where E = Enzyme, $N^+ = NADP^+$, $M = Mn^{2+}$, SH = D-isocitric acid, O = oxalosuccinic acid, and α-K = α-ketoglutaric acid. A structural representation of the above reaction mechanism is presented in the appendix of this thesis.

Attempts to determine the reaction rate by pH measurements of the fourth step failed due to the small amount of CO_2 evolved. Consequently,

little can be said about the reaction rate for step 4 (reaction 41) or for that matter whether it even occurs.

The proposed rate law for the five-step mechanism would be:

$$\text{Rate} = v_o = k[ESNM] \tag{43}$$

which is consistent with the results obtained from the number of binding sites on the enzyme molecule (based on the assumption that one of the two molecules of D-isocitrate binds to the enzyme less effectively and does not form the product). The first order dependence on the enzyme concentration can be easily shown from the rate law in the following manner:

$$\text{Rate} = v_o = k[ESNM] \tag{44}$$

From reaction 39:

$$[ESNM] = K_2(ES^-)(N^+)(M) \tag{45}$$

Substituting the value of (ESNM) in equation 44 the following result is obtained:

$$v_o = kK_2(ES^-)(N^+)(M) \tag{46}$$

From reaction 38:

$$(ES^-) = K_1(E)(SH) \tag{47}$$

Substituting the value of (ES^-) in equation 46 the following expression is obtained:

$$v_o = kK_1K_2(E)(SH)(N^+)(M) \tag{48}$$

or

$$v_o = k'(E)(SH)(N^+)(M) \tag{49}$$

where $k' = kK_1K_2$. This shows the first order dependence on the enzyme concentration.

SUMMARY

$NADP^+$-specific isocitrate dehydrogenase isolated from different sources has different Km values, and this is attributed to either different physical and chemical properties of the enzyme or the experimental conditions. The number of binding sites of the reaction components to the enzyme isolated from Phycomyces blakesleeanus as determined by the Hill equation are two for isocitrate, and one each for $NADP^+$ and Mn^{2+}. The sigmoid nature of the curve obtained from a plot of rate vs isocitrate concentration supports the fact that the enzyme contains more than one molecule of isocitrate and hence has more than one active site.

The value of the slope obtained from a plot of log (rate) vs log (enzyme-volume) is unity and indicates that the reaction is first order with respect to the enzyme.

The rate of this enzymatic reaction is dependent on ionic strength reaching a maximum in the range of 0.07 M - 0.13 M under our experimental conditions.

Kinetic results obtained in the presence of polydentate ligands indicate that not all six sites of a metal ion are required for activation of the enzymatic reaction. There is considerable activity in the presence of tetraen suggesting that only one open site on the metal ion is necessary for metal ion catalysis. Low activity in the presence of EDTA and excess terpyridine indicates that when all sites of Mn^{2+} are coordinated by ligands, the reactivity decreases sharply to zero with the metal ion losing its catalytic effect.

Different metal ions vary in their function as an activator. In this reaction, Mn^{2+} is most active among all the metal ions tested which is consistent with the kinetic results obtained on isocitrate dehydrogenase isolated from other sources (Table IV). The relatively high activity associated with the Hg^{2+} was unexpected. The order of reactivity was as follows: $Mn^{2+} > Hg^{2+} > Mg^{2+} > Pd^{2+} > Ru^{3+} > Ir^{3+}$. The metals: Rh^{3+}, Cu^{2+}, Pt^{2+}, and Zn^{2+} showed very small activity and Ag^{+}, Au^{3+}, Li^{+}, Ca^{2+}, Rb^{+}, AsO_2^{2-}, and SbO^{+} showed no catalytic effect.

Selected reagents which have an affinity for the -SH group believed to be present on isocitrate dehydrogenase act as inhibitors. The order of inhibition at inhibitor concentrations of 5×10^{-4} M is as follows: quinacrine-di-HCl > p-hydroxy mercury benzoate > N-ethylmaleimide > iodoacetic acid > semicarbazide-HCl > 3-indol glyoxylic acid.

The large difference in reactivity between $NADP^{+}$ and 3-acetyl $NADP^{+}$ indicates that the transfer of hydride ion from the substrate to the coenzyme is strongly influenced by changes in the electron density of the pyridine ring. $NADP^{+}$ is found to be much more reactive than 3-acetyl $NADP^{+}$ due to the lower electron density on the pyridine ring which results from the greater electron withdrawal of the $-NH_2$ group compared to the methyl group.

Studies of the reaction mechanism show that the proton and hydride ion transfer take place in different steps and at different rates. The proton removal is rapid followed by the slow hydride ion transfer. Mn^{2+} ion is needed for the formation of NADPH.

RECOMMENDATIONS

Some possible recommendations for future work on the kinetic properties of isocitrate dehydrogenase are as follows:

1. The pure enzyme should be prepared in order to determine the turnover number. The molecular weight of the enzyme should also be obtained.

2. Bismuth, cadmium, lead, and vanadium are reported to have an affinity for the -SH group[42] and their effectiveness as activators should provide additional information about the kinetic properties of the enzyme.

3. Studies of polydentate ligands having oxygen instead of nitrogen donor atoms should be made in order to determine their effects on the availability of the metal ion for reaction.

4. The stability constants of complexes formed by the polydentate ligands and substrate with all metal ions used during this study should be determined.

5. The effects of different cations and anions on the reaction rate should be examined.

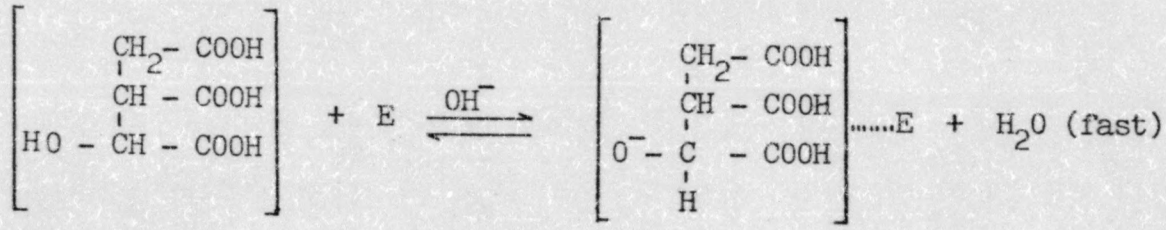
6. Oxalosuccinic acid which is the proposed intermediate in this reaction should be used as the substrate instead of isocitric acid to test the proposed reaction mechanism.

7. Manometric methods of following the reaction by measuring the $CO_2(g)$ evolved may be one technique used to gain more insight on step 4 of our proposed mechanism.

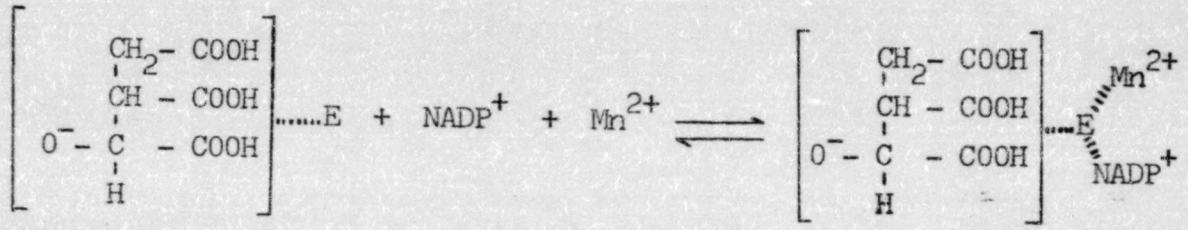
8. Examine the effect of pH on several inhibitors in order to gain more insight as to their mode of reaction.

APPENDIX

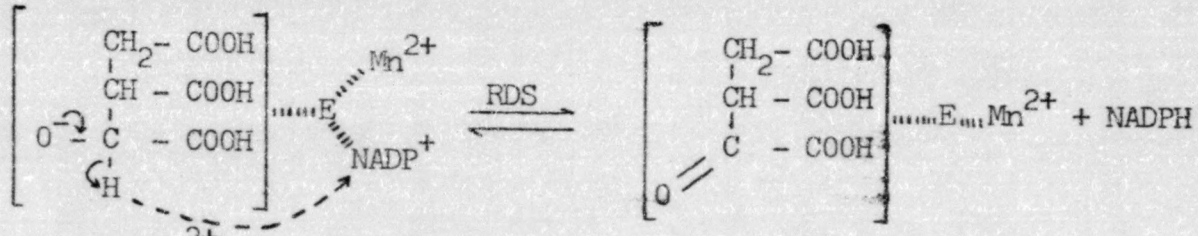
REACTION MECHANISM

1. 

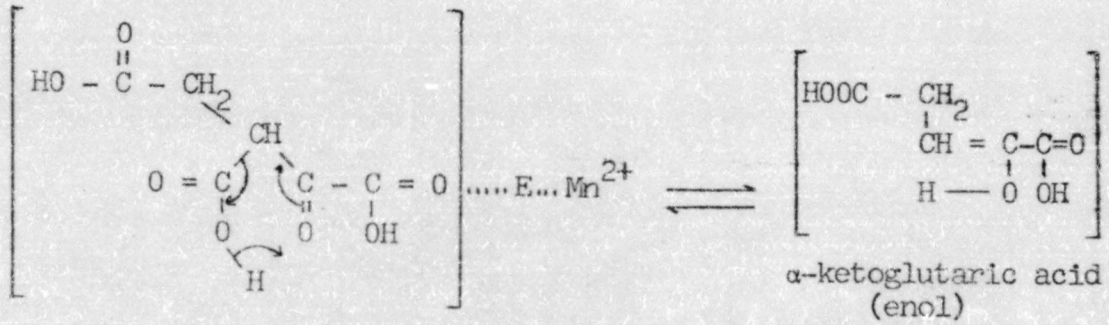
[D-Isocitric Acid] (E-Isocitric Acid)⁻

2. 

(E-Isocitric Acid)⁻ $(ESNM)^{2+}$

3. 

$(ESNM)^{2+}$ (Intermediate)

4. 

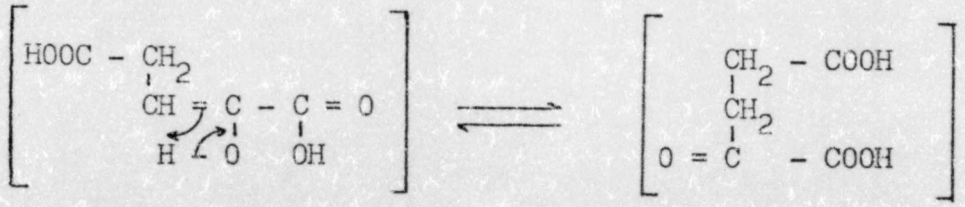
(Intermediate)

α-ketoglutaric acid (enol)

$+ Mn^{2+} + E + CO_2$

APPENDIX

(continued)

5. 

α-ketoglutaric acid (enol)

α-ketoglutaric acid (keto)

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