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Fang Jenny Zhao
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KINETIC AND CHEMICAL MODIFICATION STUDIES ON MALATE DEHYDROGENASE FROM *E. COLI*

(TITLE)

BY

Fang Jenny Zhao

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
Master of Science in Chemistry

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1994
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
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KINETIC AND CHEMICAL MODIFICATION STUDIES

ON MALATE DEHYDROGENASE FROM *E. COLI*

Thesis Approved by

Dr. N. C. Furumo	Date
Dr. M. E. Mcguire	Date
Dr. R. L. Keiter	Date
Dr. D. J. Sheeran	Date

ABSTRACT

Malate dehydrogenase (MDH) is the enzyme which catalyzes the conversion of malate to oxaloacetate in the TCA cycle. The object of this research was to discipher the mechanism of the reaction and the function of the enzyme. MDH from *E. Coli* was purified and analyzed by chemical modification studies using diethylpyrocarbonate. Results indicate the existence of a histidine residue at the active site necessary for catalysis. From kinetic studies, a group required for catalysis with a pK_a of 8.5 was observed, which we believe to be a histidine residue. These studies indicate that MDH from *E. Coli* has a chemical and kinetic mechanism similar to MDH's from other sources.

ACKNOWLEDGEMENT

I wish to express my sincere gratitude and deep appreciation to Dr. Norbert C. Furumo, my research advisor, for his guidance and extra patience during this study.

Also, I would like to thank Dr. Mark E. Mcguire for his constant encouragement and all the other faculty and staff of the Chemistry Department as well as the other members of our research group for their kind help.

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INTRODUCTION

Life is characterized by metabolism, which is defined as the sum of all the chemical reactions that take place in cells. Two general purposes of metabolism are : (1) to capture energy from the environment and convert it to a form suitable for use in cellular work; (2) to transform small organic compounds into macromolecules needed for proper functioning of cells. Metabolic reactions are carried out through several pathways; in each pathway (usually consisting of many reactions), the product of the first reaction becomes the substrate for the second reaction, and so on down the line. Each reaction is catalyzed by an enzyme. The enzyme not only makes the reaction possible, but also can be a point of regulation in the organism's response to the environment.

Among the metabolic pathways the tricarboxylic acid cycle (TCA cycle) is called the "heart" of metabolism, for many different pathways can produce as an end product, acetyl-CoA, the starting point for the TCA cycle. This cycle generates electron carriers needed for synthesis of ATP, and it also provides important intermediates for other

metabolic pathways. Briefly, the TCA cycle transfers the acetate moiety of acetyl-CoA to a carrier molecule that enters into a series of reactions that converts the acetate group to carbon dioxide and electrons in the form of hydride, eventually regenerating the original carrier molecule (see Figure 1 and Figure 2).

Figure 1. The TCA cycle in simplified form (1).

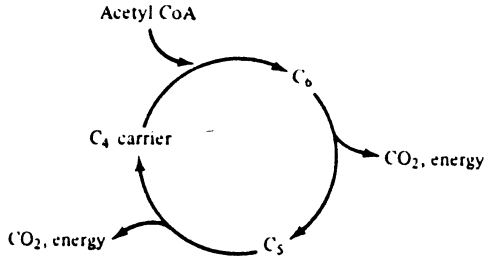
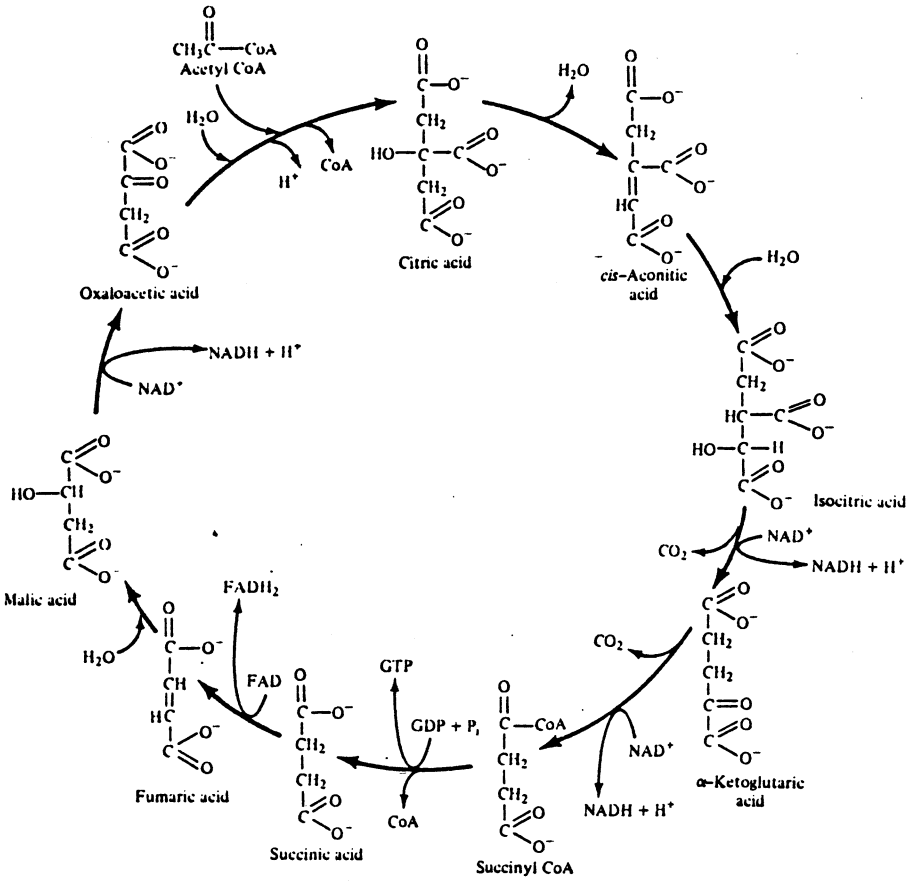


Figure 2. The TCA cycle (1).



The last step is the regeneration of oxaloacetic acid (OAA) from malate, accompanying the conversion of NAD^+ to NADH. This reaction is catalyzed by the enzyme malate dehydrogenase (MDH). This research centers on MDH. We wish to determine how it performs its catalytic function by identifying the amino acid side chains which play crucial roles in the reaction.

Enzymes are specialized proteins that assist and accelerate (catalyze) chemical reactions. Usually, catalysis by an enzyme consists of three basic steps: (1) The reactants, which are also called substrates, bind to the enzyme in a specific arrangement and form an enzyme-substrate complex. (2) Through the catalytic activity of certain groups on the enzyme surface, the substrates are converted into products. (3) The products then dissociate from the enzyme. The study of an enzyme's catalytic mechanism is basically a study of the interactions between the enzyme and its substrates. The substrates need to recognize somewhere along the convoluted surface, an indentation or crevice, whose contours have the precise shape and carry the proper charge to contain and bind the substrate molecules. At this place on the enzyme surface,called the active site, the substrate and the enzyme lock together to form the enzyme-substrate complex. The two are usually held together by weak bonds such as ionic bonds and hydrogen bonds. Enzymatic catalysis of the conversion of substrates to products depends on certain amino acid side chains which are an integral part of the active site. Most enzymes can no longer function if a single amino acid within

the active site is replaced or if other amino acid substitutions change the overall three-dimensional structure of the protein.

The catalytic activity of an enzyme is proportional to the number of active enzyme molecules. Activity is usually determined by monitoring the appearance of a reaction product or the disappearance of a substrate over a period of time. If the product or substrate absorbs light at a specific wavelength, the change in absorbance of the sample solution can be measured by a spectrophotometer. Thus, with the extinction coefficient, the change in concentration of the absorbing species can be determined. Conditions important in an enzyme assay include substrate concentration, cofactor concentration, temperature, pH and ionic strength.

The study of an enzyme usually involves the investigation of its structure and catalytic properties. A fundamental problem is the determination of the mechanism of the enzyme and how to account for their astonishingly high and specific catalytic activity in terms of their chemical structure (2).

Many physical, chemical and biological methods can be

applied to the study of enzymes. Among them, the most classical method is the kinetic study. From the effect of varying certain conditions on the rate of the enzyme catalyzed reaction, inferences may be made about the mechanism of enzyme action. Ideally, such kinetic studies should be compared with chemical and structural studies on the enzyme in order to get a more complete picture of the process. Furthermore, without a knowledge of the kinetics of an enzyme, it is not possible to solve the problem of how an enzyme works in chemical terms or how it functions in the cell. Thus, an initial study of the kinetics of the enzyme is necessary for the design of future experiments and the interpretation of their results. It can be regarded as being an essential foundation to any study of an enzyme (2).

For an enzyme catalyzed reaction, the kinetic order may vary considerably depending on the conditions used, and the rate law may not be obvious enough to evaluate. To avoid these problems, kinetic studies of enzyme catalyzed reaction are generally conducted by measuring initial reaction velocities, because a plot of substrate concentration versus time generally gives a straight line for the first 10% to 20% of the total reaction; their slopes are equal to the

initial reaction rates or velocities (3).

Usually from the kinetic study, a mathematical model is developed to describe the kinetic mechanism. In a multiple substrate reaction, such as the conversion of OAA to malate and NADH to NAD^+, catalyzed by MDH, if all reactants combine with the enzyme prior to reaction, then this is called a sequential mechanism. A sequential mechanism can either be an ordered mechanism, in which reactants combine with the enzyme and dissociate from it in a obligatory order, or it could be random mechanism, in which the order of combination and release is not obligatory. The number of kinetically important reactants in a given direction are designated by the term Uni (one), Bi (two), Ter (three), Quad (four), etc. (3). MDH from many sources follow an ordered Bi-Bi mechanism (15).

Another important tool used to study enzymes is chemical modification of amino acid residues. This is a way to alter the chemical, physical and biological properties of proteins by chemically changing their structure upon treatment with chemical reagents. Since the particular activity of an enzyme lies in its active site, the effect due to chemically changing the groups in the active site can

be used to determine the roles of the individual amino acid side chains in proteins.

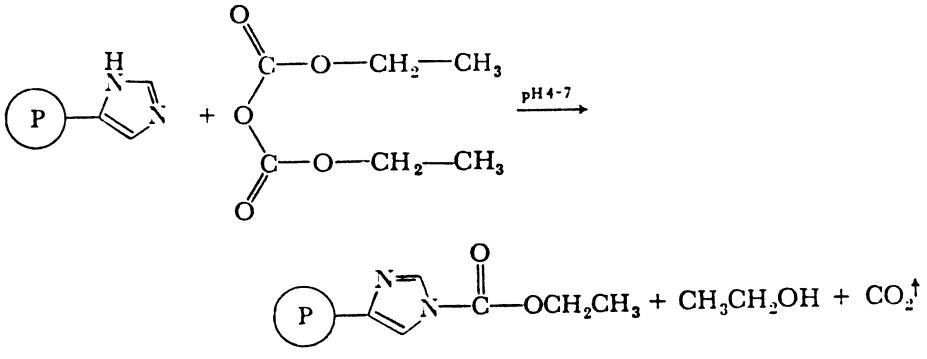
The different chemical properties of the various amino acid side chains provide a basis for their differential modification. When enzymes are treated with certain chemical reagents, changes in enzymatic activity can be attributed to the modification of active site residues. However, this is only one of several possibilities, such as, the changes in activity result indirectly in an accompanying structural change, or by modification of residues happens in allosteric effector sites. If chemical modification has no effect on the properties of interest, it is clearly reasonable to assume that the modified residues are not essential to the activity under investigation.

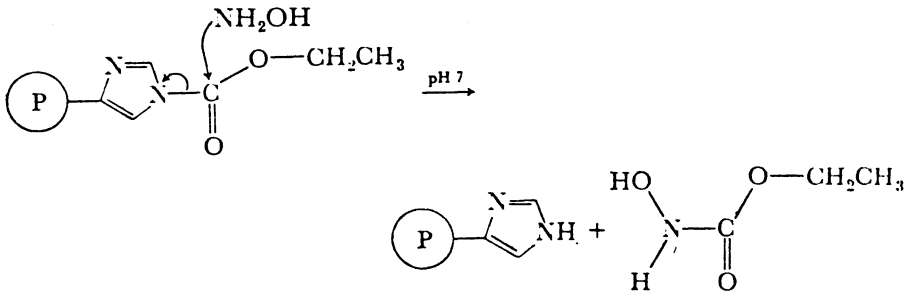
When changes in activity accompany the modification of a single residue, conformational changes are less likely to be responsible. Because of the high reactivity of many active site residues, it has been possible to prepare a relatively large number of monosubstituted enzyme derivatives. The high reactivity of some of these groups appears related to their catalytic function.

Good evidence that changes in properties of a protein

primarily are effects of chemical modification can be obtained by removing the modifying groups and demonstrating a return of the original properties. Protection by substrates or related compounds can be used to selectively prevent the modification of active center residues in many enzymes. If enzyme activity is retained following modification in the presence of substrate, but is lost in its absence, it is usually assumed that a group in the active site has been protected by substrate.

Diethylpyrocarbonate (DEPC), reacts with the imidazole of side chain histidine. It is a restored process in the presence of certain compounds, resulting in the regeneration of the original group. For example, it is rapidly decomposed at pH 7 by hydroxylamine (5).





The reaction of DEPC with imidazole groups of proteins is accompanied by an increase in absorbance at 230 nm (31). This can be used to quantitate the extent of reaction using a molar extinction coefficient of $3.2 * 10^3$ M^{-1} cm^{-1} at 242 nm for N-carbethoxyhistidyl. Its selective reaction with only accessible imidazole groups and the ease with which such reaction can be quantitated make it a convenient probe for determining the accessibility of histidine residues.

In the past 30 years, a lot of research has been done on lactate dehydrogenase (LDH), mitochondrial malate dehydrogenase (mMDH) and cytoplasmic malate dehydrogenase (cMDH). It has been found that all the above three enzymes are homologous; an invariant residue in the catalytic center of all three enzymes include a histidine group.

Crystallographic studies on cMDH revealed that two independent subunits comprising the dimeric enzyme are

nearly identical in structure and are related to each other by roughly 2-fold rotational symmetry (8). The crystal structure of MDH from *Escherichia coli* strain HB 101 (6) indicates that *E. coli* MDH is closely homologous with mMDH; it is dimeric and its subunit-subunit interface occurs through similar surface regions. Histidine 177 is found to be hydrogen bonded, in the active site, to an aspartate to form a classic His•Asp pair (5).

Site-directed mutagenesis studies on LDH indicate that a histidine at the active site has proton-donating and accepting properties and it forms a histidine-aspartate interaction (7). A comparison study of cMDH and LDH by crystallographic least square methods shows that the active site of these two enzymes contain similarly oriented His-Asp pairs linked by a hydrogen bond which may function as a proton relay system during catalysis (8).

Investigations on pig heart mMDH revealed the contribution of two highly conserved residues, Asp-43 and His-46, by construction of mutant enzymes containing Asn-43 (D43N) and Leu-46 (H46L). The H46L mutant enzyme, when compared to the wild type enzyme, exhibits a dramatic shift in the pH profile for catalysis toward an optimum at low pH

values, suggesting that His-46 may be the residue responsible for the pH-dependent dissociation of mMDH dimer. The D43N substitution results in a mutant enzyme that is essentially inactive (9). Another pH study on chicken heart mMDH concluded that a group on the enzyme with a pK_a of 7.5 is involved in the binding of malate to the enzyme-NADH complex (20).

Chemical modification studies have been done on pig heart and bovine mMDH using iodocacetamide. A histidine residue was identified to be the modified group and can be protected against modification by NADH in both cases, suggesting the histidine is at the NADH binding site (10,11,12). The modification was also done using DEPC on the pig heart cMDH and showed complete inhibition of the enzyme (13).

Many studies were done on the mechanism and the binding order of the substrates to both the mMDH and cMDH. The fact that OAA and malate do not protect against modification by iodoacetamide, but the mixture of OAA and NAD^+ does, demonstrates the formation of an abortive ternary complex (10). A vibrational analysis concluded that pig heart LDH and mMDH are not involved in enzyme-coenzyme binary

complexes (19). Most of the kinetic studies on mMDH and cMDH from pig heart under various conditions support the sequential ordered mechanism with a ternary complex (14-17), but a study on beef heart cMDH proposed the mechanism of ordered addition of substrate to the enzyme, with no kinetically active ternary complexes of substrate and enzyme (18). Studies on the chicken liver mMDH suggests an ordered Bi-Bi sequential mechanism (23,25), but studies on the chicken liver cMDH shows that it follows an ordered Bi-Bi ternary mechanism at pH 7.4 and iso Theorell-Chance Bi-Bi mechanism at pH 9.6.

Attempts have been made by various means to determine the overall reaction mechanism of the conversion from OAA to malate or vice versa catalyzed by MDH. From the kinetic studies on pig heart mMDH and cMDH, it seems that the rate determining step is not chemical transformation, but coenzyme dissociation (16,17). The pH dependence studies reveal a reaction mechanism similar to lactate dehydrogenase (20,22). The mechanism assumes that the residue involved is presumably the essential histidine residue. In the forward reaction, i.e., from OAA to malate, enzyme in the protonated form binds OAA, and a hydrogen bond between the proton on

the histidine and the carbonyl oxygen of the substrate, which causes the partially positive carbonyl carbon to accept a hydride ion from the reduced coenzyme (Figure 3A). Similarly, the formation of a hydrogen bond between the unprotonated imidazole ring nitrogen and the hydroxyl group of malate causes the adjacent carbon to become more negative, which will facilitate the leaving of the hydride ion (Figure 3B). The sequence of the reaction is summed up in Figure 4.

Although much research has been done on mMDH and cMDH, relatively little is known about MDH from *E. coli*. This research intends to compare *E. coli* MDH with MDHs from other sources in order to discover the similarities and differences of how this enzyme functions.

Figure 3. Schematic illustration of the configuration of the coenzyme and substrate at the active site of malate dehydrogenase (20).

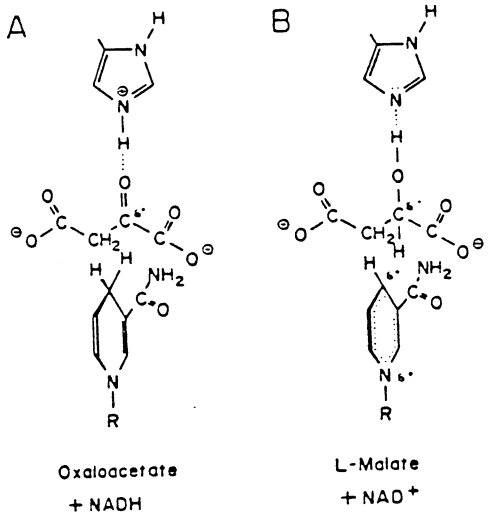
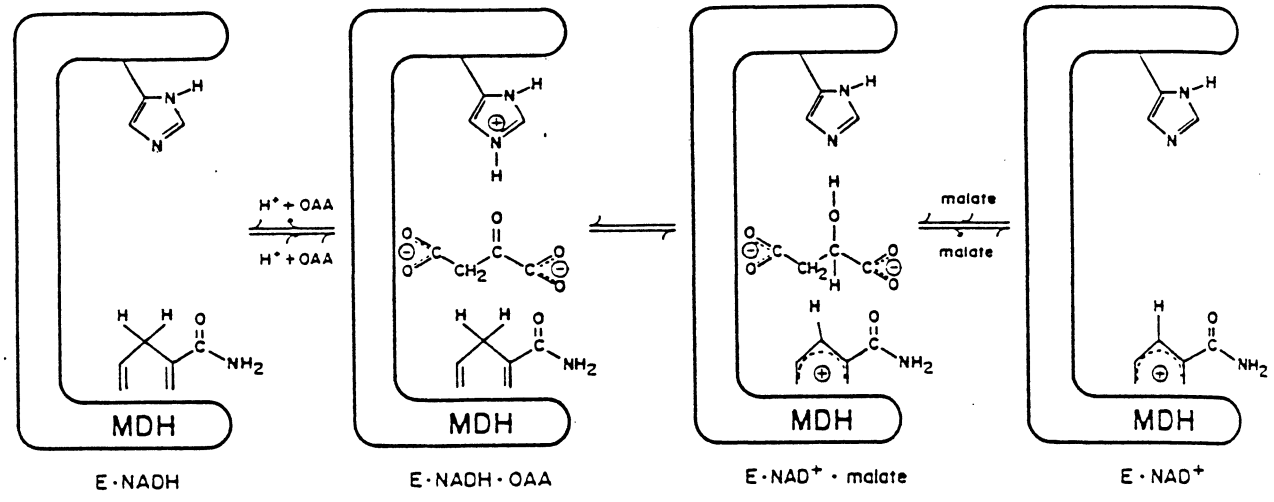


Figure 4. Sequence of events that take place during the reaction catalyzed by malate dehydrogenase (MDH) (20).



EXPERIMENTAL

Materials and Instruments

All substrates, cofactors and buffers were purchased from Sigma Chemical Company unless otherwise stated. NADH was purchased from United States Biochemical Corporation. Solutions of NADH and oxalacetate (OAA) were prepared immediately prior to use, and the concentrations were determined by UV-VIS absorbance at 340 nm for NADH and 257 nm for OAA. The Bradford dye reagent for protein determination was purchased from Bio-Rad. The affinity media, Cibacron Blue 3GA agarose (blue agarose) and diethylaminoethyl (DEAE)-Sepharose anion exchange resin was from Sigma Chemical Company.

Sonication of *E. coli* cell paste was done using a Bronwill Scientific Biosonik III sonicator. Centrifugation was performed in on a Sorvall SS-4 manual Superspeed Centrifuge at 4°C. A non-thermstated LKB Biochrom Ultrospec 4050 was used for most of the routine absorbance measurements during enzyme purification. The final assays and kinetic studies were done on a Shimadzu UV-3100 Recording Spectrophotometer with a thermostated

microprocessor controlled cell holder. Protein electrophoresis was done using a Protean II Multi-Gel from Bio-Rad.

Enzyme Assays

During the enzyme purification process, assays were performed at room temperature with 0.15 mM NADH and 0.1 mM OAA in 50 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer and 2.0 mM disodium ethylenediaminetetraacetate (EDTA), pH 7.0. The decrease in absorbance of NADH at 340 mM was measured.

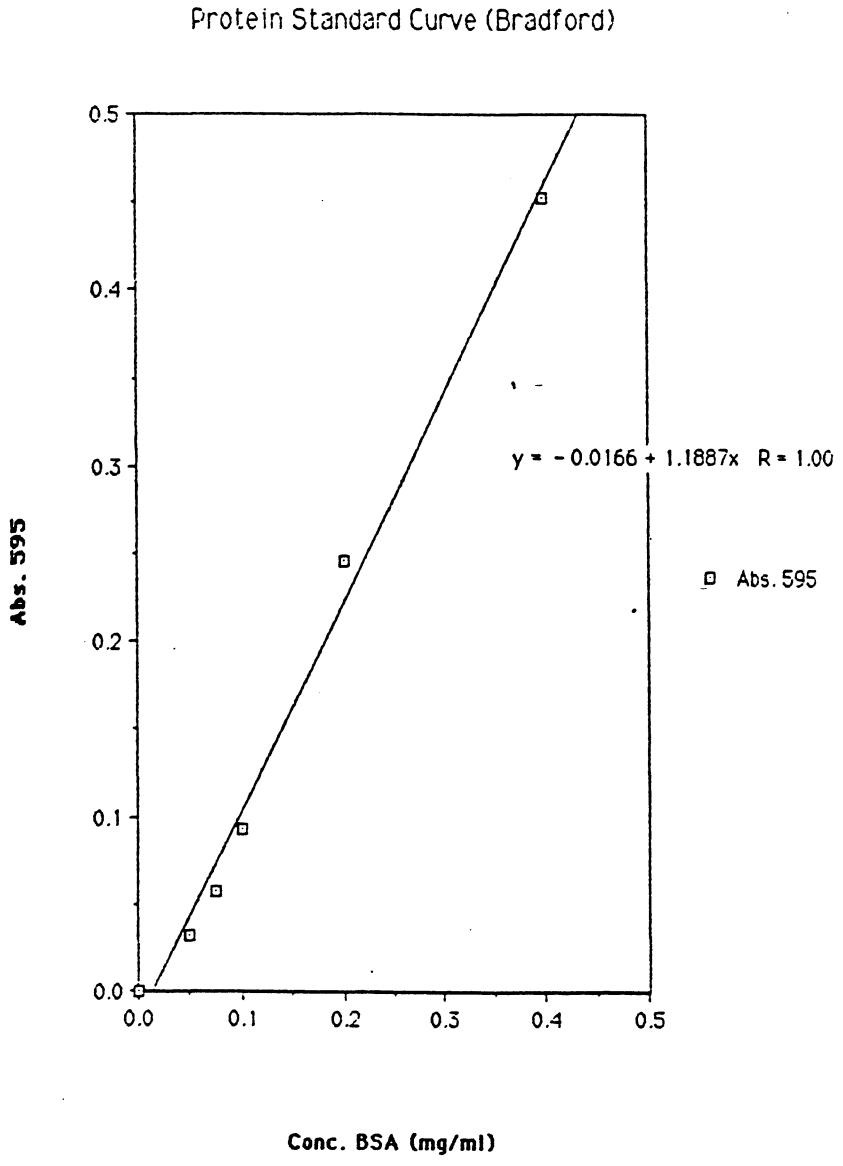
Protein Determination

Protein concentrations were determined by the Bradford method, using bovine serum albumin as standard shown in Figure 5 (28). The concentration of the purified enzyme was also measured by absorbance at 205 nm, and the extinction coefficient was determined by the formula (28):

$$E_{205}^{1\ mg/ml} = 27.0 + 120 * A_{280}/A_{205}$$

The concentration of protein during the purification process was monitored by absorbance at 280 nm.

Figure 5. Protein Standard Curve (Bradford). The blank contains 1.0 ml of 1:5 diluted (1:5 in H_2O) Bradford solution plus 20 ul sample buffer. The sample contains 1.0 ml diluted Bradford solution plus 20 ul sample. The absorbance is measured after 5 minutes at 595 nm and the concentration of the sample is calculated from the curve.



Enzyme Purification

Malate dehydrogenase was purified from *Escherichia coli*, type HB 101, containing the pEM6 plasmid which contains the MDH gene. HB 101 cells containing the plasmid were plated out on an agar plate containing 2*TY Medium (16 g/L trytone, 10 g/L yeast extract and 5 g/L NaCl) (29). Colonies were selected from a refrigerated plate, inoculated into 4 test tubes of 2 ml 2*TY medium, and allowed to grow at 37°C while shaking for 24 hours. Fifty microliter samples of these bacteria were used to inoculate a fresh 100 ml solution of 2*TY containing 1% ampicillin at 37°C while shaking and allowed to grow for another 24 hours. The above contents of these test tubes were added to 4 one liter flasks (1 test tube per flask) of minimal media (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl and 10 mg/mL ampicillin), which was autoclaved and sterile filtered using 0.22 μm 115 mL Nalgene Disposable Filterware. The shaking was done in a shaker room at 37°C for 24 hours. The growth process was monitored spectrophotometrically at 600 nm. The suspension was centrifuged in a GSA rotor and the HB 101 cell paste was collected, weighed, and then stored at -20°C.

About 1.0 gram of HB 101 paste was thawed and homogenized with a magnetic stir bar in 10 ml of 10 mM potassium phosphate/KOH, pH 7.0 (KPi buffer). The homogenate was sonicated on ice at an intensity of 35% of maximum power for 1 minute followed by 1 minute's rest to allow the protein solution to cool. This was repeated for a total of five times. The slurry then was centrifuged at maximum speed in the SS-34 rotor for 20 minutes. The pellet was discarded and the supernatant, which contains solubilized MDH, was assayed for activity and total protein.

The first purification step was performed using dye ligand chromatography on Cibacron Blue 3GA agarose. Fifty milliliters of blue agarose was washed with 1 liter of deionized water and equilibrated with 10 mM KPi buffer. The supernatant was mixed with the equilibrated blue agarose, and was allowed to stand in a beaker for 30 minutes at 4°C to allow MDH to bind to the blue agarose. A column (3.0 cm * 20 cm) was built with the MDH-bound blue agarose, and washed with KPi buffer to remove unbound protein until the absorbance at 280 nm was less than 0.1. The wash solution was then assayed for activity; 10% of total units loaded was in the wash. Elution of the enzyme from the blue agarose was

done by using a 0 to 2 M NaCl salt gradient in KPi buffer. The total volume of the gradient was 250 ml. Five milliliter fractions were collected and assayed for MDH activity and protein concentration. Fractions 12 to 18 were combined and assayed for activity and protein concentration, and 94% of total units were recovered. The enzyme was dialyzed 18 hours (overnight) against KPi buffer, with a buffer change performed every 6 hours.

Ion exchange chromatography was performed on DEAE-Sepharose. Twenty milliliters of DEAE-sepharose was equilibrated with 10 mM KPi buffer. The dialyzed sample was applied to the DEAE in a batchwise fashion and the suspension was allowed to sit for 30 minutes at 4°C to allow MDH to bind to the media. Then a column was built with the enzyme-bound DEAE-Sepharose and was washed with KPi buffer until the absorbance at 280 nm was less than 0.01. The wash was collected and assayed for total units; 8% of the total units loaded was in the wash. A 0 to 0.2 M NaCl salt gradient in KPi buffer, with total volume of 100 mL, was set up to elute the MDH from the DEAE. Fractions 11 to 21 were found to contain most of the enzyme activity, and thus were pooled and assayed. The recovery, based on enzyme activity,

was 76%.

Since the concentration of the MDH at this step is quite dilute (0.224 mg/ml), the MDH had to be concentrated before storage. The enzyme solution was brought to 75% ammonium sulfate to salt out the purified MDH. Using a standard table (27), the amount of solid ammonium sulfate was determined and added to the MDH solution slowly with constant stirring on ice. The solution was allowed to mix for 15 minutes at 4°C or on ice to allow complete precipitation of MDH. The mixture was centrifuged at maximum speed in the SS-34 rotor at 4°C for 30 minutes. The supernatant was assayed and the pellet was dissolved in 1 ml of 50 mM KPi buffer. An assay indicated that 45% of total activity was recovered.

The concentrated enzyme was dialyzed as before and clarified by centrifugation. The enzyme was sterilized using a 0.22 μm sterile filter. A final assay for activity and protein concentration was performed using the Shimadzu UV-3100 at 25°C. The protein concentration was also determined by measuring the absorbance at 205 nm in 5 mM K_2HPO_4, 50 mM Na_2SO_4 buffer, pH 7.0. Finally, a SDS polyacrylamide gel using the Laemmli method was performed to

check the purity of the enzyme (28).

Chemical Modification

Commercially available diethylpyrocarbonate (DEPC) was obtained from Aldrich Chemical Company at a purity of 97%. It was stored in a desiccator at 4°C, and when needed, it was diluted into anhydrous acetonitrile. Since DEPC can be hydrolyzed by water, it should be warmed up to room temperature before opening the desiccator. A stock solution of 1 M is stable for several months at 4°C (30). The concentration of the stock solution can be determined quantitatively by UV absorption. An aliquot of the diluted stock solution is added to 3 mL of 10 mM imidazole at pH 7.5 in a cuvette having a 1-cm light path. The increase in absorbance at 230 nm due to N-carbethoxyimidazole ($Ex = 3.0*10^3 cm^{-1} M^{-1}$) is determined.

The stock MDH solution used in the chemical modification studies had a specific activity of about 2000 u/mg, and the concentration was 64 μM. A buffer of 50 mM KPi and 100 mM KCl pH 6.5 was used in the chemical modification experiment.

Since excess DEPC can cause formation of the

dicarbethoxyhistidyl derivative, different amounts of DEPC are used in order to find the minimum concentration of DEPC necessary to bring about reversible chemical modification. The degree of modification is determined by the decrease in enzyme activity.

A typical detailed procedure for DEPC modification of MDH is as follows. A control reaction mixture is prepared from 223 μL of KPi/KCl buffer mixed with 25 ul of stock MDH solution (64 μM) and 2.5 μL of acetonitrile (instead of DEPC) in a microcentrifuge tube. This was used as a reference solution,and was placed into the constant temperature cell holder. At 5 minute intervals, 10 ul of this reference mixture was removed and diluted 100 fold and then assayed. A total of 12 samples were assayed over a period of one hour. For the modification reaction mixture, 2.5 ul of 1 M DEPC stock solution in acetonitrile was added to 223 ul of KPi/KCl buffer and 25 ul of stock MDH (64 μM) solution in the microcentrifuge tube, and was placed in the constant temperature cell holder as before. An assay of the activity was done immediately (time 0). Assays were performed at 5 minute intervals for the first 15 minutes. Then the interval was changed to 10 minutes since the rate

of chemical modification decreases with time. Data were collected for one hour.

The pseudo-first-order rate constants for inactivation by DEPC, k_{obs}, can be calculated according to

$$\text{Ln } (V_t/V_0) = -k_{obs}t$$

where V_0 and V_t represent the enzyme activity at times 0 and t, respectively.

To know exactly how much MDH was modified, the absorbance at 242 nm was measured. A 750 ul reaction mixture was prepared by mixing 75 ul stock MDH solution, 7.5 ul 300 mM DEPC and 667.5 ul KPi/KCl buffer, this was allowed to stand for one hour at 25°C. The mixture then was washed using KPi/KCl buffer through a Centricon-30 for 15 minutes at 3000 rpm. This was repeated 3 times. A dilution of the retentate was made so that it would have the same MDH concentration as before washing. An assay was done to check the remaining activity. The absorbance at 242 nm was taken to determine the concentration of the modified enzyme.

Demodification

Aliquots of sample mixtures and control mixtures were mixed with hydroxylamine at a final concentration 1 M, pH

7.5. The mixtures were allowed to incubate at 25°C for one hour. Both mixtures were washed with KPi/KCl buffer in a Centricon-30 for 45 minutes to remove unreacted DEPC and hydroxylamine. Both mixtures then were assayed for MDH activity.

Protection Tests

If the inactivation of MDH by DEPC is prevented by the presence of a substrate, it can be concluded that a histidyl residue is a catalytic group located at the active site. Thus, OAA, NADH, Malate or NAD^+ were added individually to the DEPC/MDH solution. The activity of the MDH was monitored as before.

Substrate protection of MDH against modification by DEPC was determined in the following fashion. The control consisted of 20 ul stock MDH solution, 2 ul acetonitrile and 178 ul 5 mM OAA in KPi/KCl buffer. The sample mixture contained 20 ul of stock MDH, 2 ul 300 mM DEPC and 178 ul 5 mM OAA in KPi/KCl buffer. For the control, assays were performed every 10 minutes for a total of one hour; for the sample, assays were performed every 2.5 minutes for the first 15 minutes and then every 10 minutes thereafter.

Protection tests with 5 mM NADH, NAD^+ or malate were performed in the same manner.

Kinetic Studies

2-[N-Morpholino]ethanesulfonic acid (MES) was the buffer used at pH 6 and 6.5; N-[2-Hydroxyethyl]piperazine-N'-[2-ethansulfonic acid] (HEPES) was used at pH 7.0 and 7.5; N-tris[Hydroxymethyl] methyl-3-amino-propanesulfonic acid (TAPS) was used at pH 8.0, 8.5 and 9.0.

Enzyme assays contained 128 uM active sites. Typically a 1000 dilution in KPi/KCl buffer was necessary in order to obtain measurable rates. Diluted enzyme is not very stable; 1000 fold diluted MDH would lose 2% of it original activity in half an hour, 11% in one hour, 13.5% in an hour and a half (these data were obtained at pH 7.0, 25°C). Therefore, a fresh dilution of 1000 fold of the stock enzyme was made every hour during the experiments. All reactions were carried out in quartz cells using the UV-3100 Shimadzu recording spectrophotometer.

Forward Reaction

The rate of the MDH catalyzed reaction, OAA to malate,

was measured by monitoring the change in absorption of NADH at 340 nm at 25°C at pH 6, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Reaction mixtures contained in a total volume of 1 ml: 50 mM buffer, 2.0 mM EDTA, 100 mM KCl and variable concentrations of NADH and OAA. Three stock solutions were made: 1) NADH solution contained 100 mM KCl, 50 mM buffer, 2.0 mM EDTA and 0.3 mM NADH; 2) OAA solution contained 100 mM KCl, 50 mM buffer, 2.0 mM EDTA and 1 mM OAA; 3) the buffer solution contained 100 mM KCl, 50 mM buffer and 2.0 mM EDTA. In this way, by adding different amounts of stock solutions, certain concentrations of substrates can be obtained.

The NADH solution and OAA solution were made fresh every day, and were checked by A_{340} (E_x = 6.22*10^3 M^{-1} cm^{-1}) and A_{257} (E_x = 1.1 mM^{-1} cm^{-1}) for the accurate determination of concentrations of NADH and OAA, respectively. The pH was adjusted by KOH or HCl when necessary. All the stock solutions were kept in an ice bath.

Reaction mixtures were incubated for approximately 3 min at 25°C before addition of the enzyme. The reaction was started by adding 10 μl of the diluted enzyme solution; this amount of enzyme corresponded to 0.045 μg of protein (the concentration of the active sites in the reaction mixture

was 1.28 nM). Immediately after the addition of the enzyme, the reaction mixture in the cuvette was mixed well with a plunger, and the change in absorbance at 340 nm was recorded over a period of time to ensure that the initial rate was being measured (usually in the first 5 seconds of the reaction). The initial rate of the reaction was calculated in terms of micromoles of NADH oxidized per min, using a molar absorption coefficient for NADH of $6.22*10^3$ M^{-1} cm^{-1}. For each NADH and OAA concentrations, reactions were repeated once or twice. The values appearing in the double reciprocal figures (see Results) correspond to mean estimates of the initial rates obtained from 2 or 3 replicate experiments and were used in determining the kinetic parameters.

Estimates of the kinetics parameters and of their standard errors were obtained by fitting the appropriate rate equations to data using an enzyme Kinetics program written in BASIC, developed specially for kinetics studies by R. Viola (version 1.2, 1988). All fits reported here were performed on an IBM PC.

Before the data for an entire experiment were analyzed with the kinetics program, the conformity of individual

lines was checked by a Michaelis-Menton plot and a Lineweaver-Burk plot using the Enzfitter program. Based on these preliminary plots, the proper pattern was selected, and the appropriate equations were used to fit the data. For a sequential mechanism, the following equation was used:

$$v = V[A][B]/(K_S^A K_m^B + K_m^A[B] + K_m^B[A] + [A][B])$$

where v is the initial velocity; V is the maximum velocity; K_m^A is the Michaelis constant for substrate A (NADH); K_m^B is the Michaelis constant for substrate B (OAA); K_s^A is the true dissociation constant for substrate A.

Substrate Inhibition Studies

Substrate inhibition experiments were carried out at a fixed concentration of NADH of 0.15 mM, but variable OAA concentrations (0.05 mM, 0.10 mM, 0.20 mM, 0.30 mM, 0.40 mM and 0.50 mM). The data were fitted to the Substrate Inhibition program in the Kinetics program, using the equation:

$$v = V[OAA]/(K_m' + [OAA] + K_I[OAA]^2)$$

where K_I is the equilibrium constant for the formation of ESS from ES and S, and K_m' is a modified Michaelis-Menten constant.

Another inhibition test was run to decide if the enzyme activity can be fully inhibited by increasing the concentration of OAA. At pH 7.0, OAA concentration was increased from 0.05 mM to 1.1 mM in the reaction mixture, and the corresponding initial velocity was measured and recorded.

Reverse reaction

In the reverse direction, malate to OAA, the experimental procedures were the same as the forward direction except that the reagents used in the reaction mixture were 50 mM TAPS, 100 mM KCl, 2.0 mM EDTA and varied concentrations of malate and NAD^+. MDH (4.5 mM/mg) was diluted 100 fold. The experiment was only carried out at pH 9.0.

RESULTS

Purification of MDH

Results obtained during the major steps of the purification process are shown in Table 1. The final assay was performed at 25°C, and the protein concentration was determined by both the Bradford method and by absorbance at 205 nm. The result of the SDS-PAGE analysis is shown in Figure 6. The absence or the presence of contaminating bands indicates the relative purity of the product. From Figure 7, the molecular weight of the protein can be determined. The six components in the marker lane (lane 1) have molecular weights of 974000, 66200, 45000, 31000, 21500 and 14400 daltons, and each component moved 2.6, 3.28, 5.63, 6.96, 9.14 and 10.24 cm. Since MDH in lanes 2 to 4 moved 6.90 cm, the molecular weight of the MDH monomer is calculated to be 32000 daltons.

Figure 6. SDS-polyacrylamide gel run in the Protean II Multi-Gel cell (29). The left lane is the molecular marker ranging from 974000 daltons to 14400 daltons, and the other 3 lanes are purified enzyme containing 10, 15 and 30 μgs of protein from left to right.

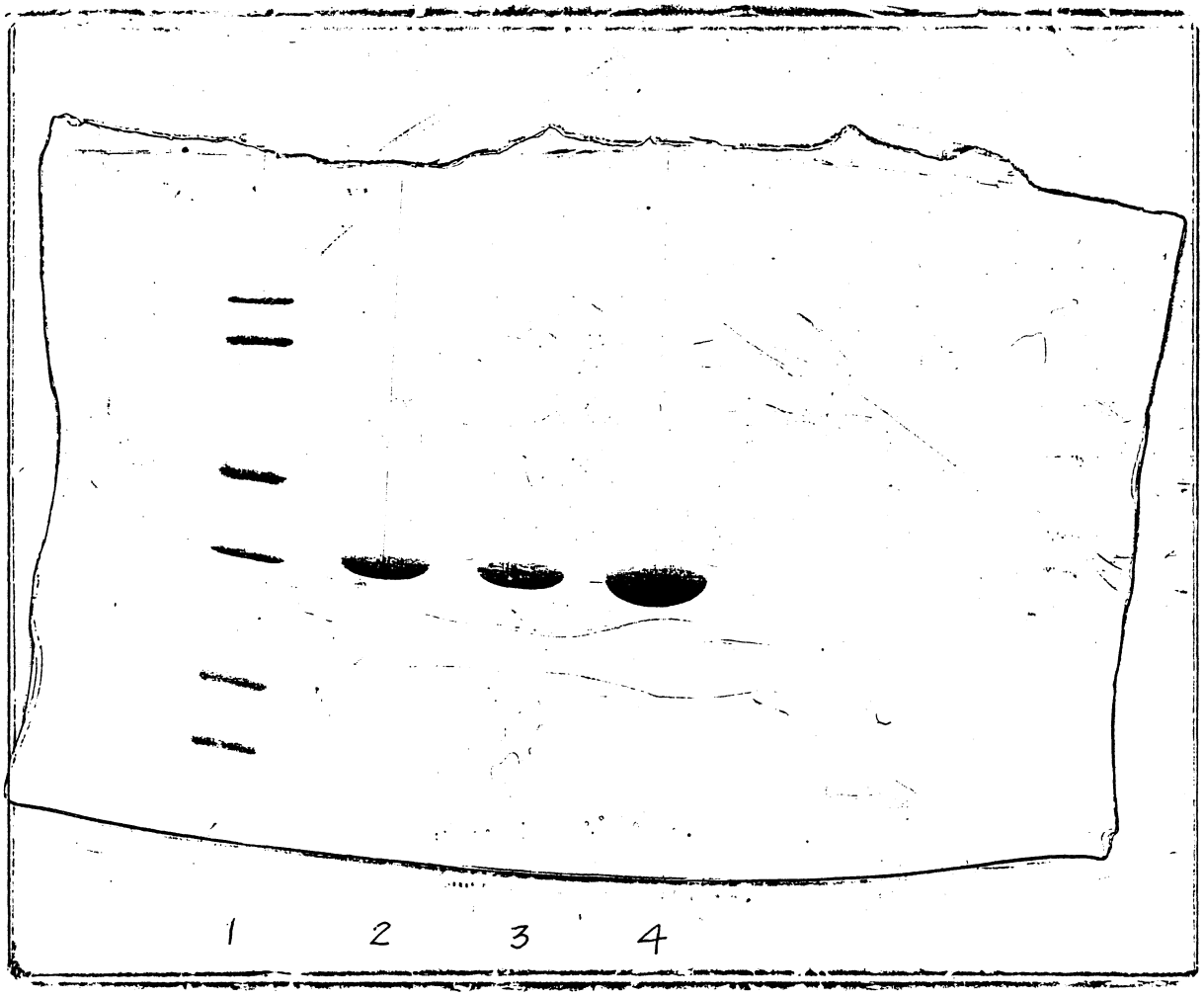
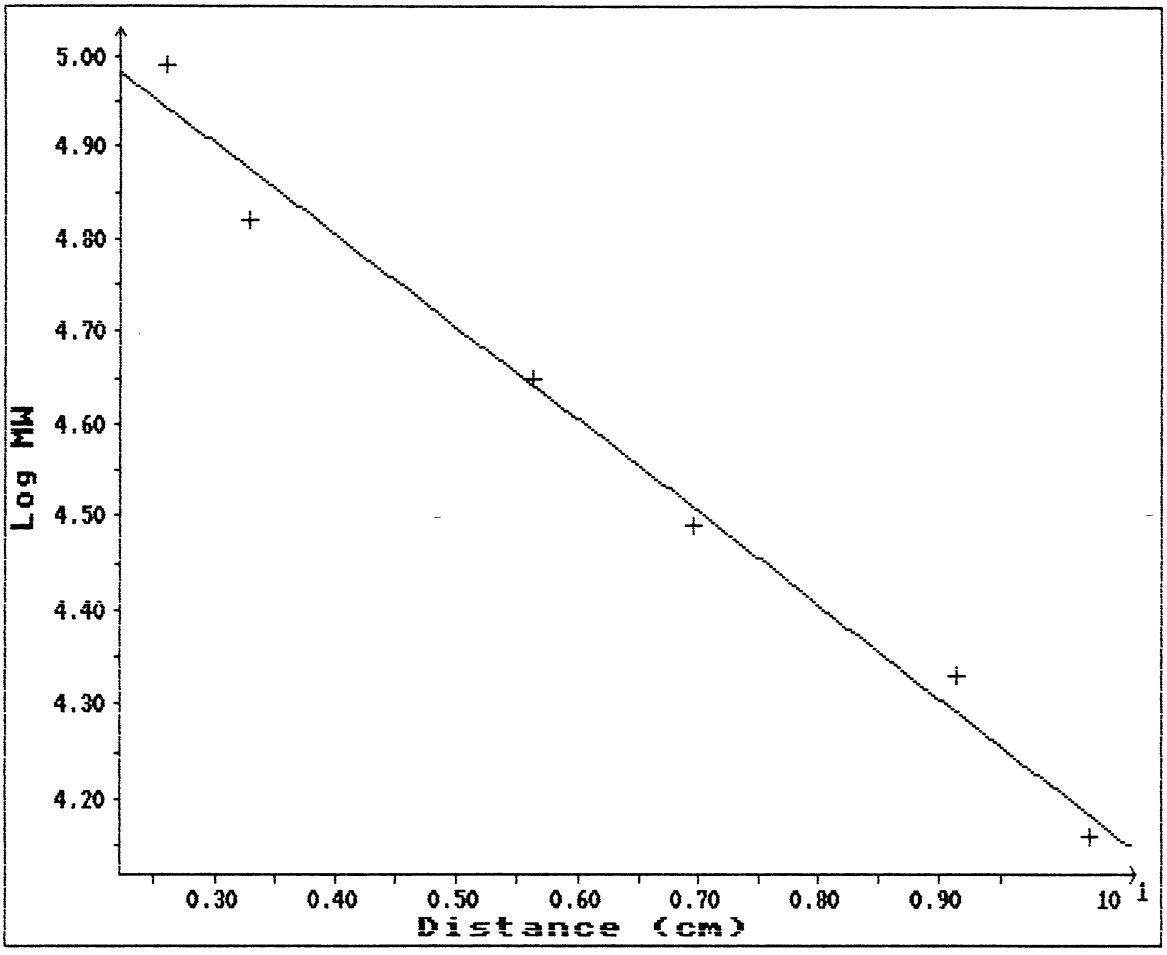


Table 1. Malate dehydrogenase purification procedure from HB 101, containing the pEM6 plasmid encoding MDH. Enzyme activity and protein concentration were determined after each purification step. The final protein determination was done by the Bradford method and by absorbance at 205 nm at 25°C. The units of activity are μmol of NADH reduced per minute.

Fraction	Total protein (mg)	Total volume (ml)	Total units (u)	S.A. (u/mg)	% Recovery of Act. (%)
Cytosol supernatant	66	15	31000	478	100
Elute from Cibacron Blue 3 GA	7.5	35	29000	3910	94
Elute from DEAE	4.7	21	22000	4730	71
Concentrated by $(NH_4)_2SO_4$ (before dialysis)	4.4	1	10000	2970	32
Final assay (at 25°C) (after dialysis)	3.4	1	9000	2750	29
Protein determined by A_{205} at 25°C	4.5	1		2050	

Figure 7. Plot of the logarithm of molecular weights of the molecular weight marker run on a 7.5% SDS-polyacrylamide gel vs. the distanced migrated from the interface of the stacking and separating gels in centimeters. The curve can be used to determine the molecular weight of the purified protein.



Chemical modification

Activity assays were performed in order to determine the effect of DEPC on the rate of the MDH catalyzed reaction. Modification of MDH by DEPC as performed at 10 mM, 1 mM and 0.1 mM DEPC. The data is shown in Table 2, Figure 8 and Figure 9.

The degree of formation of N-carbethoxyhistidyl can also be measured quantitatively by measuring the absorbance at 242 nm. MDH was incubated with 3 mM DEPC for an hour, the activity assay result was recorded at different time intervals and is shown in Table 3 and Figure 10. The absorbance result is shown in Table 4.

Table 2. Inactivation of MDH by DEPC. MDH (concentration of active sites was 12.8 μM) was incubated at 25°C with 0 mM DEPC as a control, 10 mM, 1 mM and 0.1 mM DEPC in 50 mM KPi, 100 mM KCl buffer, pH 6.5. Aliquots were removed and assayed at different time intervals as described in the Experimental Section. The activity results were recorded as a percent activity of the control at each concentration of DEPC.

Time (min)	Ctrl (mABS /min)	10mM DEPC		1mM DEPC		0.1mM DEPC	
		Act.	%Act.	Act.	%Act.	Act.	%Act.
0	652.5			601.6	92.20	558.1	85.53
5	649.7	399.1	61.43	556.7	86.69	663.7	102.1
10	629.0	98.6	15.7	513.1	81.57	629.6	100.0
15	655.7	65.9	10.1	340.1	51.87	608.1	92.89
20	614.9	3.7	0.60	451.2	73.38	609.3	99.09
25	626.3	47.0	7.50	398.2	63.58		
30	586.0	18.9	3.23	332.0	56.66		
35	584.5	12.3	2.10				
40	602.5	13.1	2.17	443.1	73.54		
45	543.7	27.2	5.00	368.8	67.83		
50	581.8	30.1	5.17	382.8	65.80		
55	543.0	16.1	2.97				
60	626.7	4.0	0.64				
65	659.9	9.3	1.4				
72						547.2	

Figure 8. Effect of various concentrations of DEPC on MDH activity as a function of time. The experimental conditions are the same as stated in the legend of Table 2.

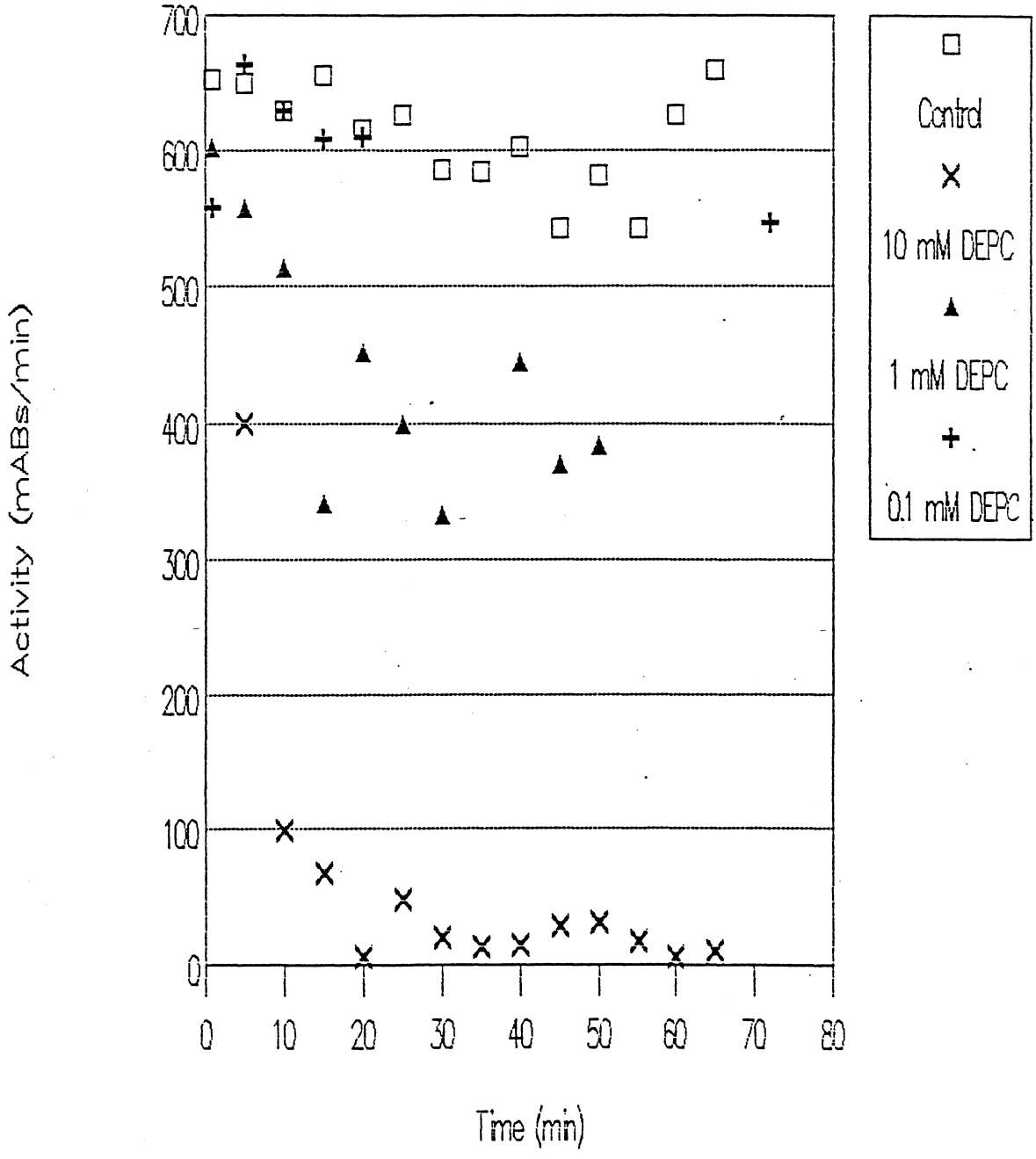


Figure 9. Effect of various concentrations of DEPC on MDH activity as a function of time. The percentage activity (sample/control) is displayed. The experimental conditions are the same as stated in the legend of Table 2.

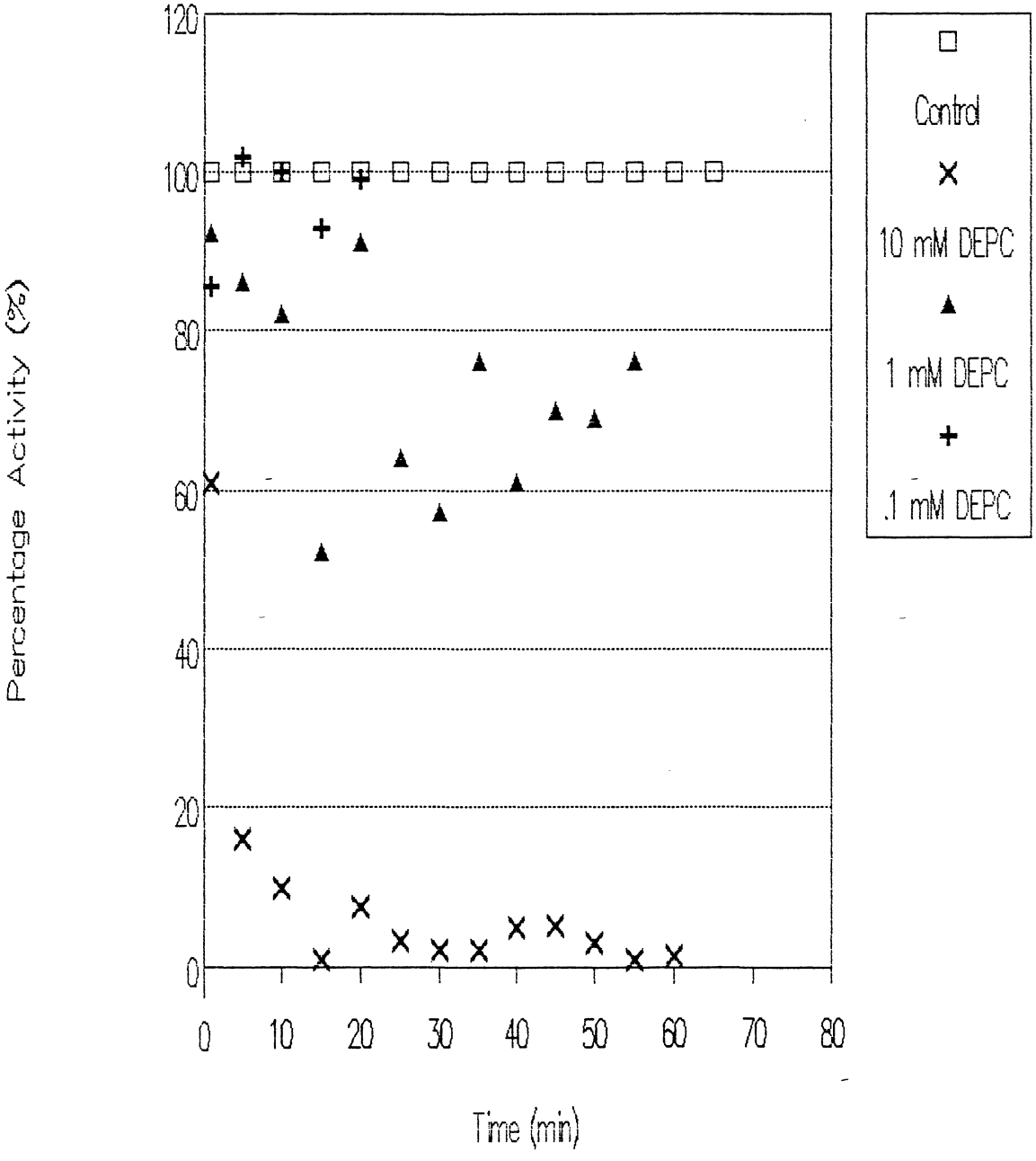


Table 3. Inactivation of MDH by 3 mM DEPC. The experimental conditions are as same as stated in the legend of Table 2.

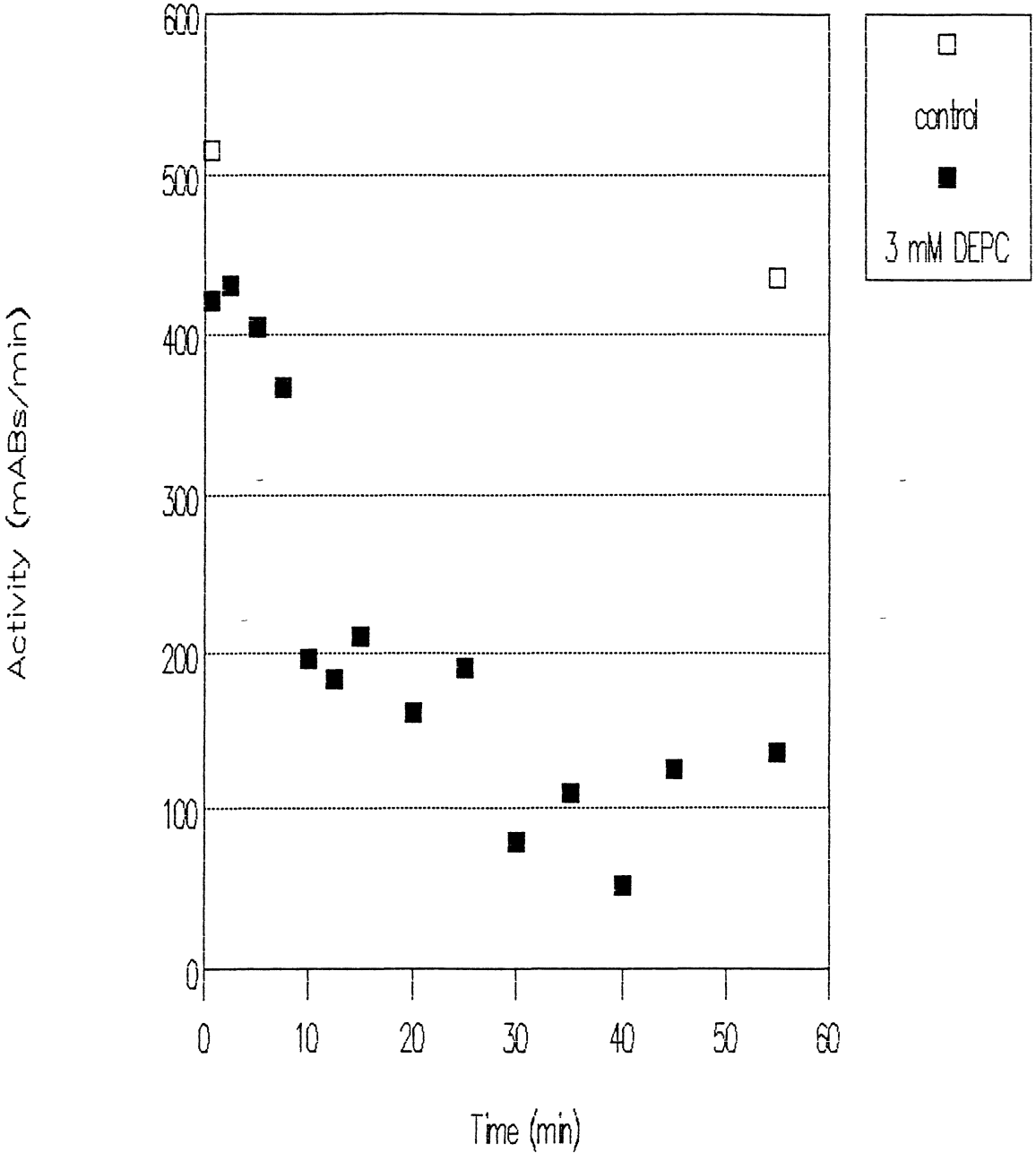
Time (min)	**Control (mABs/min)**	**3 mM DEPC (mABs/min)**	**Percentage** Activity (%)
0	515.8	421.4	81.70
2.5		430.9	
5		405.0	
7.5		367.1	
10		196.9	
12.5		184.0	
15		211.3	
20		161.9	
25		190.2	
30		78.8	
35		109.6	
40		52.4	
45		125.0	
55	436.3	136.1	31.19

Table 4. Absorbance measurement for the concentration of N-carbethoxyhistidyl. The experimental conditions are as stated in the legend of Table 2.

After 60 min incubation with 3 mM DEPC	Activity = 112.5 (mABs/min)
After removal of unreacted DEPC	Activity = 86.4 (mABs/min)
Absorbance at 242 nm	0.280*
[N-carbethoxyhistidyl] (Extinction coefficient = 3200 cm^{-1} M^{-1})	0.0875 mM
[MDH] in the above mixture	0.0064 mM
[histidyl] in the above mixture	0.0256 mM

*Note: There was no enzyme in the blank when the absorbance was measured.

Figure 10. Inactivation of MDH by 3 mM DEPC. The activity was monitored at different time intervals shown in Table 3. The experimental conditions are the same as stated in the legend of Table 2.



Demodification of MDH

Experiments were carried out in order to determine the extent by which MDH activity can be restored by treatment with hydroxylamine. The enzyme, which had been modified by DEPC, was assayed after one hour's incubation in the presence of hydroxylamine. Both the control and the sample were mixed with hydroxylamine at a final concentration of 1 M. After washing the mixture in a Centricon-30 to remove the unreacted hydroxylamine and DEPC, both the control and the sample were assayed for activity and protein concentration. The results are shown in Table 5.

Table 5. Reactivation of MDH. After reaction for one hour with 1 M hydroxylamine, control and sample (MDH which had been treated with 3 mM DEPC followed by treatment with hydroxylamine) were tested for activity. Protein concentration was determined by Bradford assay. The experimental conditions are as stated in the legend of Table 2.

	Control	Sample	Sample/control
Activity (unit/ml)	348.1	230.9	66.33%
[protein] (mg/ml)	0.127	0.134	100%
	0.109	0.105	100%
Specific Activity (unit/mg)	2740	1720	62.8%
	3190	2200	69.0%

Protection against Modification by DEPC

Oxalacetate (OAA), NADH, malate and NAD^+ were individually tested as protecting agents against modification by DEPC. The results of activity tests are shown in Tables 6A, 6B and Figures 11 to 14.

Table 6A. Protection of MDH by substrates against modification by DEPC. Three sets of data are shown: at different time intervals, the assay results of MDH with 3 mM DEPC, MDH only with each protecting agent as control and MDH with both 3 mM DEPC and a protecting agent as sample. The experimental conditions are as stated in the legend of Table 2.

Time (min)	MDH Act. (mABs /min)	5 mM OAA		5 mM NADH	
		Control (mAbs/min)	Sample (mABs/min)	Control (mAbs/min)	Sample (mABs/min)
0	421.4	504.6	373.0	541.2	612.2
2.5	431.9		448.9		533.5
5	405.0	471.8	433.3		554.6
7.5	367.1		440.7		547.3
10	196.9	423.0	365.0	648.7	563.1
12.5	184.0		510.3		552.0
15	211.3	384.4	351.1		445.1
20	161.9	496.8	339.3	453.2	518.5
25	190.2		298.0		465.5
30	78.8	420.3		605.3	444.6
35	109.6		288.7		
40	52.4	489.7		583.5	461.3
45	125.0		275.5		
50		503.1		479.0	472.9
55	136.1		286.0		
60		501.0		591.5	

Table 6B.

Time (min)	MDH Act. (mABs/ min)	15 mM Malate		5 mM NAD^+	
		Control (mAbs/min)	Sample (mAbs/min)	Control (mABs/min)	Sample (mABs/min)
0	421.4	364.4	373.6	522.4	
2.5	430.9		439.7		607.6
5	405.0		467.7		410.0
7.5	367.1		484.8		444.4
10	196.9	351.9	407.4		453.3
12.5	184.0		345.1		366.0
15	211.3		357.6		342.7
17.5			234.4		
20	161.9	347.6	247.8	490.6	442.0
25	190.2				425.6
30	78.8	371.7	218.5	538.5	371.8
35	109.6				
40	52.4	326.9	217.1	574.7	232.5
45	125.0				
50		363.6	232.0		
55	136.1				303.8
60		334.7	200.4		

Figure 11. Protection of MDH modification by 5 mM OAA. Three activity tests were performed: MDH with 3 mM DEPC (empty square), MDH only with 5 mM OAA as control (plus) and MDH with both 3 mM DEPC and 5 mM OAA as sample (filled triangle). The experimental conditions are the same as stated in the legend of Table 2.

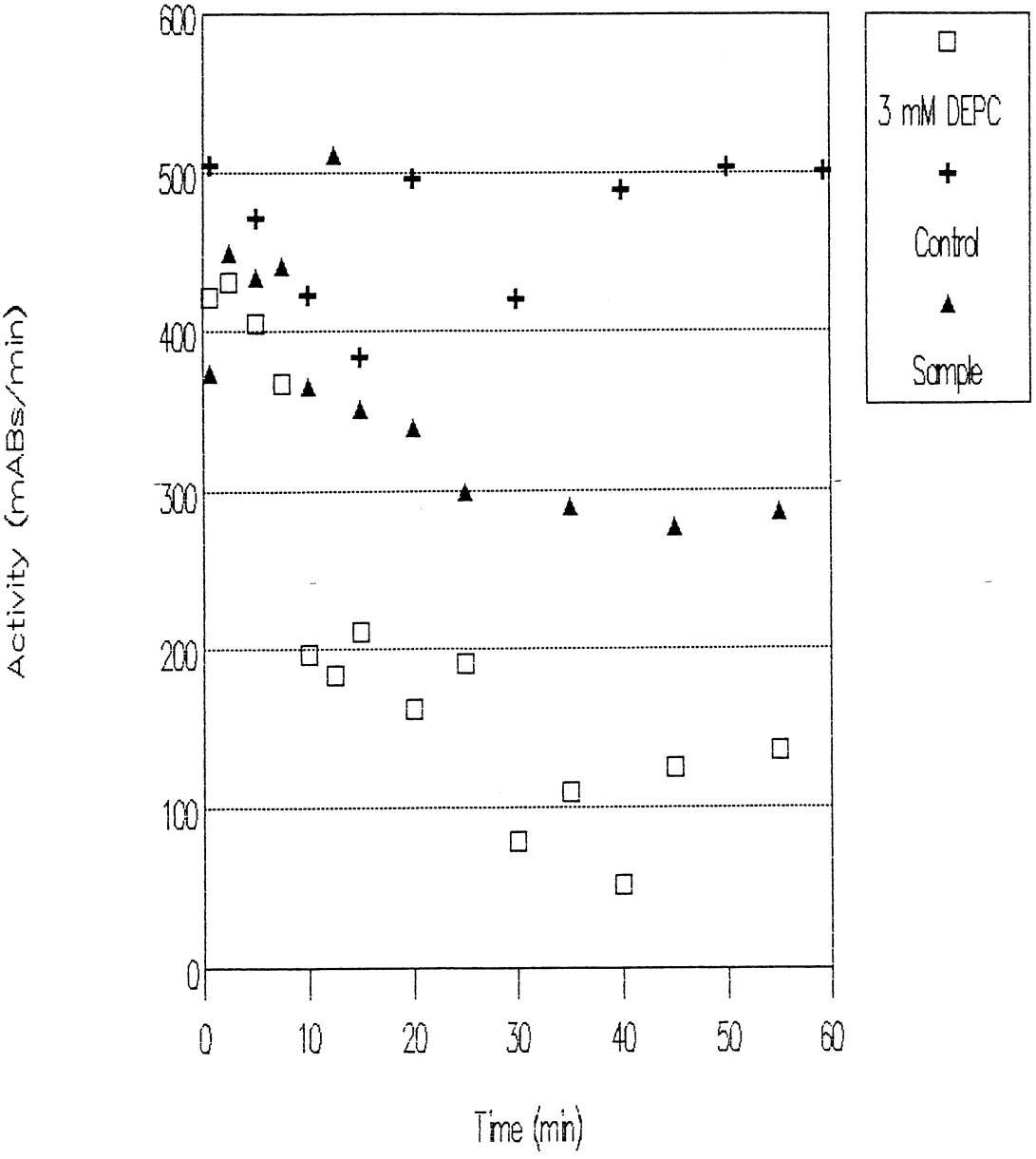


Figure 12. Protection of MDH modification by 5 mM NADH. Three activity tests were performed: MDH with 3 mM DEPC (empty square), MDH only with 5 mM NADH (plus) and MDH with both 3 mM DEPC and 5 mM NADH (filled triangle). The experimental conditions are the same as stated in the legend of Table 2.

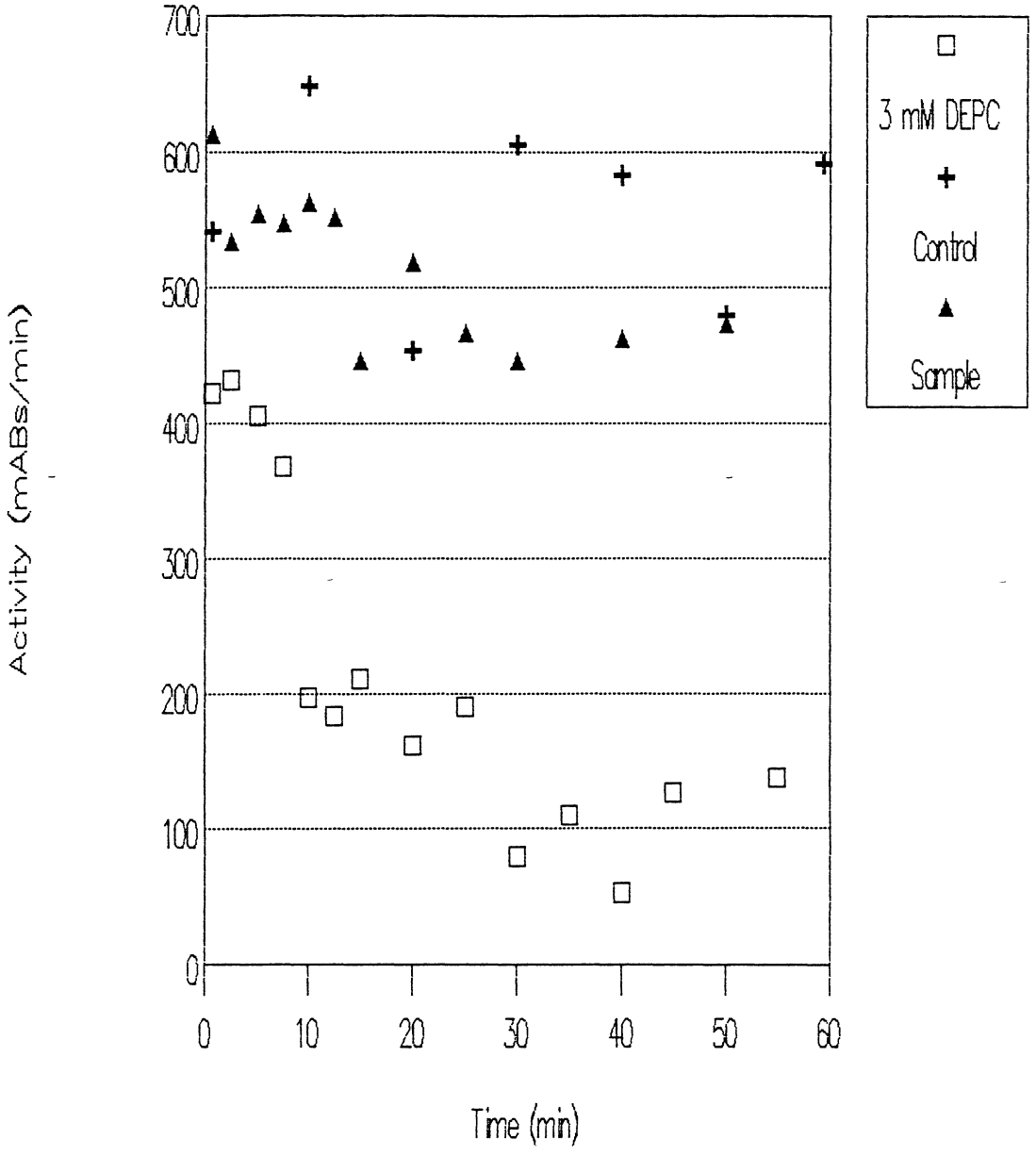


Figure 13. Protection of MDH modification by 15 mM malate. Three activity tests were performed: MDH with 3 mM DEPC (empty square), MDH only with 15 mM malate as control (plus) and MDH with both 3 Mm DEPC and 15 mM malate as sample (filled triangle). The experimental conditions are the same as stated in the legend of Table 2.

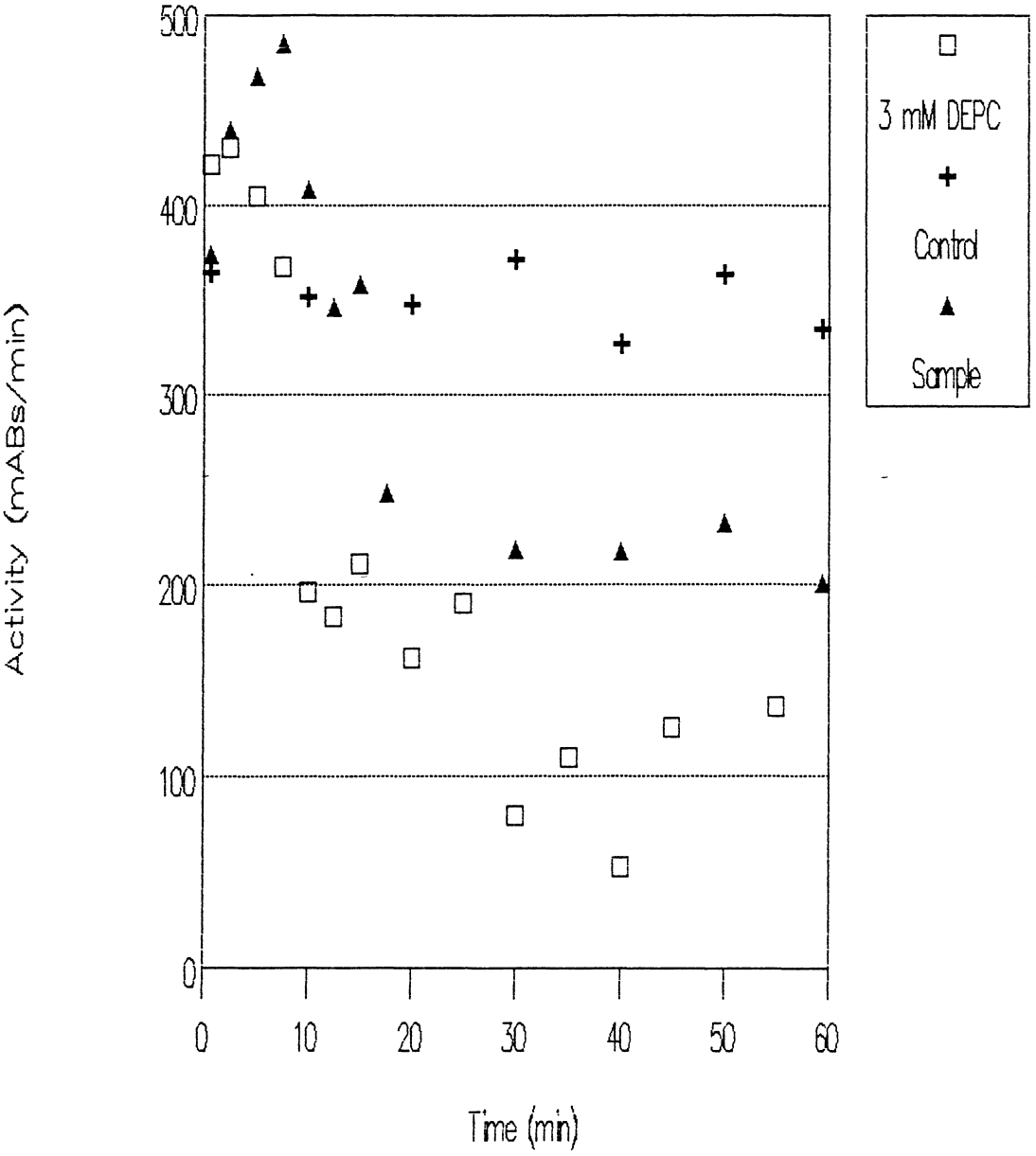
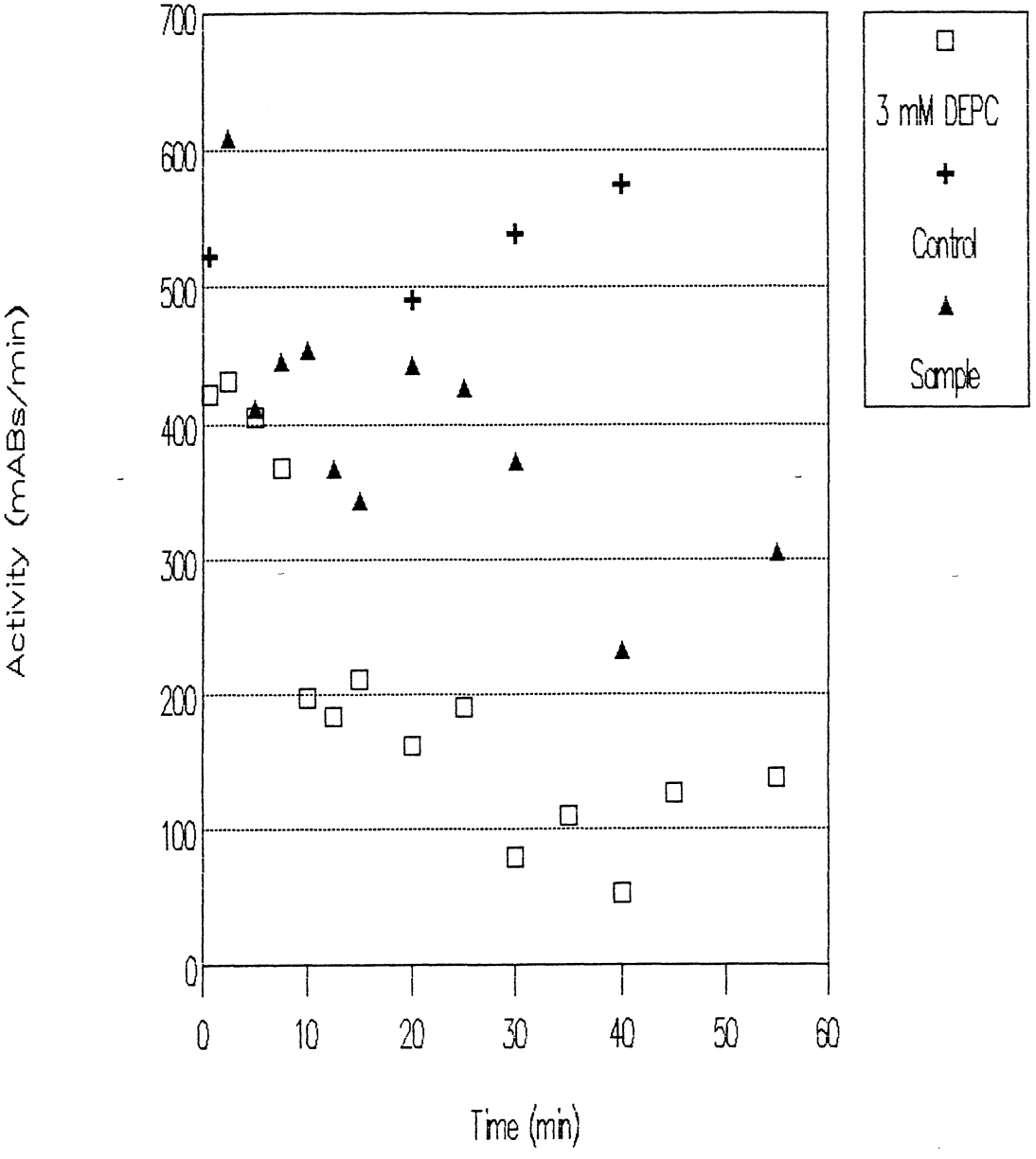


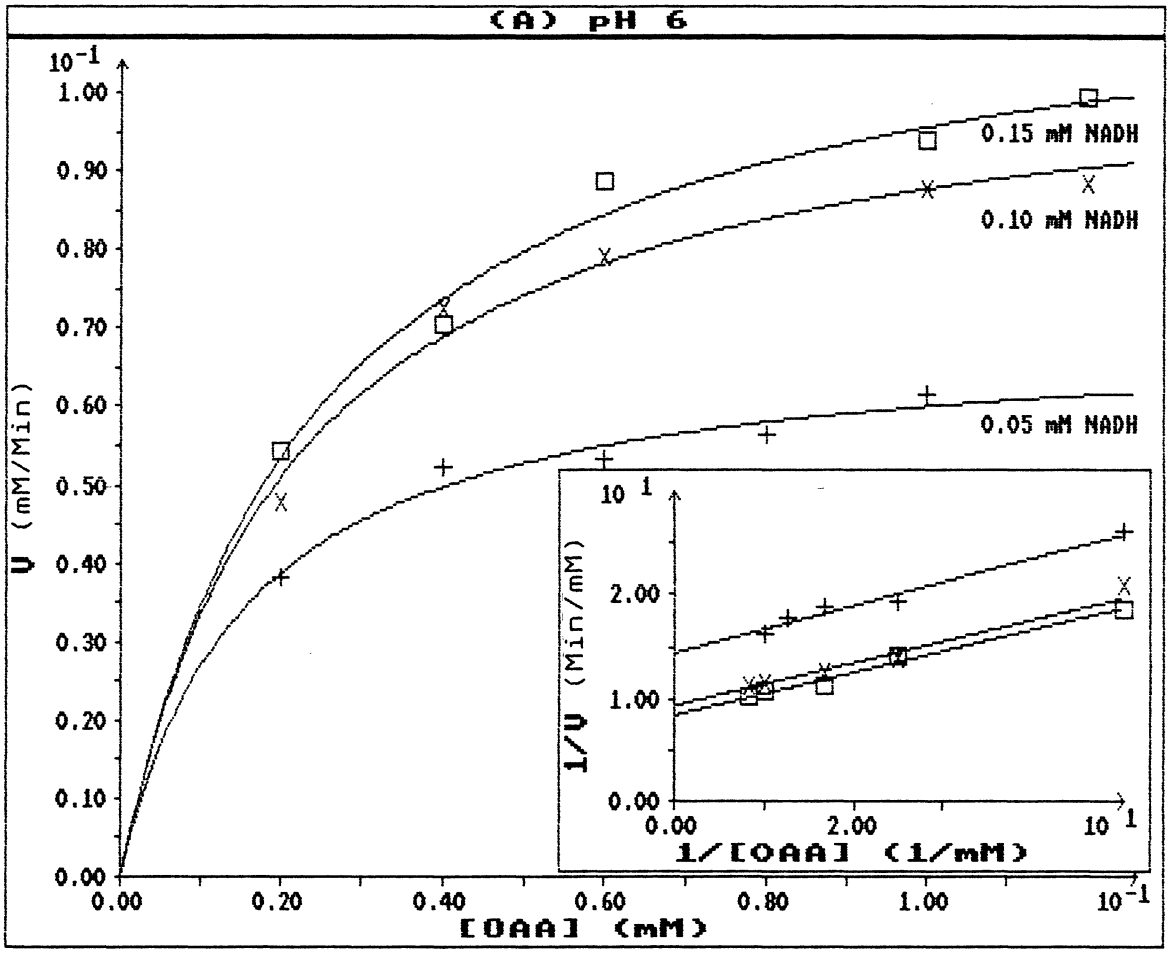
Figure 14. Protection of MDH modification by 5 mM NAD^+. Three activity tests were performed: MDH with 3 mM DEPC (empty square), MDH only with 5 mM NAD^+ as control (plus) and MDH with both 3 mM DEPC and 5 mM NAD^+ (filled triangle) as sample. The experimental conditions are the same as stated in the legend of Table 2.

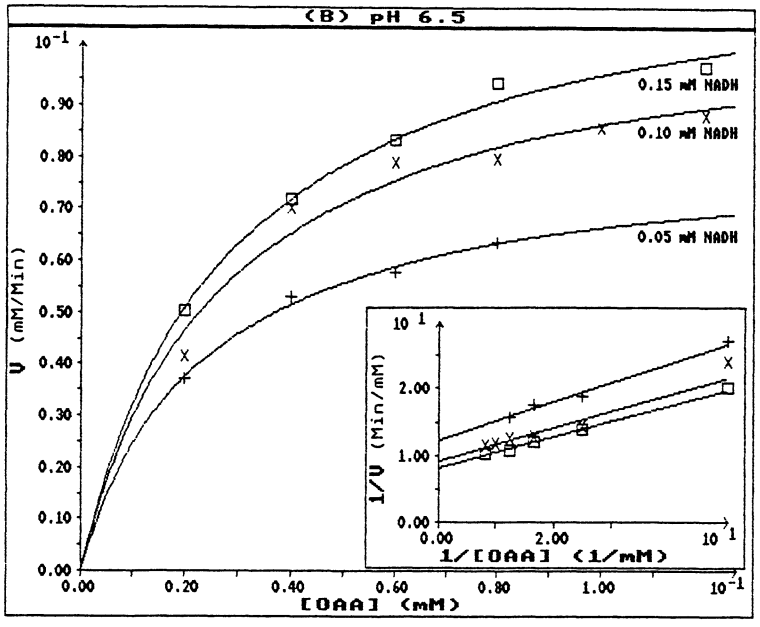


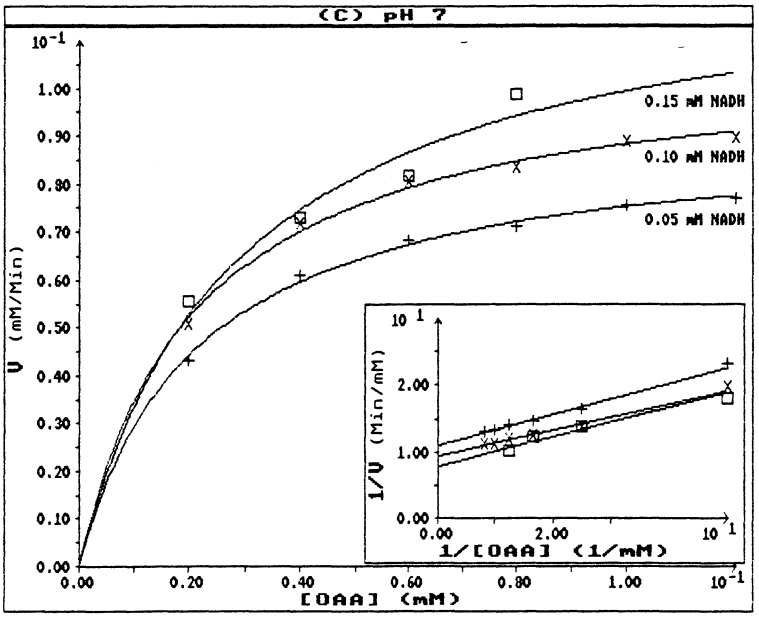
Kinetic Studies

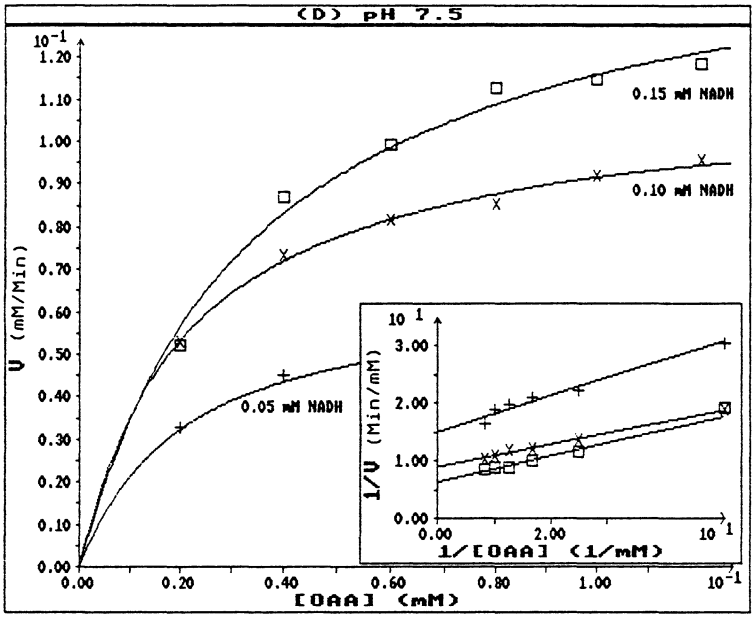
Initial velocity studies were performed as a function of OAA and NADH concentrations at various pH values. All the raw data are listed in the appendix. At each pH value, the initial velocity plot versus OAA concentration and double-reciprocal plots of 1/v versus 1/[OAA] at a series of concentrations of NADH are shown in Figures 15A through 15G. The figures were generated by the non-linear regression data analysis program Enzfitter by Robin J. Leather-Barrow. At low concentrations of OAA and NADH, when inhibition by OAA didn't occur, the family of straight lines intersected to the left of the 1/v axis. Using the sequential mechanism equation, kinetic parameters were obtained as well as standard errors which are included in Table 7. The pH-dependent profiles of Log(Vm/Km) are plotted in Figure 16. The data were fitted to a pH-profile kinetics program in which a pK_a value of 8.5 was obtained(the data are included in the appendix).

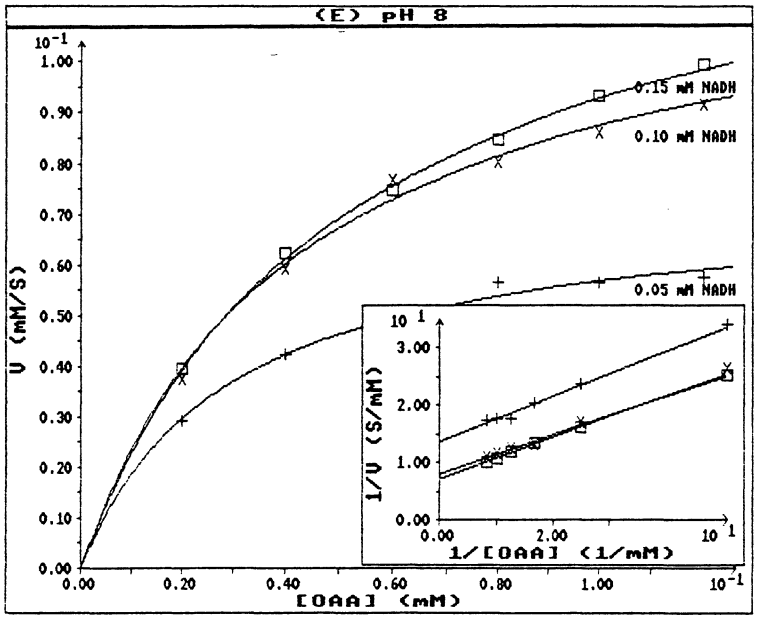
Figure 15. Effect of [OAA] and [NADH] on the initial velocity. The data were fitted into Michaelis-Menten equation, and the Lineweaver-Burk double reciprocal plots were also shown at different pH values. The initial velocity is expressed as mM of NADH oxidized per min. (A) pH 6, (B) pH 6.5, (C) pH 7, (D) pH 7.5, (E) pH 8, (F) pH 8.5 and (G) pH 9.

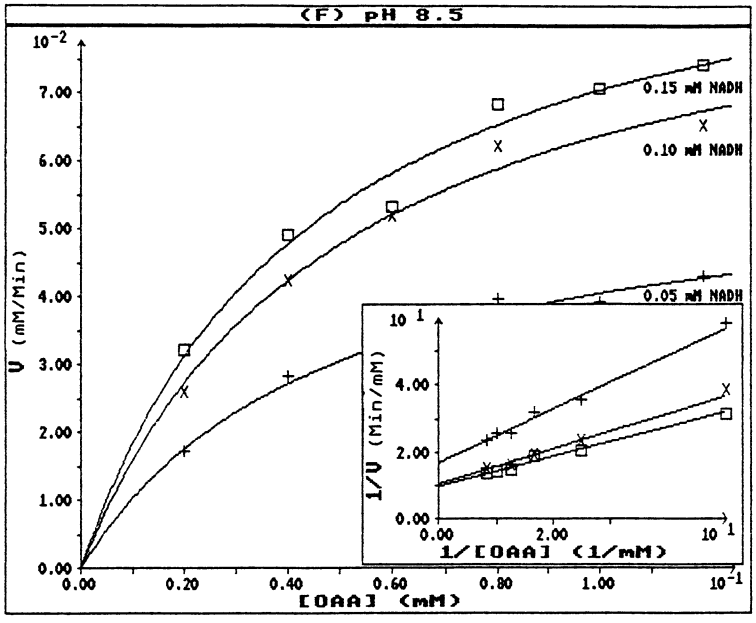



(B) pH 6.5
V (mM/Min)
[OAA] (mM)
0.15 mM NADH
0.10 mM NADH
0.05 mM NADH
1/V (Min/mM)
1/[OAA] (1/mM)


(C) pH 7
V (mM/Min)
[OAA] (mM)
0.15 mM NADH
0.10 mM NADH
0.05 mM NADH
1/V (Min/mM)
1/[OAA] (1/mM)


(D) pH 7.5
V (mM/Min)
[OAA] (mM)
0.15 mM NADH
0.10 mM NADH
0.05 mM NADH
1/V (Min/mM)
1/[OAA] (1/mM)


(E) pH 8
V (mM/S)
[OAA] (mM)
0.15 mM NADH
0.10 mM NADH
0.05 mM NADH
1/V (S/mM)
1/[OAA] (1/mM)


(F) pH 8.5
0.15 mM NADH
0.10 mM NADH
0.05 mM NADH
V (mM/Min)
[OAA] (mM)
1/V (Min/mM)
1/[OAA] (1/mM)

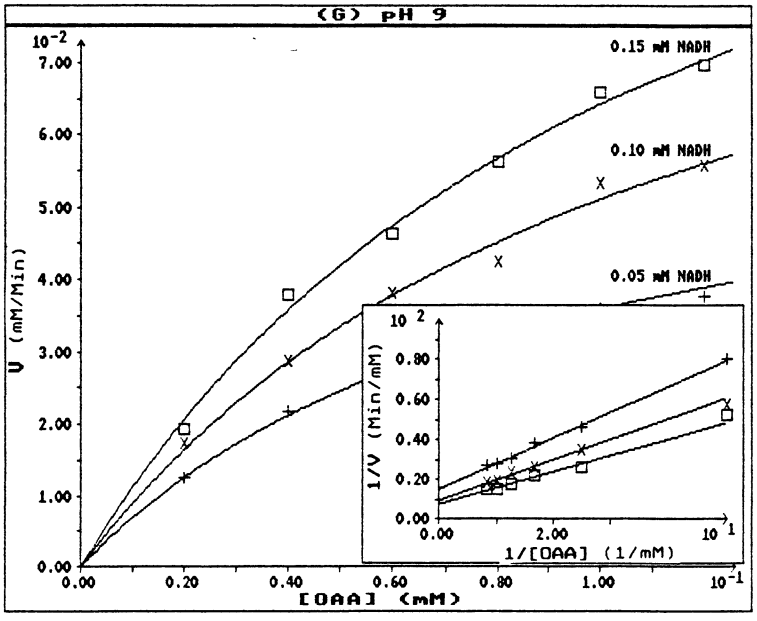

(G) pH 9
0.15 mM NADH
0.10 mM NADH
0.05 mM NADH
V (mM/Min)
[OAA] (mM)
1/V (Min/mM)
1/[OAA] (1/mM)

Table 7A. Estimates of kinetic parameters derived from initial velocity studies using the sequential mechanism of the reduction of OAA by NADH catalyzed by MDH at different pH values. The values were estimated from the experimental data depicted in Figure 15.

pH	V±SE (mM/min)	% ERR	K_A±SE (mM)	% ERR	K_B±SE (mM)	% ERR
6	0.19±0.03	15	0.082±0.027	33	0.039±0.014	36
6.5	0.16±0.02	10	0.045±0.014	30	0.032±0.009	29
7.0	0.15±0.01	9	0.029±0.010	35	0.030±0.008	27
7.5	0.36±0.11	31	0.22±0.10	45	0.082±0.032	39
8.0	0.27±0.06	23	0.092±0.037	40	0.13±0.05	40
8.5	0.17±0.03	18	0.102±0.036	35	0.080±0.026	33
9.0	0.21±0.07	33	0.12±0.07	58	0.15±0.07	47

pH	K_{IA}±SE (mM)	%ERR	K_{IB}±SE (mM)	%ERR
6	0.00053±0.0138	2589	0.00026±0.00999	3900
6.5	0.023±0.021	94	0.016±0.015	95
7.0	0.030±0.008	27	0.0051±0.017	333
7.5	0.0031±0.0157	506	-0.0023±0.0059	257
8.0	0.0038±0.013	349	0.0052±0.018	3475
8.5	0.029±0.035	121	0.013±0.020	154
9.0	0.047±0.023	49	0.077±0.050	65

Table 7B.

pH	$LogV_{max}$	$LogV_{max}/K_A$	$LogV_{max}/K_B$
6	-0.72	0.37	0.69
6.5	-0.80	0.55	0.70
7.0	-0.84	0.70	0.68
7.5	-0.44	0.21	0.65
8.0	-0.58	0.33	0.46
8.5	-0.78	0.21	0.32
9.0	-0.67	0.26	0.15

Figure 16 (A). The pH dependence of Log(V_{max}/K_{NADH}).

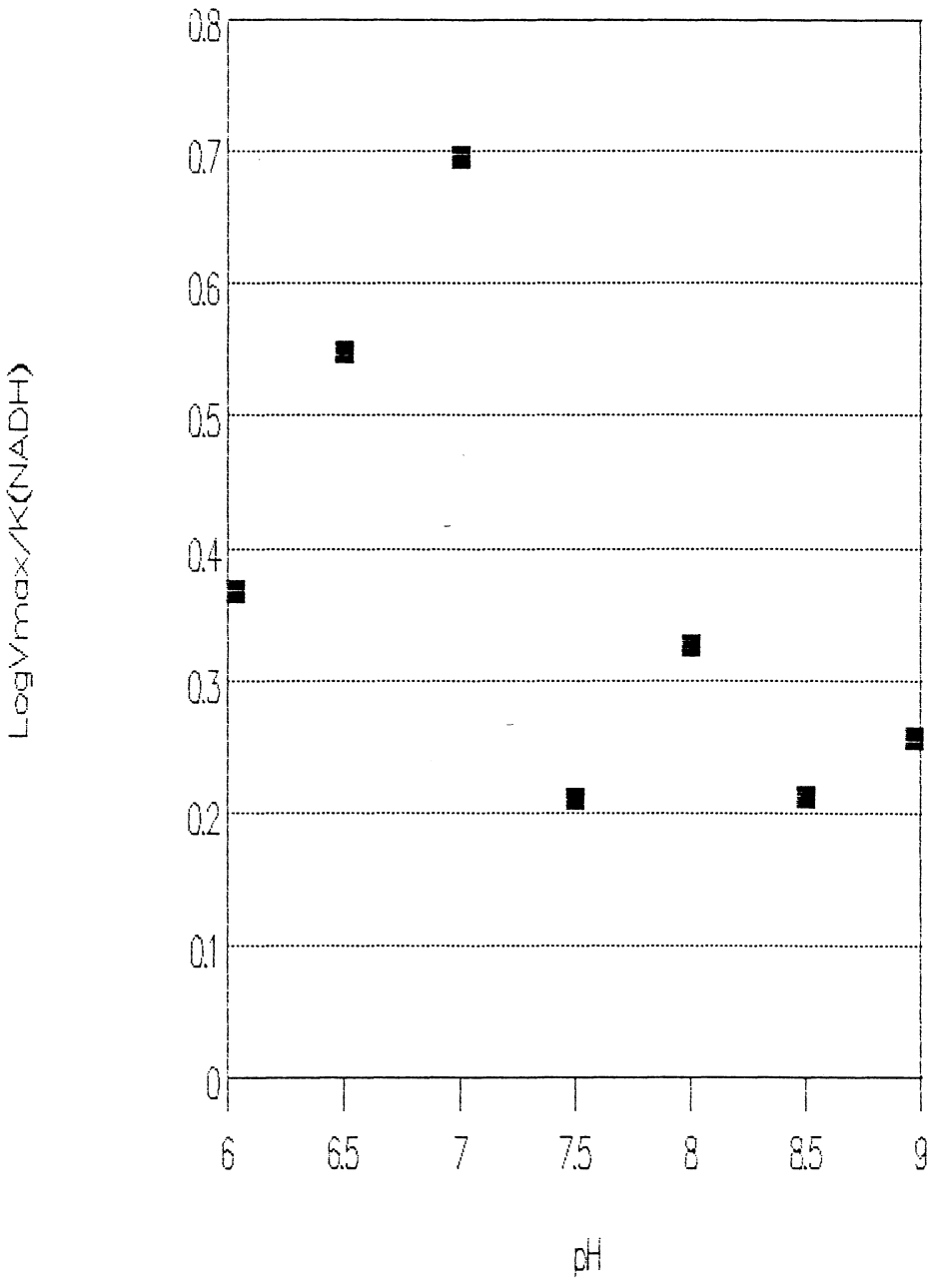
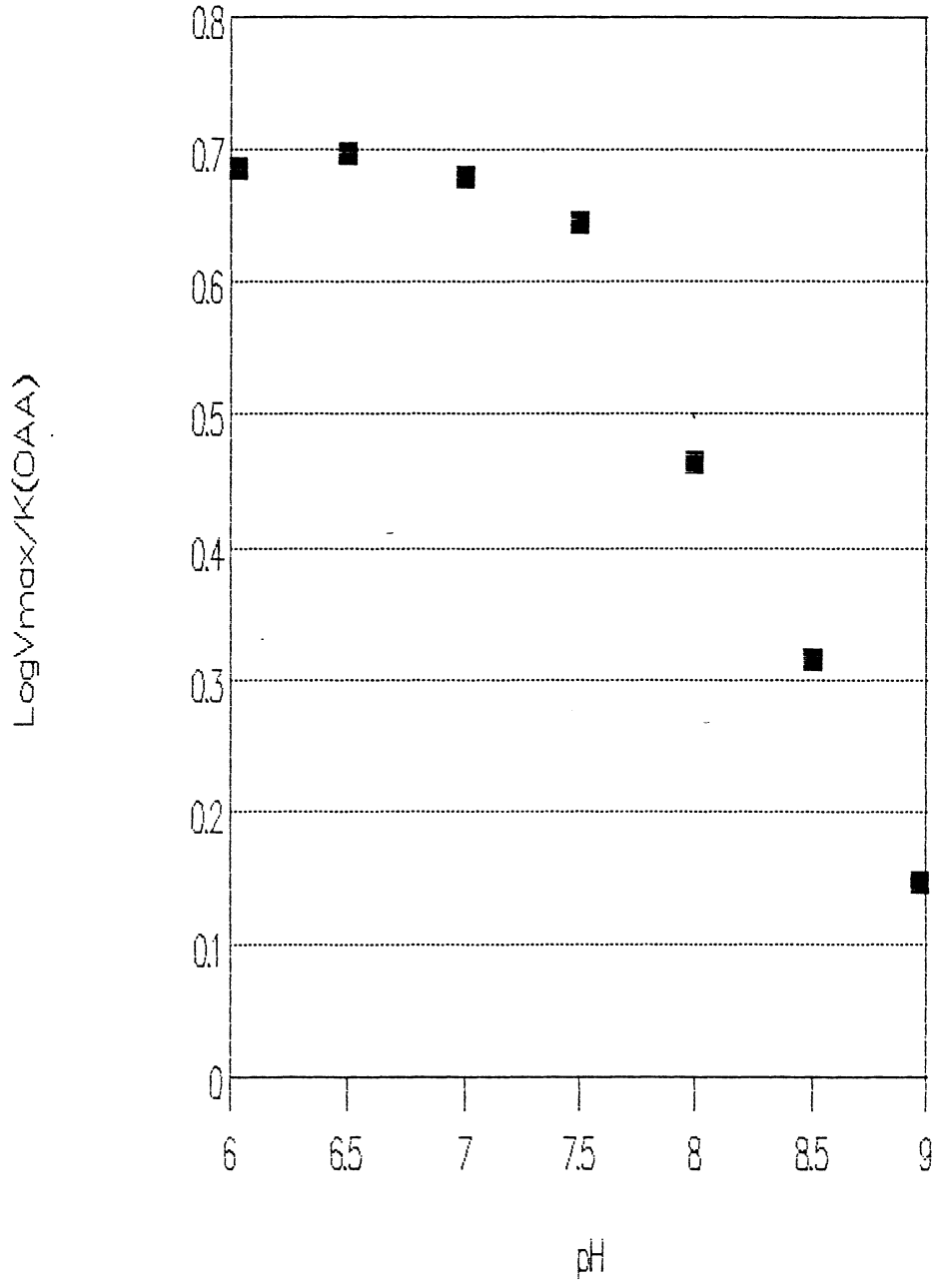


Figure 16 (B). The pH dependence of Log(V_{max}/K_{OAA}).



Substrate Inhibition Studies

In the direction of OAA reduction, with increasing OAA concentration, the initial velocity of the reaction decreased. The data (tabulated in the appendix) is shown graphically in Figure 17. Inhibition parameters, obtained from the substrate inhibition program, are shown in Table 8.

Another inhibition experiment was run to decide if the enzyme activity can be completely inhibited by OAA. At pH 7.0, OAA concentration was increased from 0.05 mM to 1.1 mM in the reaction mixture, and the corresponding initial velocity of each reaction was measured and plotted (Figure 18).

Table 8. Estimates of inhibition parameters from initial velocity studies using the substrate inhibition model of the reduction of OAA by NADH catalyzed by MDH at different pH values. The values were estimated from the experimental data included in the appendix.

pH	V±SE (mM/min)	%	K_m±SE (mM)	%	K_I±SE (mM)	%
6	0.155±0.003	2	0.050±0.002	4	0.56±0.02	4
6.5	0.31±0.02	6	0.074±0.007	9	0.44±0.04	9
7.0	0.132±0.001	0.8	0.042±0.001	2	1.09±0.04	4
7.5	0.138±0.006	4	0.061±0.006	10	0.61±0.07	11
8.0	0.15±0.01	7	0.082±0.014	17	0.51±0.13	25
8.5	0.092±0.007	8	0.045±0.011	24	14.4±30.8	214
9.0	0.11±0.01	9	0.13±0.02	8	24.8±10.2	41

Figure 17. Substrate inhibition by OAA.

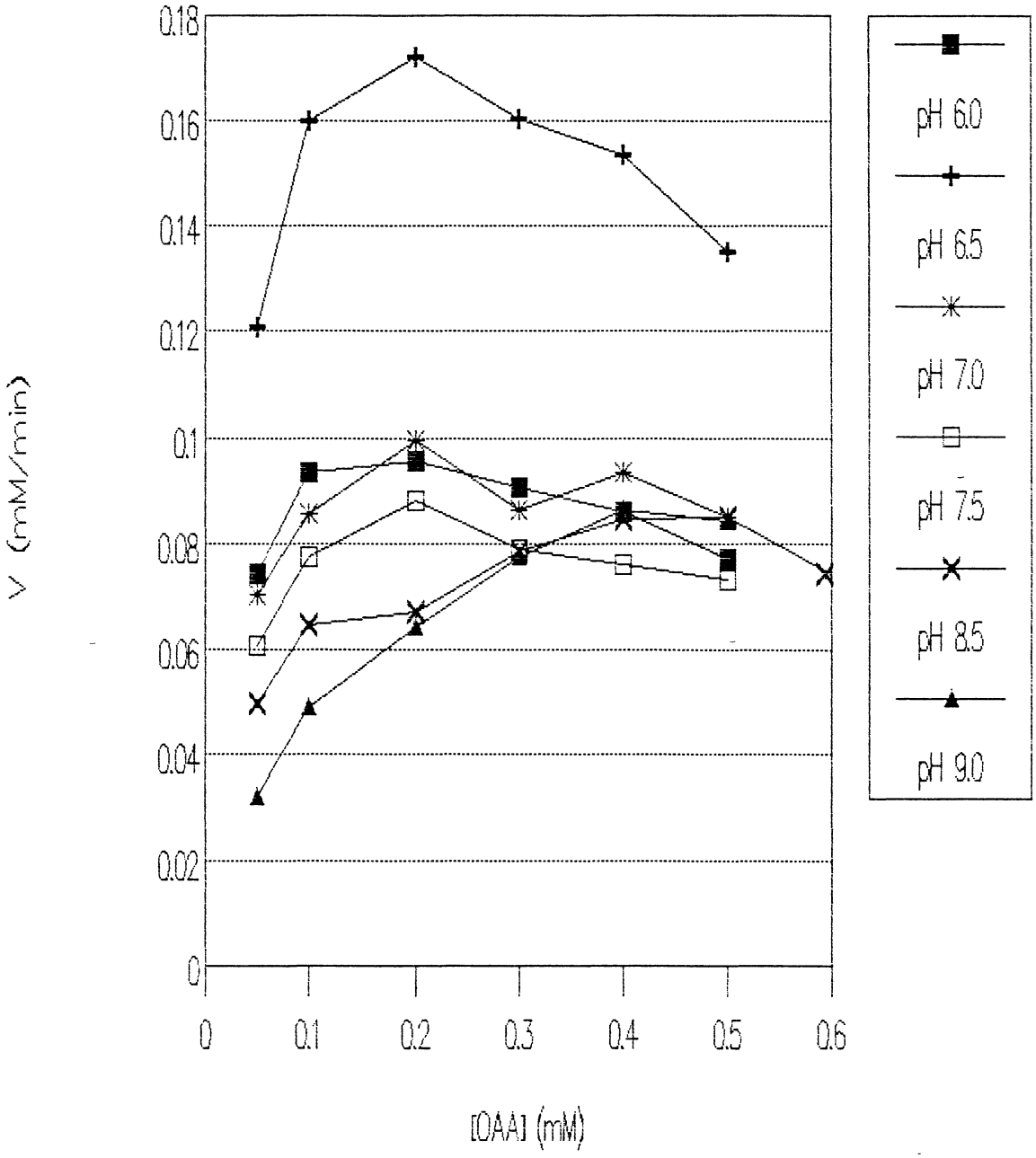
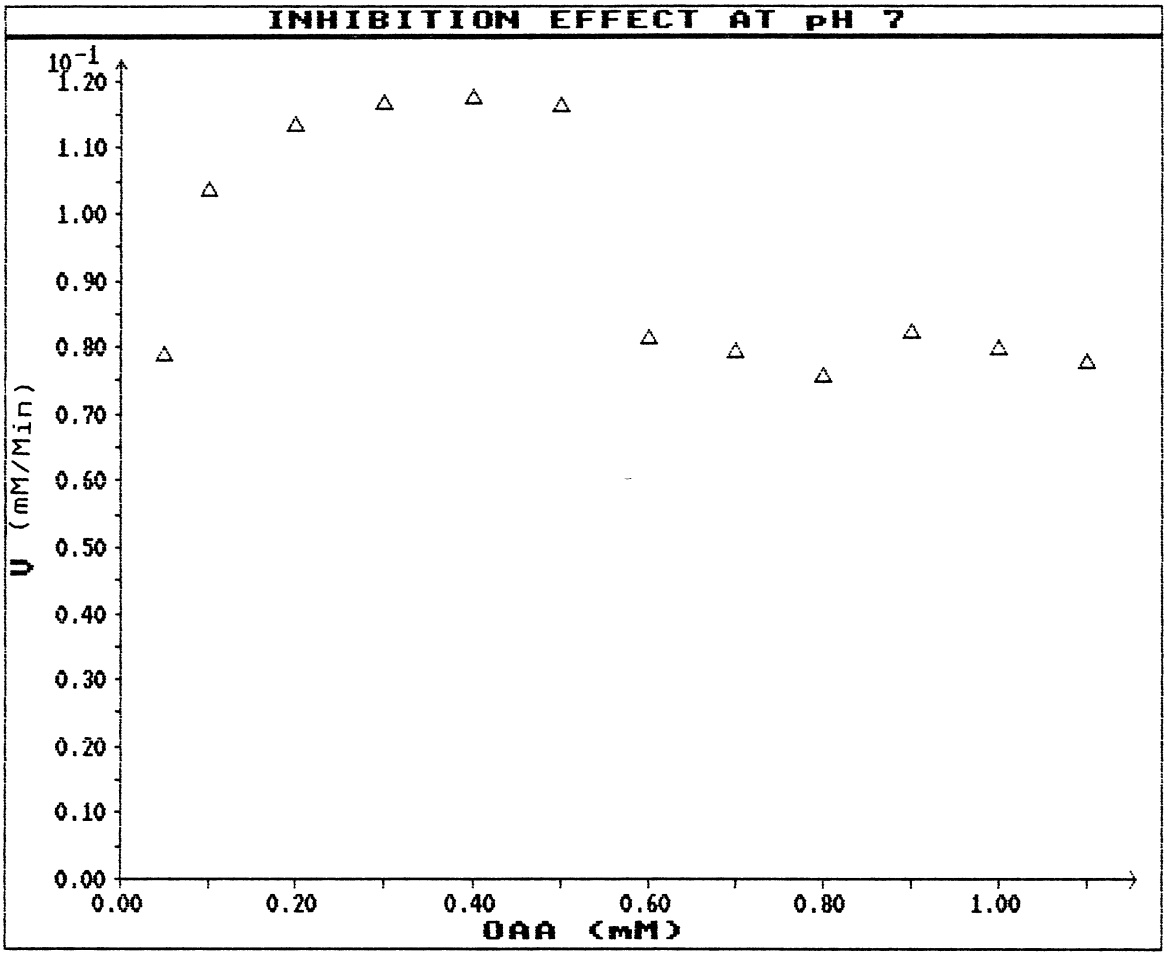


Figure 18. The degree of inhibition of MDH by OAA. The concentration of OAA was varied from 0.05 mM to 1.1 mM and the inhibitory effect on the initial velocity was determined. The concentration of NADH was 0.15 mM.

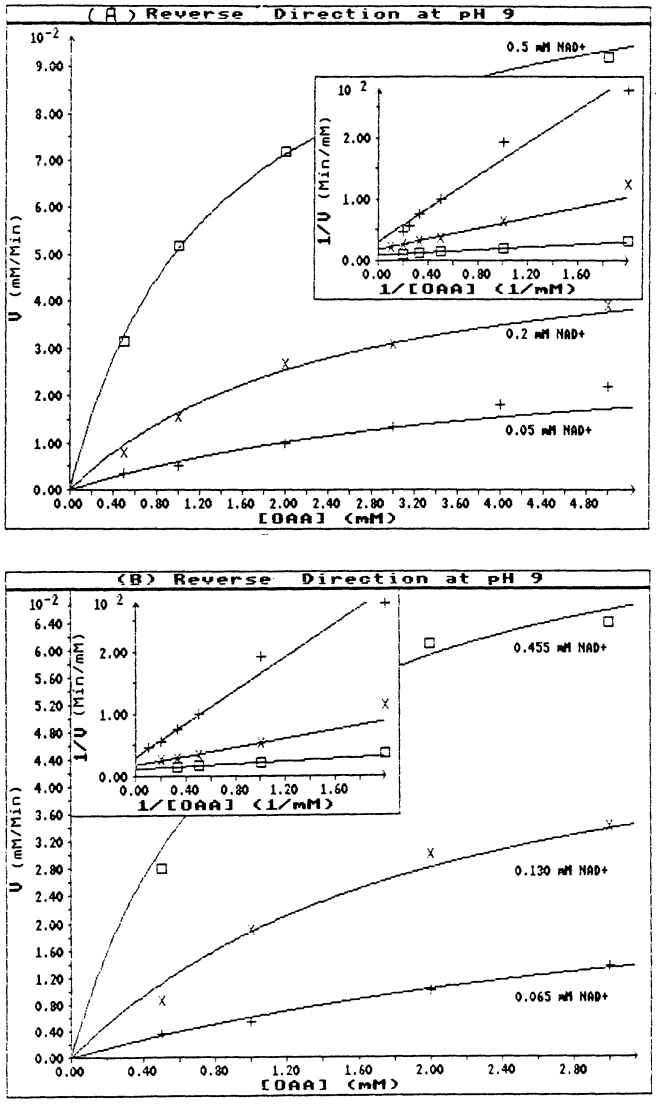


Reverse Reaction Initial velocity studies were also performed in the direction of malate oxidation at pH 9.0. The initial velocity vs. OAA concentration and the double-reciprocal plots of the initial velocity vs. 1/[OAA] at fixed NAD^+ concentration are plotted in Figure 19. The raw data are included in the appendix. Kinetic parameters were obtained in the same way as the forward direction and are listed in Table 9.

Table 9. Estimates of kinetic parameters from the initial velocity study using an ordered sequential mechanism of the oxidation of malate by NAD^+ catalyzed by MDH at pH 9.0. The values were estimated from the experimental data.

Kinetics Parameters	Experiment (A)	Experiment (B)	Average
$V_m \pm$ SE (mM/min)	0.28 ± 0.12	0.20 ± 0.91	0.24 ± 0.46
% ERROR	43	455	192
$K_A \pm$ SE (mM)	0.72 ± 0.47	0.90 ± 3.9	0.81 ± 1.96
% ERROR	65	433	241
$K_B \pm$ SE (mM)	-2.87 ± 1.04	1.2 ± 4.7	-0.84 ± 2.41
% ERROR	36	392	287
$K_{IA} \pm$ SE (mM)	-1.03 ± 0.32	-1.95 ± 0.07	-1.49 ± 0.32
% ERROR	31	4	16
$K_IB \pm$ SE (mM)	4.09 ± 2.03	-2.6 ± 1.9	1.49 ± 1.39
% ERROR	50	73	93

Figure 19. Effect of [malate] and [NAD^+] on the initial velocity of the malate oxidation direction reaction at pH 9. Two experiments were performed at different [NAD^+] under the same condition as shown in (A) and (B). The data were fitted to the Michaelis-Menten equation, and the Lineweaver-Burk double reciprocal plots are also shown in the window. The initial velocity is expressed as mM of NAD^+ reduced per min.



DISCUSSION

Chemical modification and pH dependence of kinetic parameters are two widely used techniques for the identification of the functional groups within the active sites of enzymes. A pH-dependence in the V/K profile gives information regarding groups on the enzyme, or on the substrate, which are involved in either binding on catalysis. The V/K profile reveals information concerning changes in the state of ionization of essential functional groups that affect activity. Modification of the amino acid residues on the enzyme by group-specific reagents augments the information obtained through kinetic studies. Protection against inactivation by substrates and products of the reaction can give an indication of the location of these residues. These approaches may allow an assignment of the functional groups involved in the catalytic mechanism of an enzyme. These techniques have been used on dehydrogenases including MDH from other sources, but not on MDH from *E. coli*..

In the V/K profile shown in Figure 16, a change in $\log(V_m/K)$ on the basic side of pH is seen. A pK_a of 8.5 was

calculated from this data. This pK_a is a group on the enzyme, but to know exactly which group is resposible for this pK_a, chemical modification experiments were performed to clarify the group's identity.

An enzyme, upon treatment with a certain modifying reagent, can lose its catalytic activity due to the covalent modification of active site residues. This may be the result of alteration of the chemical activities of groups at the active site, or a change in conformation of the enzyme secondary to the covalent modification.

Figures 8 and 9 show the activity reduction upon the treatment of MDH with DEPC. The reduction pattern conforms to a pseudo-first order process. Increasing the concentration of DEPC can cause a quicker loss of MDH activity. Thus, it can be concluded that the presence of DEPC reduces the MDH activity. From the previous studies, DEPC shows a selectivity for the modification of histidine residues in proteins, but it has also been reported to react with other types of residues. Of the residues that can potentially be modified by DEPC, only histidine and tyrosine can be regenerated by treatment with hydroxylamine (31).

The reactivation experiment with hydroxylamine shows

66% recovery of a activity (Table 5). This provides evidence that the loss of enzyme activity is due to the modification of histidine, rather than lysine or cysteine. In the event that disubstituted histidine is produced during prolonged incubation, treatment with hydroxylamine results in the scission of the imidazole ring (31). This reaction may account for the incomplete recovery of activity of the DEPC-inactivated enzyme.

Modification of histidine and tyrosine by DEPC can be distinguished spectrophotometrically. Formation of N-carbethoxyhistidine results in an increased absorbance 242 nm, whereas acetylation of tyrosine causes a decrease in absorbance at 278 nm (31). Table 4 shows the increase of absorbance at 242 nm of MDH, and from the spectrum obtained after the incubation of MDH with DEPC (not shown in the results), no decrease at 278 nm was observed, eliminating the possibility that the inactivation might have been due to the modification of tyrosine residues.

Modification by DEPC was carried out in the absence and presence of substrates and products in order to determine if the inactivation is due to modification of a residue at the active site of the enzyme. From the results of the

protection tests shown in Table 6 and Figures 6 through 9, it can be seen that OAA and NADH can protect MDH from modification to a certain degree, but the results from malate and NAD^+ are difficult to explain due to scatter in the data. This might have been caused by a conformational change by ligand-binding relayed to the active site. At least we can conclude that OAA and NADH are bound more tightly than malate and NAD^+, because the enzyme-OAA or enzyme-NADH complex is harder to alkylate than the enzyme-malate/NAD^+ complex. The fact that MDH being protected by OAA and NADH shows that the modification occurs at the active site.

An absorbance measurement at 242 nm was performed to determine the number of histidine residues per active sites. It was found that out of 0.0128 mM MDH active sites, 0.0875 mM of N-carbethoxyhistidine was formed, i.e., 14 mole of DEPC reacted per mole of enzyme. Since there are 4 histidine in one molecule of enzyme, the larger numbers of moles of modified residues calculated from the A_{242} might be caused by disubstituted histidine.

The Lineweaver-Burk double reciprocal plot (Figure 10) shows the intersection of lines at a point to the left of

the vertical axis at each pH values. According to enzyme kinetic theory, the addition of the substrates abide by the sequential mechanism. The reaction in the reverse direction shows the same pattern. From the inhibition study, it can be seen that OAA is the substrate inhibitor: when the concentration of OAA increases, the activity reaches a maximum value, then it decreases. However, the inhibition pattern shows partial inhibition, but not complete inhibition, which can result from allosteric combination of the substrate, but more commonly, it may represent some randomness in the mechanism (32).

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SEQUENTIAL KINETICS FIT

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FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

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#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	2.000E-02	4.200D-02	4.130E-02	2.421E+01	6.953D-04
2	5.000E-02	4.000E-02	5.500D-02	5.262E-02	1.900E+01	2.380D-03
3	5.000E-02	6.000E-02	6.000D-02	5.791E-02	1.727E+01	2.093D-03
4	5.000E-02	8.000E-02	5.500D-02	6.097E-02	1.640E+01	-5.971D-03
5	5.000E-02	1.000E-01	6.100D-02	6.297E-02	1.588E+01	-1.970D-03
6	5.000E-02	2.000E-02	3.800D-02	4.130E-02	2.421E+01	-3.305D-03
7	5.000E-02	4.000E-02	5.400D-02	5.262E-02	1.900E+01	1.380D-03
8	5.000E-02	6.000E-02	5.300D-02	5.791E-02	1.727E+01	-4.907D-03
9	5.000E-02	8.000E-02	5.500D-02	6.097E-02	1.640E+01	-5.971D-03
10	5.000E-02	1.000E-01	6.500D-02	6.297E-02	1.588E+01	2.030D-03
11	1.000E-01	2.000E-02	5.400D-02	5.032E-02	1.987E+01	3.681D-03
12	1.000E-01	4.000E-02	7.800D-02	6.805E-02	1.469E+01	9.947D-03
13	1.000E-01	6.000E-02	8.200D-02	7.711E-02	1.297E+01	4.889D-03
14	1.000E-01	1.000E-01	9.000D-02	8.630E-02	1.159E+01	3.699D-03
15	1.000E-01	1.200E-01	9.800D-02	8.895E-02	1.124E+01	9.049D-03
16	1.000E-01	2.000E-02	4.700D-02	5.032E-02	1.987E+01	-3.319D-03
17	1.000E-01	4.000E-02	6.500D-02	6.805E-02	1.469E+01	-3.053D-03
18	1.000E-01	6.000E-02	8.200D-02	7.711E-02	1.297E+01	4.889D-03
19	1.000E-01	1.200E-01	8.900D-02	8.895E-02	1.124E+01	4.890D-05
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27	1.500E-01	6.000E-02	8.400D-02	8.669E-02	1.153E+01	-2.694D-03
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V/KB=	4.8533D+00	1.0441D+00	9.1736E-01

SIGMA= 4.993029D-03

VARIANCE= 2.493033D-05

SEQUENTIAL MECHANISM FIT-NADH VS OAA 01-01-1980 II152 PH6

SEQUENTIAL KINETICS FIT

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FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

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#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	2.000E-02	3.500D-02	3.771E-02	2.652E+01	-2.708D-03
2	5.000E-02	4.000E-02	5.800D-02	5.196E-02	1.924E+01	6.037D-03
3	5.000E-02	6.000E-02	6.100D-02	5.946E-02	1.682E+01	1.545D-03
4	5.000E-02	8.000E-02	6.400D-02	6.407E-02	1.561E+01	-7.474D-05
5	5.000E-02	1.000E-01	6.600D-02	6.721E-02	1.488E+01	-1.208D-03
6	5.000E-02	1.200E-01	7.200D-02	6.947E-02	1.439E+01	2.528D-03
7	5.000E-02	2.000E-02	4.100D-02	3.771E-02	2.652E+01	3.292D-03
8	5.000E-02	4.000E-02	5.400D-02	5.196E-02	1.924E+01	2.037D-03
9	5.000E-02	6.000E-02	6.000D-02	5.946E-02	1.682E+01	5.446D-04
10	5.000E-02	6.000E-02	6.400D-02	5.946E-02	1.682E+01	4.545D-03
11	5.000E-02	1.000E-01	6.600D-02	6.721E-02	1.488E+01	-1.208D-03
12	5.000E-02	2.000E-02	3.600D-02	3.771E-02	2.652E+01	-1.708D-03
13	5.000E-02	4.000E-02	4.700D-02	5.196E-02	1.924E+01	-4.963D-03
14	5.000E-02	6.000E-02	5.200D-02	5.946E-02	1.682E+01	-7.455D-03
15	5.000E-02	8.000E-02	6.300D-02	6.407E-02	1.561E+01	-1.075D-03
16	1.000E-01	2.000E-02	4.100D-02	4.666E-02	2.143E+01	-5.664D-03
17	1.000E-01	4.000E-02	7.300D-02	6.543E-02	1.528E+01	7.570D-03
18	1.000E-01	6.000E-02	7.900D-02	7.556E-02	1.323E+01	3.441D-03
19	1.000E-01	8.000E-02	8.600D-02	8.190E-02	1.221E+01	4.102D-03
20	1.000E-01	1.000E-01	9.200D-02	8.624E-02	1.160E+01	5.761D-03
21	1.000E-01	1.200E-01	8.900D-02	8.940E-02	1.119E+01	-3.980D-04
22	1.000E-01	2.000E-02	4.200D-02	4.666E-02	2.143E+01	-4.664D-03
23	1.000E-01	4.000E-02	6.700D-02	6.543E-02	1.528E+01	1.570D-03
24	1.000E-01	6.000E-02	7.900D-02	7.556E-02	1.323E+01	3.441D-03
25	1.000E-01	8.000E-02	7.300D-02	8.190E-02	1.221E+01	-8.898D-03
26	1.000E-01	1.000E-01	7.900D-02	8.624E-02	1.160E+01	-7.239D-03
27	1.000E-01	1.200E-01	8.700D-02	8.940E-02	1.119E+01	-2.398D-03
28	1.500E-01	2.000E-02	4.900D-02	5.068E-02	1.973E+01	-1.676D-03
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30	1.500E-01	6.000E-02	8.800D-02	8.306E-02	1.204E+01	4.942D-03
31	1.500E-01	8.000E-02	9.300D-02	9.027E-02	1.108E+01	2.732D-03
32	1.500E-01	1.200E-01	9.600D-02	9.885E-02	1.012E+01	-2.848D-03
33	1.500E-01	2.000E-02	5.100D-02	5.068E-02	1.973E+01	3.239D-04
34	1.500E-01	4.000E-02	6.700D-02	7.162E-02	1.396E+01	-4.617D-03
35	1.500E-01	6.000E-02	7.800D-02	8.306E-02	1.204E+01	-5.058D-03
36	1.500E-01	8.000E-02	9.600D-02	9.027E-02	1.108E+01	5.732D-03
37	1.500E-01	1.000E-01	9.300D-02	9.523E-02	1.050E+01	-2.228D-03
38	1.500E-01	1.200E-01	9.800D-02	9.885E-02	1.012E+01	-8.484D-04
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CONST.	VALUE	S.E.	Weight
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KA=	4.4914D-02	1.3460D-02	5.5200E+03
KB=	3.1830D-02	9.1052D-03	1.2062E+04
KIA=	2.2503D-02	2.1226D-02	2.2196E+03
KIB=	1.5947D-02	1.5160D-02	4.3511E+03
V/KA=	3.5311D+00	7.2716D-01	1.8912E+00
V/KB=	4.9828D+00	9.6883D-01	1.0654E+00

SIGMA= 4.303981D-03

VARIANCE= 1.852425D-05

SEQUENTIAL MECHANISM FIT-NADH VS OAA 01-01-1980 PH6.5

SEQUENTIAL KINETICS FIT

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FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

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#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	2.000E-02	4.400D-02	4.452E-02	2.246E+01	-5.197D-04
2	5.000E-02	4.000E-02	6.300D-02	5.988E-02	1.670E+01	3.125D-03
3	5.000E-02	6.000E-02	6.700D-02	6.765E-02	1.478E+01	-6.534D-04
4	5.000E-02	8.000E-02	7.100D-02	7.235E-02	1.382E+01	-1.353D-03
5	5.000E-02	1.000E-01	8.000D-02	7.550E-02	1.325E+01	4.500D-03
6	5.000E-02	1.200E-01	7.900D-02	7.775E-02	1.286E+01	1.246D-03
7	5.000E-02	2.000E-02	4.200D-02	4.452E-02	2.246E+01	-2.520D-03
8	5.000E-02	4.000E-02	6.200D-02	5.988E-02	1.670E+01	2.125D-03
9	5.000E-02	6.000E-02	6.600D-02	6.765E-02	1.478E+01	-1.653D-03
10	5.000E-02	8.000E-02	6.800D-02	7.235E-02	1.382E+01	-4.353D-03
11	5.000E-02	1.000E-01	7.000D-02	7.550E-02	1.325E+01	-5.500D-03
12	5.000E-02	1.200E-01	8.200D-02	7.775E-02	1.286E+01	4.246D-03
13	5.000E-02	2.000E-02	4.300D-02	4.452E-02	2.246E+01	-1.520D-03
14	5.000E-02	4.000E-02	5.800D-02	5.988E-02	1.670E+01	-1.875D-03
15	5.000E-02	6.000E-02	7.100D-02	6.765E-02	1.478E+01	3.347D-03
16	5.000E-02	8.000E-02	7.500D-02	7.235E-02	1.382E+01	2.647D-03
17	5.000E-02	1.000E-01	7.600D-02	7.550E-02	1.325E+01	5.002D-04
18	1.000E-01	2.000E-02	5.400D-02	5.021E-02	1.992E+01	3.791D-03
19	1.000E-01	4.000E-02	7.600D-02	6.934E-02	1.442E+01	6.660D-03
20	1.000E-01	6.000E-02	8.500D-02	7.943E-02	1.259E+01	5.571D-03
21	1.000E-01	8.000E-02	8.800D-02	8.566E-02	1.167E+01	2.340D-03
22	1.000E-01	1.000E-01	9.100D-02	8.989E-02	1.112E+01	1.109D-03
23	1.000E-01	1.200E-01	9.400D-02	9.295E-02	1.076E+01	1.048D-03
24	1.000E-01	2.000E-02	5.300D-02	5.021E-02	1.992E+01	2.791D-03
25	1.000E-01	4.000E-02	6.900D-02	6.934E-02	1.442E+01	-3.403D-04
26	1.000E-01	6.000E-02	7.900D-02	7.943E-02	1.259E+01	-4.285D-04
27	1.000E-01	8.000E-02	8.300D-02	8.566E-02	1.167E+01	-2.660D-03
28	1.000E-01	1.000E-01	9.900D-02	8.989E-02	1.112E+01	9.109D-03
29	1.000E-01	1.200E-01	8.700D-02	9.295E-02	1.076E+01	-5.952D-03
30	1.000E-01	2.000E-02	4.500D-02	5.021E-02	1.992E+01	-5.209D-03
31	1.000E-01	4.000E-02	7.200D-02	6.934E-02	1.442E+01	2.660D-03
32	1.000E-01	6.000E-02	7.800D-02	7.943E-02	1.259E+01	-1.429D-03
33	1.000E-01	8.000E-02	8.000D-02	8.566E-02	1.167E+01	-5.660D-03
34	1.000E-01	1.000E-01	7.600D-02	8.989E-02	1.112E+01	-1.389D-02
35	1.000E-01	1.200E-01	8.800D-02	9.295E-02	1.076E+01	-4.952D-03
36	1.500E-01	2.000E-02	5.600D-02	5.244E-02	1.907E+01	3.557D-03
37	1.500E-01	4.000E-02	8.100D-02	7.320E-02	1.366E+01	7.803D-03
38	1.500E-01	6.000E-02	9.000D-02	8.432E-02	1.186E+01	5.679D-03
39	1.500E-01	8.000E-02	1.100D-01	9.125E-02	1.096E+01	1.875D-02
40	1.500E-01	1.000E-01	1.000D-01	9.599E-02	1.042E+01	4.010D-03
41	1.500E-01	1.200E-01	1.100D-01	9.943E-02	1.006E+01	1.057D-02
42	1.500E-01	2.000E-02	4.400D-02	5.244E-02	1.907E+01	-8.443D-03
43	1.500E-01	4.000E-02	6.800D-02	7.320E-02	1.366E+01	-5.197D-03
44	1.500E-01	6.000E-02	8.100D-02	8.432E-02	1.186E+01	-3.321D-03
45	1.500E-01	8.000E-02	9.000D-02	9.125E-02	1.096E+01	-1.254D-03
46	1.500E-01	1.000E-01	9.000D-02	9.599E-02	1.042E+01	-5.990D-03
47	1.500E-01	1.200E-01	9.200D-02	9.943E-02	1.006E+01	-7.430D-03
48	1.500E-01	2.000E-02	5.100D-02	5.244E-02	1.907E+01	-1.443D-03
49	1.500E-01	4.000E-02	7.000D-02	7.320E-02	1.366E+01	-3.197D-03
50	1.500E-01	6.000E-02	7.400D-02	8.432E-02	1.186E+01	-1.032D-02
51	1.500E-01	8.000E-02	9.500D-02	9.125E-02	1.096E+01	3.746D-03
52	1.500E-01	1.000E-01	9.100D-02	9.599E-02	1.042E+01	-4.990D-03

CONST.	VALUE	S.E.	Weight
V=	1.4467D-01	1.3013D-02	5.9058E+03
KA=	2.9141D-02	1.0283D-02	9.4574E+03
KB=	3.0263D-02	8.1560D-03	1.5033E+04
KIA=	5.0766D-03	1.6868D-02	3.5145E+03
KIB=	5.2720D-03	1.7866D-02	3.1329E+03
V/KA=	4.9645D+00	1.3363D+00	5.6000E-01
V/KB=	4.7805D+00	8.9580D-01	1.2462E+00

SIGMA= 5.774999D-03

VARIANCE= 3.335061D-05

SEQUENTIAL MECHANISM FIT-NADH VS OAA 01-01-1980 PH7

SEQUENTIAL KINETICS FIT

****

FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

****

#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	2.000E-02	5.000E-02	3.120D-02	3.477E-02	2.876E+01	-3.574D-03
2	2.000E-02	5.000E-02	3.420D-02	3.477E-02	2.876E+01	-5.738D-04
3	4.000E-02	5.000E-02	4.210D-02	4.511E-02	2.217E+01	-3.009D-03
4	4.000E-02	5.000E-02	4.810D-02	4.511E-02	2.217E+01	2.991D-03
5	6.000E-02	5.000E-02	4.680D-02	5.007E-02	1.997E+01	-3.270D-03
6	6.000E-02	5.000E-02	4.900D-02	5.007E-02	1.997E+01	-1.070D-03
7	8.000E-02	5.000E-02	5.050D-02	5.298E-02	1.887E+01	-2.483D-03
8	1.200E-01	5.000E-02	6.040D-02	5.626E-02	1.778E+01	4.144D-03
9	1.000E-01	5.000E-02	5.300D-02	5.490E-02	1.822E+01	-1.899D-03
10	2.000E-02	1.000E-01	5.410D-02	4.968E-02	2.013E+01	4.424D-03
11	4.000E-02	1.000E-01	7.710D-02	6.932E-02	1.443E+01	7.780D-03
12	6.000E-02	1.000E-01	8.150D-02	7.985E-02	1.252E+01	1.655D-03
13	1.200E-01	1.000E-01	9.600D-02	9.414E-02	1.062E+01	1.861D-03
14	2.000E-02	1.000E-01	5.180D-02	4.968E-02	2.013E+01	2.124D-03
15	4.000E-02	1.000E-01	6.980D-02	6.932E-02	1.443E+01	4.796D-04
16	8.000E-02	1.000E-01	8.510D-02	8.640E-02	1.157E+01	-1.305D-03
17	1.000E-01	1.000E-01	9.200D-02	9.088E-02	1.100E+01	1.115D-03
18	1.200E-01	1.000E-01	9.470D-02	9.414E-02	1.062E+01	5.615D-04
19	1.000E-01	5.000E-02	5.280D-02	5.490E-02	1.822E+01	-2.099D-03
20	2.000E-02	1.500E-01	5.200D-02	5.795E-02	1.725E+01	-5.955D-03
21	4.000E-02	1.500E-01	7.720D-02	8.442E-02	1.184E+01	-7.225D-03
22	6.000E-02	1.500E-01	9.420D-02	9.959E-02	1.004E+01	-5.386D-03
23	1.000E-01	1.500E-01	1.091D-01	1.163E-01	8.599E+00	-7.194D-03
24	1.200E-01	1.500E-01	1.183D-01	1.214E-01	8.238E+00	-3.086D-03
25	2.000E-02	1.500E-01	5.230D-02	5.795E-02	1.725E+01	-5.655D-03
26	4.000E-02	1.500E-01	9.640D-02	8.442E-02	1.184E+01	1.198D-02
27	6.000E-02	1.500E-01	1.040D-01	9.959E-02	1.004E+01	4.414D-03
28	8.000E-02	1.500E-01	1.128D-01	1.094E-01	9.140E+00	3.389D-03
29	1.000E-01	1.500E-01	1.201D-01	1.163E-01	8.599E+00	3.806D-03

CONST.	VALUE	S.E.	Weight
V=	5.3693D-01	2.1314D-01	2.2012E+01
KA=	1.0353D-01	6.2752D-02	2.5395E+02
KB=	3.6826D-01	1.8929D-01	2.7910E+01
KIA=	5.1561D-03	7.6027D-03	1.7301E+04
KIB=	1.8340D-02	2.9055D-02	1.1846E+03
V/KA=	5.1861D+00	1.1891D+00	7.0725E-01
V/KB=	1.4580D+00	1.7878D-01	3.1286E+01

SIGMA= 4.763900D-03

VARIANCE= 2.269474D-05

SEQUENTIAL MECHANISM FIT-OAA VS NADH 01-01-1980 PH7.5

SEQUENTIAL KINETICS FIT

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FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

****

#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	2.000E-02	5.000E-02	3.560D-02	3.894E-02	2.568E+01	-3.338D-03
2	4.000E-02	5.000E-02	5.270D-02	5.104E-02	1.959E+01	1.661D-03
3	6.000E-02	5.000E-02	5.580D-02	5.694E-02	1.756E+01	-1.137D-03
4	8.000E-02	5.000E-02	5.840D-02	6.043E-02	1.655E+01	-2.029D-03
5	1.000E-01	5.000E-02	6.190D-02	6.274E-02	1.594E+01	-8.374D-04
6	1.200E-01	5.000E-02	6.380D-02	6.438E-02	1.553E+01	-5.769D-04
7	2.000E-02	5.000E-02	3.800D-02	3.894E-02	2.568E+01	-9.379D-04
8	4.000E-02	5.000E-02	5.180D-02	5.104E-02	1.959E+01	7.610D-04
9	6.000E-02	5.000E-02	5.370D-02	5.694E-02	1.756E+01	-3.237D-03
10	8.000E-02	5.000E-02	5.770D-02	6.043E-02	1.655E+01	-2.729D-03
11	1.000E-01	5.000E-02	5.560D-02	6.274E-02	1.594E+01	-7.137D-03
12	2.000E-02	1.000E-01	4.260D-02	4.237E-02	2.360E+01	2.317D-04
13	4.000E-02	1.000E-01	6.600D-02	6.046E-02	1.654E+01	5.542D-03
14	6.000E-02	1.000E-01	7.620D-02	7.049E-02	1.419E+01	5.710D-03
15	8.000E-02	1.000E-01	8.930D-02	7.687E-02	1.301E+01	1.243D-02
16	1.000E-01	1.000E-01	8.580D-02	8.128E-02	1.230E+01	4.520D-03
17	1.200E-01	1.000E-01	8.640D-02	8.451E-02	1.183E+01	1.886D-03
18	2.000E-02	1.000E-01	4.810D-02	4.237E-02	2.360E+01	5.732D-03
19	4.000E-02	1.000E-01	6.090D-02	6.046E-02	1.654E+01	4.422D-04
20	6.000E-02	1.000E-01	7.500D-02	7.049E-02	1.419E+01	4.510D-03
21	8.000E-02	1.000E-01	7.520D-02	7.687E-02	1.301E+01	-1.667D-03
22	1.000E-01	1.000E-01	8.880D-02	8.128E-02	1.230E+01	7.520D-03
23	1.200E-01	1.000E-01	8.760D-02	8.451E-02	1.183E+01	3.086D-03
24	2.000E-02	1.500E-01	4.470D-02	4.365E-02	2.291E+01	1.050D-03
25	4.000E-02	1.500E-01	6.720D-02	6.442E-02	1.552E+01	2.779D-03
26	6.000E-02	1.500E-01	7.980D-02	7.656E-02	1.306E+01	3.235D-03
27	8.000E-02	1.500E-01	8.950D-02	8.453E-02	1.183E+01	4.967D-03
28	1.000E-01	1.500E-01	9.040D-02	9.016E-02	1.109E+01	2.378D-04
29	1.200E-01	1.500E-01	9.190D-02	9.435E-02	1.060E+01	-2.451D-03
30	2.000E-02	1.500E-01	4.240D-02	4.365E-02	2.291E+01	-1.250D-03
31	4.000E-02	1.500E-01	5.970D-02	6.442E-02	1.552E+01	-4.721D-03
32	6.000E-02	1.500E-01	7.390D-02	7.656E-02	1.306E+01	-2.665D-03
33	8.000E-02	1.500E-01	7.340D-02	8.453E-02	1.183E+01	-1.113D-02
34	1.000E-01	1.500E-01	8.780D-02	9.016E-02	1.109E+01	-2.362D-03
35	2.000E-02	1.500E-01	4.030D-02	4.365E-02	2.291E+01	-3.350D-03
36	4.000E-02	1.500E-01	6.150D-02	6.442E-02	1.552E+01	-2.921D-03
37	6.000E-02	1.500E-01	7.110D-02	7.656E-02	1.306E+01	-5.465D-03
38	8.000E-02	1.500E-01	8.130D-02	8.453E-02	1.183E+01	-3.233D-03
39	1.000E-01	1.500E-01	8.590D-02	9.016E-02	1.109E+01	-4.262D-03

CONST.	VALUE	S.E.	Weight
V=	1.8339D-01	2.5122D-02	1.5845E+03
KA=	5.8943D-02	1.6165D-02	3.8270E+03
KB=	7.3823D-02	2.2639D-02	1.9512E+03
KIA=	-9.6692D-03	9.1005D-03	1.2074E+04
KIB=	-1.2110D-02	1.1641D-02	7.3792E+03
V/KA=	3.1113D+00	4.5353D-01	4.8617E+00
V/KB=	2.4842D+00	4.3877D-01	5.1943E+00

SIGMA= 4.609269D-03

VARIANCE= 2.124536D-05

SEQUENTIAL MECHANISM FIT-OAA VS NADH 01-01-1980 II155PH7.5

SEQUENTIAL KINETICS FIT

****

FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

****

#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	2.000E-02	5.000E-02	2.940D-02	3.108E-02	3.217E+01	-1.684D-03
2	4.000E-02	5.000E-02	4.600D-02	4.409E-02	2.268E+01	1.906D-03
3	6.000E-02	5.000E-02	5.220D-02	5.124E-02	1.951E+01	9.565D-04
4	1.000E-01	5.000E-02	5.590D-02	5.888E-02	1.698E+01	-2.981D-03
5	1.200E-01	5.000E-02	5.850D-02	6.116E-02	1.635E+01	-2.659D-03
6	2.000E-02	5.000E-02	3.010D-02	3.108E-02	3.217E+01	-9.845D-04
7	4.000E-02	5.000E-02	3.860D-02	4.409E-02	2.268E+01	-5.494D-03
8	6.000E-02	5.000E-02	4.560D-02	5.124E-02	1.951E+01	-5.643D-03
9	8.000E-02	5.000E-02	5.570D-02	5.576E-02	1.793E+01	-6.400D-05
10	1.000E-01	5.000E-02	5.680D-02	5.888E-02	1.698E+01	-2.081D-03
11	1.200E-01	5.000E-02	5.670D-02	6.116E-02	1.635E+01	-4.459D-03
12	2.000E-02	1.000E-01	3.760D-02	3.764E-02	2.657E+01	-4.034D-05
13	4.000E-02	1.000E-01	6.200D-02	5.708E-02	1.752E+01	4.924D-03
14	6.000E-02	1.000E-01	7.670D-02	6.894E-02	1.451E+01	7.758D-03
15	8.000E-02	1.000E-01	7.820D-02	7.694E-02	1.300E+01	1.261D-03
16	1.000E-01	1.000E-01	8.940D-02	8.270E-02	1.209E+01	6.705D-03
17	1.200E-01	1.000E-01	9.620D-02	8.704E-02	1.149E+01	9.164D-03
18	2.000E-02	1.000E-01	4.030D-02	3.764E-02	2.657E+01	2.660D-03
19	4.000E-02	1.000E-01	5.620D-02	5.708E-02	1.752E+01	-8.757D-04
20	6.000E-02	1.000E-01	8.170D-02	6.894E-02	1.451E+01	1.276D-02
21	8.000E-02	1.000E-01	8.220D-02	7.694E-02	1.300E+01	5.261D-03
22	1.000E-01	1.000E-01	8.280D-02	8.270E-02	1.209E+01	1.048D-04
23	1.200E-01	1.000E-01	8.650D-02	8.704E-02	1.149E+01	-5.361D-04
24	2.000E-02	1.500E-01	3.710D-02	4.049E-02	2.470E+01	-3.387D-03
25	4.000E-02	1.500E-01	6.250D-02	6.329E-02	1.580E+01	-7.862D-04
26	6.000E-02	1.500E-01	7.490D-02	7.791E-02	1.284E+01	-3.011D-03
27	8.000E-02	1.500E-01	8.110D-02	8.809E-02	1.135E+01	-6.989D-03
28	1.000E-01	1.500E-01	9.710D-02	9.558E-02	1.046E+01	1.519D-03
29	1.200E-01	1.500E-01	1.047D-01	1.013E-01	9.869E+00	3.373D-03
30	2.000E-02	1.500E-01	3.970D-02	4.049E-02	2.470E+01	-7.866D-04
31	4.000E-02	1.500E-01	6.190D-02	6.329E-02	1.580E+01	-1.386D-03
32	6.000E-02	1.500E-01	7.440D-02	7.791E-02	1.284E+01	-3.511D-03
33	8.000E-02	1.500E-01	8.790D-02	8.809E-02	1.135E+01	-1.894D-04
34	1.000E-01	1.500E-01	8.900D-02	9.558E-02	1.046E+01	-6.581D-03
35	1.200E-01	1.500E-01	9.390D-02	1.013E-01	9.869E+00	-7.427D-03

CONST.	VALUE	S.E.	Weight
V=	2.6588D-01	6.0905D-02	2.6958E+02
KA=	9.1480D-02	3.6476D-02	7.5162E+02
KB=	1.2531D-01	5.0364D-02	3.9424E+02
KIA=	3.7781D-03	1.3154D-02	5.7795E+03
KIB=	5.1752D-03	1.8045D-02	3.0710E+03
V/KA=	2.9065D+00	5.2593D-01	3.6153E+00
V/KB=	2.1218D+00	3.8500D-01	6.7465E+00

SIGMA= 4.823276D-03

VARIANCE= 2.326399D-05

SEQUENTIAL MECHANISM FIT-OAA VS NADH 01-01-1980 PH8B

SEQUENTIAL KINETICS FIT

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FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

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#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	2.500E-02	5.000E-02	3.640D-02	2.929E-02	3.414E+01	7.105D-03
2	5.000E-02	5.000E-02	3.310D-02	3.596E-02	2.781E+01	-2.859D-03
3	7.500E-02	5.000E-02	3.560D-02	3.891E-02	2.570E+01	-3.310D-03
4	1.000E-01	5.000E-02	4.250D-02	4.058E-02	2.465E+01	1.925D-03
5	1.500E-01	5.000E-02	4.400D-02	4.239E-02	2.359E+01	1.611D-03
6	2.500E-02	5.000E-02	3.030D-02	2.929E-02	3.414E+01	1.005D-03
7	5.000E-02	5.000E-02	3.600D-02	3.596E-02	2.781E+01	4.064D-05
8	7.500E-02	5.000E-02	4.020D-02	3.891E-02	2.570E+01	1.290D-03
9	1.000E-01	5.000E-02	3.940D-02	4.058E-02	2.465E+01	-1.175D-03
10	1.500E-01	5.000E-02	4.260D-02	4.239E-02	2.359E+01	2.114D-04
11	2.500E-02	5.000E-02	2.320D-02	2.929E-02	3.414E+01	-6.095D-03
12	5.000E-02	5.000E-02	3.410D-02	3.596E-02	2.781E+01	-1.859D-03
13	7.500E-02	5.000E-02	3.630D-02	3.891E-02	2.570E+01	-2.610D-03
14	1.000E-01	5.000E-02	3.900D-02	4.058E-02	2.465E+01	-1.575D-03
15	1.500E-01	5.000E-02	4.030D-02	4.239E-02	2.359E+01	-2.089D-03
16	2.500E-02	1.000E-01	2.870D-02	2.928E-02	3.415E+01	-5.792D-04
17	5.000E-02	1.000E-01	3.660D-02	4.154E-02	2.407E+01	-4.944D-03
18	7.500E-02	1.000E-01	4.450D-02	4.829E-02	2.071E+01	-3.786D-03
19	1.000E-01	1.000E-01	5.040D-02	5.255E-02	1.903E+01	-2.150D-03
20	1.500E-01	1.000E-01	5.680D-02	5.764E-02	1.735E+01	-8.396D-04
21	2.500E-02	1.000E-01	3.380D-02	2.928E-02	3.415E+01	4.521D-03
22	5.000E-02	1.000E-01	4.560D-02	4.154E-02	2.407E+01	4.056D-03
23	7.500E-02	1.000E-01	5.350D-02	4.829E-02	2.071E+01	5.214D-03
24	1.000E-01	1.000E-01	7.460D-02	5.255E-02	1.903E+01	2.205D-02
25	1.500E-01	1.000E-01	5.790D-02	5.764E-02	1.735E+01	2.604D-04
26	2.500E-02	1.000E-01	3.230D-02	2.928E-02	3.415E+01	3.021D-03
27	5.000E-02	1.000E-01	3.870D-02	4.154E-02	2.407E+01	-2.844D-03
28	7.500E-02	1.000E-01	4.690D-02	4.829E-02	2.071E+01	-1.386D-03
29	2.500E-02	1.500E-01	2.980D-02	2.927E-02	3.416E+01	5.258D-04
30	5.000E-02	1.500E-01	4.050D-02	4.381E-02	2.283E+01	-3.312D-03
31	7.500E-02	1.500E-01	5.610D-02	5.250E-02	1.905E+01	3.598D-03
32	1.000E-01	1.500E-01	5.910D-02	5.828E-02	1.716E+01	8.170D-04
33	1.500E-01	1.500E-01	6.940D-02	6.549E-02	1.527E+01	3.906D-03
34	2.500E-02	1.500E-01	2.750D-02	2.927E-02	3.416E+01	-1.774D-03
35	5.000E-02	1.500E-01	4.470D-02	4.381E-02	2.283E+01	8.885D-04
36	7.500E-02	1.500E-01	4.890D-02	5.250E-02	1.905E+01	-3.602D-03
37	1.000E-01	1.500E-01	5.420D-02	5.828E-02	1.716E+01	-4.083D-03
38	1.500E-01	1.500E-01	6.240D-02	6.549E-02	1.527E+01	-3.094D-03
39	2.500E-02	1.500E-01	3.210D-02	2.927E-02	3.416E+01	2.826D-03
40	5.000E-02	1.500E-01	4.190D-02	4.381E-02	2.283E+01	-1.912D-03
41	7.500E-02	1.500E-01	4.850D-02	5.250E-02	1.905E+01	-4.002D-03
42	1.000E-01	1.500E-01	5.420D-02	5.828E-02	1.716E+01	-4.083D-03

CONST.	VALUE	S.E.	Weight
V=	1.5398D-01	3.7957D-02	6.9411E+02
KA=	1.0655D-01	4.2944D-02	5.4224E+02
KB=	1.1539D-01	4.8696D-02	4.2171E+02
KIA=	-2.5059D-02	8.9532D-03	1.2475E+04
KIB=	-2.7141D-02	9.6825D-03	1.0667E+04
V/KA=	1.4452D+00	2.4562D-01	1.6576E+01
V/KB=	1.3344D+00	2.4845D-01	1.6201E+01

SIGMA= 4.800803D-03

VARIANCE= 2.304771D-05

SEQUENTIAL MECHANISM FIT-OAA VS NADH 01-01-1980 PH8.5

SEQUENTIAL KINETICS FIT

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FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

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#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	2.000E-02	5.000E-02	1.530D-02	1.788E-02	5.594E+01	-2.577D-03
2	4.000E-02	5.000E-02	2.900D-02	2.797E-02	3.575E+01	1.027D-03
3	6.000E-02	5.000E-02	3.070D-02	3.446E-02	2.902E+01	-3.759D-03
4	8.000E-02	5.000E-02	4.230D-02	3.898E-02	2.565E+01	3.321D-03
5	1.000E-01	5.000E-02	4.120D-02	4.231E-02	2.364E+01	-1.108D-03
6	1.200E-01	5.000E-02	4.570D-02	4.486E-02	2.229E+01	8.372D-04
7	2.000E-02	5.000E-02	1.890D-02	1.788E-02	5.594E+01	1.023D-03
8	4.000E-02	5.000E-02	2.740D-02	2.797E-02	3.575E+01	-5.728D-04
9	6.000E-02	5.000E-02	3.200D-02	3.446E-02	2.902E+01	-2.459D-03
10	8.000E-02	5.000E-02	3.670D-02	3.898E-02	2.565E+01	-2.279D-03
11	1.000E-01	5.000E-02	3.700D-02	4.231E-02	2.364E+01	-5.308D-03
12	1.200E-01	5.000E-02	3.960D-02	4.486E-02	2.229E+01	-5.263D-03
13	2.000E-02	1.000E-01	2.390D-02	2.614E-02	3.825E+01	-2.241D-03
14	4.000E-02	1.000E-01	4.300D-02	4.096E-02	2.441E+01	2.038D-03
15	6.000E-02	1.000E-01	5.090D-02	5.051E-02	1.980E+01	3.925D-04
16	8.000E-02	1.000E-01	6.120D-02	5.717E-02	1.749E+01	4.031D-03
17	1.000E-01	1.000E-01	7.230D-02	6.208E-02	1.611E+01	1.022D-02
18	2.000E-02	1.000E-01	2.800D-02	2.614E-02	3.825E+01	1.859D-03
19	4.000E-02	1.000E-01	4.170D-02	4.096E-02	2.441E+01	7.381D-04
20	6.000E-02	1.000E-01	5.260D-02	5.051E-02	1.980E+01	2.092D-03
21	8.000E-02	1.000E-01	6.270D-02	5.717E-02	1.749E+01	5.531D-03
22	1.000E-01	1.000E-01	6.830D-02	6.208E-02	1.611E+01	6.219D-03
23	2.000E-02	1.500E-01	3.160D-02	3.090E-02	3.236E+01	6.981D-04
24	4.000E-02	1.500E-01	5.070D-02	4.846E-02	2.063E+01	2.237D-03
25	6.000E-02	1.500E-01	5.570D-02	5.979E-02	1.673E+01	-4.089D-03
26	8.000E-02	1.500E-01	6.600D-02	6.770E-02	1.477E+01	-1.700D-03
27	1.000E-01	1.500E-01	7.050D-02	7.354E-02	1.360E+01	-3.038D-03
28	1.200E-01	1.500E-01	7.400D-02	7.802E-02	1.282E+01	-4.023D-03
29	2.000E-02	1.500E-01	3.260D-02	3.090E-02	3.236E+01	1.698D-03
30	4.000E-02	1.500E-01	4.740D-02	4.846E-02	2.063E+01	-1.063D-03
31	6.000E-02	1.500E-01	5.040D-02	5.979E-02	1.673E+01	-9.389D-03
32	8.000E-02	1.500E-01	7.050D-02	6.770E-02	1.477E+01	2.800D-03

CONST.	VALUE	S.E.	Weight
V=	1.7916D-01	4.9552D-02	4.0726E+02
KA=	5.3713D-02	3.4131D-02	8.5840E+02
KB=	8.9392D-02	5.1997D-02	3.6986E+02
KIA=	5.0879D-02	3.9052D-02	6.5572E+02
KIB=	8.4675D-02	6.9859D-02	2.0491E+02
V/KA=	3.3355D+00	1.2476D+00	6.4248E-01
V/KB=	2.0042D+00	6.3738D-01	2.4616E+00

SIGMA= 4.059573D-03

VARIANCE= 1.648013D-05

SEQUENTIAL MECHANISM FIT-OAA VS NADH 01-01-1980 PH8.5B

SEQUENTIAL KINETICS FIT

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FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

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#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	2.500E-02	1.000E-01	1.860D-02	1.602E-02	6.242E+01	2.580D-03
2	5.000E-02	1.000E-01	3.330D-02	2.685E-02	3.724E+01	6.446D-03
3	7.500E-02	1.000E-01	4.020D-02	3.467E-02	2.884E+01	5.532D-03
4	1.500E-01	1.000E-01	6.190D-02	4.890E-02	2.045E+01	1.300D-02
5	2.500E-02	1.000E-01	1.770D-02	1.602E-02	6.242E+01	1.680D-03
6	5.000E-02	1.000E-01	2.640D-02	2.685E-02	3.724E+01	-4.535D-04
7	7.500E-02	1.000E-01	3.910D-02	3.467E-02	2.884E+01	4.432D-03
8	1.000E-01	1.000E-01	4.680D-02	4.057E-02	2.465E+01	6.228D-03
9	1.500E-01	1.000E-01	5.320D-02	4.890E-02	2.045E+01	4.301D-03
10	2.500E-02	1.000E-01	1.790D-02	1.602E-02	6.242E+01	1.880D-03
11	5.000E-02	1.000E-01	2.610D-02	2.685E-02	3.724E+01	-7.535D-04
12	7.500E-02	1.000E-01	3.610D-02	3.467E-02	2.884E+01	1.432D-03
13	1.000E-01	1.000E-01	4.120D-02	4.057E-02	2.465E+01	6.279D-04
14	1.500E-01	1.000E-01	5.060D-02	4.890E-02	2.045E+01	1.701D-03
15	2.500E-02	1.500E-01	1.550D-02	1.761E-02	5.680E+01	-2.106D-03
16	5.000E-02	1.500E-01	2.720D-02	2.996E-02	3.338E+01	-2.762D-03
17	7.500E-02	1.500E-01	4.020D-02	3.911E-02	2.557E+01	1.088D-03
18	1.000E-01	1.500E-01	4.570D-02	4.616E-02	2.166E+01	-4.596D-04
19	1.500E-01	1.500E-01	5.600D-02	5.631E-02	1.776E+01	-3.063D-04
20	2.500E-02	1.500E-01	1.340D-02	1.761E-02	5.680E+01	-4.206D-03
21	5.000E-02	1.500E-01	2.680D-02	2.996E-02	3.338E+01	-3.162D-03
22	7.500E-02	1.500E-01	3.040D-02	3.911E-02	2.557E+01	-8.712D-03
23	1.000E-01	1.500E-01	4.660D-02	4.616E-02	2.166E+01	4.404D-04
24	1.500E-01	1.500E-01	4.680D-02	5.631E-02	1.776E+01	-9.506D-03
25	2.500E-02	1.500E-01	1.770D-02	1.761E-02	5.680E+01	9.416D-05
26	5.000E-02	1.500E-01	2.900D-02	2.996E-02	3.338E+01	-9.619D-04
27	7.500E-02	1.500E-01	4.440D-02	3.911E-02	2.557E+01	5.288D-03
28	1.000E-01	1.500E-01	4.570D-02	4.616E-02	2.166E+01	-4.596D-04
29	1.500E-01	1.500E-01	5.220D-02	5.631E-02	1.776E+01	-4.106D-03
30	2.500E-02	5.000E-02	1.220D-02	1.261E-02	7.929E+01	-4.117D-04
31	5.000E-02	5.000E-02	1.910D-02	2.048E-02	4.883E+01	-1.380D-03
32	7.500E-02	5.000E-02	2.360D-02	2.586E-02	3.868E+01	-2.256D-03
33	1.000E-01	5.000E-02	2.720D-02	2.976E-02	3.360E+01	-2.564D-03
34	1.500E-01	5.000E-02	3.210D-02	3.506E-02	2.852E+01	-2.962D-03
35	2.500E-02	5.000E-02	1.200D-02	1.261E-02	7.929E+01	-6.117D-04
36	5.000E-02	5.000E-02	1.880D-02	2.048E-02	4.883E+01	-1.680D-03
37	7.500E-02	5.000E-02	2.290D-02	2.586E-02	3.868E+01	-2.956D-03
38	1.000E-01	5.000E-02	2.820D-02	2.976E-02	3.360E+01	-1.564D-03
39	1.500E-01	5.000E-02	2.960D-02	3.506E-02	2.852E+01	-5.462D-03

CONST.	VALUE	S.E.	Weight
V=	1.7407D-01	9.1671D-02	1.1900E+02
KA=	1.7324D-01	1.3920D-01	5.1611E+01
KB=	1.0986D-01	1.1156D-01	8.0349E+01
KIA=	4.1824D-02	6.8022D-02	2.1612E+02
KIB=	2.6522D-02	3.7920D-02	6.9543E+02
V/KA=	1.0048D+00	2.9565D-01	1.1440E+01
V/KB=	1.5845D+00	8.0374D-01	1.5480E+00

SIGMA= 4.334628D-03

VARIANCE= 1.878900D-05

SEQUENTIAL MECHANISM FIT-OAA VS NADH 01-01-1980 PH9

SEQUENTIAL KINETICS FIT

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FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

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#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	2.000E-02	1.350D-02	1.231E-02	8.125E+01	1.193D-03
2	5.000E-02	4.000E-02	1.980D-02	2.062E-02	4.850E+01	-8.196D-04
3	5.000E-02	6.000E-02	2.790D-02	2.661E-02	3.758E+01	1.289D-03
4	5.000E-02	8.000E-02	3.500D-02	3.113E-02	3.212E+01	3.866D-03
5	5.000E-02	1.000E-01	3.590D-02	3.467E-02	2.884E+01	1.230D-03
6	5.000E-02	2.000E-02	1.140D-02	1.231E-02	8.125E+01	-9.071D-04
7	5.000E-02	1.200E-01	3.870D-02	3.751E-02	2.666E+01	1.191D-03
8	5.000E-02	4.000E-02	2.350D-02	2.062E-02	4.850E+01	2.880D-03
9	5.000E-02	6.000E-02	2.490D-02	2.661E-02	3.758E+01	-1.711D-03
10	5.000E-02	8.000E-02	3.120D-02	3.113E-02	3.212E+01	6.617D-05
11	5.000E-02	1.000E-01	3.590D-02	3.467E-02	2.884E+01	1.230D-03
12	5.000E-02	1.200E-01	3.660D-02	3.751E-02	2.666E+01	-9.094D-04
13	1.000E-01	2.000E-02	1.660D-02	1.739E-02	5.751E+01	-7.888D-04
14	1.000E-01	4.000E-02	3.110D-02	2.988E-02	3.346E+01	1.218D-03
15	1.000E-01	6.000E-02	3.630D-02	3.929E-02	2.545E+01	-2.992D-03
16	1.000E-01	8.000E-02	4.200D-02	4.663E-02	2.144E+01	-4.635D-03
17	1.000E-01	1.000E-01	5.440D-02	5.252E-02	1.904E+01	1.876D-03
18	1.000E-01	1.200E-01	5.780D-02	5.735E-02	1.744E+01	4.472D-04
19	1.000E-01	2.000E-02	1.790D-02	1.739E-02	5.751E+01	5.112D-04
20	1.000E-01	4.000E-02	2.610D-02	2.988E-02	3.346E+01	-3.782D-03
21	1.000E-01	6.000E-02	4.010D-02	3.929E-02	2.545E+01	8.079D-04
22	1.000E-01	8.000E-02	4.290D-02	4.663E-02	2.144E+01	-3.735D-03
23	1.000E-01	1.000E-01	5.210D-02	5.252E-02	1.904E+01	-4.242D-04
24	1.000E-01	1.200E-01	5.350D-02	5.735E-02	1.744E+01	-3.853D-03
25	1.500E-01	2.000E-02	1.690D-02	2.016E-02	4.959E+01	-3.264D-03
26	1.500E-01	4.000E-02	3.790D-02	3.514E-02	2.845E+01	2.756D-03
27	1.500E-01	6.000E-02	4.750D-02	4.671E-02	2.141E+01	7.876D-04
28	1.500E-01	8.000E-02	6.290D-02	5.591E-02	1.788E+01	6.985D-03
29	1.500E-01	1.000E-01	7.170D-02	6.341E-02	1.577E+01	8.291D-03
30	1.500E-01	1.200E-01	7.130D-02	6.963E-02	1.436E+01	1.668D-03
31	1.500E-01	2.000E-02	2.160D-02	2.016E-02	4.959E+01	1.436D-03
32	1.500E-01	4.000E-02	3.790D-02	3.514E-02	2.845E+01	2.756D-03
33	1.500E-01	6.000E-02	4.520D-02	4.671E-02	2.141E+01	-1.512D-03
34	1.500E-01	8.000E-02	4.920D-02	5.591E-02	1.788E+01	-6.715D-03
35	1.500E-01	1.000E-01	5.980D-02	6.341E-02	1.577E+01	-3.609D-03
36	1.500E-01	1.200E-01	6.780D-02	6.963E-02	1.436E+01	-1.832D-03

CONST.	VALUE	S.E.	Weight
V=	3.2239D-01	1.9440D-01	2.6461E+01
KA=	2.0374D-01	1.8525D-01	2.9140E+01
KB=	1.9770D-01	1.6785D-01	3.5495E+01
KIA=	5.6835D-02	4.1697D-02	5.7516E+02
KIB=	5.5149D-02	4.3826D-02	5.2065E+02
V/KA=	1.5824D+00	5.0793D-01	3.8761E+00
V/KB=	1.6308D+00	4.2140D-01	5.6313E+00

SIGMA= 3.206776D-03

VARIANCE= 1.028341D-05

SEQUENTIAL MECHANISM FIT-NADH VS OAA 01-01-1980 PH9A

SEQUENTIAL KINETICS FIT

****

FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

****

#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	2.000E-02	5.000E-02	1.340D-02	1.321E-02	7.568E+01	1.866D-04
2	4.000E-02	5.000E-02	2.240D-02	2.274E-02	4.398E+01	-3.373D-04
3	6.000E-02	5.000E-02	2.910D-02	2.993E-02	3.341E+01	-8.277D-04
4	8.000E-02	5.000E-02	3.370D-02	3.555E-02	2.813E+01	-1.849D-03
5	1.000E-01	5.000E-02	4.070D-02	4.006E-02	2.496E+01	6.365D-04
6	1.200E-01	5.000E-02	4.400D-02	4.377E-02	2.285E+01	2.306D-04
7	2.000E-02	1.000E-01	1.970D-02	1.783E-02	5.609E+01	1.871D-03
8	4.000E-02	1.000E-01	3.410D-02	3.039E-02	3.291E+01	3.712D-03
9	6.000E-02	1.000E-01	3.860D-02	3.971E-02	2.518E+01	-1.113D-03
10	8.000E-02	1.000E-01	4.470D-02	4.691E-02	2.132E+01	-2.210D-03
11	1.000E-01	1.000E-01	5.610D-02	5.263E-02	1.900E+01	3.466D-03
12	1.200E-01	1.000E-01	5.730D-02	5.729E-02	1.745E+01	5.449D-06
13	2.000E-02	1.500E-01	1.720D-02	2.018E-02	4.956E+01	-2.978D-03
14	4.000E-02	1.500E-01	3.410D-02	3.423E-02	2.922E+01	-1.263D-04
15	6.000E-02	1.500E-01	4.800D-02	4.457E-02	2.244E+01	3.430D-03
16	8.000E-02	1.500E-01	4.860D-02	5.250E-02	1.905E+01	-3.904D-03
17	1.000E-01	1.500E-01	5.770D-02	5.878E-02	1.701E+01	-1.082D-03
18	1.200E-01	1.500E-01	6.480D-02	6.387E-02	1.566E+01	9.262D-04

CONST.	VALUE	S.E.	Weight
V=	1.3942D-01	3.5581D-02	7.8989E+02
KA=	8.1771D-02	4.1733D-02	5.7418E+02
KB=	3.5604D-02	3.3665D-02	8.8234E+02
KIA=	1.3343D-01	1.5182D-01	4.3384E+01
KIB=	5.8097D-02	4.0672D-02	6.0453E+02
V/KA=	1.7050D+00	4.5022D-01	4.9335E+00
V/KB=	3.9158D+00	2.7657D+00	1.3074E-01

SIGMA= 2.361086D-03

VARIANCE= 5.574728D-06

SEQUENTIAL MECHANISM FIT-OAA VS NADH 01-01-1980 II158PH9

pH PROFILE FIT - HBBELL

****

FIT TO LOG (Y) = LOG [C / (1 + Kb/H)]

****

	pH	Yobs	Ycalc	Diff	LOGYobs	LOGYcal	Diff
1	6.00	2.300D+00	2.674D+00	-3.741D-01	3.617D-01	4.272D-01	-6.546D-02
2	6.50	3.500D+00	2.670D+00	8.302D-01	5.441D-01	4.265D-01	1.176D-01
3	7.00	5.000D+00	2.656D+00	2.344D+00	6.990D-01	4.242D-01	2.747D-01
4	7.50	1.620D+00	2.613D+00	-9.935D-01	2.095D-01	4.172D-01	-2.077D-01
5	8.00	2.130D+00	2.488D+00	-3.575D-01	3.284D-01	3.958D-01	-6.739D-02
6	8.50	1.620D+00	2.159D+00	-5.385D-01	2.095D-01	3.342D-01	-1.246D-01
7	9.00	1.800D+00	1.522D+00	2.780D-01	2.553D-01	1.824D-01	7.286D-02

CONST.	VALUE	S.E.
C	2.6762D+00	2.2282D-01
1/C	3.7367D-01	3.1111D-02
pKb	9.1201D+00	1.9992D-01

SIGMA = 1.8007D-01

VARIANCE = 3.2424D-02

The variable V/K decreases as the pH increases 01-01-1980 VKNADH

pH PROFILE FIT - HBBELL

****

FIT TO LOG (Y) = LOG [C / (1 + Kb/H)]

****

	pH	Yobs	Ycalc	Diff	LOGYobs	LOGYcal	Diff
1	6.00	4.900D+00	4.677D+00	2.231D-01	6.902D-01	6.700D-01	2.024D-02
2	6.50	5.000D+00	4.648D+00	3.524D-01	6.990D-01	6.672D-01	3.174D-02
3	7.00	4.800D+00	4.558D+00	2.425D-01	6.812D-01	6.587D-01	2.251D-02
4	7.50	4.500D+00	4.294D+00	2.057D-01	6.532D-01	6.329D-01	2.032D-02
5	8.00	2.900D+00	3.631D+00	-7.311D-01	4.624D-01	5.600D-01	-9.764D-02
6	8.50	2.100D+00	2.440D+00	-3.396D-01	3.222D-01	3.873D-01	-6.510D-02
7	9.00	1.400D+00	1.197D+00	2.027D-01	1.461D-01	7.820D-02	6.793D-02

CONST.	VALUE	S.E.
C	4.6905D+00	1.4988D-01
1/C	2.1320D-01	6.8123D-03
pKb	8.5350D+00	3.9965D-02

SIGMA = 6.4379D-02

VARIANCE = 4.1447D-03

The variable V/K decreases as the pH increases 01-01-1980 VKOAA

SUBSTRATE INHIBITION

****

FIT TO Y = V*A/(K+A+A^2/KI)

****

#	[A]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	7.860D-02	7.449E-02	1.342E+01	4.106D-03
2	1.000E-01	1.006D-01	9.259E-02	1.080E+01	8.005D-03
3	2.000E-01	9.920D-02	9.661E-02	1.035E+01	2.587D-03
4	3.000E-01	9.310D-02	9.118E-02	1.097E+01	1.924D-03
5	4.000E-01	8.910D-02	8.438E-02	1.185E+01	4.723D-03
6	5.000E-01	7.790D-02	7.787E-02	1.284E+01	3.108D-05
7	5.000E-02	6.970D-02	7.449E-02	1.342E+01	-4.794D-03
8	1.000E-01	8.630D-02	9.259E-02	1.080E+01	-6.295D-03
9	2.000E-01	9.220D-02	9.661E-02	1.035E+01	-4.413D-03
10	3.000E-01	8.840D-02	9.118E-02	1.097E+01	-2.776D-03
11	4.000E-01	8.300D-02	8.438E-02	1.185E+01	-1.377D-03
12	5.000E-01	7.610D-02	7.787E-02	1.284E+01	-1.769D-03

CONST.	VALUE	S.E.	Weight
V=	1.5504D-01	2.6869D-03	1.3851E+05
K=	4.9604D-02	1.9767D-03	2.5592E+05
K/V=	3.1994D-01	7.4838D-03	1.7855E+04
1/V=	6.4499D+00	1.1178D-01	8.0034E+01
KI=	5.6063D-01	2.2737D-02	1.9343E+03
1/KI=	1.7837E+00	7.2340D-02	1.9109E+02

SIGMA= 5.896387D-04

VARIANCE= 3.476738D-07

SUBSTRATE INHIBITION-OAA 01-01-1980 INHIBPH6

SUBSTRATE INHIBITION

****

FIT TO Y = V*A/(K+A+A^2/KI)

****

#	[A]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	1.159D-01	1.212E-01	8.251E+00	-5.301D-03
2	1.000E-01	1.641D-01	1.596E-01	6.264E+00	4.455D-03
3	2.000E-01	1.751D-01	1.719E-01	5.816E+00	3.167D-03
4	3.000E-01	1.593D-01	1.625E-01	6.152E+00	-3.246D-03
5	4.000E-01	1.664D-01	1.496E-01	6.684E+00	1.679D-02
6	5.000E-01	1.404D-01	1.371E-01	7.294E+00	3.301D-03
7	5.000E-02	1.261D-01	1.212E-01	8.251E+00	4.899D-03
8	1.000E-01	1.561D-01	1.596E-01	6.264E+00	-3.545D-03
9	2.000E-01	1.686D-01	1.719E-01	5.816E+00	-3.333D-03
10	3.000E-01	1.614D-01	1.625E-01	6.152E+00	-1.146D-03
11	4.000E-01	1.409D-01	1.496E-01	6.684E+00	-8.714D-03
12	5.000E-01	1.297D-01	1.371E-01	7.294E+00	-7.399D-03

CONST.	VALUE	S.E.	Weight
V=	3.1392D-01	1.5437D-02	4.1964E+03
K=	7.3793D-02	6.9446D-03	2.0735E+04
K/V=	2.3507D-01	1.1072D-02	8.1572E+03
1/V=	3.1855D+00	1.5665D-01	4.0753E+01
KI=	4.3777D-01	4.3670D-02	5.2436E+02
1/KI=	2.2843E+00	2.2788D-01	1.9258E+01

SIGMA= 2.466204D-03

VARIANCE= 6.082160D-06

SUBSTRATE INHIBITION-OAA 01-01-1980 INHIBPH6.5

SUBSTRATE INHIBITION

****

FIT TO Y = V*A/(K+A+A^2/KI)

****

#	[A]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	7.040D-02	6.999E-02	1.429E+01	4.072D-04
2	1.000E-01	8.560D-02	8.729E-02	1.146E+01	-1.693D-03
3	2.000E-01	9.960D-02	9.469E-02	1.056E+01	4.912D-03
4	3.000E-01	8.630D-02	9.323E-02	1.073E+01	-6.932D-03
5	4.000E-01	9.350D-02	8.964E-02	1.116E+01	3.860D-03
6	5.000E-01	8.500D-02	8.553E-02	1.169E+01	-5.322D-04

CONST.	VALUE	S.E.	Weight
V=	1.3189D-01	1.4541D-03	4.7296E+05
K=	4.1926D-02	1.2078D-03	6.8548E+05
K/V=	3.1789D-01	5.8638D-03	2.9084E+04
1/V=	7.5821D+00	8.3593D-02	1.4311E+02
KI=	1.0914D+00	4.1986D-02	5.6727E+02
1/KI=	9.1627E-01	3.5249D-02	8.0483E+02

SIGMA= 3.072806D-04

VARIANCE= 9.442138D-08

SUBSTRATE INHIBITION-OAA 01-01-1980 INHIBPH7

SUBSTRATE INHIBITION

****

FIT TO Y = V*A/(K+A+A^2/KI)

****

#	[A]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	6.060D-02	6.043E-02	1.655E+01	1.697D-04
2	1.000E-01	7.740D-02	7.838E-02	1.276E+01	-9.811D-04
3	2.000E-01	8.820D-02	8.515E-02	1.174E+01	3.054D-03
4	3.000E-01	7.900D-02	8.203E-02	1.219E+01	-3.034D-03
5	4.000E-01	7.620D-02	7.692E-02	1.300E+01	-7.212D-04
6	5.000E-01	7.320D-02	7.165E-02	1.396E+01	1.549D-03

CONST.	VALUE	S.E.	Weight
V=	1.3878D-01	6.1280D-03	2.6629E+04
K=	6.0747D-02	5.6871D-03	3.0919E+04
K/V=	4.3773D-01	2.2622D-02	1.9541E+03
1/V=	7.2058D+00	3.1819D-01	9.8772E+00
KI=	6.1323D-01	6.5476D-02	2.3326E+02
1/KI=	1.6307E+00	1.7411D-01	3.2986E+01

SIGMA= 8.942098D-04

VARIANCE= 7.996113D-07

SUBSTRATE INHIBITION-OAA 01-01-1980 INHIBPH7.5

SUBSTRATE INHIBITION

****

FIT TO Y = V*A/(K+A+A^2/KI)

****

#	[A]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	5.500D-02	5.409E-02	1.849E+01	9.114D-04
2	1.000E-01	7.160D-02	7.345E-02	1.361E+01	-1.853D-03
3	2.000E-01	8.410D-02	8.212E-02	1.218E+01	1.977D-03
4	3.000E-01	7.850D-02	7.945E-02	1.259E+01	-9.504D-04

CONST.	VALUE	S.E.	Weight
V=	1.4836D-01	1.4347D-02	4.8582E+03
K=	8.2201D-02	1.3557D-02	5.4412E+03
K/V=	5.5406D-01	3.9111D-02	6.5375E+02
1/V=	6.7404D+00	6.5183D-01	2.3536E+00
KI=	5.0563D-01	1.2565D-01	6.3338E+01
1/KI=	1.9777E+00	4.9147D-01	4.1400E+00

SIGMA= 9.504434D-04

VARIANCE= 9.033427D-07

SUBSTRATE INHIBITION-OAA 01-01-1980 INHIBPH8

SUBSTRATE INHIBITION

****

FIT TO Y = V*A/(K+A+A^2/KI)

****

#	[A]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	4.960D-02	4.832E-02	2.069E+01	1.276D-03
2	1.000E-01	6.450D-02	6.312E-02	1.584E+01	1.384D-03
3	2.000E-01	8.390D-02	7.421E-02	1.348E+01	9.691D-03
4	3.000E-01	8.840D-02	7.851E-02	1.274E+01	9.885D-03
5	4.000E-01	9.020D-02	8.061E-02	1.240E+01	9.585D-03
6	5.000E-01	8.880D-02	8.173E-02	1.224E+01	7.073D-03
7	2.000E-01	5.600D-02	7.421E-02	1.348E+01	-1.821D-02
8	3.000E-01	6.840D-02	7.851E-02	1.274E+01	-1.011D-02
9	4.000E-01	7.920D-02	8.061E-02	1.240E+01	-1.415D-03
10	5.000E-01	8.090D-02	8.173E-02	1.224E+01	-8.272D-04
11	6.000E-01	7.420D-02	8.232E-02	1.215E+01	-8.115D-03

CONST.	VALUE	S.E.	Weight
V=	9.1906D-02	7.0977D-03	1.9850E+04
K=	4.4919D-02	1.0835D-02	8.5176E+03
K/V=	4.8875D-01	8.3588D-02	1.4312E+02
1/V=	1.0881D+01	8.4030D-01	1.4162E+00
KI=	1.4407D+01	3.0850D+01	1.0507E-03
1/KI=	6.9409E-02	1.4862D-01	4.5271E+01

SIGMA= 2.869127D-03

VARIANCE= 8.231891D-06

SUBSTRATE INHIBITION-OAA 01-01-1980 INHIBPH8.5

SUBSTRATE INHIBITION

****

FIT TO Y = V*A/(K+A+A^2/KI)

****

#	[A]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-02	3.200D-02	3.125E-02	3.200E+01	7.528D-04
2	1.000E-01	4.910D-02	4.861E-02	2.057E+01	4.932D-04
3	2.000E-01	6.430D-02	6.718E-02	1.489E+01	-2.878D-03
4	3.000E-01	7.730D-02	7.684E-02	1.301E+01	4.627D-04
5	4.000E-01	8.640D-02	8.266E-02	1.210E+01	3.736D-03
6	5.000E-01	8.420D-02	8.650E-02	1.156E+01	-2.295D-03

CONST.	VALUE	S.E.	Weight
V=	1.1003D-01	9.5318D-03	1.1006E+04
K=	1.2596D-01	1.9575D-02	2.6099E+03
K/V=	1.1448D+00	8.3460D-02	1.4356E+02
1/V=	9.0887D+00	7.8738D-01	1.6130E+00
KI=	2.4834D+01	1.0186D+02	9.6387E-05
1/KI=	4.0268E-02	1.6516D-01	3.6659E+01

SIGMA= 1.325228D-03

VARIANCE= 1.756229D-06

SUBSTRATE INHIBITION-OAA 01-01-1980 INHIBPH9

SEQUENTIAL KINETICS FIT

****

FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB)

****

#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	5.000E-01	5.000E-01	2.900D-02	3.315E-02	3.017E+01	-4.146D-03
2	5.000E-01	1.000E+00	5.220D-02	5.156E-02	1.939E+01	6.360D-04
3	5.000E-01	2.000E+00	6.730D-02	7.140E-02	1.401E+01	-4.102D-03
4	5.000E-01	3.000E+00	8.520D-02	8.191E-02	1.221E+01	3.295D-03
5	5.000E-01	5.000E+00	8.850D-02	9.283E-02	1.077E+01	-4.330D-03
6	5.000E-01	5.000E-01	3.410D-02	3.315E-02	3.017E+01	9.541D-04
7	5.000E-01	1.000E+00	5.130D-02	5.156E-02	1.939E+01	-2.640D-04
8	5.000E-01	2.000E+00	7.630D-02	7.140E-02	1.401E+01	4.898D-03
9	5.000E-01	3.000E+00	8.320D-02	8.191E-02	1.221E+01	1.295D-03
10	5.000E-01	5.000E+00	9.510D-02	9.283E-02	1.077E+01	2.270D-03
11	2.000E-01	5.000E-01	8.100D-03	9.957E-03	1.004E+02	-1.857D-03
12	2.000E-01	1.000E+00	1.560D-02	1.714E-02	5.835E+01	-1.539D-03
13	2.000E-01	2.000E+00	2.670D-02	2.681E-02	3.730E+01	-1.074D-04
14	2.000E-01	3.000E+00	3.100D-02	3.302E-02	3.029E+01	-2.016D-03
15	2.000E-01	5.000E+00	3.910D-02	4.052E-02	2.468E+01	-1.424D-03
16	2.000E-01	1.000E+01	4.370D-02	4.886E-02	2.047E+01	-5.157D-03
17	5.000E-02	5.000E-01	3.600D-03	2.214E-03	4.518E+02	1.386D-03
18	5.000E-02	1.000E+00	5.200D-03	3.951E-03	2.531E+02	1.249D-03
19	5.000E-02	2.000E+00	9.900D-03	6.502E-03	1.538E+02	3.398D-03
20	5.000E-02	3.000E+00	1.340D-02	8.286E-03	1.207E+02	5.114D-03
21	5.000E-02	5.000E+00	1.820D-02	1.062E-02	9.420E+01	7.584D-03
22	5.000E-02	1.000E+01	2.200D-02	1.345E-02	7.433E+01	8.547D-03

CONST.	VALUE	S.E.	Weight
V=	2.8387D-01	1.1541D-01	7.5075E+01
KA=	7.2309D-01	4.7197D-01	4.4892E+00
KB=	-2.8667D+00	1.0439D+00	9.1758E-01
KIA=	-1.0335D+00	-3.2403D-01	9.5244E+00
KIB=	4.0976D+00	2.0316D+00	2.4228E-01
V/KA=	3.9258D-01	9.9164D-02	1.0169E+02
V/KB=	-9.9023D-02	-5.7207D-02	3.0556E+02

SIGMA= 4.123697D-03

VARIANCE= 1.700487D-05

SEQUENTIAL MECHANISM FIT-NAD VS MALATE 01-01-1980 RPH9A

SEQUENTIAL KINETICS FIT

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FIT TO Y = V*A*B/(KA*B+KB*A+A*B + KIA*KB).

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#	[A]	[B]	Vobs	Vcal	1/Vcal	Diff
1	6.500E-02	5.000E-01	3.600D-03	-6.244E-03	-1.602E+02	9.844D-03
2	6.500E-02	1.000E+00	5.200D-03	-2.310E-02	-4.329E+01	2.830D-02
3	6.500E-02	2.000E+00	9.900D-02	6.603E-02	1.514E+01	3.297D-02
4	6.500E-02	3.000E+00	1.340D-02	2.888E-02	3.462E+01	-1.548D-02
5	6.500E-02	5.000E+00	1.820D-02	1.992E-02	5.021E+01	-1.718D-03
6	6.500E-02	1.000E+01	2.200D-02	1.616E-02	6.189E+01	5.843D-03
7	1.300E-01	5.000E-01	7.500D-03	-5.175E-02	-1.932E+01	5.925D-02
8	1.300E-01	1.000E+00	2.200D-02	1.003E-01	9.968E+00	-7.832D-02
9	1.300E-01	2.000E+00	3.190D-02	4.063E-02	2.461E+01	-8.726D-03
10	1.300E-01	3.000E+00	3.250D-02	3.390E-02	2.950E+01	-1.402D-03
11	1.300E-01	5.000E+00	4.240D-02	2.994E-02	3.340E+01	1.246D-02
12	1.300E-01	5.000E-01	9.800D-03	-5.175E-02	-1.932E+01	6.155D-02
13	1.300E-01	1.000E+00	1.600D-02	1.003E-01	9.968E+00	-8.432D-02
14	1.300E-01	2.000E+00	2.840D-02	4.063E-02	2.461E+01	-1.223D-02
15	1.300E-01	3.000E+00	3.480D-02	3.390E-02	2.950E+01	8.982D-04
16	1.300E-01	5.000E+00	3.560D-02	2.994E-02	3.340E+01	5.662D-03
17	4.550E-01	5.000E-01	3.020D-02	1.230E-02	8.127E+01	1.790D-02
18	4.550E-01	5.000E-01	2.580D-02	1.230E-02	8.127E+01	1.350D-02
19	4.550E-01	1.000E+00	4.730D-02	2.083E-02	4.801E+01	2.647D-02
20	4.550E-01	1.000E+00	4.930D-02	2.083E-02	4.801E+01	2.847D-02
21	4.550E-01	2.000E+00	5.710D-02	3.187E-02	3.138E+01	2.523D-02
22	4.550E-01	2.000E+00	6.430D-02	3.187E-02	3.138E+01	3.243D-02
23	4.550E-01	3.000E+00	6.260D-02	3.871E-02	2.584E+01	2.389D-02
24	4.550E-01	3.000E+00	6.500D-02	3.871E-02	2.584E+01	2.629D-02

CONST.	VALUE	S.E.	Weight
V=	2.0229D-01	9.0725D-01	1.2149E+00
KA=	9.0250D-01	3.9365D+00	6.4533E-02
KB=	1.1791D+01	4.6673D+01	4.5906E-04
KIA=	-1.9534D-01	6.5453D-02	2.3342E+02
KIB=	-2.5519D+00	1.8854D+00	2.8131E-01
V/KA=	2.2415D-01	5.3834D-02	3.4506E+02
V/KB=	1.7157D-02	1.0178D-02	9.6526E+03

SIGMA= 3.753881D-02

VARIANCE= 1.409162D-03

SEQUENTIAL MECHANISM FIT-NAD VS MALATE 01-01-1980 RPH9B