THE PARTIAL PURIFICATION AND CHARACTERIZATION OF SOLUBLE AND MITOCHONDRIAL MALATE DEHYDROGENASE FROM THE WHITE SHRIMP (PENAEUS SETIFERUS)

A Thesis

Presented to

the Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Joseph Edwin Evans

~~December, 1970~~

May, 1971

558150

THE PARTIAL PURIFICATION AND CHARACTERIZATION OF SOLUBLE AND MITOCHONDRIAL MALATE DEHYDROGENASE FROM THE WHITE SHRIMP (PENAEUS SETIFERUS)

An Abstract

Presented to

the Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Joseph Edwin Evans

~~December, 1970~~

May, 1971

ACKNOWLEDGMENTS

I wish to thank my advisor Dr. A.H. Bartel and my co-advisor Dr. J.E. Evans for their advice and help throughout the course of this research. I thank Dr. H. B. Gray, Jr. and Daryl Ostrander for their guidance and assistance during the molecular weight determination. I also wish to thank my wife, Elaine, for typing this thesis.

I especially wish to thank the Robert A. Welch Foundation for grant number E-195 without which this research could not have been possible.

ABSTRACT

Malate dehydrogenase was extracted from the muscle tissue of the White shrimp, Penaeus setiferus, and partially purified by successive ammonium sulfate fractionation and negative adsorption on carboxymethyl cellulose. The enzyme was separated into the soluble and mitochondrial form by diethylaminoethyl cellulose chromatography.

Soluble and mitochondrial malate dehydrogenase were then characterized by a variety of physical-chemical and catalytic tests. Some of these tests included: polyacrylamide gel electrophoresis, Michaelis constants, NAD analogue ratios, substrate inhibition, thermostability, and molecular weight determinations.

The results of these tests indicate that White shrimp malate dehydrogenase is in many ways similar to the malate dehydrogenases isolated from other organisms but differs markedly in respect to molecular weight and inhibition by oxalacetate and malate.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE PAGE

LIST OF TABLES

LIST OF PLATES

INTRODUCTION

Throughout the early 1900's an enzyme was regarded as a single entity. This hypothesis was not seriously questioned until 1943 when Warburg and Christian demonstrated that yeast aldolase differed in several respects from that obtained from animal tissues. Even within the same species, enzymes with similar catalytic properties were found to differ markedly in their physical-chemical properties.[1]

The term isoenzyme or isozyme was coined to describe enzymatically active proteins, catalyzing the same reaction and occurring in the same species, but differing in their catalytic and physical-chemical properties.[1] Interest in these enzymes showing multiple molecular forms has become widespread in the past two decades. Isoenzymes such as lactate dehydrogenase (LDH) and alkaline phosphatase have been used as diagnostic agents in clinical chemistry while others such as cholinesterase and malate dehydrogenase (MDH) have been used as a tool in genetic research.

The isoenzyme malate dehydrogenase, or L-malate: NAD oxidoreductase E. C. 1.1.1.37, was discovered by Thumberg, Batelli, and Stern in 1910 and was first isolated in the pure state from pig heart by Straub.[2] Since human serum malate dehydrogenase was separated by starch-block electrophoresis into three distinct fractions by Vesell and Bearn in 1958, many other investigators have demonstrated its heterogeneity in tissues from various species of animals, plants, and micro-organisms.[1]

The enzyme is a member of the tricarboxylic acid cycle (TCA) and catalyzes the following reaction:

$$\text{L-malate} + \text{DPN} \underset{\text{sMDH}}{\overset{\text{mMDH}}{\rightleftharpoons}} \text{oxalacetate} + \text{DPNH} + \text{H}^{+}$$

Studies during the late 1950's indicated that mammalian MDH from the cytoplasm differed from that isolated from mitochondria in electrophoretic mobility, kinetics, and relative ability to utilize co-enzyme analogues. [3,4,5]

Researchers devised many tests to prove that the mitochondrial and soluble form of MDH were separate entities differing in their physical-chemical and kinetic properties. These tests included the susceptibility to inhibition by high oxalacetate concentrations, comparison of electrophoretic mobilities, thermostability tests, and Michaelis constants. The vast majority of MDH's tested showed a definite pattern when subjected to these tests. Of these, the most consistant were: that the mitochondrial enzyme is inhibited to a greater extent by high concentration of oxalacetate than is the soluble enzyme and that the mitochondrial enzyme is more sensitive to heat than is the soluble form.

The Michaelis constants and turnover numbers calculated by Grimm and Doherty in 1961 for ox heart mMDH and sMDH are shown in Table 1. [1] These marked differences suggest that the mitochondrial enzyme is better suited for the oxidation of malate, while the soluble enzyme is a more efficient catalyst for the reduction of oxalacetate. These findings led Kaplan to conclude that these

charcteristics may prevent the reduction of oxalacetate in mitochondria and the oxidation of malate in the soluble fraction.

Figure 1[6] shows Kaplan's model proposing possible functions of mitochondrial and soluble malate dehydrogenase. He envisions the oxidation of soluble $NADH_2$ by oxalacetate to form malate, which enters the mitochondria and is again oxidized by the mitochondrial enzyme and mitochondrial bound NAD. The oxalacetate produced might then be released into the cytoplasm and the cycle repeated.

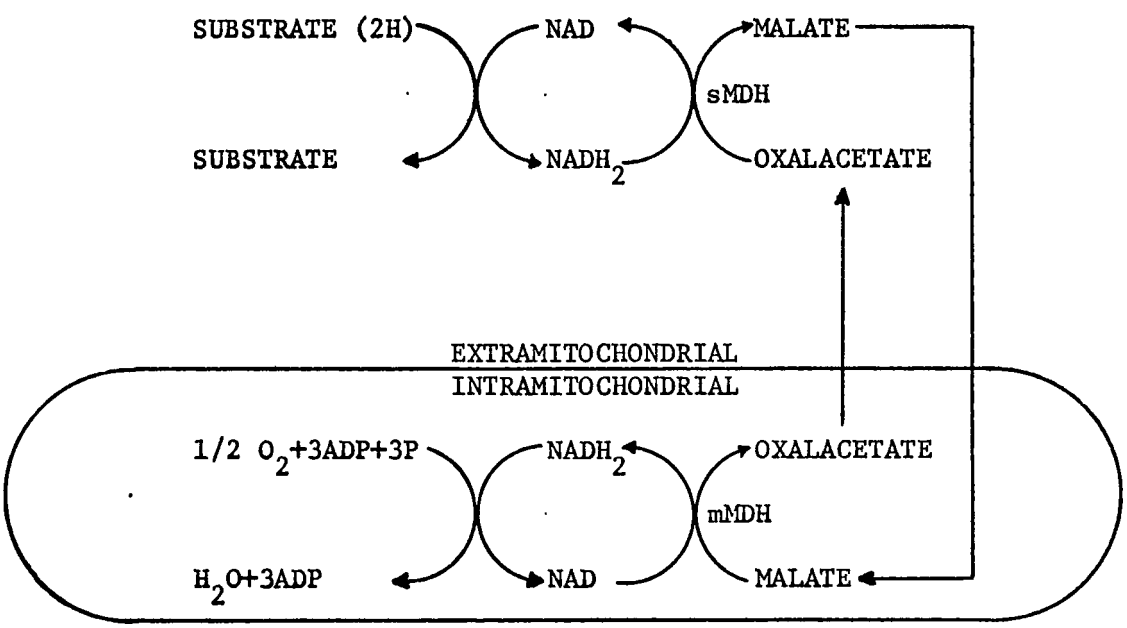
Table 1

Michaelis constants and turnover numbers for purified mitochondrial and soluble ox-heart malate dehydrogenase.[1]

	mMDH	sMDH
Km (malate)	9.9×10^{-4}	5.4×10^{-4}
Km (oxalacetate)	4.0×10^{-5}	5.1×10^{-5}
Turnover numbers (malate and NAD) (moles NAD reduced/min./mole enzyme)	35,000	20,000
Turnover numbers (oxalacetate and $NADH_2$) (moles $NADH_2$ oxidized/min./mole enzyme)	59,000	72,000

Figure 1

Possible function of mitochondrial and soluble malate dehydrogenase as proposed by Kaplan.


SUBSTRATE (2H)
NAD
MALATE
sMDH
SUBSTRATE
NADH2
OXALACETATE
EXTRAMITOCHONDRIAL
INTRAMITOCHONDRIAL
1/2 O2+3ADP+3P
NADH2
OXALACETATE
mMDH
H2O+3ADP
NAD
MALATE

For the past three years our laboratory has been investigating the comparative protein biochemistry of three closely related species of Gulf Coast shrimp, the white Penaeus setiferus, the brown Penaeus aztecus, and the pink Penaeus duorarum.

These three species can be easily identified and separated in the adult stages of their life cycle,[7] but are hard to differentiate in the post larval and juvenile stages. Due to their commercial importance as a food product,[7] fish biologists must keep records of population density so they will be able to predict the relative numbers of shrimp available for a given shrimping season. Differentiation of these species is done microscopically which is slow and requires a highly trained observer.

In 1967 Rodgers [8] demonstrated that these three species could be differentiated by the electrophoretic patterns obtained from a gross protein extract of each species. Later, Wiersema[9] attempted to differentiate these species by their electrophoretic patterns when stained for the isoenzyme LDH and MDH. Her results, which demonstrated that shrimp MDH consisted of many electrophoretic components, gave rise to the study of shrimp MDH which is now in progress in our laboratory.

The primary purpose of this research was to determine if shrimp mitochondrial and soluble MDH differed, and, if so, to perfect a method of isolation, separation, and purification of soluble and mitochondrial MDH from the Gulf Coast shrimp.

Because of seasonal availability, the White shrimp (Penaeus setiferus) was chosen for this primary investigation.

A secondary purpose of this research was to use the isolated and purified enzymes to study the kinetic and physical aspects of White shrimp soluble and mitochondrial MDH. By satisfying these two purposes, later studies of the brown and pink shrimp will be greatly simplified. The ultimate goal of this study will be the comparison of the catalytic and physical data obtained by studying all three species of shrimp.

MATERIALS AND METHODS

Shrimp

Live shrimp were purchased from bait dealers in Galveston and Clear Lake. White shrimp (Penaeus setifer) were identified by the presence of dorsal grooves on the abdomen and the absence of dorsal grooves on the tail segment.[7] The shrimp were maintained in tanks containing Instant Ocean (Aquarium Systems, Inc.) and only live shrimp were used in extraction of the enzyme.

Buffers

Potassium phosphate buffers were prepared by mixing appropriate amounts of 0.5M K_2HPO_4 and 0.5M KH_2PO_4 and diluting with double distilled water.[10] Disodium EDTA and 2-Mercaptoethanol were added as indicated for each procedure.

Mitochondrial Isolation

A three to one (volume to weight) ratio of ice cold 0.25M sucrose and shrimp muscle was blended in a Waring blender for 30 seconds. After blending the mixture was diluted to ten to one (volume to weight) by the addition of more ice cold 0.25M sucrose. The diluted mixture was then homogenized with two strokes of a motor driven Teflon homogenizer. Upon homogenization, the homogenate was centrifuged at 500 X g for ten minutes in an International PR-2 centrifuge (International Equipment Co.) and the supernatent decanted. The supernatent was again centrifuged at 500 X g for

twenty minutes. The supernatent was carefully decanted without disturbing the precipitate and was centrifuged at 38000 X g in a Beckman Model-L preparative ultracentrifuge (Beckman Instruments, Belmont, California) for thirty minutes. The supernatent (soluble proteins) was retained and the mitochondrial pellet was resuspended in fresh ice cold 0.25M sucrose. The pellet was washed by recentrifuging at 38000 X g for thirty minutes. Again the pellet was resuspended in fresh ice cold 0.25M sucrose and layered on a sucrose step gradient consisting of layers of 0.75M, 1.0M, 1.30M, and 1.75M sucrose. The gradients were centrifuged 20,600 rpm (SW-25.1 rotor) for one hour. The mitochondria layered upon the 1.30M sucrose step and were collected from a hole punched in the bottom of the tube. The mitochondria collected from the 1.30M step were layered on a second sucrose step gradient prepared in the same manner and centrifuged under the same conditions. Again the mitochondria layered on the 1.30M sucrose step and were collected in the same manner as before.

Detection of MDH from Isolated Mitochondria

After microscopic observation of mitochondria stained with Janus Green-B, the remaining preparation was dialyzed overnight against three changes of 0.005M K_2HPO_4-0.001M EDTA-0.001M 2-mercaptoethanol buffer to remove the sucrose. The mitochondrial suspension was then subjected to three freeze-thaw cycles using acetone and dry ice. After thawing the suspension was centrifuged to remove all particulate matter. After centrifugation, the mitochondrial extract

was disc electrophoresed on 7.5 percent polyacrylamide gels at pH 8.3. The gels were then stained specifically for malate dehydrogenase and succinic dehydrogenase.

Detection of MDH from the Cytoplasm

The supernatent retained from the mitochondrial isolation was dialyzed overnight against three changes of 0.005M K_2HPO_4-0.001M EDTA-0.001M 2 mercaptoethanol buffer to remove the sucrose. After dialysis the supernatent was disc electrophoresed on 7.5 percent polyacrylamide gels at pH 8.3 and stained specifically for malate dehydrogenase and succinic dehydrogenase.

Polyacrylamide Gel Electrophoresis

7.5 percent polyacrylamide gels were prepared according to Davis and electrophoresed in Canalco Model 1200 electrophoresis apparatus using stock glycine buffer pH 8.3.[11]

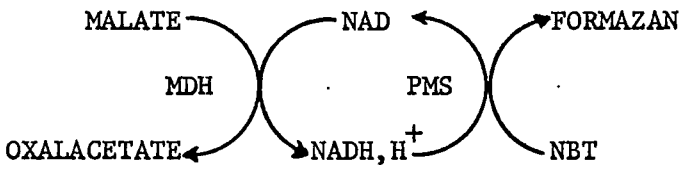
Gross proteins were stained with Amido Black and destained in a Canalco Quick Gel destainer using 7 percent acetic acid as a destaining and storage solution.

Malate dehydrogenase was detected with the use of a specific stain consisting of the following:

1.0M Na L-malate	10ml
0.1M KCN	5ml
0.5M Tris HCl pH 7.1	15ml
Water	70ml
NAD	50mg
NBT	30mg
PMS	2mg

After electrophoresis, the gels were incubated in the staining solution at 37°C in the absence of light. The reaction takes from

five to fifteen minutes and appears in the form of purple bands corresponding to the location of MDH activity. The purple bands are the result of NBT receiving protons from $NADH,H^+$ and the subsequent formation of a dark purple formazan precipitate. The mechanism of the reaction is illustrated below.



MDH Activity Assay

Two assay methods were used, one of which has already been explained in the previous paragraph. This was the colormetric reaction used to locate MDH activity in polyacrylamide gels.

The other method used was the ultraviolet determination of MDH at 340mu by Siegel and Bing.[12] The principle of the assay is shown in the following reaction:

$$\underset{\text{(high OD 340mu)}}{\text{Oxalacetic acid + B-DPNH}} \xrightleftharpoons{\text{MDH}} \underset{\text{(low OD 340mu)}}{\text{malic acid + B-DPN}}$$

It should be noted that although the cytoplasmic enzyme is most efficient in catalyzing the forward reaction, the mitochondrial enzyme will catalyze it.

Preweighed vials containing 0.256μ moles of B-DPNH were purchased from Sigma Chemical Company as was oxalacetic acid and phosphate buffer pH 7.5. To perform the assay, pipette directly into the vial 2.8ml of phosphate buffer and 0.1ml of the sample to be tested. After twenty minutes incubation at 25°C, transfer

the contents of the vial to a cuvette and add 0.1ml of a 10mg/10ml oxalacetate solution. Read and record the change in optical density at 340mμ versus water as a reference. Select a period where the change in optical density is linear with time and calculate the change in optical density per minute for this period. The number of optical units of MDH/ml of sample can be calculated by substitution in the following equation:

$$\frac{1000 \text{ X change in OD/minute}}{\text{ml of sample used}} = \text{optical units of MDH/ml}$$

One optical unit of MDH activity is described as the amount that will cause a decrease in OD_{340} of 0.001 per minute at 25°C of a 3ml reaction mixture in a cuvette of one centimeter light-path.[12]

For catalytic studies of soluble MDH the above method was used with the following change: The amount of substrate (oxalacetic acid) was varied.

The procedure for catalytic studies of mitochondrial MDH was similar to the above procedure. Exceptions were that the substrate used was L-malic acid and the co-factor was NAD. This time the increase in optical density per unit time was measured.

Protein Determination

Protein concentration was determined by the method of Lowry et al.[13]

Bovine serum albumin was used to prepare standard curves for the Lowry test. This solution consisted of 0.12 grams of

bovine serum albumin dissolved in 20ml of 0.02N HCl. a 10:1 dilution of the BSA solution was made with 0.02N HCl and the absorbance at 278mμ was determined against a blank containing 0.02N HCl. The amount of protein in the standard was calculated as mg/ml by dividing the optical density of the original BSA solution by 0.64.[14] The BSA solution was diluted 1:1 with water and made 0.4N in respect to NaOH by adding nine parts of BSA solution to one part 4.0N NaOH. This solution was then diluted 10:1 with 0.4N NaOH and appropriate dilutions of this solution were made.

Lowry reagents were prepared as follows:

Reagent A (Alkaline Tartrate)

4.40% Na CO_3
0.04% Na tartrate
0.11M NaOH

Reagent B ($CuSO_4$ Solution)

0.10% $CuSO_4$ $5H_2O$

Reagent C (Phenol reagent)

Purchased from W. H. Curtin Company (Houston, Texas)

To perform the test, nine parts A were mixed with one part B. Five ml of this solution was added to 0.5ml of the BSA standard solution. The solution was left at room temperature for thirty minutes. After incubation, the optical density at 700mμ was read against a blank containing 0.4N NaOH and reagents A, B, and C. A standard curve was made using different dilutions

of the BSA standard, and unknown protein solutions were tested as indicated for the standard.

MDH Isolation, Separation, and Purification

Muscle Preparation

Live shrimp (approximately 200g) were beheaded, peeled, and cleaned and exposed to three freeze thaw cycles using acetone and dry ice. The thawed muscle tissue was blended in a Waring blender for one minute and diluted 6:1 (volume to weight) with 0.005M K_2HPO_4-0.001M EDTA-0.001M 2-mercaptoethanol buffer. The suspension was then homogenized in a motor driven Teflon homogenizer. The homogenate was stirred in the cold for one hour and then centrifuged at 10,000 X g for thirty minutes. The supernatent was assayed for MDH activity.

Ammonium Sulfate Fractionation

Solid ammonium sulfate was slowly added to the supernatent at 4°C pH 7.5 to give a saturation of 40%[15] and allowed to sit overnight in the cold. The precipitated protein was centrifuged out, assayed, and discarded. The supernatent was raised to 80%[15] saturation and again allowed to sit overnight. The precipitated protein was centrifuged out and dissolved in a small amount of 0.005M K_2HPO_4-0.001M EDTA-0.001M 2-mercaptoethanol buffer. The supernatent was assayed and discarded.

Dialysis

Dialysis tubing was prepared by boiling in 0.001M EDTA for thirty minutes and rinsed three times with double distilled

water. Samples were dialyzed against three changes (three liters each) of buffer for thirty-six hours at 4°C.

Concentration

Dialyzed samples were concentrated before chromatography by packing the dialysis tubing in Aquacide II (Sigma) at 4°C. Samller volumes were concentrated by adding Lyphogel (Gelman Instrument Company, Ann Arbor, Michigan) directly to the sample. After concentration, all samples were redialyzed to insure correct ionic strength of the buffer.

Column Chromatography

Diethylaminoethyl cellulose and carboxymethyl cellulose were purchased from Bio Rad Laboratories (Richmond, California) and prepared by two methods.

DEAE and CM cellulose were washed three times (fifteen vols each time) with the buffer to be used for column elution and the fines were decanted. The cellulose was packed by gravity in a 30cm Glenco column (Houston, Texas) and equilibrated by passing ten volumes of the elution buffer through the column.

DEAE and CM cellulose was precycled[16] by mixing with fifteen volumes of 0.5M HCl for DEAE-cellulose or 0.5M NaOH for CM-cellulose and allowed to sit for thirty minutes. The supernatent was decanted and the cellulose was washed in a Buchner funnel until pH 4 for DEAE-cellulose or pH 8 for CM-cellulose was reached. The cellulose was then subjected to a second treatment consisting of 0.5M NaOH for DEAE-cellulose or 0.5N HCl for CM-cellulose. After thirty

minutes the supernatent was decanted and the cellulose was washed in a Buchner funnel until the pH of the effluent was near neutral. The cellulose was mixed with elution buffer, degassed, and the fines removed by decantation. The cellulose was packed by gravity in a 30cm Glenco column and equilibrated by passing ten volumes of the elution buffer through the column. All columns were eluted in the cold.

Table 2 is a summary of the aforementioned isolation and purification procedure.

Thermostability

Soluble and mitochondrial MDH were diluted with 0.05M phosphate buffer to give about 5000 optical units of activity each and placed in a waterbath at 45°C $\pm$ 0.5°C. Samples were withdrawn from each at five minute intervals, cooled immediately, and assayed for remaining activity.

Substrate Inhibition

The effect of oxalacetate concentration on soluble and mitochondrial MDH activity was determined by assaying for activity when enzyme and co-factor were held constant and the oxalacetate concentration was varied. The reaction mixture contained a constant amount of enzyme, 0.256μ moles NADH, and from 2.52×10^{-5}M to 2.52×10^{-2}M oxalacetate.

The effect of malate concentration on soluble and mitochondrial MDH was determined in a similar way. The reaction mixture contained a constant amount of enzyme, 1.24μ moles NAD, and from 1.0×10^{-4}M to 1.0×10^{-1}M L-malic acid.

Table 2

A summary of the procedure for the isolation and purification of shrimp MDH

1. Clean shrimp
2. Freeze-thaw three times in acetone and dry ice
3. Blend muscle
4. Dilute 6:1 with phosphate buffer and homogenize
5. Clarify by centrifugation
6. Bring supernatent to 40% saturation with respect to ammonium sulfate and leave in the cold overnight.
7. Centrifuge out precipitated protein and bring supernatent to 80% saturation with ammonium sulfate.
8. Leave overnight in the cold and centrifuge out precipitaed protein.
9. Dissolve protein precipitant in small amount of phosphate buffer and dialyze against phosphate buffer.
10. Concentrate solution and apply to a DEAE-cellulose column and elute with a 0.005M to 0.05M phosphate gradient.
11. Dialyze and concentrate both peaks and apply to separate CM-cellulose column. Elute MDH with 0.005M PO_4 buffer.

Km Determinations

Michaelis constants for oxalacetate and L-malate were calculated by the double reciprocal plot method of Lineweaver and Burk[17] using a program of least squares on an Olivetti-Underwood Programma 101.

Soluble and mitochondrial MDH were assayed for activity while holding enzyme and co-factor constant and varying the substrates. Oxalacetate concentration varied from 2.5×10^{-5}M to 1.9×10^{-4}M and L-malate concentration varied from 4.2×10^{-5}M to 4.2×10^{-4}M.

Utilization of D-malic Acid

D-malate utilization was assayed by the method previously described for the conversion of L-malate to oxalacetate by soluble and mitochondrial MDH. The only change was that D-malic acid was substituted for L-malic acid.

NAD Analogue Studies

NAD analogues were purchased from Sigma Chemical Company and studies were performed using the spectrophotometric assay previously described for the conversion of L-malate to oxalacetate by soluble and mitochondrial MDH. The method described by Kitto[18] was used with the following changes. The (H) or high concentration of malate of 1.0×10^{-1}M L-malic acid used by Kitto inhibited both forms of the enzyme so the (H) concentration was changed to 3.2×10^{-2}M. Consequently the (L) or low concentration of

L-malic acid of 6.0 X 10^{-3}M was changed to 1.0 X 10^{-3}M so there would be a sufficient difference in the two malate concentrations.

The conversion of DPN to DPNH and DeDPN to DeDPNH was measured at 340mu while the conversion of APDPN to APDPNH was measured at 365mu.[18]

Molecular Weight

A molecular weight determination of sMDH was made by the sedimentation equilibrium method using the Rayleigh optical system as described by Van Holde.[19]

sMDH was concentrated and diluted with phosphate buffer pH 7.5 to give a final concentration of 2.5mg/ml. The experiment was performed at 6,400 rpm and the temperature maintained at 20°C.

Interference photographs were taken at the beginning of the run and after 36 hours. A synthetic boundary experiment was then performed by layering solvent over the solution and recentrifuging. Another photograph was taken as soon as fringes could be resolved in the boundary.

The correction factor for the change in the base line was calculated by measurements of the plate taken at the beginning of the Rayleigh run. The photograph taken at the end of the run was then analyzed by measuring the distance from the miniscus to each fringe crossed in going to the bottom of the cell. These results were used to plot a curve of corrected fringe number

versus actual distance from the axis of rotation. The ends of this curve were extrapolated to obtain fractional fringes crossed.

A preliminary molecular weight was then calculated by substitution in the following equation:

$$M = \frac{2RT}{\omega^2(1 - \bar{v}p)(b^2 - a^2)} \quad \frac{\Delta jeq}{\Delta jsb}$$

in which:

R = Gas constant ergs/M/°K = 8.317×10^7

T = Temperature °K = 293.2°

ω^2 = Angular velocity radians^2/sec^2 = 4.492×10^5

$\bar{v}$ = Partial specific volume = 0.74ml/g

p = Density g/ml = 1.0031

a = Distance from axis of rotation of the top of the liquid column = 6.861cm

b = Distance from axis of rotation of the bottom of the liquid column = 7.135cm

jeq = Number of fringes crossed in going from top to bottom of cell = 18.80

jsb = Number of fringes proportional to the initial concentration = 8.4032

In order to obtain a more accurate molecular weight and to check on homogeneity of the enzyme a point plot of ln j(r) versus r^2 was made where j(r) is the absolute fringe number at any point and r is the distance from the axis of rotation. The slope of

plot was calculated and substituted in the following equation to obtain a molecular weight.

$$M = \frac{2RT}{\omega^2(1 - \bar{v}p)} \cdot \text{Slope}$$

RESULTS

Mitochondrial Isolation

Due to the nature of shrimp muscle tissue, none of the published mitochondrial isolation procedures we attempted were satisfactory in obtaining a pure preparation of shrimp mitochondria. The first method tried was that of Kitiyakara and Harman,[20] but this procedure yielded few mitochondria and much cellular debris. Other published isoaltion procedures attempted were defeated by two major problems; first, the methods outlined for rupturing muscle cells were found to be inadequate for shrimp muscle; and secondly, the shrimp muscle homogenate required a much greater volume of solvent than reported for other types of tissue. Therefore it was necessary to devise our own isolation procedure using Kitiyakara and Harman's as a guide. The procedure finally employed for the isolation of shrimp mitochondria was described earlier in the section on materials and methods. Although this procedure still is not completely satisfactory, a relatively pure preparation of mitochondria can be observed microscopically when stained with Janus Green-B, a dye specific for mitochondria.

Polyacrylamide gel electrophoresis of the mitochondrial and supernatent fractions obtained by this procedure clearly showed that mMDH and sMDH differed electrophoretically. Plate 1 shows

the electrophoretic patterns of mMDH and sMDH stained specifically with Nitro Blue Tetrazolium. The mitochondrial fraction appears to have one major and one minor electrophoretic component while the soluble fraction appears to have two major and two minor electrophoretic components.

As a criterion for purity of the mitochondrial isolation both electrophoretic fractions were stained specifically for succinic dehydrogenase, a FAD dependent enzyme found only in the mitochondria.[21] The result of this experiment is shown in Plate 2. As shown in the plate, only one SDH electrophoretic component was detected and that only in the mitochondrial fraction.

These results show that sMDH and mMDH of shrimp muscle do differ in their electrophoretic properties. However, the methodology of mitochondrial isoaltion is too difficult to provide enough of the two enzymes to initiate a study of their physical-chemical and catalytic properties. Therefore a large scale isolation procedure, using salt fractionation and column chromatography, had to be devised.

MDH Isolation, Separation, and Purification

The procedure for the isolation and purification of chicken heart mMDH and sMDH of Kitto and Kaplan[22] was used as a guide for the isolation and purification of shrimp mMDH and sMDH.

Plate 1

Electrophoretic patterns, from left to right, of White shrimp sMDH, White shrimp mMDH, Brown shrimp sMDH, and Brown shrimp mMDH.

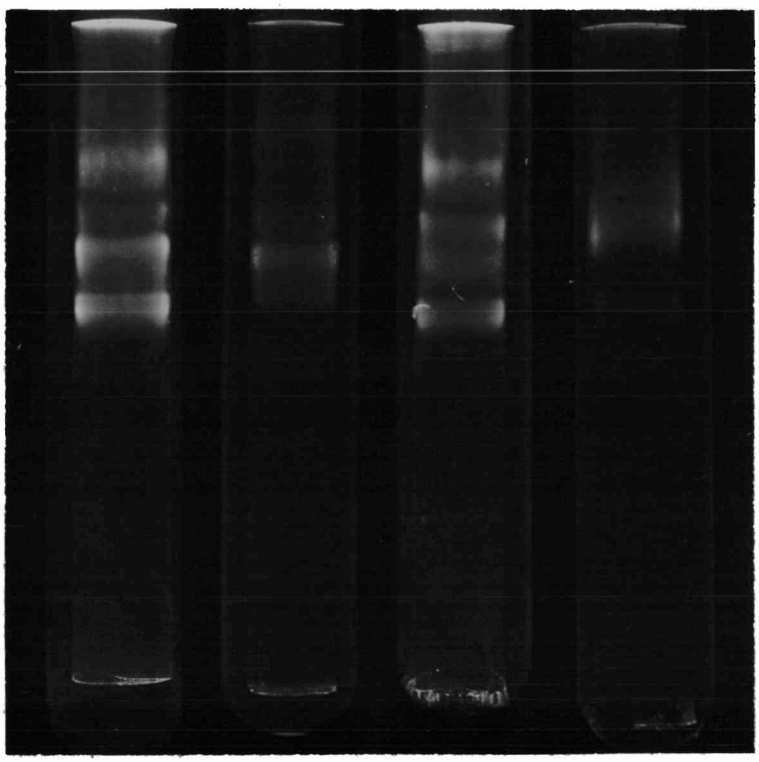
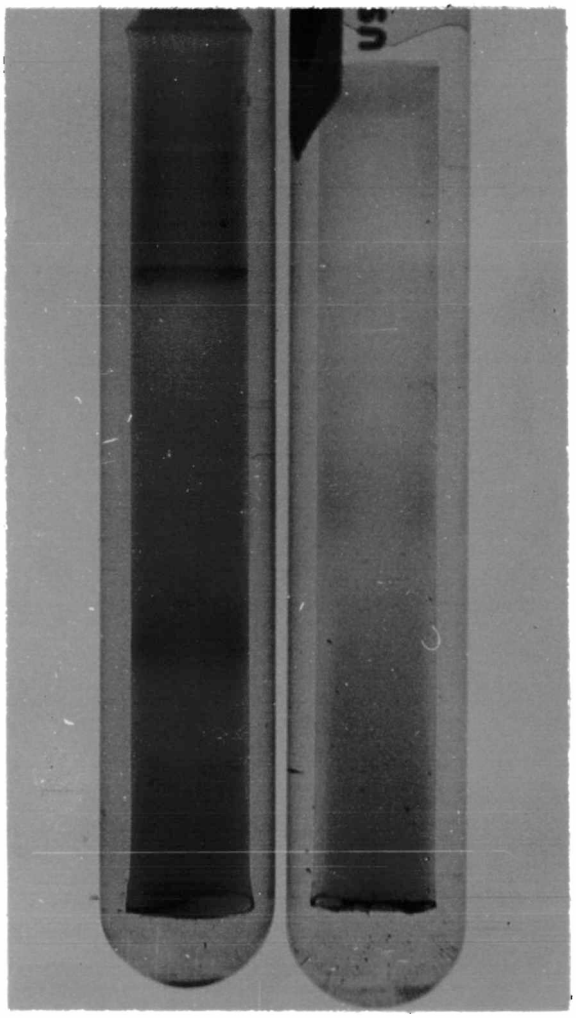


Plate 2

Electrophoretic patterns, from left to right, of White shrimp mitochondrial and soluble proteins stained specifically for SDH.



By the use of small scale experiments at first it was found that more MDH activity/gm of muscle could be obtained when shrimp tissue was freeze-thawed in acetone and dry ice as opposed to freezing in a freezer. One of two things may account for this. Either the lower temperature attained by acetone and dry ice yielded a high burst rate of mitochondria or the speed at which acetone and dry ice freezes tissue causes less damage to the MDH molecule.

It also was found that a large volume to weight ration (i.e. buffer to shrimp) had to be employed during homogenization in order for preparative centrifugation to be successful. This hydrophilic characteristic of homogenized shrimp tissue was one of the determining factors of the scale of our experiments. Since high dilutions were required, approximately 200 grams (1200ml solution) of tissue was the upper limit our equipment could handle.*

Muscle preparation was carried out according to the scheme set forth in the section on materials and methods. The enzyme was then precipitated from the clarified supernatent by adding solid ammonium sulfate.[15]

* A new preparative centrifuge of much greater capacity is on order. This will allow the centrifugation of up to 600g of tissue.

Although the precipitation was carried out at 4°C, the nomogram used to determine amounts of ammonium sulfate to be added was calculated for room temperature.[20] Although this will not provide correct solubility characteristics of the enzyme in ammonium sulfate, most workers have followed this procedure because it is operational, convenient, and the aim is to provide a preliminary isolation.[18,22] During ammonium sulfate fractionation the pH was held at pH 7.5 by the addition of 0.5N ammonium hydroxide.

Figure 2 shows the percent of the total MDH activity found in the different ammonium sulfate fractions. This shows that 57.14% of the MDH activity occurs in the 50 to 60% saturated fraction. Figure 3 shows that over 90% of the total MDH activity is contained in the 40 to 80% fraction. This fraction was the one used for further purification.

Table 3 shows the results of partial purification of shrimp MDH. As shown in the table, ammonium sulfate fractionation removes large amounts of contaminating protein while retaining most of the original MDH activity.

During dialysis of the 40 to 80% ammonium sulfate fraction, a large amount of buffer was taken up. Therefore it became necessary to concentrate the fraction with Aquacide II before applying it to a column. During this step from 5 to 10% of the total activity was usually lost, but the future use of ultrafiltration may alleviate this problem.

Figure 2

Percent of total MDH activity found in different ammonium sulfate fractions.

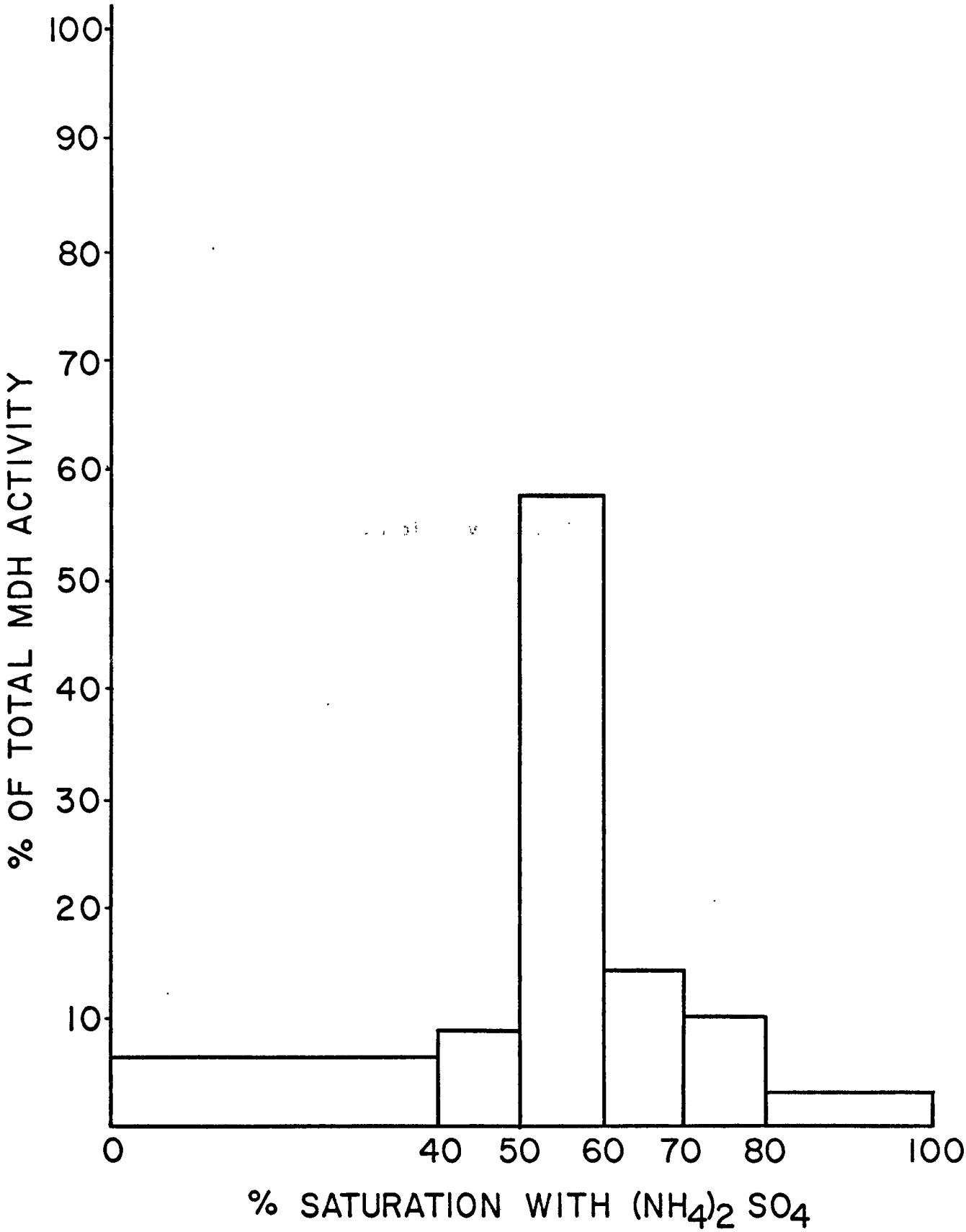

100
90
80
70
60
50
40
30
20
10
% OF TOTAL MDH ACTIVITY
0
40
50
60
70
80
100
% SATURATION WITH $(NH_4)_2 SO_4$

Figure 3

Percent of total MDH activity found in the 40 to 80% ammonium sulfate fraction.

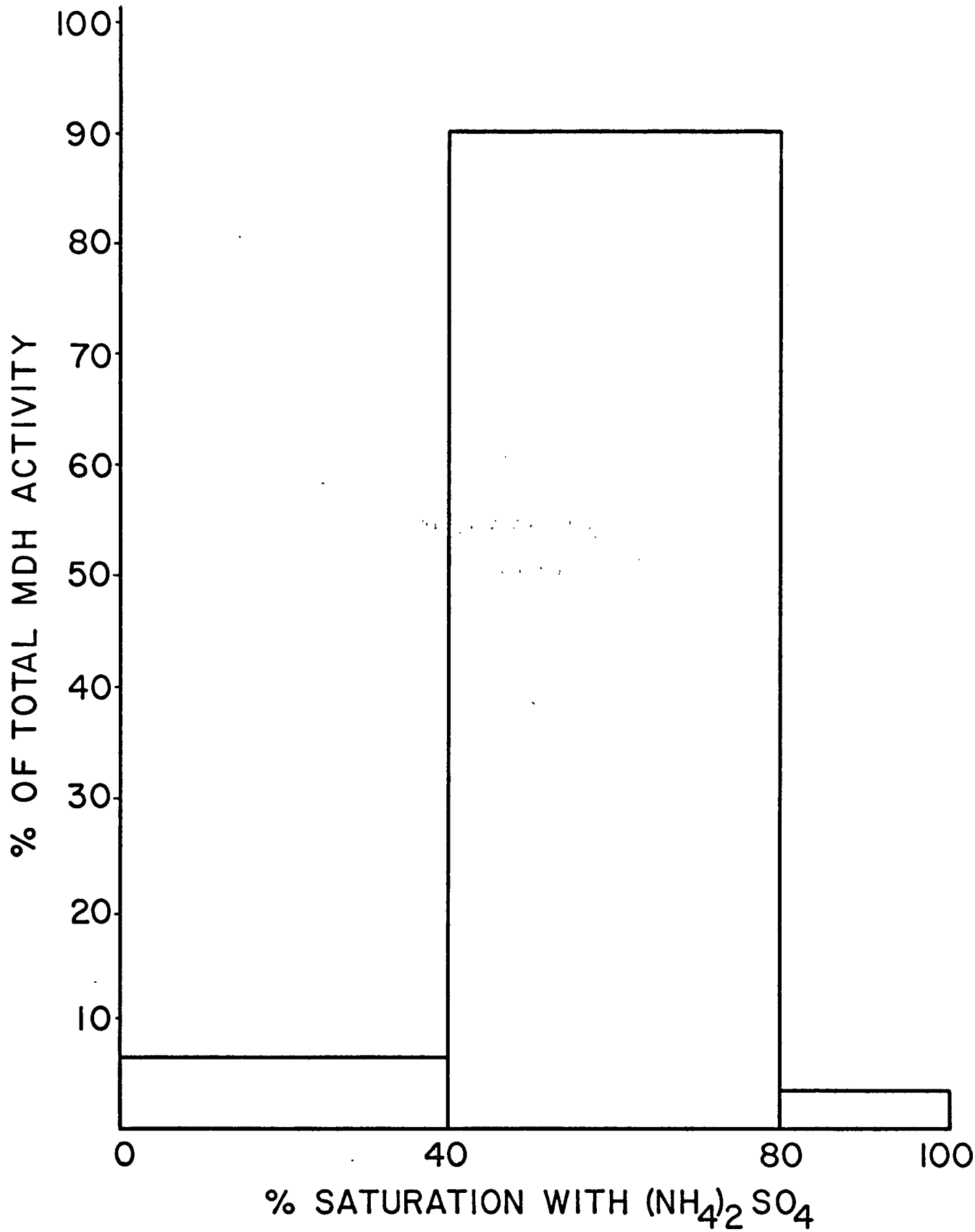

100
90
80
70
60
50
40
30
20
10
% OF TOTAL MDH ACTIVITY
0
40
80
100
% SATURATION WITH $(NH_4)_2SO_4$

Table 3

Partial purification of shrimp MDH
by ammonium sulfate fractionation.

Fraction	Protein (mg)	Activity (OD units)	Specific Activity	% Recovery
Crude extract	1482	3.19×10^6	2152	100
0 - 40%	1089	2.0×10^5	183	6.2
40 - 80%	290	2.88×10^6	9931	90.4
80 - 100%	100	1.10×10^5	1100	3.6

In the first small scale experiment, shrimp MDH could not be separated into mMDH and sMDH using the method described by Kitto and Kaplan for chicken heart MDH. Their method consisted of negative adsorption to DEAE-cellulose to remove contaminating proteins and then separation of the two forms by CM-cellulose chromatography. Neither form of shrimp MDH was found to adsorb to CM-cellulose when eluted with 0.005M phosphate buffer, but one form did adsorb to DEAE-cellulose with this buffer. This information suggested, therefore, that two fractions of shrimp MDH could be obtained and further purified with respect to other proteins by preliminary separation by DEAE-cellulose chromatography and further purification by negative adsorption on CM-cellulose. Figures 4a and 4b show the elution pattern obtained when the dialyzed and concentrated 40 to 80% ammonium sulfate fraction is applied to a DEAE-cellulose column equilibrated with 0.005M K_2HPO_4, 0.001M EDTA, 0.001M 2-mercapto-ethanol buffer and eluted with a linear gradient established between 150ml of 0.005M K_2HPO_4, 0.001M EDTA, 0.001M 2-mercapto-ethanol and 150ml of 0.05M K_2HPO_4, 0.001M EDTA, 0.001M 2-mercaptoethanol.

When MDH peak I and peak II are applied to separate CM-cellulose columns, the MDH does not adsorb and is eluted with 0.005M phosphate buffer. Figure 5 shows the elution pattern when peak I is applied to a CM-cellulose column and eluted with a step

Figure 4a

Elution pattern of White shrimp MDH (fractions 1 - 40) from DEAE-cellulose using a 0.005M to 0.05M phosphate gradient and collecting 3ml fractions at a flow rate of 1ml/min.

MDH ▲----

Protein ●——

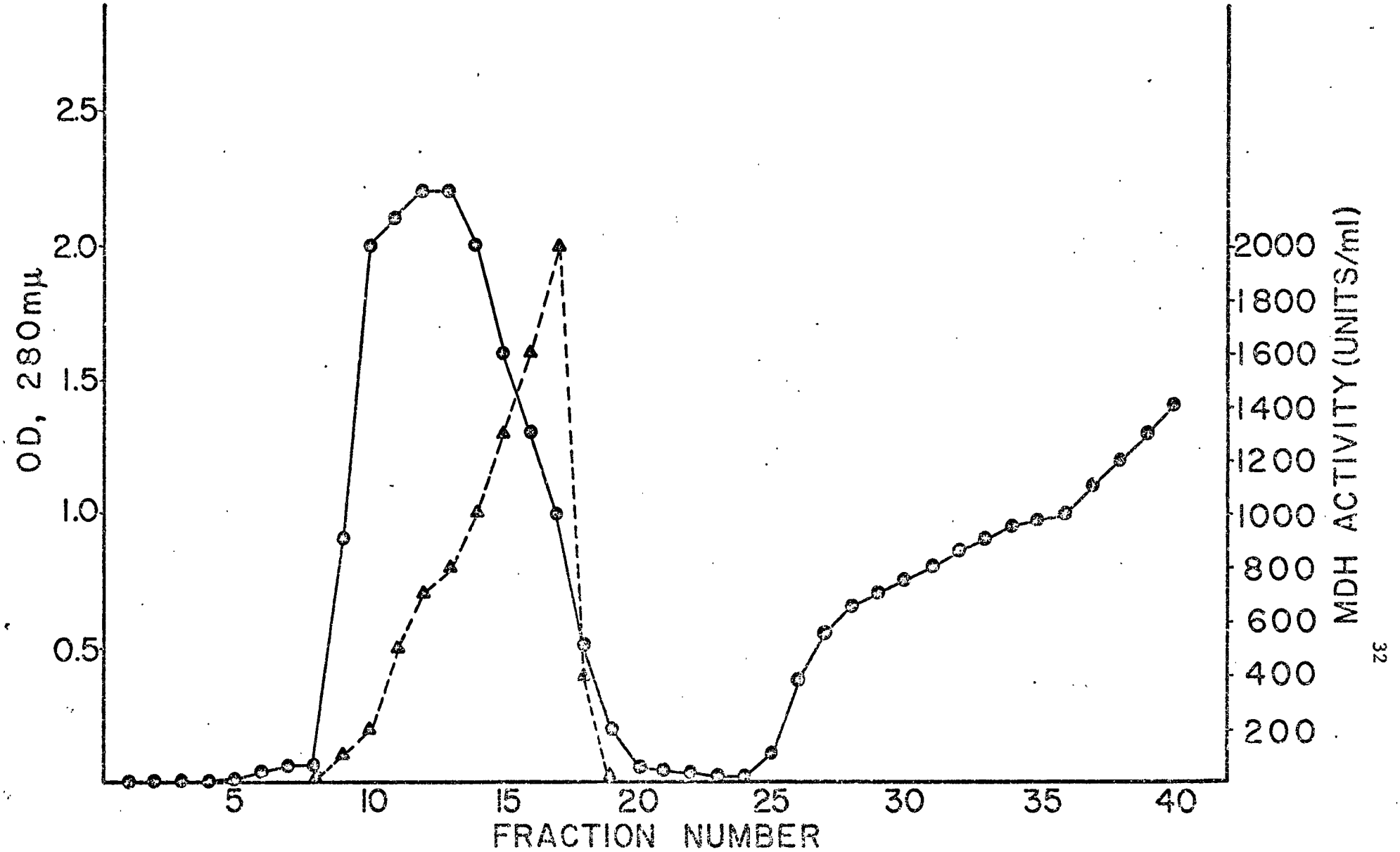
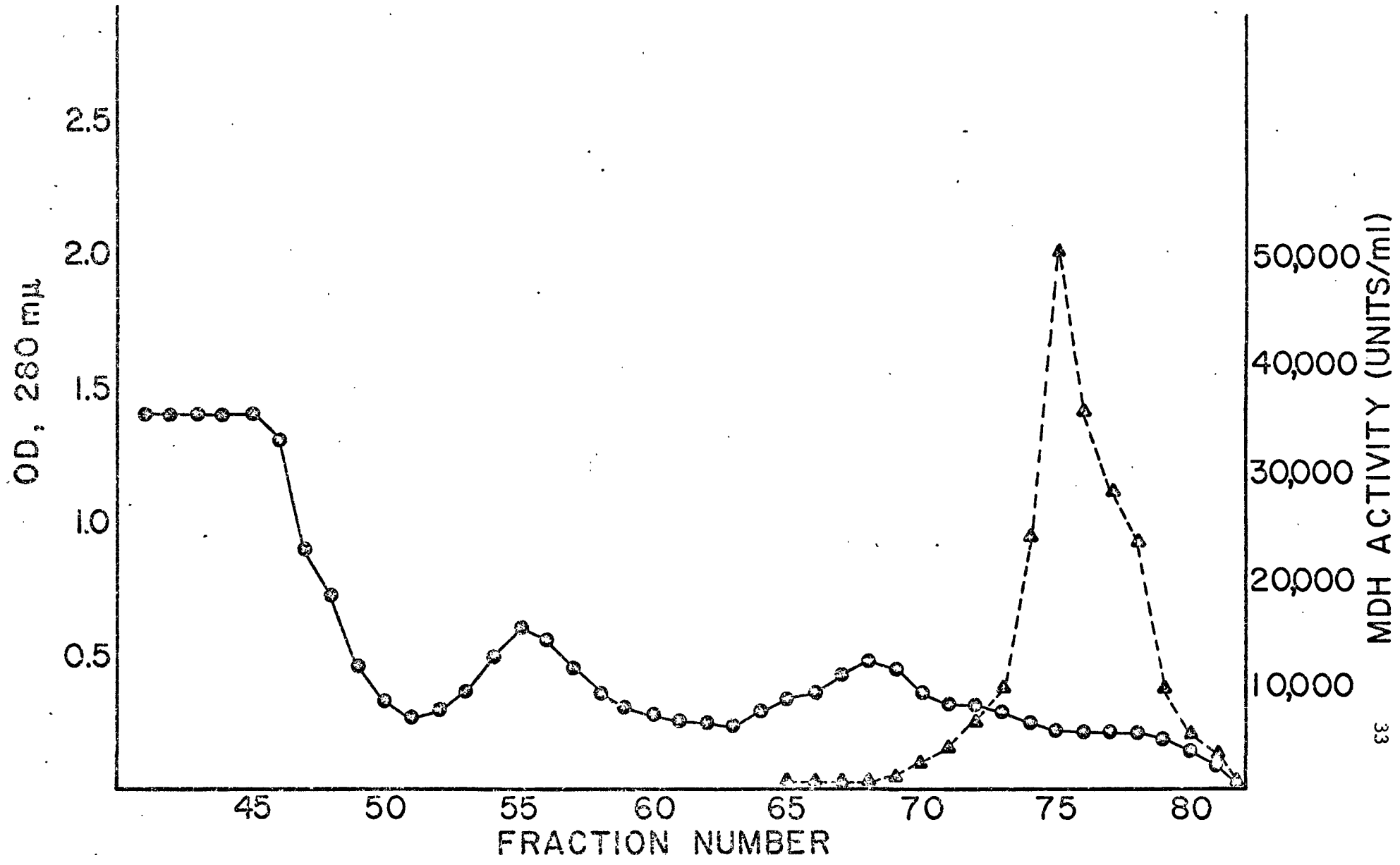

OD, 280mμ
2.5
2.0
1.5
1.0
0.5
5
10
15
20
25
30
35
40
FRACTION NUMBER
MDH ACTIVITY (UNITS/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200

Figure 4b

Elution pattern of White shrimp MDH (fractions 41 - 80) from DEAE-cellulose using a 0.005M to 0.05M phosphate gradient and collecting 3ml fractions at a flow rate of 1ml/min.

MDH ▲----

Protein ●——


OD, 280 mμ
2.5
2.0
1.5
1.0
0.5
45
50
55
60
65
70
75
80
FRACTION NUMBER
50,000
40,000
30,000
20,000
10,000
MDH ACTIVITY (UNITS/ml)

gradient consisting of first, 75ml 0.005M K_2HPO_4, 0.001M EDTA, 0.001M 2-mercaptoethanol, and second, 75ml 0.20M K_2HPO_4, 0.001M EDTA, 0.001M 2-mercaptoethanol. Figure 6 shows the elution pattern of peak II (DEAE) under the same conditions.

After CM-cellulose chromatography DEAE peak I and DEAE peak II were disc electrophoresed on 7.5% polyacrylamide gels pH 8.3. Plate 3 shows sMDH and gross protein patterns obtained from peak I. As shown in the plate, peak I has four major MDH components and compares favorably with the soluble fraction of the earlier mitochondrial isolation. Also shown in Plate 3 is the relative purity of sMDH. No additional bands were detected when the gel containing peak I was stained with Amido Black.

Plate 4 shows mMDH and gross protein patterns obtained from peak II. Peak II has two major MDH components and compares to the mitochondrial fraction previously electrophoresed. However, three minor contaminants were detected when peak II was stained with Amido Black. Upon purification and concentration of the two forms of MDH by chromatography, it appears that the minor component found in the earlier mitochondrial step is now concentrated and shows up as a major component. Likewise, the two minor MDH components found in the earlier soluble fraction after mitochondrial isolation also appear as major components. Therefore, based on electrophoretic evidence alone, it appears that peak I corresponds to the soluble enzyme (sMDH) and peak II corresponds to the mitochondrial enzyme (mMDH).

Figure 5

Elution pattern of White shrimp sMD (peak I DEAE) from CM-cellulose using a 0.005M and 0.20M phosphate step gradient and collecting 3ml fractions at a flow rate of 1ml/min.

sMDH ▲- - - -

Protein ○———

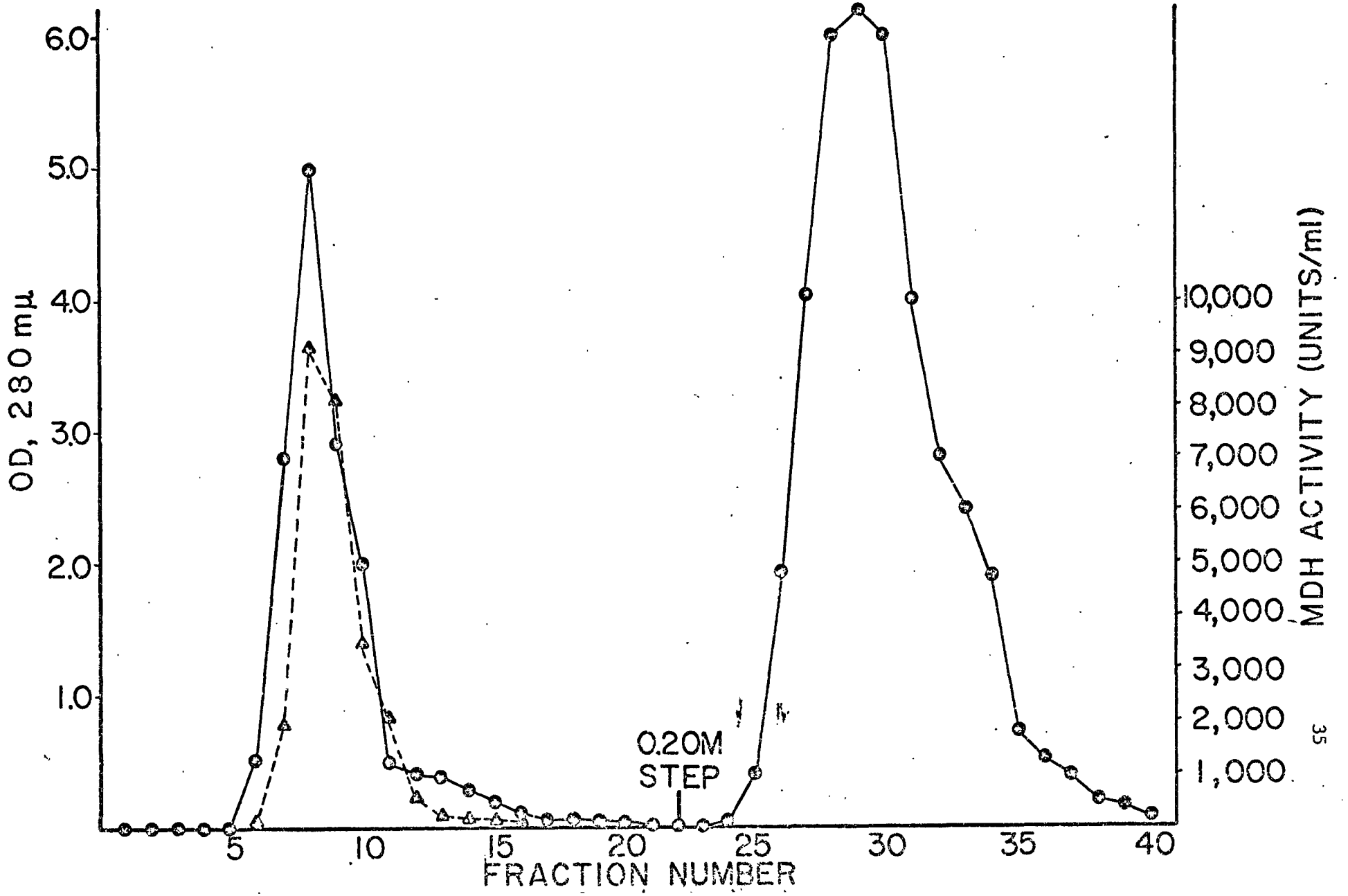

OD, 280 mμ
6.0
5.0
4.0
3.0
2.0
1.0
0.20M
STEP
5
10
15
20
25
30
35
40
FRACTION NUMBER
MDH ACTIVITY (UNITS/ml)
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000

Figure 6

Elution pattern of White shrimp mMDH (peak II DEAE) from CM-cellulose using a 0.005M and 0.20M phosphate step gradient and collecting 3ml fractions at a flow rate of 1ml/min.

mMDH ▲----

Protein O——

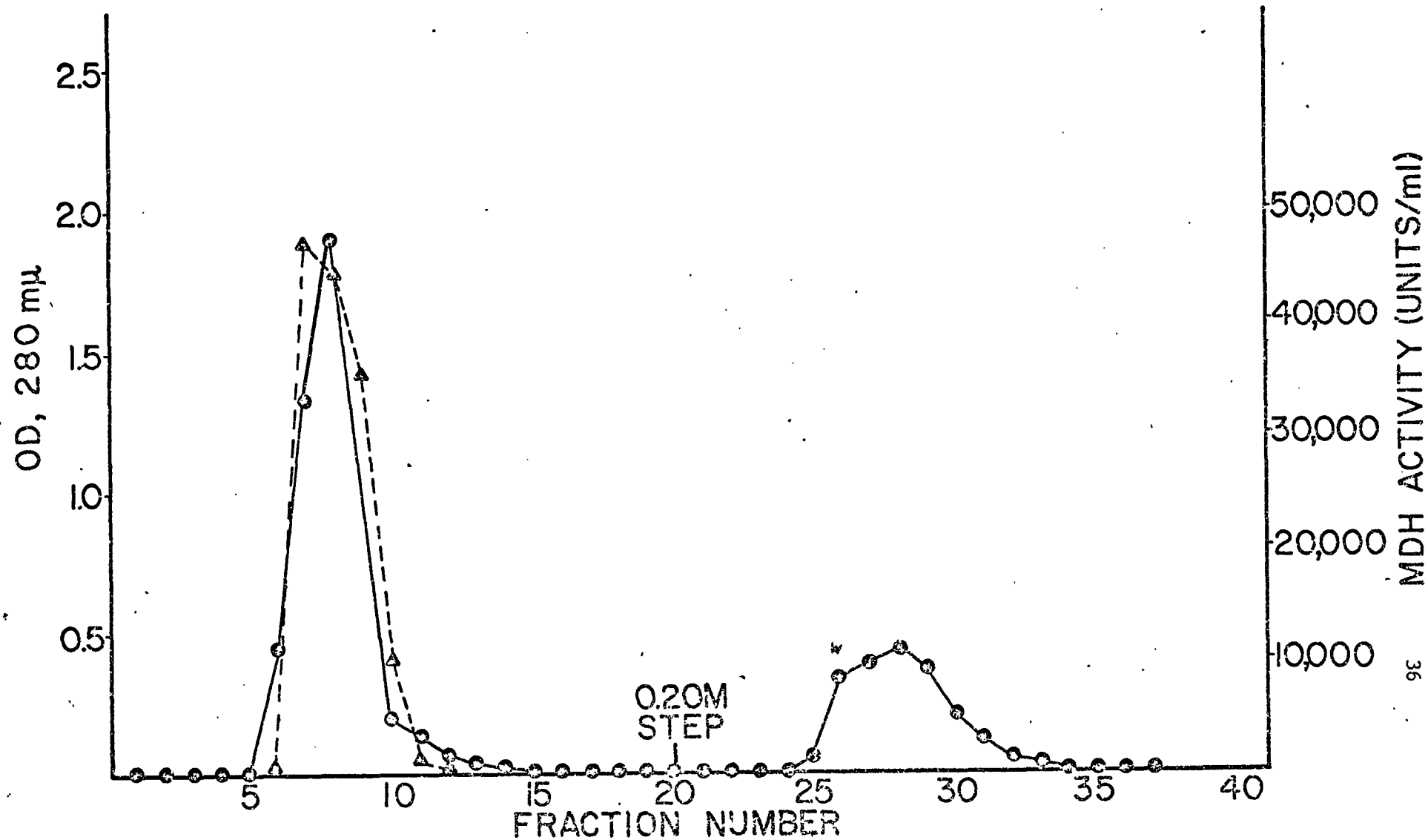

OD, 280 mμ
2.5
2.0
1.5
1.0
0.5
MDH ACTIVITY (UNITS/ml)
50,000
40,000
30,000
20,000
10,000
0.20M
STEP
5
10
15
20
25
30
35
40
FRACTION NUMBER

Plate 3

From left to right, sMDH and gross protein electrophoretic patterns obtained from peak I.

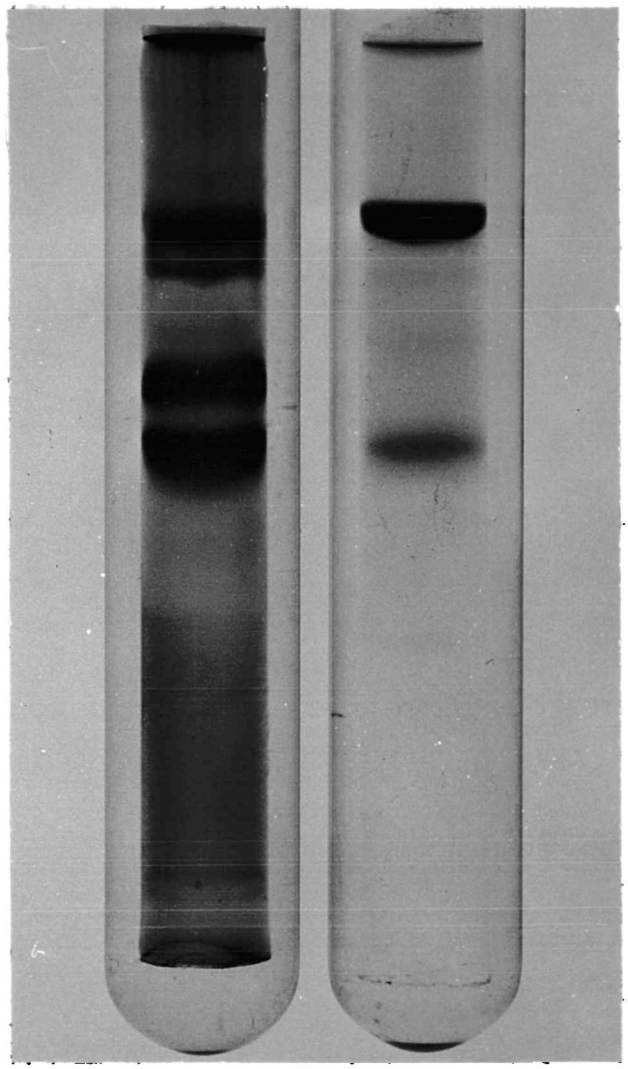
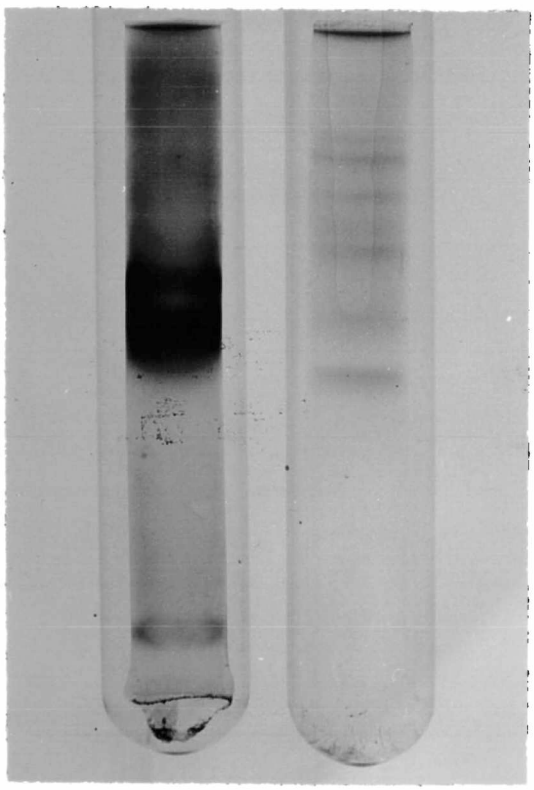


Plate 4

From left to right, mMDH and gross protein electrophoretic patterns obtained from peak II.



Physical-chemical and Catalytic Tests

The two fractions obtained by salt fractionation and chromatography were then used for physical-chemical and catalytic studies. Table 4 lists the results of these studies.

Although a complete pH curve was not determined for the reduction of oxalacetate and the oxidation of malate, an optimum pH was estimated from measure activities in a pH range that included published values of other MDH as the mean.[22] For the reduction of oxalacetate, activities were measured at pH 6, 7, 7.5, and 8. For the oxidation of malate, activities were measured at pH 6, 7, 7.5, 8, 9, 10, and 11. These measures indicated the optimum pH for the reduction of oxalacetate to be about 7.5 for both sMDH and mMDH, and the optimum pH for the oxidation of malate to be about 10.0 for both sMDH and mMDH.

Due to the feedback inhibition of MDH, enzyme activity as a function of substrate concentration was determined (Figures 10 and 11). The data also allowed an estimate of an "optimum molarity of substrate" to be used in measurements of activity. These values are shown in Table 4. As expected, these concentrations were approximately twice the K_m values shown in Table 4.

Figure 7 shows the results of a double reciprocal Lineweaver-Burk plot of 1/V versus 1/oxalacetate concentration where mMDH and sMDH concentration was held constant throughout the experiment.

Table 4

Results of physical-chemical and catalytic studies of sMDH and mMDH

Test	Peak I (sMDH)	Peak II (mMDH)
Optimum pH (oxalacetate reduction)	≅ 7.5	≅ 7.5
Optimum pH (malate oxidation)	≅ 10.0	≅ 10.0
Optimum molarity of oxalacetate for enzyme assay	2.0×10^{-4} M	1.0×10^{-4} M
Optimum molarity of malate for enzyme assay	9.0×10^{-4} M	1.0×10^{-3} M
K_m (oxalacetate)	1.03×10^{-4} M	5.65×10^{-5} M
K_m (malate)	3.89×10^{-4} M	4.92×10^{-4} M
Oxalacetate inhibition	Slightly inhibited	Slightly inhibited
Malate inhibition	Marked	Marked
Termostability 45°	Stable	Unstable
Ability to use D-malate	Nil	Nil
Ability to use DPN analogues		
a) APDPN	Good	Good
b) DeDPN	Poor	Poor
Molecular weight	270,652	-

The K_m of oxalacetate for both enzyme forms was calculated by taking the reciprocal of the X intercepts. The K_m (oxalacetate) for sMDH was found to be 1.03×10^{-4}M while the K_m (oxalacetate) for mMDH was 5.65×10^{-5}M. Figure 8 is a similar plot of 1/V versus 1/malate concentration. The reciprocal of the X intercepts yielded a K_m (malate) for sMDH of 3.98×10^{-4}M and a K_m (malate) of 4.92×10^{-4}M for mMDH. These values are comparable to those found for MDH from other species.[18,22,25]

Figure 9 shows the change in activity of sMDH and mMDH when exposed to a temperature of 45°C at 5 minute intervals up to 30 minutes. Both forms of the shrimp enzyme denature fairly rapidly although the soluble form retains more of its original activity than does the mitochondrial form. The denaturation of both enzymes appears linear with time.

A similar experiment performed at 55°C yielded complete denaturation of both sMDH and mMDH at the end of 5 minutes.

Figure 7

A double reciprocal Lineweaver-Burk plot of 1/V versus 1/oxalacetate concentration for sMDH and mMDH.

sMDH ○——

mMDH △- - - -

sMDH

y at x = 0 = 0.811
x at y = 0 = -9684

mMDH

y at x = 0 = 1.560
x at y = 0 = -17687

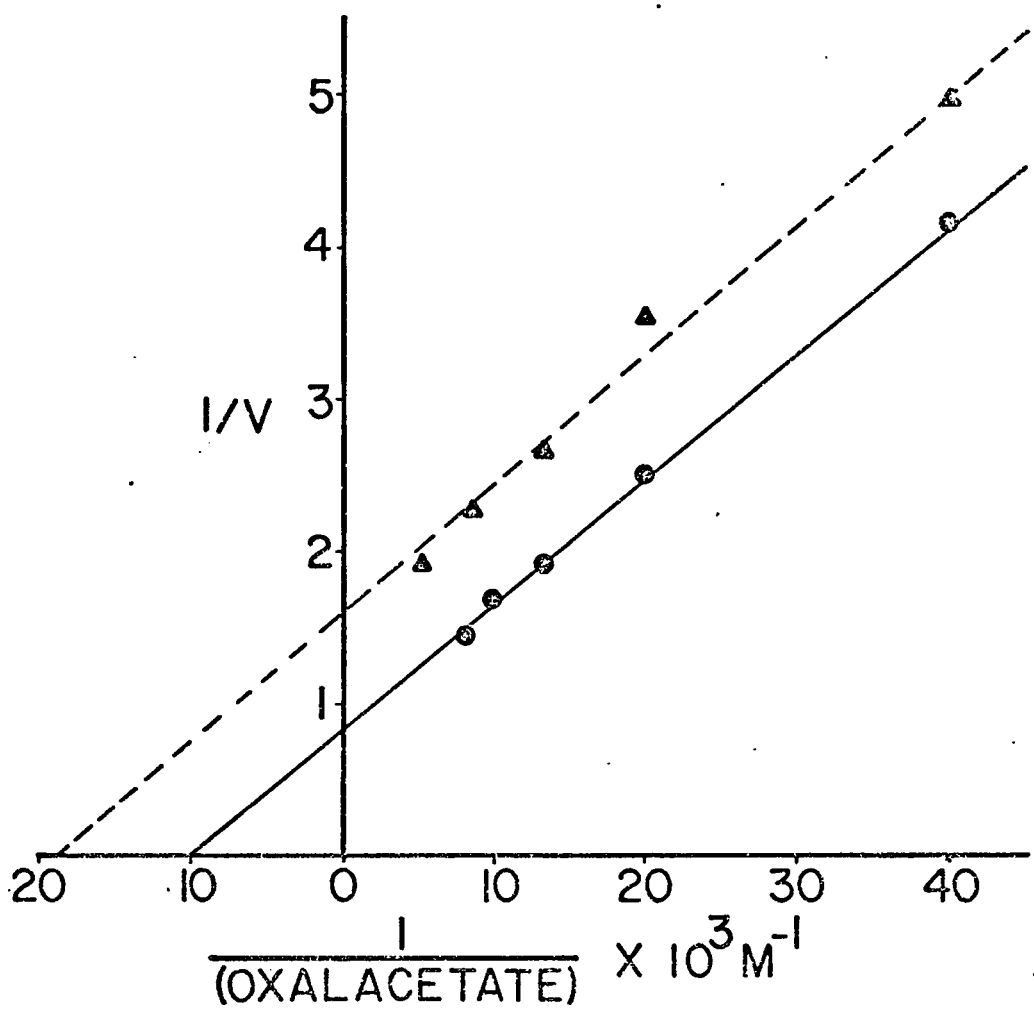


Figure 8

A double reciprocal Lineweaver-Burk plot of 1/V versus 1/malate concentration for sMDH and mMDH.

sMDH ●———

mMDH ▲- - - - -

sMDH	mMDH
x at y = 0 = 2567	x at y = 0 = 2029
y at x = 0 = 0.952	y at x = 0 = 0.771

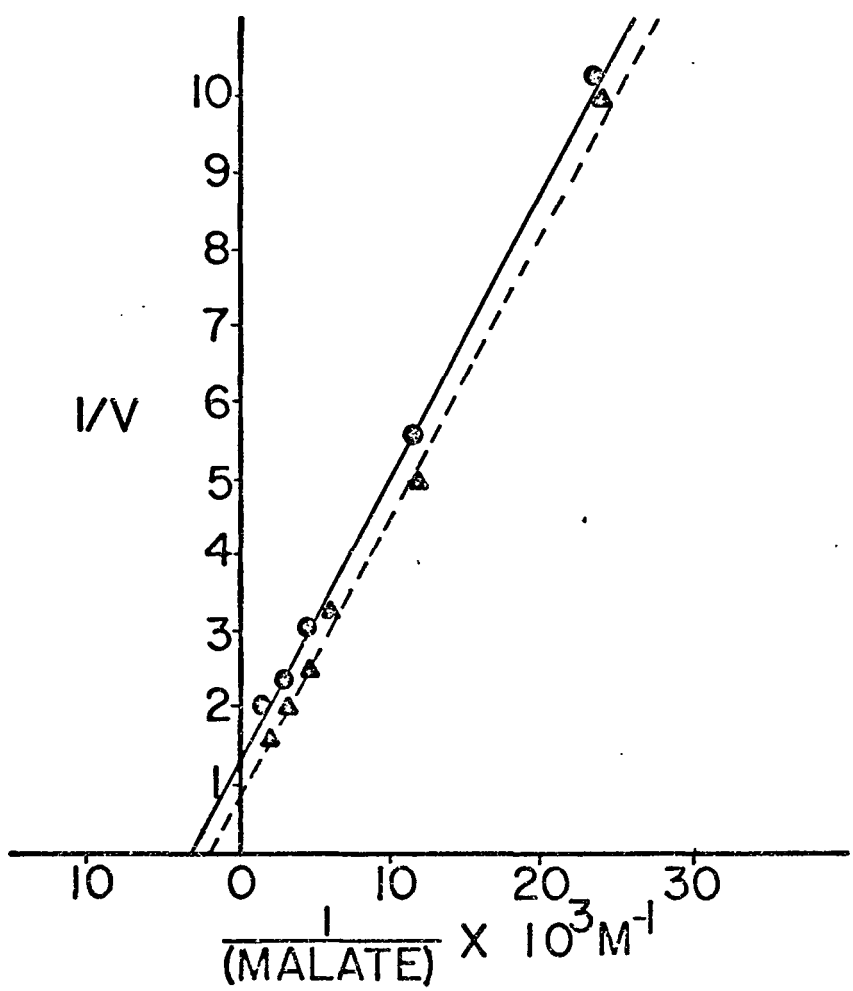


Figure 9

Thermostability of sMDH and mMDH at 45°C.

sMDH ○———

mMDH △- - - -

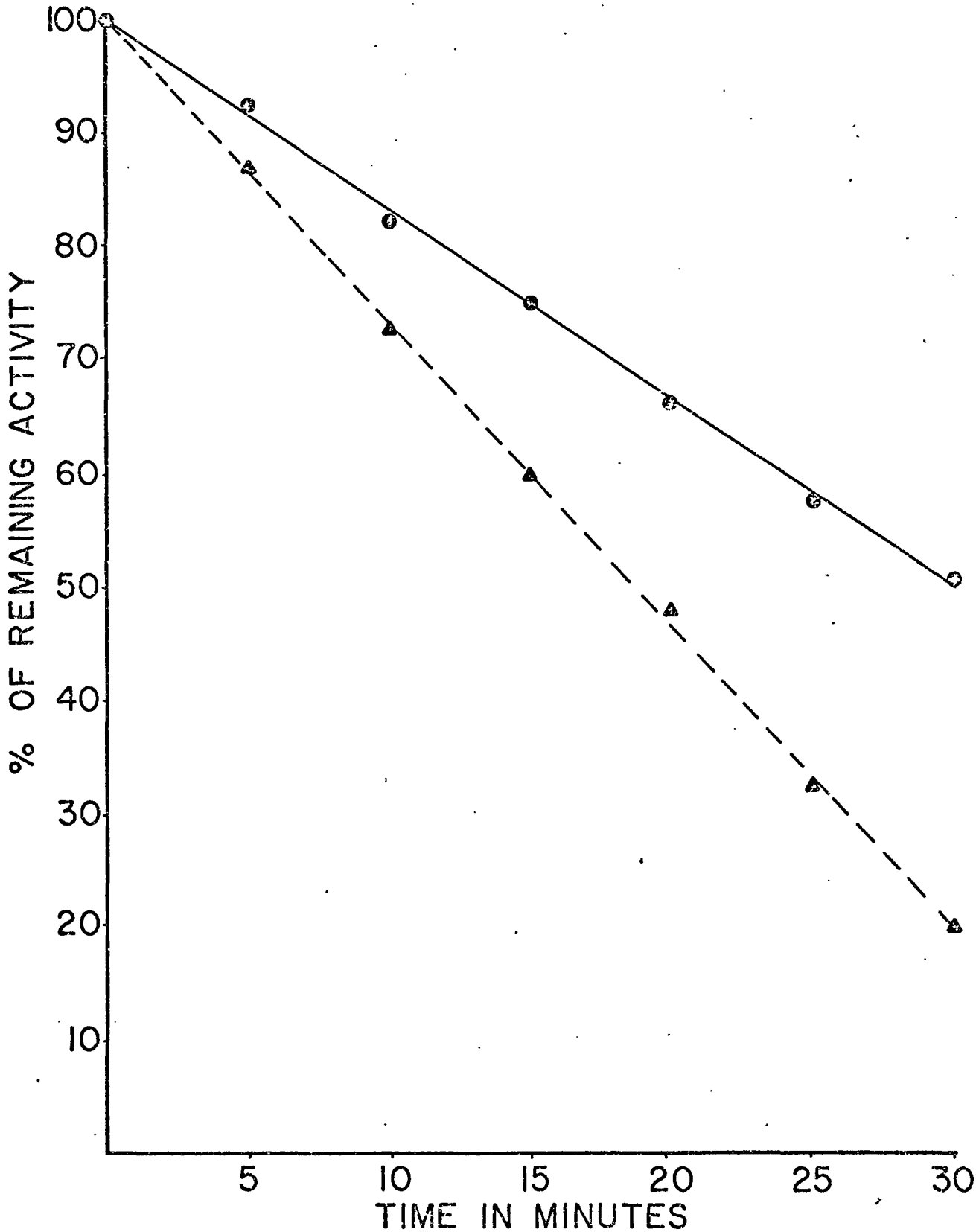

100
90
80
70
60
50
40
30
20
10
% OF REMAINING ACTIVITY
5
10
15
20
25
30
TIME IN MINUTES

Figure 10 shows the effect of increasing oxalacetate concentration on the activity of sMDH and mMDH. Both forms of the enzyme are partially inhibited by high concentrations of oxalacetate, but the inhibition is much less than reported for sMDH and mMDH of other species at similar concentrations of substrate.[22] The enzyme tends to follow a classical distinguishing characteristic of MDH in that the soluble form is slightly less inhibited by oxalacetae than is the mitochondrial form.

Figure 11 is a similar experiment showing the effect of L-malate concentration on the activity of sMDH and mMDH. In contrast to the data reported by other researchers,[18,22] both sMDH and mMDH show a marked inhibition in the presence of high concentrations of L-malate. Again, classical lines are followed in that the mitochondrial form is less inhibited than the soluble form of the enzyme.

The upper half of Table 5 shows the relative activities (change in OD/min.) of sMDH and mMDH when DPN, APDPN, and DeDPN were used as co-factors. It is apparent that both forms of the enzyme yield a higher activity when APDPN is substituted for DPN. Conversely both enzyme forms show a decrease in activity when DPN is replaced with DeDPN.

The use of analogue ratios has been shown to be effective in demonstrating the heterogeneity of enzymes catalyzing the

Figure 10

Effect of oxalacetate concentration on the activity of sMDH and mMDH.

sMDH ●————

mMDH ▲------

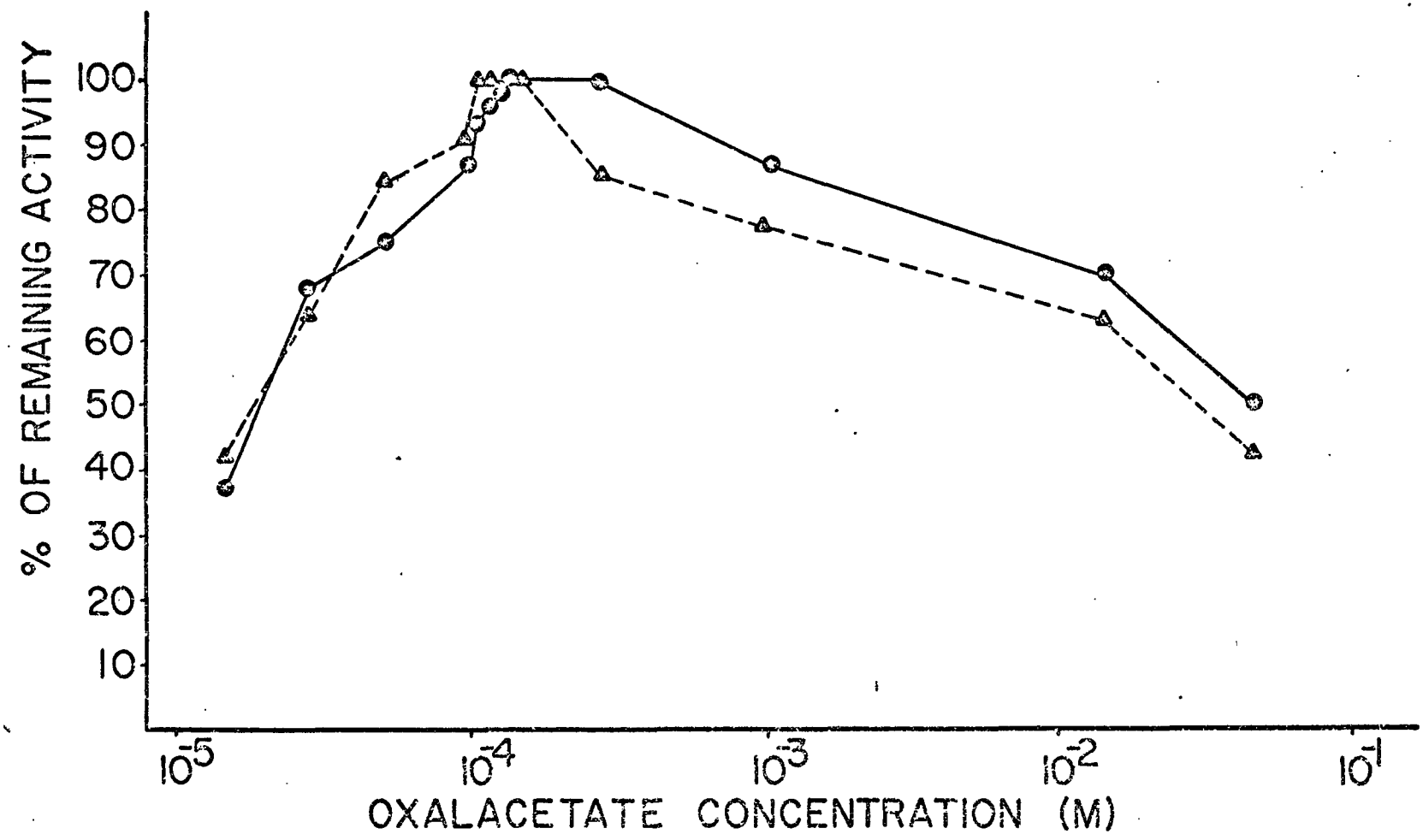

% OF REMAINING ACTIVITY
100
90
80
70
60
50
40
30
20
10
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
OXALACETATE CONCENTRATION (M)

Figure 11

Effect of malate concentration on the activity of sMDH and mMDH.

sMDH ●———

mMDH ▲- - - - -

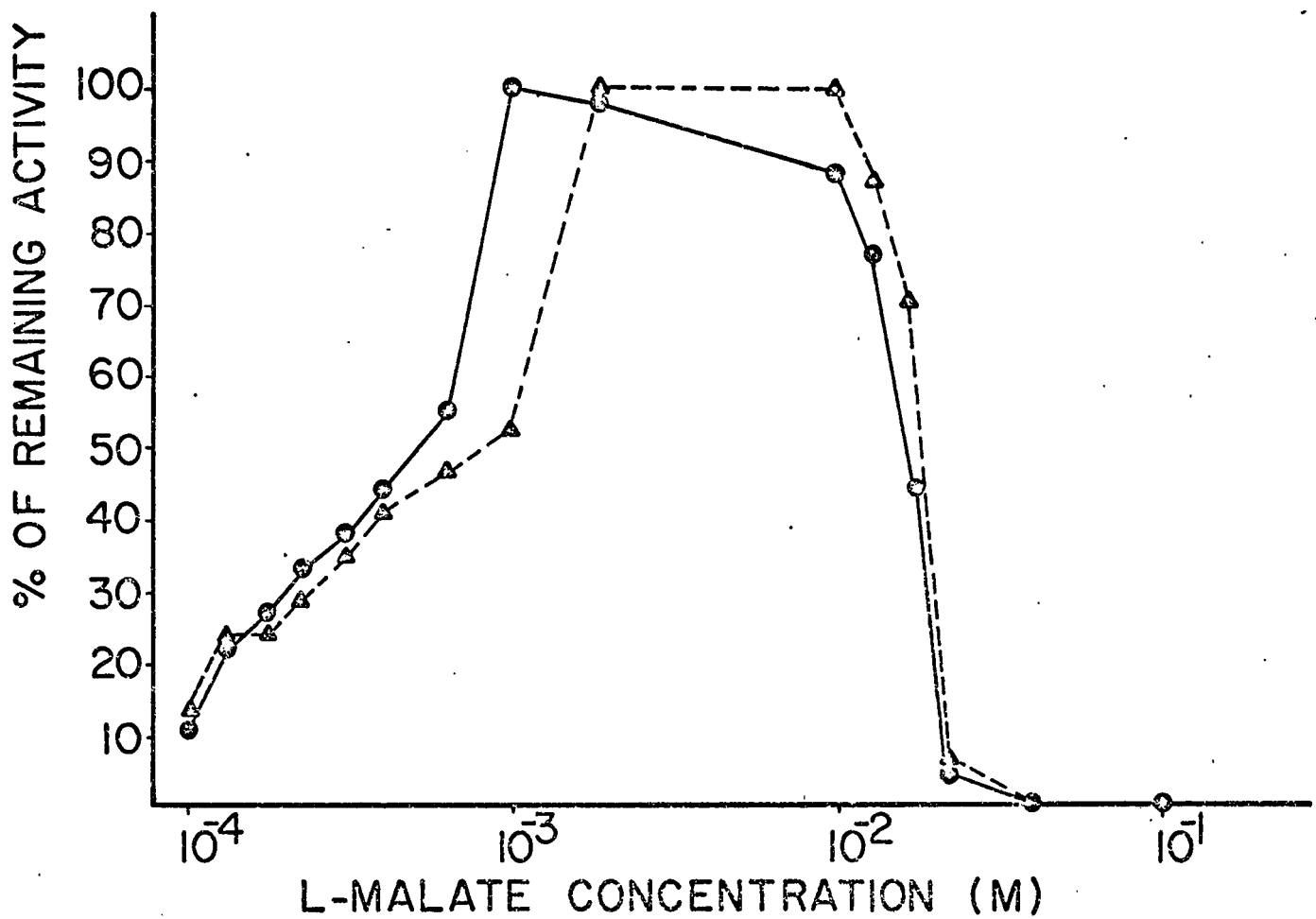

% OF REMAINING ACTIVITY
100
90
80
70
60
50
40
30
20
10
10^{-4}
10^{-3}
10^{-2}
10^{-1}
L-MALATE CONCENTRATION (M)

Table 5

NAD analogue ratio studies

(L) = 1.0×10^{-3} M malate
(H) = 3.2×10^{-2} M malate
DPN = 1.24 u moles
APDPN = 1.24 u moles
DeDPN = 1.24 u moles

Analogue	OD/min. Peak I (sMDH)	OD/min. Peak II (mMDH)
DPN (H)	0.08	0.12
DPN (L)	0.18	0.09
APDPN (L)	0.25	0.12
DeDPN (L)	0.115	0.054

Analogue Ratio	Peak I (sMDH)	Peak II (mMDH)
$\frac{\text{DPN}}{\text{DPN}}$ (L/H)	2.25	0.75
$\frac{\text{APDPN}}{\text{DPN}}$ (L/L)	1.38	1.33
$\frac{\text{DPN}}{\text{DeDPN}}$ (L/L)	1.55	1.66
$\frac{\text{APDPN}}{\text{DPN}}$ (L/H)	3.12	1.0
$\frac{\text{DeDPN}}{\text{DPN}}$ (L/H)	1.43	0.45

same reaction.[18] These ratios are shown in the lower half of Table 5. These results clearly show that the low (L) to high (H) ratios can be used to differentiate soluble and mitochondrial malate dehydrogenase. The (L) to (L) ratios, however, do not differ enough to be conclusive.

Molecular Weight Determination

Figure 12 is a plot of corrected fringe number versus actual distance from the axis of rotation. Extrapolation of this curve resulted in a total of 18.80 fringes crossed in going from top to bottom of the liquid column. Substitution into the equation $M = \frac{2RT}{\omega^2(1 - \bar{v}p)(b^2 - a^2)} \cdot \frac{\Delta jeq}{\Delta jsb}$ gave a preliminary molecular weight of 245, 498. Since this molecular weight was approximately 4 times those reported for soluble MDH from mammalian cells, and approximately 2 times that reported for some bacteria, a plot of ln j(r) versus r^2 was made. This point evaluation is shown in Figure 13. This plot serves two purposes in that the slope can be used to calculate a more accurate molecular weight and that linearity indicates a homogenous sample. As shown in Figure 13 the plot is relatively linear except for the first two points which were obtained by extrapolation. This would suggest that the sMDH preparation was relatively homogenous. A program of least squares was used to calculate a slope of 0.64 and when this value was substituted in the equation $M = \frac{2RT}{\omega^2(1 - vp)} \cdot$ slope, a molecular weight of

Figure 12

Plot of corrected fringe number versus actual distance from the axis of rotation.

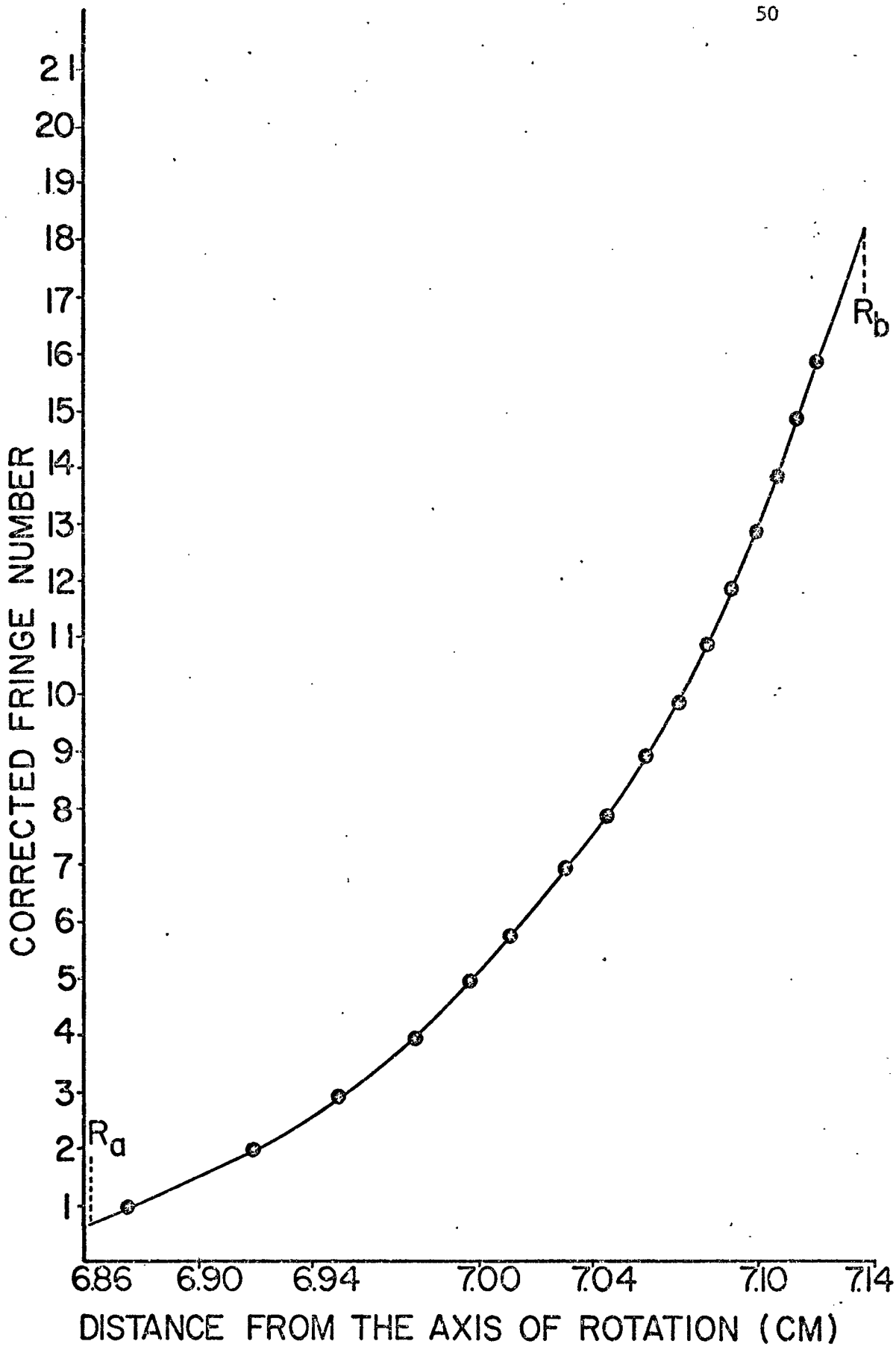
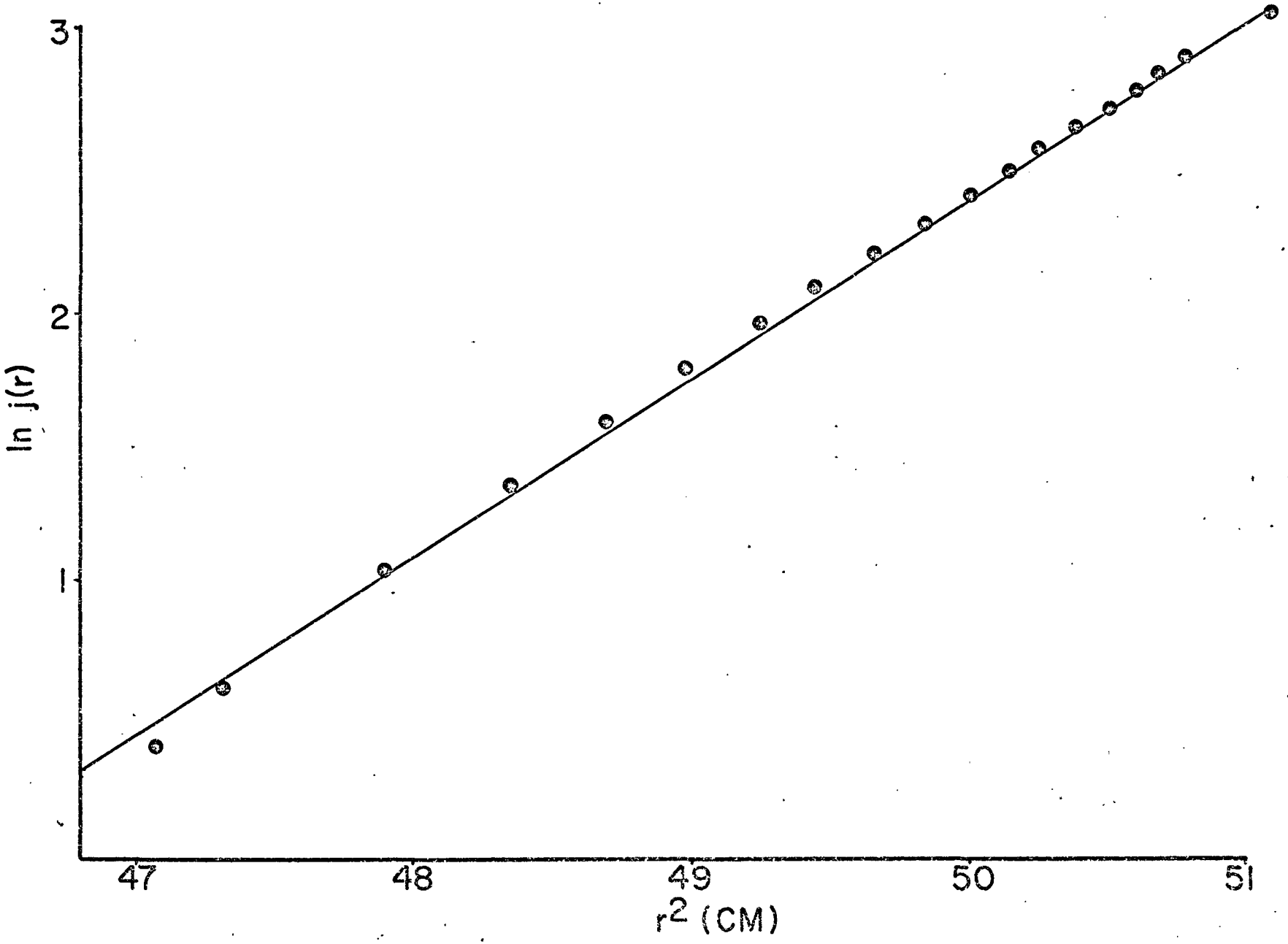

CORRECTED FRINGE NUMBER
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
R_a
R_b
6.86
6.90
6.94
7.00
7.04
7.10
7.14
DISTANCE FROM THE AXIS OF ROTATION (CM)

Figure 13

Plot of ln $j(r)$ versus r^2.


3
2
1
ln j(r)
47
48
49
50
51
r2 (CM)

270,652 was obtained. This weight is considered to be more accurate because more emphasis is put on the calculated points when the slope is determined by least squares as opposed to the calculation of Δjsb by extrapolation.

DISCUSSION

Table 6 compares some of the physical-chemical and catalytic data of soluble and mitochondrial MDH isolated from organisms which compose a representative cross section of the evolutionary scale. It is apparent that although certain similarities are present in molecular weights, Michaelis constants, oxalacetate inhibition, and thermostability, there also exist marked differences. In a number of respects shrimp soluble and mitochondrial malate dehydrogenase resembles those isolated from many vertebrates.

The Michaelis constants of the shrimp enzymes, for example, agree with the general trend found for others, in that K_m (oxalacetate) is higher for the soluble form while the K_m (malate) is higher for the mitochondrial form of the enzyme. Shrimp mitochondrial malate dehydrogenase also shows less thermostability than the soluble malate dehydrogenase. This is true of the chicken[22] and tuna[18] heart malate dehydrogenases, in which the mitochondrial enzyme is the most heat labile form. An exception to this trend is malate dehydrogenase isolated from Drosophila virilis.[27] The most heat labile form isolated from this insect is the soluble enzyme.

One of the most marked differences of shrimp malate dehydrogenases as compared with those isolated from other organisms, is

Table 6

A comparison of the physical-chemical and catalytic properties of sMDH and mMDH isolated from different organisms.

Source	MW	Km (oxalacetate)	Km (malate)	Oxalacetate Inhibition	Thermostability	Reference
White shrimp (s)	270,000	1.03×10^{-4}M	3.89×10^{-4}M	Slight	Stable 45°	
White shrimp (m)	-	5.65×10^{-5}M	4.92×10^{-4}M	Slight	Unstable 45°	
Tuna heart (s)	67,000	-	-	Slight	Stable 48°	18
Tuna heart (m)	67,000	-	-	Slight	Unstable 48°	18
Chicken heart (s)	67,000	5.0×10^{-5}M	8.0×10^{-4}M	Slight	Stable 55°	22
Chicken heart (m)	67,000	3.8×10^{-5}M	9.0×10^{-4}M	marked	Unstable 55°	22
Beef heart (s)	52,000	4.2×10^{-5}M	4.7×10^{-4}M	Slight	-	23
Beef heart (m)	62,000	3.4×10^{-5}M	3.7×10^{-4}M	Marked	-	24
Ostrich heart (s)	67,000	3.0×10^{-5}M	-	Slight	Stable 48°	25
Ostrich heart (m)	67,000	-	-	Marked	Unstable 48°	25
Human erythrocyte	-	9.5×10^{-6}M	3.8×10^{-4}M	-	Stable	26
Drosophila virilis (s)	68,000	4.0×10^{-5}M	8.0×10^{-4}M	Slight	Unstable 55°	27
Drosophila virilis (m)	68,000	4.7×10^{-5}M	1.1×10^{-3}M	Marked	Stable 55°	27
Neurospora crassa (s)	67,000	-	-	-	Stable 60°	28
Neurospora crassa (m)	67,000	-	-	-	Stable 60°	28
Physarium flavicomum (s)	-	1.4×10^{-5}M	7.8×10^{-4}M	Slight	Stable 55°	29
Physarium flavicomum (m)	-	1.8×10^{-5}M	1.1×10^{-3}M	Marked	Unstable 55°	29
Bacillus subtilis	148,000	6.1×10^{-5}M	9.0×10^{-4}M	Slight	Unstable 58°	30
E. coli	62,000	-	-	Slight	-	31

the effect of substrate concentration upon catalytic activity. In the case of malate dehydrogenases isolated from chicken heart, beef heart, and ostrich heart, the mitochondrial form is greatly inhibited by high concentrations of oxalacetate while the soluble enzyme is only slightly inhibited.[22,32,25] At an oxalacetate concentration of 1×10^{-3}M, chicken heart malate dehydrogenase exhibited 60% of its original activity compared with 35% for the mitochondrial enzyme. In contrast, neither form of shrimp malate dehydrogenase is greatly inhibited at a concentration of 1×10^{-3}M oxalacetate. The soluble malate dehydrogenase retained almost 90% of its original activity while the mitochondrial form retained almost 80% of its activity. In order to decrease the activity to less than 50%, almost 1×10^{-1}M oxalacetate was required. Even so, mMDH may be more inhibited by oxalacetate than the sMDH as indicated by Figure 11. However, before a definite conclusion can be drawn, the two should be compared on the basis of specific activities in order to clarify the 100% activity point. These results closely parallel those reported by Kitto and Lewis[18] in their study of tuna heart malate dehydrogenase.

The inhibition of shrimp MDH by malate resembles tuna MDH also in that the range of activity is limited to about a 10 - 100 fold difference of malate concentration. Inhibition by oxalacetate, on the other hand, covers a range of 1000 - 10,000 fold difference.

One striking difference does exist, however, in that both forms of the shrimp enzyme are completely inhibited at a malate concentration of 5.2×10^{-2}M. The mitochondrial form of chicken and tuna heart malate dehydrogenase retained over 90% of their original activity at this concentration.[18,22]

The unusually high molecular weight of 270,652 found for shrimp sMDH might be explained by one of two reasons. Either shrimp sMDH exists in vitro in a multimeric form such as a dimer or tetramer, or sMDH forms a high molecular weight complex with another protein. The first reason may be the correct once since 270,652 is exactly four times the molecular weight reported for chicken and tuna sMDH.[18,22] Further research including co-factor binding studies and sedimentation equilibrium studies under dissociating conditions should answer this question.

Although the isolation and purification procedure described in this research yields electrophoretically pure sMDH, the mitochondrial form of the enzyme shows slight contamination by three other proteins when electrophoresed and stained with Amido black. Therefore, before sedimentation-equilibrium and immunological studies can be performed on mMDH, it must be further purified. The use of gel filtration after CM-cellulose chromatography may remove these contaminants.

Recently Mann and Vestling[33] have reported evidence for the existance of nonidentical subunits in rat liver mitochondrial

malate dehydrogenase. The techniques used in their study include hybridization, two-dimensional tryptic fingerprinting, and acrylamide gel electrophoresis under dissociating conditions.

Since shrimp mitochondrial malate dehydrogenase consists of two major electrophoretic components, it would be interesting to utilize a study of this type to determine if the mitochondrial form of the shrimp enzyme is composed of two nonidentical subunits. A similar study should be performed on the soluble form of shrimp malate dehydrogenase which exhibits four electrophoretic components.

A study of the immunological properties of shrimp soluble and mitochondrial malate dehydrogenase could also prove enlightening. The work of Kitto et al.[18,22] has shown that chicken heart and tuna heart soluble and mitochondrial malate dehydrogenase are immunologically distinct. If this is also true for shrimp, it could be used as a tool to compare the three closely related species of which the White shrimp is a member.

SUMMARY

Malate dehydrogenase was extracted from the muscle tissue of the White shrimp, Penaeus setiferus, and partially purified by successive ammonium sulfate fractionation and negative adsorption to carboxymethyl cellulose. The malate dehydrogenase was then separated into the soluble and mitochondrial form by diethylaminoethyl cellulose chromatography.

A variety of physical-chemical and kinetic tests were performed on the two forms of the enzyme and the results were compared to those reported for malate dehydrogenase isolated from other organisms. The results of this comparison indicated that White shrimp soluble and mitochondrial malate dehydrogenase was, in many respects, similar to the two enzyme forms isolated from other organisms, but differed markedly in molecular weight and with respect to inhibition by oxalacetate and malate.

REFERENCES

1. Wilkinson, Henry J. 1965. Isoenzymes. J. B. Lippincott Co. Philadelphia, Pa.

2. Straub, F. B. 1942. Reinigung der Apfelsauredehydrase und die Bedeutung der Zellstruktur in der Apfelsaure-dehydrierung. Hoppe-Zyler's Z. Physiol Chem. 275: 63 - 72.

3. Delbruck, A., E. Zebe, and T. Bucher. 1959. Uber Verteilungsmuster von Enzymen des Energie liefernden Stoff wechsels im Flugmuskel, Sprungmuskel und Fettkorper von Locusta migratoria und ihre cytologische Zuordnung. Biochem. Z. 331: 273 - 296.

4. Wieland, T., G. Pfleiderer, I. Haupt, and W. Worner. 1959. Uber die Verschiedenheit der Milchsauredehydrogenasen. III. Vergleiche der Milchsauredehydrogenasen aus verschiedenen Ratten organen. Biochem. Z. 331: 103 - 109.

5. Thorne, C. J. R. 1960. Characterization of Two Malic Dehydrogenases from Rat Liver. Biochim. Biophys. Acta. 42: 175 - 176.

6. Kaplan, N. O. and M. M. Ciotti. 1961. Evolution and Differentiation of Dehydrogenases. Ann. N. Y. Acad. Sci. 94: 701 - 722.

7. Moffett. A. W. 1967. The Shrimp Fishery in Texas. Bulletin #50. Texas Parks and Wildlife Dept. Austin, Texas.

8. Rodgers, A. V. 1968. A Preliminary Investigation of the Myogens of Adult Commercial Shrimp. Masters Thesis. Department of Biology. University of Houston, Houston, Texas.

9. Wiersema, Donna S. 1969. Enzyme Studies of Commercial Shrimp. Masters Thesis. Department of Biology. University of Houston. Houston, Texas.

10. Gomori, G. 1955. Preparation of Buffers For Use in Enzyme Studies. Methods in Enzymology. Vol. 1. Academic Press. New York, N. Y. 138 - 146.

11. Davis, N. C. 1963. Gel Electrophoresis. Ann. N. Y. Acad. Sci. 121: 405.

12. Sigma Technical Bulletin #340-UV. 1966. Sigma Chemical Co. St. Louis, Mo.

13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193: 265 - 275.

14. Kaziro, Y., S. Ochoa, R. C. Warner, and J. Chen. 1961. Metabolism of Propionic Acid in Animal Tissues. J. Biol. Chem. 236: 1917 - 1923.

15. Dixon, M. 1953. Nomogram For Obtaining Amounts of Ammonium Sulfate to be Added. Biochem. J. 54: 457.

16. Whatman Information Leaflet #IL2. W. and R. Balston Ltd., London, England.

17. Lineweaver, H. and D. Burk. 1934. The Determination of Enzyme Dissociation Constants. J. Am. Chem. Soc. 56: 685 - 666.

18. Kitto, G. B. and Richard G. Lewis. 1967. Purification and Properties of Tuna Supernatent and Mitochondrial Malate Dehydrogenases. Biochim. Biophys. Acta. 139: 1 - 15.

19. Van Holde, K. E. 1967. Sedimentation Equilibrium. Fractions. Vol. 1. Beckman Instruments, Inc. Palo Alto, Calif.

20. Kitiyakara, A. and J. W. Harman. 1953. Isolation of Muscle Mitochondria. J. Exp. Med. 97: 553.

21. Handbook of Biochemistry. Ed. - Herbert A. Sober. The Chemical Rubber Co. Cleveland, Ohio.

22. Kitto, G. B., and N. O. Kaplan. 1966. Purification and Properties of Chicken Heart Mitochondrial and Supernatent Malic Dehydrogenases. Biochem. 5: 3966 - 3980.

23. Englard, Sasha, and Hilda H. Breiger. 1962. Beef Heart Malic Dehydrogenases II. Preparation and Properties of Crystalline Supernatent Malic Dehydrogenase. Biochim. Biophys. Acta. 56: 571 - 583.

24. Englard, Sasha, and Lewis Siegel. 1969. Mitochondrial L-malate Dehydrogenase of Beef Heart. Methods in Enzymology. Vol. 13. Ed. - J. M. Lowenstein. Academic Press. New York, N. Y. 99 - 106.

25. Kitto, G. B. 1967. Purification and Properties of Ostrich Heart Malate Dehydrogenases. Biochim. Biophys. Acta. 139: 16 - 23.

26. Shrago, E. and A. B. Falcone. 1963. Purification adn Properties of Human-Erythrocyte Malic Dehydrogenase. Biochim. Biophys. Acta. 73: 7 - 16.

27. McReynolds, M. S. and G. B. Kitto. 1970. Purification and Properties of Drosophila Malate Dehydrogenases. Biochim. Biophys. Acta. 198: 165 - 175.

28. Kitto, G. B., M. E. Kottke, L. H. Bertland, W. H. Murphey, and N. O. Kaplan. 1967. Sutdies on Malate Dehydrogenases and Aspartate Aminotransferoses from Neurospora crassa. Arch. Biochem. Biophys. 121: 224 - 232.

29. Nordyke, E. L. 1970. The Partial Purification and Characterization of the Isozymes of Malate Dehydrogenase in the Myxomycete Physarum flavicomum Variety I. Masters Thesis. Department of Biology. University Of Houston. Houston, Texas.

30. Yoshida, A. 1965. Purification and Chemical Characterization of Malate Dehydrogenase of Bacillus subtilis. J. Biol. Chem. 240: 1113 - 1117.

31. Murphey, William H., G. B. Kitto, Johannes Everse, and N. O. Kaplan. 1967. Malate Dehydrogenases I. A Survey of Molecular Size Measured by Gel Filtration. Biochem. 6: 603 - 609.

32. Englard, Sasha, Lewis Siegel, and Hilda H. Breiger. 1960. Purification and Properties of Beef Heart Muscle Cytoplasmic Malic Dehydrogenase. Biochim. Biophys. Research Comm. 3: 323 - 327.

33. Mann, K. G. and C. S. Vestling. 1970. Isozymes of Rat Liver Mitochondrial Malate Dehydrogenase. Evidence of Nonidentical Subunits. Biochem. 9: 3020 - 3025.