# PURIFICATION AND SOME KINETIC STUDIES OF MALATE DEHYDROGENASE FROM WHITE YAM (DIOSCOREA ROTUNDATA) TUBERS

BY

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## THESIS APPROVAL

This thesis by ANDREW, Jonathan Nok meets the regulations governing the degree of Master of Science of Ahmadu Bello University and is approved for its contribution to scientific knowledge.

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## DECLARATION

I declare that this work is wholly the author's and has not been part of any presentation for any other qualification.

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## ABSTRACT

Malate dehydrogenases [L-Malate $NAD^+$ oxidoreductase E.C. 1.1.1.371 have been purified from white yam (Diosoorea rotundata) tuber by a purification protocol involving ammonium sulphate fractionation, ion exchange chromatography on DEAE-cellulose, and Sephadex gel chromatography.

The enzyme was resolved into two forms $MDH_1$ and $MDH_2$ on DEAE-cellulose. Both enzyme forms were homogenous with different electrophoretic mobilities on polycrylamide gels. Their mobilities on S.D.S.-polycrylamide gel were however the same.

The molecular weights of the yam tuber malate dehydrogenases determined by gel filtration were 61,300 ± 150 and 63,000 ± *400* for $MDH_1$ and $MDH_2$ respectively while the subunit molecular weight of 31,000 was determined by SDS-PAGE for both enzymes.

The activation energy (Ea) and Arhenius constant (A) for $MDH_1$ were 33.bkJ/mole and 1.94 x $10^5 dm^3 m$ $mole^{-1} min$ in the same respect, the values for $MDH_2$ were 47.8kJ/mole and 4.92.x $10^7 dm^3 m$ mole-$^1$min".

The $MDH_1$ showed two pH maxima at 6.5 and 7.5 assayed at 30°C. Groups associated with enzyme catalysis and binding of substrate have pKa values of 6.8 and 7.6. A single pH maximum of

6.5 associated to pKa of 6.8 was manifested by $MDH_2$.

Both forms of the enzyme were inhibited by oxaloacetic acid concentration at or greater than 0.25mM at pH 7.5. The half life of $MDH_1$ was found to be 122 days in the freezer, 20 days in the refrigerator, and 8.3 days at ambient temperature. The half life of $MDH_2$ in the freezer, refrigerator and at ambient temperature were: 5 days, $3^1/2$ days and $1^1$ /4 days respectively.

The initial velocity studies with the enzymes indicated an ordered sequential mechanism. The Km values obtained from secondary plots were 0.05mM 0.08mM 0.48mM, 2.56mM for NADH, OAA, $NAD^+$ and 1-malate respectively for $MDH_1$. The values fcr $HDH_2$ were found to be 0.02mM, 0.083mM, 0.25mM and 0.83mM in the same respect.

Product inhibition analysis in both forward and backward reactions to decipher the kinetic meohanism for $MDH_1$ revealed an ordered bi-bi sequential-mechanism. ATP inhibition on both enzymes was competitive with respect to OAA. Both enzymes were inhibited by naturally occuring metabolites like -ketoglutarate, citrate, suooinate. Inhibition studies with group specific reagents: Parahydroxy meroury benzoate, KCN, $NaN_3$, strongly inhibited both enzymes, thus implicating the thiol group (-SH) in the catalytic potency of the enzyme.

Chapter 1

# 1.0 INTRODUCTION AND LITERATURE REVIEW

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Chapter 1

# 1.0 INTRODUCTION AND LITERATURE REVIEW

## 1.1 Yam Tuber

Yams are tropical crops produced mainly in West Africa, South East Asia and the Carribean to the extent of 20 million tons per annum (Coursey, 1961).

Yam tubers are storage organs second only to cassava in carbohydrate content. This makes the plant a major source of energy. The shortcomings associated with yam production are numerous. Some of these are long growing season, low yield, yam rot, and limited technology for storage.

Once harvested, the yam tuber goes into dormancy, a period of metabolic quiescence during which the rate of respiration may drop to as low as 20% of the pre-harvest value, and remains at this level until sprouting (Ikediobi, 1985). Ugochukwu (1977) and Ikediobi and Oti (1983) observed that there are changes in the concentrations of some metabolites and activities of certain enzymes in yam during storage.

It is imperative to note that dormancy is a necessary and unavoidable stage in the life cycle of the yam tuber, and it is precisely because of it that tubers must be stored before they

can be used as planting materials. Owing to the potential benefits, the temptation to successfully manipulate dormancy cannot be overemphasized.

It would appear that any dramatic success in this area must await more knowledge and understanding of the biochemistry of the yam tuber dormancy and sprouting than is presently available (Ikediobi, 1985). Since dormancy is critial for yam storage both for food and planting, its control is relevant to the efficient and optimal utilization of the crop by man. However, the minimal available information on the mechanism of dormancy makes its control difficult.

Sprouting or germination is characterized by increased metabolic activity in the yam tuber and that signals the termination of dormancy. When sprouting commences, there is loss in dry matter at about 0.21% $\pm$ 0.02% daily at 25$^{o}$C, and 35% of this loss is accounted for by respiratory activity (Passam, & Read 1978). The loss in weight has been shown to be linear with a first order rate constant of 1.4 x $10^{-2}$/week ($t_{1/2}$ = 1 year) and also corresponds to an increase in entropic work of the tuber. A weight loss of 1g is accompanied by an entropic work of 3.81Kcal (Osuji and Umezurike 1985). This entropic energy could be readily utilized for activating some cellular components e.g. enzymes.

The argument is frequently made that the proper understanding of the biochemical and physiological processes occuring

in yam tuber from harvest through dormancy and senescence is essential for an efficient and economic planning of yam storage and utilization. For example, knowledge of the carbohydrate metabolism would enable a yam merchant/exporter to benefit from accurate information about the rate of moisture loss by tubers stored under conditions similar to those in the warehouse.

Since our ability to utilize yam for food and industrial purposes centres on a clear understanding of the tuber as a plant organ, work of largely basic nature must continue on the yam tuber at both chemical and biochemical levels and should involve: studies on the enzymology of the yam tuber in relation to respiration, dormancy, breakage of dormancy, including the identification of the enzyme system involved.

Research investigations on carbohydrate metabolism of the yam tuber has been exclusively on the glycolytic and pentose phosphate pathways. No detailed work on the tricarboxylic acid cycle has been reported. Glycolytic pathway and the TCA cycle are the main energy generating systems for the metabolism of any tissue. The conversion of 1 mole of glucose to 2 moles of pyruvic acid accounts for 2 high energy phosphates (2ATP) as a result of substrate level phosphorylation in the glycolytic sequence. The further oxidation of 2 moles of the pyruvic acid in the TCA cycle generates 30 high energy phosphates (30 ATP). In addition 6 more ATP are added to make up 38 ATP.

From the foregoing, it is obvious that the TCA cycle is an

active energy source for the respiratory activity of the yam tuber. It is in view of this, that this research work was started with an objective to isolate, purify and characterize Malate dehydrogenase (MDH). Its kinetics and mechanism of action will enable definitive role(s) to be assigned to the enzyme in the metabolism of the yam tuber. Its findings can be exploited in manipulating the physiological activities of the yam tuber towards ameliorating the problem of yam production and storage.

## 1.2 Enzyme Compartmentation in Yam Tubers

Physiologically there seems to be a difference between the head, middle and tail regions of the yam tuber. Oluoha (1982) have undertaken simple studies on pH variation in the different regions of the yam, as well as on distribution of some key enzymes of carbohydrate metabolism in these regions. The enzymes studied were phosphorylase, hexokinase, phosphofructokinase, pyruvate kinase and glucose-6-phosphate dehydrogenase.

The results obtained showed a general pH increase from tail to head for D rotundata and D alata while in D cayenesis the highest pH was obtained in the middle region of the tuber. With the exception of hexokinase, the highest activities of the glycolytic enzymes examined in old yam tubers are located in the regions of intermediate pH values in the tuber, while the lowest activities are coincident with the lowest pH values. In D

cayenensis, apart from hexokinase, the activity distribution of phosphorylase, phosphofructokinase and glucose-6-phosphate dehydrogenase is essentially the same in both middle and tail regions of the tuber. Ujam (1983) has studied the distribution of hexokinase within the tubers of D rotundata, D cayenensis and D alata. Using four different tubers in each case he had shown that hexokinase activity increases from head through middle to tail in D rotundata and to some extent in D alata while the distribution in D cayenesis is less specific. The establishment of pH difference between the different physiological regions of the yam tuber is interesting and is suggestive of some definite compartmentation in the seemingly dormant tuber. The role of the minute, though significant, pH variation in parts of the tuber has been postulated to be for the control of the rate of enzymic reactions in the various regions (Davies, et al 1972).

## 1.3 Detailed studies of some enzymes of carbohydrate metabolism

**Alcohol dehydrogenase:**

Detailed studies of Alcohol dehydrogenase has been conducted in D rotundata. The enzyme exhibits broad substrate specificity. The enzyme from D rotundata was found by Ujam (1979) to oxidise ethanol, n-propanol, n-butanol, n-pentanol and n-hexanol as well as benzyl alcohol, - p-OH-benzylalcohol, p-methoxy benzyl alcohol. $NAD^+$ is the preferred co-enzyme for the

oxidation ofthese substrates, unlike in potato tubers where there are separate aliphatic and aromatic alcohol dehydrogenases, using $NAD^+$ and $NADP^+$ respectively Davies et al (1972). Like the potato enzyme, the D-rotundata enzyme neither oxidizes methanol nor reduces formaldehyde.

**Hexokinase**

Ujam (1983) has purified and characterized this enzyme from white yam (D-rotundata). He observed that D-rotundata contains more of this enzyme than D-alata and D-cayenensis. The pH optimum of the D-rotundata hexokinase is 7.5 and its optimum temperature is 38°C. Substrate specificity indicates that hexokinase from D-rotundata has broad specificity. Glucose is the best substrate amongst the sugars with a Km of 0.065mM at saturating ATP concentration. The following order of reactivity for various sugar substrates was obtained glucose > mannose > fructose > ribose > xylose > glucosamine > galactose. Phosphorylation coefficient was the standard reference for this measurement.

Nucleotide specificity indicates that ATP is the preferred nucleotide, even though minimal activities were obtained with GTP and ITP as phosphoryl donors. Inhibition studies indicate that ADP and 5-AMP competitively inhibit the enzyme with ATP as the variable substrate and with glucose at a fixed concentration of 5mM. The Ki values of ADP and AMP as determined by Dixon plot

and slope of primary plots were found to be 2.3 and 3.8mM respectively.

$Mg^{2+}$ and $Mn^{2+}$ were found to be obligatory for D-rotundata hexokinase activity. $Ca^{2+}$ could not replace either $Mg^{2+}$ or $Mn^{2+}$. $Na^{+}$ and $K^{+}$ in the presence of $Mg^{2+}$ or $Mn^{2+}$ had no observable effects and could not replace either cation.

Succinate and glucose-6-phosphate at 2, 5, and 10mM concentrations have no observable effects on the activity of white yam hexokinase. Citrate and EDTA slightly increased the enzymatic activity. The enzyme has been shown to have a molecular weight of about 102,000 ± 3,000.

**Invertase:**

Passam and Harret (1977) have reported results of preliminary studies on the nature of invertase in yam tubers. According to them, acid invertase develops in tissue discs of D. alata, D. rotundata and D. cayenensis during ageing. The pH optimum for yam invertase activity was found to be 4.5. Activity was found to be dependent on temperature and to be located in both cell wall and soluble fractions. Passam and Harret (1977) did not observe differences in activity. The suggested reason for non-regional distribution of invertase by these workers is that acid invertase is an enzyme produced primarily in response to wounding unlike respiratory enzymes.

**Glucose-6-Phosphate Dehydrogenase:**

Ationu and Ikediobi (1983) characterised this enzyme from D. rotundata tubers. They found that their enzyme had a molecular weight of 163,000 ± 28,000 and performed extensive kinetic studies on they enzyme. This finding confirms previous reports by Ugochukwu et al (1977) that G6PD occurs in yam tubers and implicates the HMP pathway in carbohydrate dissimilation in yam tubers.

## 1.4 Malate dehydrogenase

Malate dehydrogenase MDH (L.- Malate $NAD^+$: Oxidoreductase (E.C. 1.1.1.37) plays a prominent role in carbohydrate metabolism. It catalyses the reversible oxidation of L.- malate to oxaloacetic acid in the mitochondrial tricarboxylic acid cycle (TCA cycle).

This is the fourth and final redox reaction encountered in the cycle with Keq of $1.3 \times 10^{-5}$, which seemingly favours the reverse reaction, (i.e. the reduction of OAA to L-malate). However, the condensation reaction between OAA and acetyl CoA to form citrate, catalysed by citrate synthase, is highly exergonic ( G -9000 cal, Keq = $2.2 \times 10^{6}$) and this therefore displaces the equilibrium in favour of OAA formation by its continuous removal to form citrate.

Malate dehydrogenase has been shown to promote the rapid

reduction of the keto tautomer of OAA to l.-malate by the reduced nicotinamide adenine dinucleotide (NADH). The reduction is accompanied by direct transfer of hydrogen atom from the co-enzyme to OAA so that the hydrogen of the solvent does not become attached to the carbon atom of L. malate which holds the hydroxyl-group (Tchen, 1955).

**Iso-enzymes of Malate dehydrogenase:**

Malate dehydrogenase has been shown to exist in iso-enzymic forms. Davies (1957) separated two forms of malate dehydrogenase from pea epicotyl associated with cytoplasm and mitochondria. Two forms of the enzyme, cytosolic and mitochondrial from Neurospora were reported by Benveriste & Munkres (1970). There were equally variant forms of mitrochondrial MDH population. Hayden, (1972) characterized two major forms of malate dehydrogenase from maize endosperm. Russel and Irwin (1973) reported for the first time the purification and crystallization of MDH iso-enzymes from Zea mays by a co-purification protocol involving thirteen steps. The immunological proof indicated that both enzymes were similar, as they were affected to the same extent by the anti-mito-chondrial MDH antibodies prepared.

Human serum malate dehydrogenase was separated by starch-block electrophoresis into three distinct fractions by Vessel and Bearn (1958).

The mitochondrial MDH of mammalian tissues has been shown

to differ from the corresponding cell sap enzyme in electrophoretic mobility (Delbruk et al 1959). The purified mitochondrial enzyme consists of several electrophoretically distinct components. Two active fractions have been detected by the moving-boundry technique in phosphate buffer pH 7.5, but ultracentrifuge studies indicated only a single symmetrical peak (Grim and Doherty, 1961).

Substrate analogue studies with the monofluoro- and difluoro-derivatives of oxaloacetate have provided a means not only for distinguishing between the cytoplasmic and mitochondrial forms of the enzyme but also for differentiating between the enzymes of different tissues. Kun and Volfin, (1966) found the rat liver cytoplasmic enzyme to utilize the fluoro-derivatives almost as effetively as oxaloacetate, while the corresponding Kidney enzyme is appreciably less active with oxalomonofluoroacetate, but readily reduced the difluoro-derivative.

The mitochondrial and cytoplasmic mammalian malate dehydrogenases differ from each other in many respects, and must be regarded as separate enzymes which differ in their primary structure (Wieland, Pfleiderer, Haupt and Worner, 1959; Delbruck, Zebe and Bucher 1959; Thorne 1960; Grim and Doherty 1961). Each form is however heterogenous, and has similar catalytic properties (Thorne, 1960).

### 1.4.1. Malate dehydrogenase isoenzymes in insect development

Changes in the pattern of malate dehydrogenase isoenzymes occur during development in insects (Laufer, 1961). Blood from the diapausing pupa of cecropia contains at least two isoenzymes, while that of the developing adult contains three or more; there is also a shift in the relative activities towards more acidic fractions. During the larval development of Cynthia there is gradual increase in an electrophoretically fast anodic isoenzyme, while a fraction of intermediate mobility diminishes and is replaced by a slow anodic fraction shortly before pupation. During diapause enzyme activity falls to low levels, but subsequently there occurs an increase in the fast and slow osoenzymes which alone are found in the adult insect.

Multiple forms of MDH also occur in the embryos of sea urchins, which exhibit three bands on acrylamide-gel electrophoresis. Unfertilized eggs, on the the other hand, contain five fractions (Moore and Villee, 1963). The change occurs within 90 min. of fertilization, and Villee (1968) has suggested that it may be related to changes in respiration and protein synthesis before cell division in the embryo.

### 1.4.2 Physiological importance of Malate dehydrogenases

An important metabolic function of cyloplasmic MDH in plant tissues is the generation of L-malate which plays a role in ionic

adjustment. Osmotic adjustment, and perhaps NADPH production Irwin and Dugger (1965).

Mitochondrial MDH in plants like other living eucaryotic organisms have a functional TCA cycle localized in the mitochondria. There can be little doubt, therefore that a major role of mitochondrial MDH is the oxidation of L-malate to oxaloacetate in the tricarboxylic acid cycle.

Microbody MDH associated with microbodies or peroxisomes are known to play an important role in photorespiration (Tolbert, 1971). In the photosynthetic related metabolic pathway oxidizing glycolate, Tolbert proposed a specific role for microbody MDH in the regeneration of reducing power i.e. NADH, for the reduction of hydroxy pyruvate to glycerate. In glyoxysomes with glyoxylate cycle, a specific role of mb MDH is known in the oxidation of l-malate to oxaloacetate similar to the TCA cycle Victor and Irwin (1971). It may be of significance that algae appear to lack the peroxisomal microbody MDH and also do not have glycolate oxidase, but glycolate dehydrogenase and not MDH, could be the redox couple with hydroxypyruvate reductase Victor, and Irwin( 1971).

Chloroplast MDH has been thought to catalyse the reversible reduction of oxaloacetate in the leaf.

The Michaelis constants and turn over numbers of the mitochondrial and soluble forms of malate dehydrogenase studied by Grim and Doherty, (1961) from Ox-heart showed marked

differences, which suggest that the mitochondrial enzyme is better suited for oxidation of malate, while the soluble enzyme is a more efficient catalyst for the reverse reaction. These observation might well be related to their biological functions, since both forms are strongly inhibited by excess substrate, but whereas the mitochondrial enzyme is paticularly sensitive to oxaloacetate, excess malate has a greater inhibitory effect upon the soluble enzyme. Kaplan (1961, 1963) has concluded therefore that these characteristics may prevent the reduction of oxaloacetate in mitochondria and the oxidation of malate in the soluble fraction.

These possibilities may play an important part in the energy relationships of mitochondria, and Kaplan envisages oxidation of soluble NADH by oxaloacetate to form malate, which enters the mitochondria, where it is again oxidized by the mitochondrial enzyme. The oxaloacetate produced might then be released into the cytoplasm, so enabling the cycle to be repeated.

### 1.4.3 Assay of Malate dehydrogenase

Malate dehydrogenase is assayed spectrophotometrically in the direction of OAA reduction by following the disppearance of NADH at 340nm. It is also assayed in the direction of l-malate oxidation at pH 8.5 by measuring the increase in absorbance at 340nm.

One unit of MDH activity is defined as that amount of enzyme catalysing the oxidation of 1 micromole of NADH/min at 30°C in the OAA reduction. Specific activity is expressed as units/mg protein. Victor and Irwin (1970).

1.4.4 **Molecular weight**

An extensive gel filtration survey of malate dehydrogenase from animal, plant, and microbial sources published by Murphey and Barnaby (1967) documented a single molecular weight of about 60,000 from all species studied except certain gram positive bacteria.

Cole et al (1971) reported a molecular weight of 61,000 for MDH isolated from chlorella pyrenoidosa. Three malate dehydrogenases isolated from spinacea oleracea associated with mitochondria cytoplasm and chloroplast were all shown to have molecular weights of 66,000 (Victor and Irwin, 1971). Toshihisa and Haruhiko (1986) observed two identical sub-units each with a molecular weight of nearly 35,000.

1.4.5 **Kinetic properties**

The Km values as reported by most research workers are within the range $10^{-4}$M-$10^{-5}$M. Cole et al (1971) determined Km for MDH from chlorella pyrenoidosa to be 4 x $10^{-5}$M and 5 x $10^{-5}$1 for NADH and OAA respectively. They also reported substrate inhibition at OAA level above 3.45 x $10^{-4}$M.

Kinetic constants reported by Toshihisa and Haruhiko (1986) for their enzyme from R. capsulata were 2.1mM, 0.048mM, 0.085mM, 0.025mM for l-malate, $NAD^+$ OAA, and NADH respectively. Victor and Irwin (1971) reported Km values of 5.9mM 0.57mM, 0.08mM, 0.04mM for l-malate, $NAD^+$, OAA and NADH for mitochondrial MDH isolated from spinach leaves.

1.4.6 **Effect of pH**

The pH optimum determined in the direction of L-malate oxidation was 9.75 and that for oxaloacetarte reduction was 6:8-7.4 (Toshihisa and Haruhiko, 1986). Victor and Irwin (1971) reported pH optimum values of 7.5 and 8.5-9.5 for OAA reduction and L-malate oxidation respectively. The kinetics of L-malate oxidation is always followed at pH 8.5-9.5 while that of OAA reduction is monitored at pH 68-7.4.

1.4.7 **Thermostability**

Malate dehydrogenase from R capsulata is susceptible to thermal inactivation and loses its activity completely at 40°C for 10 min (Toshihisa and Haruhiko, 1986). However addition of $NAD^+$, NADH or OAA to the enzyme stabilizes it. In the presence of 1mM $NAD^+$ the enzyme retained its full activity up to 35°C and 75% of the original activity after incubation at 40°C for 10 min.

Victor and Irwin (1971) reported the thermal stabilities of three MDH isoenzymes from Zea mays after heating at 54°C ± 1°C. They observed after heating for one minute that no microbody MDH

was present, mitochondrial MDH retained 73% of its initial activity, while 93% residual activity was retained by cytoplasmic MDH.

The thermostabilities of malate dehydrogenases from green stem tissue of Opuntia at 63° were equally distinctive (Murkeji and Irwin, 1969).

1.4.8 **Substrate specificity**

Bovine heart mitochondrial MDH catalyses the reduction of L-malate, meso-tartarate, L-tartarate oxaloglycollate, tartronate (Grim and Doherty, 1961).

Toshihisa and Haruhiko, (1986) reported that MDH from R capsulata required exclusively $NAD^+$ for L-malate oxidation and NADH for OAA reduction. $NADP^+$ and NADPH were inert co-factors for the respective processes. The enzyme was equally shown to be highly specific for L-malate. However, 10mM meso-tartarate was slightly oxidized (0.82% rate of L-malate oxidation). None of the following substrates (D-malate, L-tartarate, L & D lactate, dihydroxyl furmarate showed any reactivity.

Russel and Irwin (1973) reported that cytoplasmic malate dehydrogenase from zea mays was twice as active in the presence of thioniconinamide analog of $NAD^+$ than in the presence of $NAD^+$ when assayed at 0.1mM L-malate pH 8.5.

1.4.9 **Inhibitors**

Malate dehydrogenase is inactivated by p-hydroxy mercury benzoate (PHMB), potassium cyanide (KCN) etc. only when all groups that will react with the denaturing agent (i.e. 7-SH mole of enzyme) have completely reacted. Unlike other enzymes, the inactivation is irreversible by (GSH) glutathione reductase (Raval and Wolfe 1926b).

Chapter 2

# 2.0 MATERIALS AND METHODS

## 2.1 Source of yam tuber

Twelve healthy tubers of Dioscorea rotundata were purchased from Samaru market, Zaria.

## 2.2 Chemicals

Marker proteins, alcohol dehydrogenase, ovalbumin, and myoglobin were purchased from Sigma Chemical Company, St. Louis, U.S.A.

Nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced nicotinamide adenine dinucleotide phsophate (NADPH) l-malate, D-malate, oxaloacetate, ketoglutarate, citric acid, pyruvic acid, isocitric acid succinic acid, adenonsine triphospharte (ATP) adenosine diphosphate (ADP) were obtained from BDH Chemicals, Poole, England.

Tris-(hydroxymethyl) aminomethane, glycine, Blue dextran 2000, Sephadex G-200 and DEAE Cellulose 52 were obtained from Pharmacia - Fine Chemicals, Upsala, Sweden.

Acrylamide, N.N. methyl bis acrylamide (BIS), ammonium per sulphate, bromophenol blue, sucrose, N, N, N' N' tetramethylethylene liamine (TEMED), Coomasie blue and sodium dodecylsulphate (S.D.S.) were obtained from Shandon Products Ltd., Chesire, England. All other chemicals are of analytical grade.

## 2.3 Equipment

High speed refrigerated centrifuge, Beckmann model J-25, D.C. power supply for electrophoresis: Biorad Laboratory Model 500 power supply.

## 2.4 Extraction of Malate dehydrogenase

A whole yam tuber (Dioscorea rotundata) weighing about 300g was peeled, cut into slices, and soaked in 0.1% solution of sodium metabisulphite ($Na_2S_2O_3$).

A hundred gram portion of the slices was homogenized in 200ml of 25mM phosphate buffer pH 7.2 containing 5mM EDTA, and 2mM - 2-Mercaptoethanol with a warring blender. The slurry was squeezed between two cheese cloths, and the filtrate was used to re-extract two more hundred-gram portions of the yam tissue. The final filtrate was assayed for enzyme activity and protein. This formed the crude extract which was used for the subsequent experiments.

## 2.5 Assay for enzyme activity

Malate dehydrogenase was assayed spectrophotometri-cally in the direction of oxaloacetate (OAA) reduction, by following the disappearance of NADH at 340nm. The assay medium in a Cuvette (1cm path length) contained 0.134mM NADH, 0.2mM OAA in 25mM phosphate buffer (pH 7.2) in a total volume of three milliliter. One unit of MDH activity was defined as that amount of enzyme catalysing the oxidation of 1 micromole NADH per minute at 30°C in the OAA reduction. Specific activity was expressed as units/ mg protein.

## 2.6 Determination of protein

Protein concentrations were determined by the Biuret method, using Bovine serum albumin as standard.

## 2.7 Ammonium sulphate fractionation

Initial experiments to determine the ammonium sulphate fractionation range for the enzyme was performed with 30ml of the crude extract at the following salt saturation 20, 30, 50, 70, 80, 90%. The enzyme exhibited reasonable activity between 30-80% $(NH_4)_2SO_4$ saturation.

The remaining portion of the crude extract was brought up to 30% $(NH_4)_2SO_4$ saturation. Sedimentation was aided by

centrifugation at 2000g for 15 min. The pellet was discarded and the supernatant solution taken to 80% $(NH_4)_2SO_4$ saturation and centrifuged at 5000g for 15 min. The pellet was dissolved in 40ml of 25mM phosphate buffer (pH 7.2) containing 5mM EDTA and 2mM 2-mercaptoethanol.

## 2.8 Gel filtration

Sephadex G.200 (10gm) was swollen in distilled water for 2-days and packed in a chromatographic column (2.6 x 40cm). The gel was equilibrated with 25mM phosphate buffer (pH 7.2) containing 5mM EDTA and 2mM 2-mercaptoethanol. A cooling device (Grant coolant) was used to equilibrate the column to $4^{o}C$.

Five milliliter of the ammonium sulfate concentrate was layered on the sephadex-bed and allowed to drain. The column was eluted with the equilibrating buffer. Eighty fractions of 5ml each were collected and assayed for MDH and protein.

## 2.9 Ion exchange chromatography

DEAE-cellulose DE (52) 25gm was precycled with 0.5N HCl and 0.5N NaOH successively for 30 min. The slurry was charged with 0.25M phosphate buffer, pH 7.2. The ion exchanger was packed into a chromatographic column and equilibrated with 0.025M phosphate buffer, pH 7.2. The column was chilled to $4^{o}C$ by the cooling device as in 2.8 above.

Ten milliliters from the active peak of the preceding step was applied on the ion exchanger. The enzyme was eluted by a linear phosphate gradient of 25mM - 250mM. The flow rate was maintained at 2.5ml/min. Eighty fractions of 5ml each were collected and assayed for MDH activity and protein at 280nm. Active enzyme fractions were pooled and concentrated with dry Sephadex G-50.

2500 0.025
1m

## 2.10 Disc gel electrophoresis

Analytical polycrylamide gel electrophoresis was carried out as described by Ornstein (1964). Gels (7.4%) were run in Tris-glycine buffer, pH 8.4. The enzyme extract (0.2ml) was mixed with 1.0ml of the stacking buffer, 0.2ml of 2M sucrose, and 0.1ml Bromophenol blue and 0.1ml of the preparation was dispensed on the gels.

A current of 2mA/gel tube was applied until the tracking dye (bromophenol blue) entered the running gel, and the current was increased to 5mA/gel tube. The power pack was switched off when the tracking dye was 2mm to the gel end. The gels were removed and soaked in 12% TCA (trichloroacetic acid) for 30 min. to fix the proteins, and later transferred to Coomasie blue stain for 1 hour. The gels were destained in 7% acetic acid and stored in the same solution for observation.

## 2.11 Gel filtration chromatography for M. wt. determination

The basic principle behind gel filtration is the separation of macromolecules on the basis of their molecular size.

A column (2.6 x 60cm) packed with pre-swollen sephadex G.200 was equilibrated with 25mM phosphate buffer, pH 7.2.

Five milliliter of 0.02% (w/v) Blue dextran was applied to the sephadex bed and eluted with equilibrating buffer.

The flow rate was controlled at 1.5ml/min. and 5ml fractions were collected until blue-dextran was completely eluted. The void volume Vo of the blue dextran was $65cm^3$.

Two milligrams each of the following marker proteins: aldolase, alcohol dehydrogenase, BSA, ovalbumin and mygolobin were dissolved in 10ml phosphate buffer. An aliquot of 1ml of each marker protein solution was mixed to give a final volume of 5ml. The mixed marker protein solution was applied on the sephadex bed and eluted with 25mM phosphate buffer. Fractions were collected as earlier described.

The elution pattern was determined by taking absorbance at 280nm. The various marker proteins emerged in order of decreasing molecular weights as follows: aldolase, ADH, BSA, Ovalbumin, and myoglobin.

Five milliliters of the purifed enzyme was then applied to

the same column. The elution pattern of the enzyme was determined by assaying each fraction for MDH activity as earlier described.

## 2.12 Polyacrylamide gel electrophoresis

Sub-unit molecular weight was determined by SDS polyacrylamide gel electrophoresis, according to Weber et al (1964) on 10% gels.

One milliliter of potassium phosphate buffer pH 7.0 containing 1% SDS and 1% 2-mercaptoethanol was added to one vial of molecular weight marker (14,300 - 71,000). While 2-mercaptoethanol prevent the oxidation of essential sulfyhydryl groups, SDS is an active detergent which denatures the proteins and make them move as discs of homogenous macromolecules.

The vial was incubated at 100°C for 2 min. A Similar protocol was employed for MDH purified enzymes.

Suitable gel loads were prepared from 0.3ml treated marker solution (0.1ml treated MDH) 0.1ml glycerol, 0.3ml phosphate buffer (containing 0.1% SDS, 0.1% 2-mercaptoethanol) 0.02ml-2-mercaptoethanol and 0.02ml of 0.05% Bromophenol blue.

Aliquots (0.1ml) of the gel load were dispensed on the gels and electrophoresed at 5mA/gel tube, until the dye bands are about 2mm to the end of the gel tubes. The gels were removed

from the tubes by using a hypodermic syringe, and the distance moved by the dye measured. The gels were then placed in Coomasie blue stain for 12hr, and destained in 7% acetic acid. The gels were finally stored in 7% acetic acid.

## 2.13 Effect of pH on the purified enzymes

The activity profiles of the purified enzymes were determined as a function of pH using 0.2mM OAA and 0.134mM NADH as substrate and co-enzyme, respectively.

The following buffers were used in the pH analysis; glycine-HCl pH 5-6. Phosphate buffer pH 6.5-8.0, glycine NaOH pH 8.5-9.5. Buffers of similar grades to the aforementioned were also prepared in the analysis of effect on pH on Km and Vmax.

The activity of MDH was assayed as earlier described. NADH was used as variable substrate while maintaining OAA at sub-saturating level. The pH was varied from 5-9. The Lineweaver Burk plots were made to obtain values for Km and Vmax.

The logarithmic values of Vmax, Vmaz/Km as functions of pH were plotted as described by Dixon and Webb (1964).

## 2.14 Effect of temperature on MDH activity and stability

The purified enzyme (0.2ml) was dispensed into each of 6 different test-tubes and incubated at the following temperatures 5°C, 17°C, 25°C, 30°C, 37°C, and 46°C for 10 minutes.

The enzyme reaction medium was also incubated at the various temperatures for the given time. At the end of the incubation period, the enzyme was quickly transferred into the reaction medium and the activity measured as earlier described.

The energy of activation (Ea) and Arrhenius frequency factor of collision (A) were determined from Arhenius plot of log velocity as ordinate and reciprocal of absolute tempeature (K) as aboissa.

A study was conducted on the stability of the purified enzymes on storage. Aliquots of 0.5ml of the purified MDH were introduced into 15 tubes and sealed with aluminium foil. Three batches of 5-tubes each were left at laboratory temperature (28°C), refrigerator (6°C) and the deep freezer -(15°C) for a period of 14 days. The residual MDH activity was monitored for the specified period.

## 2.15 Substrate specifity

D-mallate, DL-lactate, and -ketoglutarate were used with

$NAD^+$. L-malate and $NADP^+$, OAA and NADPH were tested for substrate specificity.

## 2.16 Initial velocity studies

The pure enzyme was assayed at varying concentrations of NADH (0.02-0.2mM) while keeping OAA as the changing fixed substrate at concentrations of (0.05-0.4mM). OAA and NADH were also used as variable and changing fixed substrates, respectively.

In the reverse reaction conducted at pH 9.0 L-malic and $NAD^+$ were used as changing fixed and variable substrates respectively, and vice versa. The enzyme was assayed in the direction of reduction of $NAD^+$ i.e. increase in absorbance at 340nm.

## 2.17 Product inhibition studies

The experimental protocol entailed holding one substrate at subsaturating level and varying the other substrate in the region of its Km, at different fixed concentration of one of the products.

Initial velocity experiment was conducted in the absence of the inhibitor ($NAD^+$) NADH was used as variable substrate while OAA remained fixed and saturating. 1.5mM - 4.0mM $NAD^+$ was used

as product inhibitor with the aforementioned conditions unchanged.

L-Malic acid (4.4mM - 8.8mM) was also used as the other product inhibitor with similar experimental condition. In the reverse reaction $NAD^+$/L-Malate were interchangibly used as variable/fixed substrates, while administering different levels of OAA/NADH as product inhibitor in each case.

### 2.18 Inhibition studies using naturally occurring metabolites

A fixed concentration of 10mM of each of the following inhibitors; citric acid, isocitric acid, ketoglutaric acid, asparagine and glutamic acid was incubated with the enzyme assay medium (i.e. 0.2mM OAA and 0.134mM NADH).

Detailed inhibition studies were conducted with citric acid, ketoglutaric acid using NADH and OAA as the variable and fixed substrate respectively.

### 2.19 Inhibition studies with ATP, ADP

Initial inhibition analysis was conducted with 3mM ATP, and 3mMADP to determine percentage inhibition.

Further inhibition studies were done with ATP, ADP in the concentration range of 2mM - 4mM. In each case NADH, OAA were

used as variable and fixed substrates interchangeably. Kinetic analysis were performed and Ki, Km, Kapp, Vmax were determined.

## 2.20 Inhibition studies using group specific reagents

Inhibition using $NaN_3$, KCN, iodoacetamide, p-hydroxy-mercury benzoate (PHMB) as group specific reagents was conducted. The protocols involved are similar to all other inhibition studies discussed.

Chapter 3

# 3.0 RESULTS AND DISCUSSION

## 3.1 Enzyme purification

The purification profile of malate dehydrogenase (MDH) from D. rotundata is summarised in table 1. Each purification step was followed with a routine assay of malate dehydrogenase in the direction of oxaloacetic acid reduction at the expense of NADH.

Chromatographic separation of malate dehyudrogenase (MDH) iso-enzymes by elution on D.E.A.E. cellulose column with linear gradient (25mM - 250mM sodium phosphate buffer, pH 7.2) resulted in two distinct active peaks (Fig. 2). The active peaks were re-chromatographed on separate D.E.A.E.-cellulose columns (Fig. 3) which gave a final purification fold of 147 and 129 for $MDH_1$ and $MDH_2$ respectively.

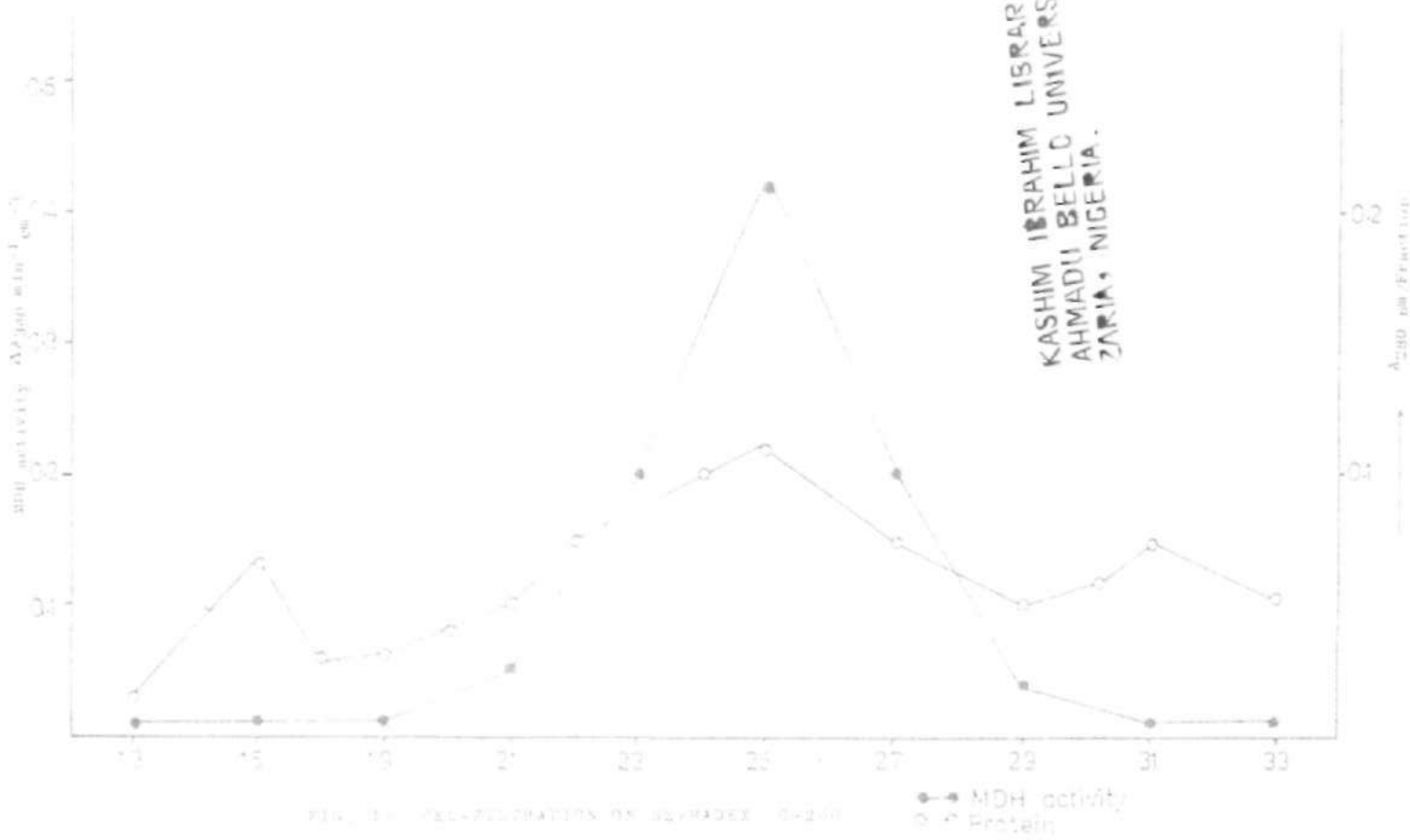
Polyacrylamide gel electrophoresis (P.A.G.E.) of the resolved malate dehydrogenases (Plate 1) indicated that the active peaks from Figure 2 are two molecular forms of the enzyme.

Malate dehydrogenase isoenzymes have been described in plants and animals. Davies and Kun (1957) separated two MDH isoenzymes from Pea epicotyl using D.E.A.E.-cellulose

Table 3.1: Purification Profile

| Purification Step | Volume ($CM^3$) | Protein (mg) | Total Activity (Units) | Specific Activity (Units/mg) | Yield | Purification Factor |
|---|---|---|---|---|---|---|
| Crude Extract | 250 | 2638 | 950 | 0.36 | 100 | '1' |
| 30-80% $(NH_4)_2SO_4$ Fractionation | 40 | 492 | 700 | 1.42 | 73.6 | 4.0 |
| Sephadex G-200 Chroma-tography | 25 | 13 | 500 | 41.0 | 52.6 | 114 |
| DEAE-Celluose Chroma-tography (Peak 1) | 40 | 5.4 | 254 | 46.5 | 26.7 | 130 |
| DEAE-Cellulose Chroma-tography (Peak 2) | 40 | 5.3 | 228 | 42.5 | 24.0 | 118 |
| 2nd DEAE-Cellulose Chroma-tography (PEAK 1) | 15 | 3.0 | 158 | 52.7 | 16.6 | 147 |
| 2nd DEAE-Cellulose Chroma-tography (Peak 2) | 20 | 3.2 | 149 | 46.4 | 15.6 | 129 |

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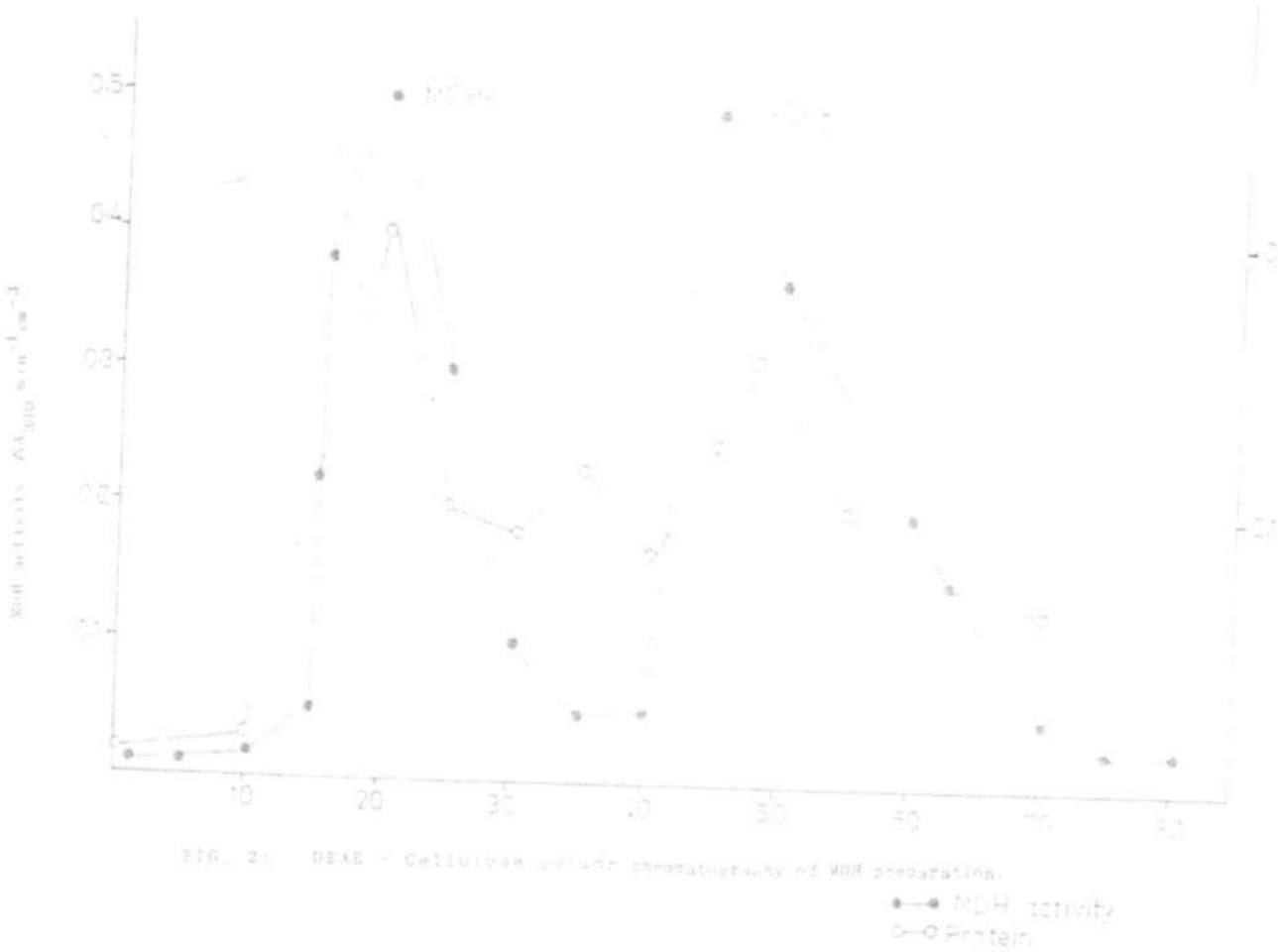
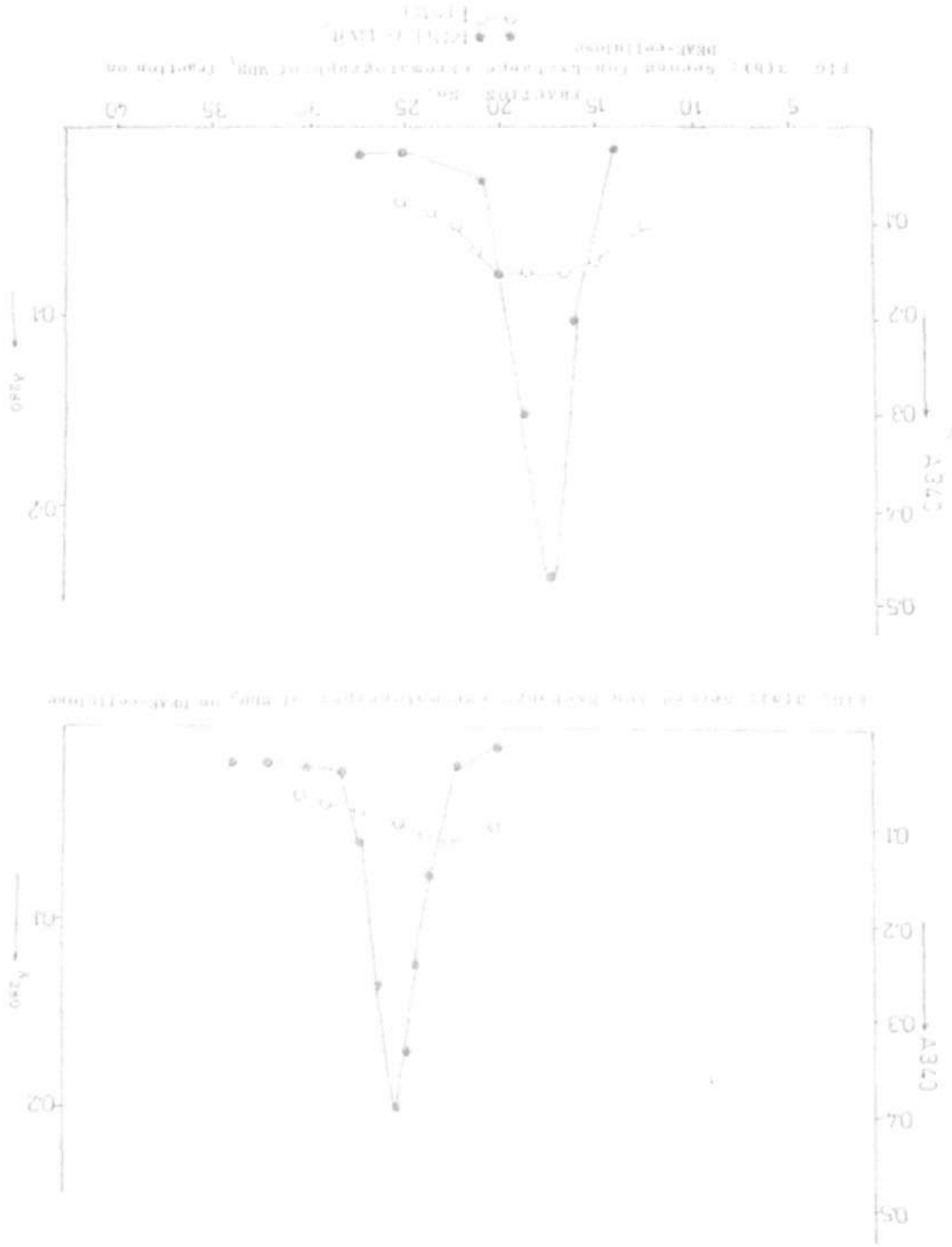



FIG. 2: DEAE - Cellulose column chromatography of MDH preparation.



chromatography. Victor and Irwin, (1971) reported the separation of Malate dehydrogenases of leaf tissue from Slinacea oleracea associated with chloroplast, mitochondria, and cytoplasm. Examining the MDH system of chlorella pyrenoidos, Cole et al (1968) demonstrated the existence of multiple electrophoretic forms. We also report here MDH isoenzymes from yam tuber (D rotundata).

Since multiple forms of this enzyme appear prevalent in biological systems, understanding their biological significance cannot be over emphasized. Through careful examination of the iso-enzymes, some insight can be gained on the question of their biological role.

In this work, the homogeneity of the Malate dehydrogenases, which is an index of purity was tested on Disc gel at pH 8.3. Both peaks from Fig. 2 corresponding to $MDH_1$ and $MDH_2$ moved as single homogenous bands (Plate 1) and corresponded to the two resolved bands of total MDH obtained from gel filtration (Fig. 1). The electrophoretic mobility of $MDH_2$ on P.A.G.E. gel was greater than $MDH_1$. However, $MDH_1$ was eluted first before $MDH_2$ on D.E.A.E. - cellulose column at pH 7.2. Previously, Victor and Ting, (1968) reported a similar pattern of migration of Malate dehydrogenases from Spinacea Oleracea. Murkeji and Ting (1969) also reported the migration of three homogenious bands of MDHs associated with different cell organelles from green stem tissue of Opuntia ficus. These reports are compatible with our findings

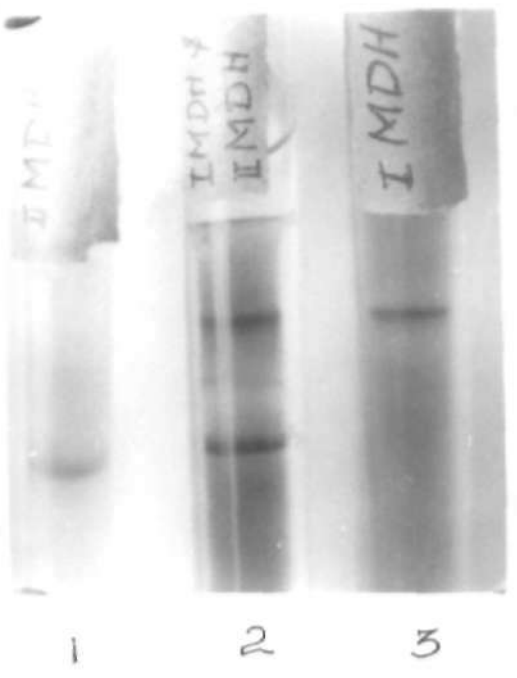



Plate I: Gel-electrophoresis of separate MDH

Gel (1). The band on gel (1) represents $MDH_2$ from peak 2 of Fig. 2. This is an exact match of the second band on gel 2.

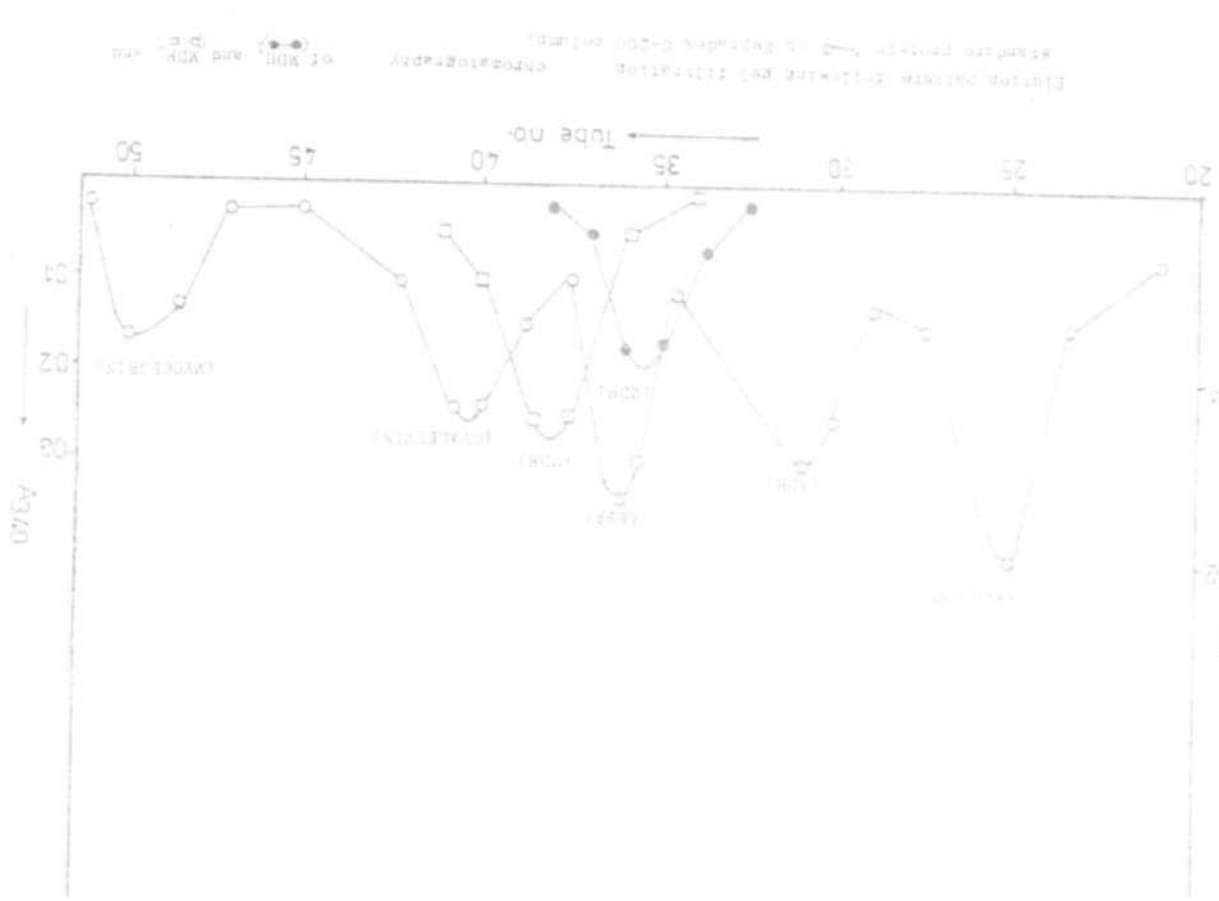
Gel (2). The two bands represent total MDH after the gel filtration step shown in Fig. 2.

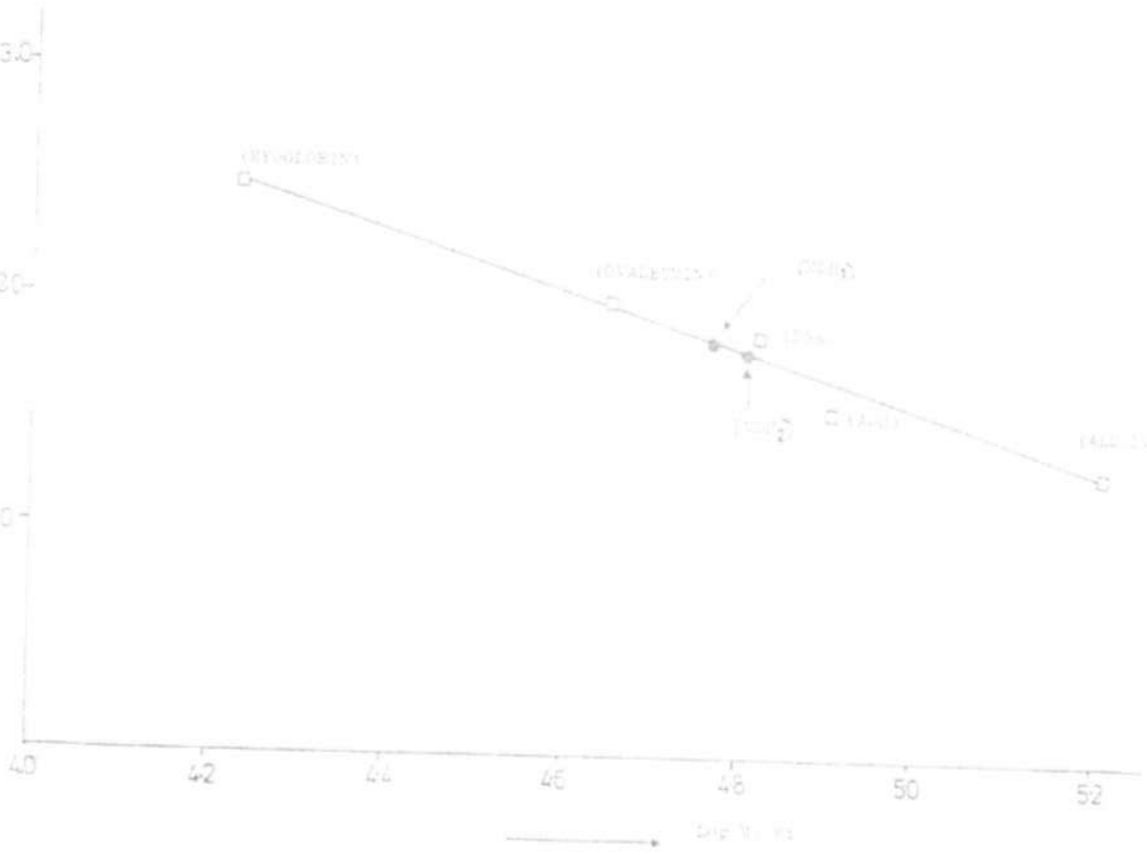
Gel (3). The band on gel 3 is an exact match of the first band on gel 2 and represent $MDH_1$ from peak 1 of Fig. 2.

on malate dehydrogenase iso-enzymes from the yam tuber D. rotundata.

### 3.2 Molecular weight determination

The molecular weights of the enzymes determined by gel filtration on Sephadex G-200 (2.6 x 60 cm) were 61,200 ± 150 for $MDH_1$ and 63,000 ± 400 for $MDH_2$ (Fig. 4 and 5). Both enzymes on treatment with sodium dodecyl sulphate (S.D.S.) migrated as single bands with the same electrophoretic mobility (Plate 2) and the estimated molecular weight was 31,000 (Fig. 6). This observation suggests that the enzymes exist in two forms and each form is made up of two identical sub units. Some bacteria have been described to contain Malate dehydrogenases with molecular weight of 120,000 which are thought to be tetramers (Kaplan, 1963). Murphey and Barnaby (1967) also reported from gel filtration survey a single molecular weight of 60,000 for all Malate dehydrogenases isolated from animal, plant and microbial sources. In 1971 Cole et al (1971) estimated the molecular weight of the enzyme from chlorella to be 61,000. Three Malate dehydrogenases isolated from Spinacea olerarea associated with mitochondria, chloroplast and cytoplasm were shown to have molecular weights of about 66,000 (Victor and Ting 1971). In the same vein, Toshihisa and Haruhiko (1985) published the molecular weight of the enzyme from R. capsulata to be 80,000 by gel filtration, and a subunit molecular weight of 35,000 by S.D.S. - P.A.G.E.


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Log Vt. Vo

Plot of elution volume (Ve) Vs Log M.wt. of calibration proteins for the determination of Molecular Wt. for VCM.

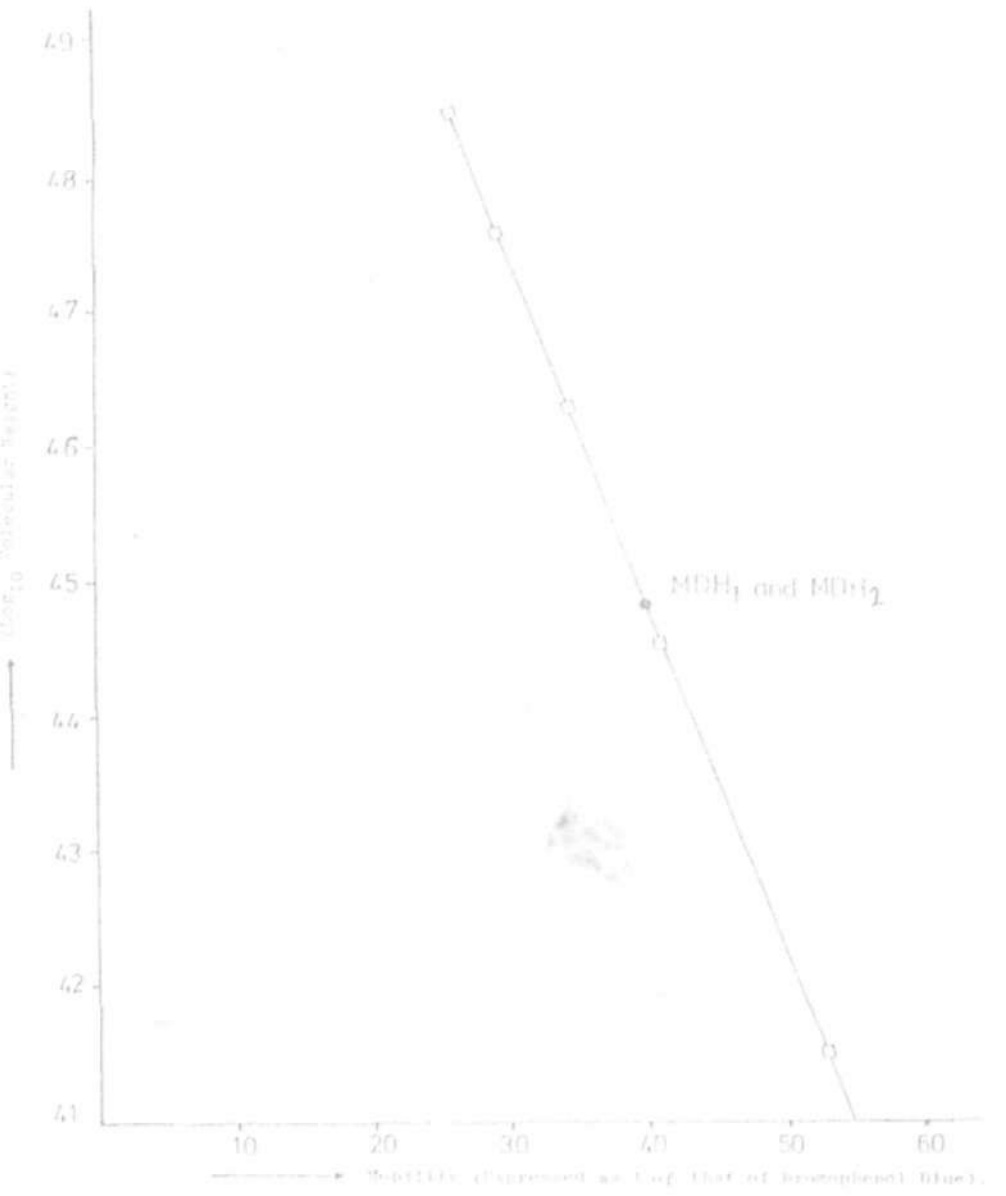



Fig. 3.2.2. Polyacrylamide Gel Electrophoresis - calibration curve for the determination of the molecular weight of $MDH_1$ and $MDH_2$.




Plate 2: Sodium dodecyl sulphate polycrylamide gel electrophoresis (SDS-PAGE) of $MDH_1$ and $MDH_2$.

Gel (1). The band on gel 1 represents $MDH_2$

Gel (2). The band on gel 2 represents $MDH_1$

Gel (3). Represents the standard marker proteins for molecular weight determination.

These data compare, well with malate dehydrogenases from Yam tuber. Since it was observed that the molecular weights of both enzymes are about the same, aggregation into polymeric forms may not be a molecular mechanism for generating heterogeneity of these enzymes in the yam tissue. Genetic evidence had earlier shown that malate dehydrogenase isoenzymes are different gene products (Kurimatsu 1968),

### 3.3 Effect of temperature on rate of reaction

Temperature dependent studies on the enzymes indicated that both forms of Malate dehydrogenases increase their rates of reaction with temperature. The observed increase was about twice per $10^{o}C$ increase within $0^{o}C$ - $40^{o}C$ temperature range. The temperature optimum for the enzyme catalysed reactions was $37^{o}C$. Denaturation was observed at $46^{o}C$. Arhenius plots of log velocity against $^{1}/T$ for both enzymes gave activation energy (Ea) and Arhenius frequency factor of collision (A) of 33.5kJ/mole and $1.94 \times 10^{5} dm^{3}m\ mole^{-1}\ min^{-1}$ for $MDH_1$ and 47.8 kJ/mole and $4.92 \times 10^{7} dm^{3}m\ mole^{-1}\ min^{-1}$ for $MDH_2$ (Fig. 7).

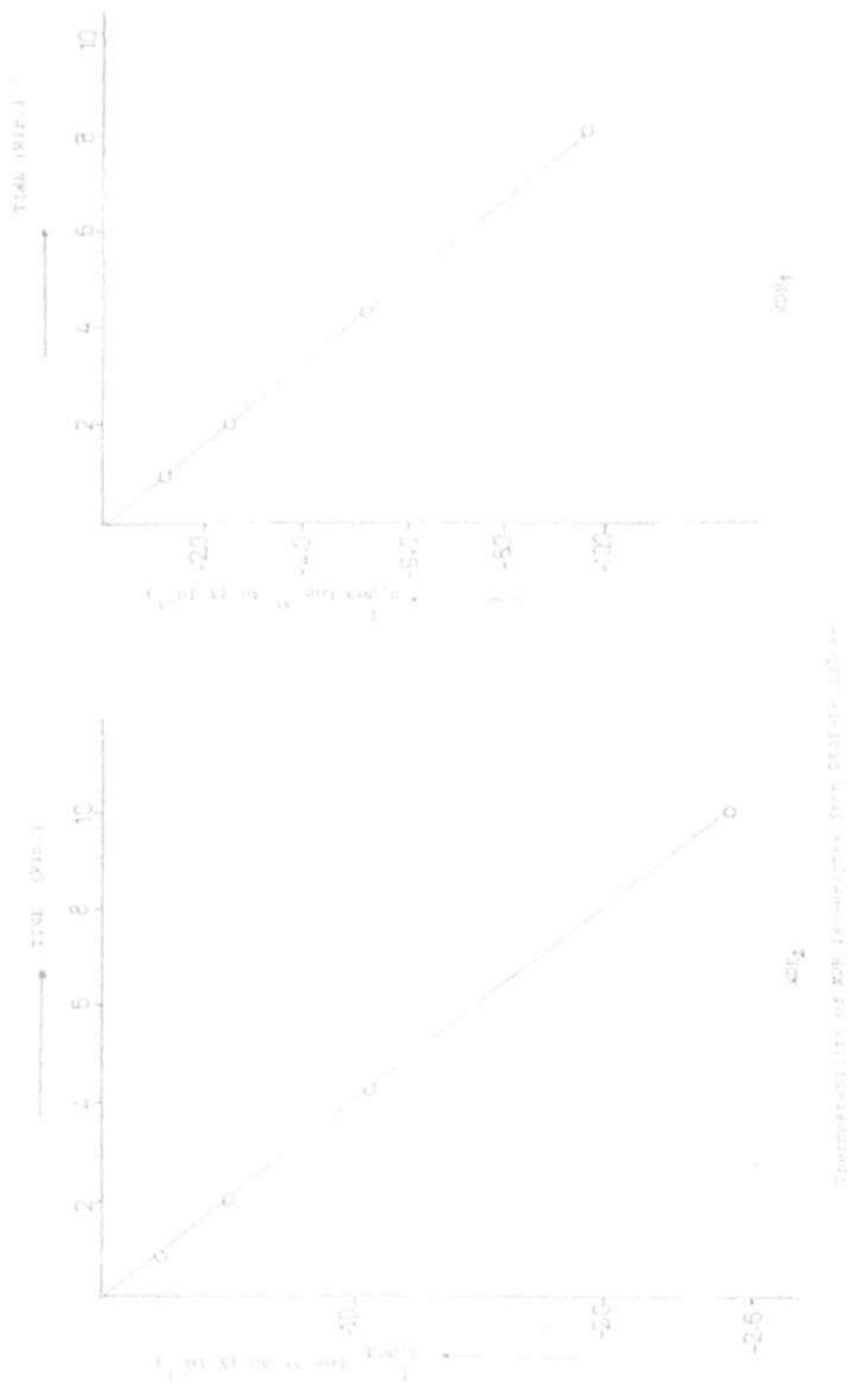
Increase in temperature generally affects two independent processes (i) The catalysed and reaction (ii) Inactivation of the enzyme. In the lower temperature region (up to $37^{o}C$), inactivation is extremely slow and negligible, thus producing no

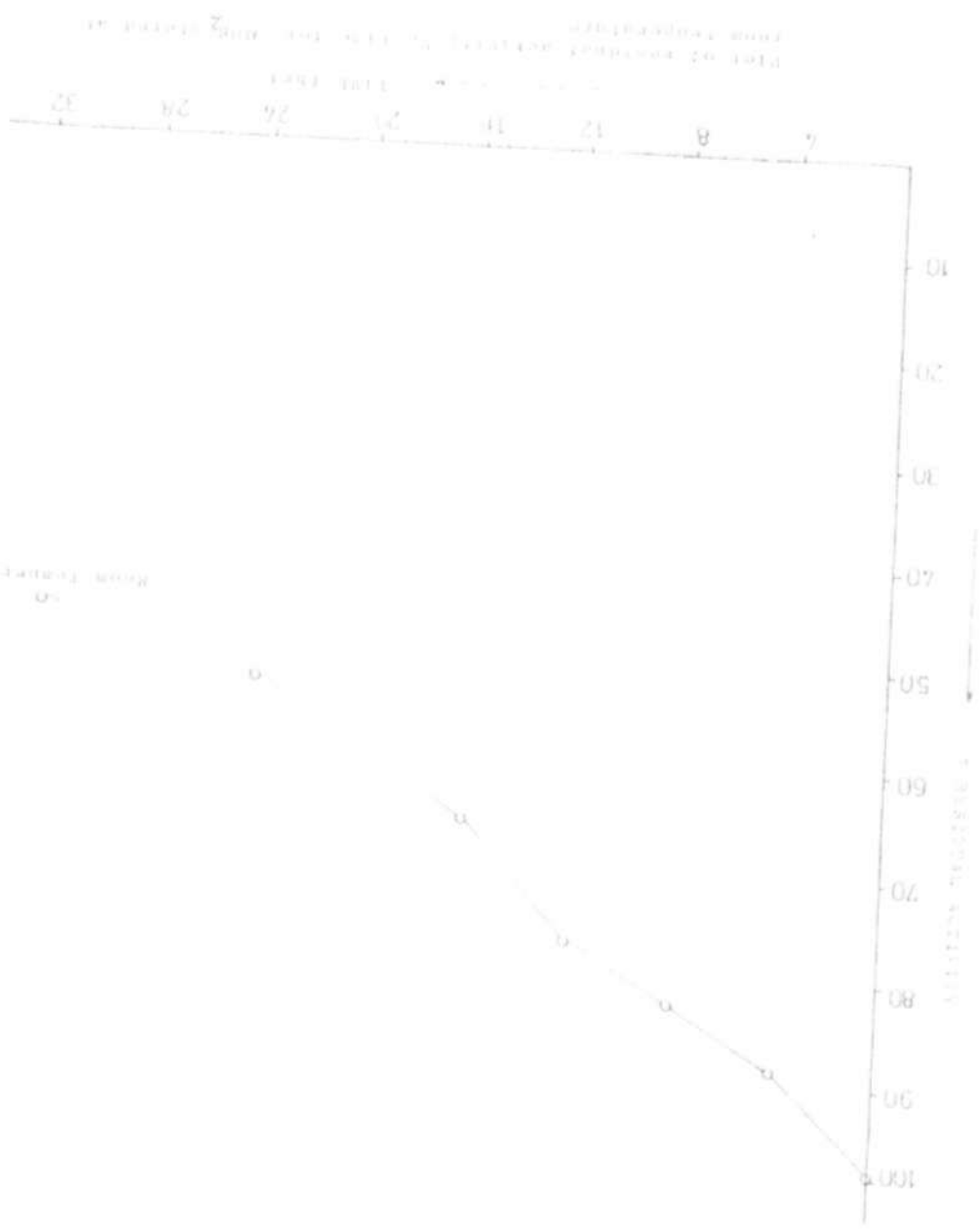

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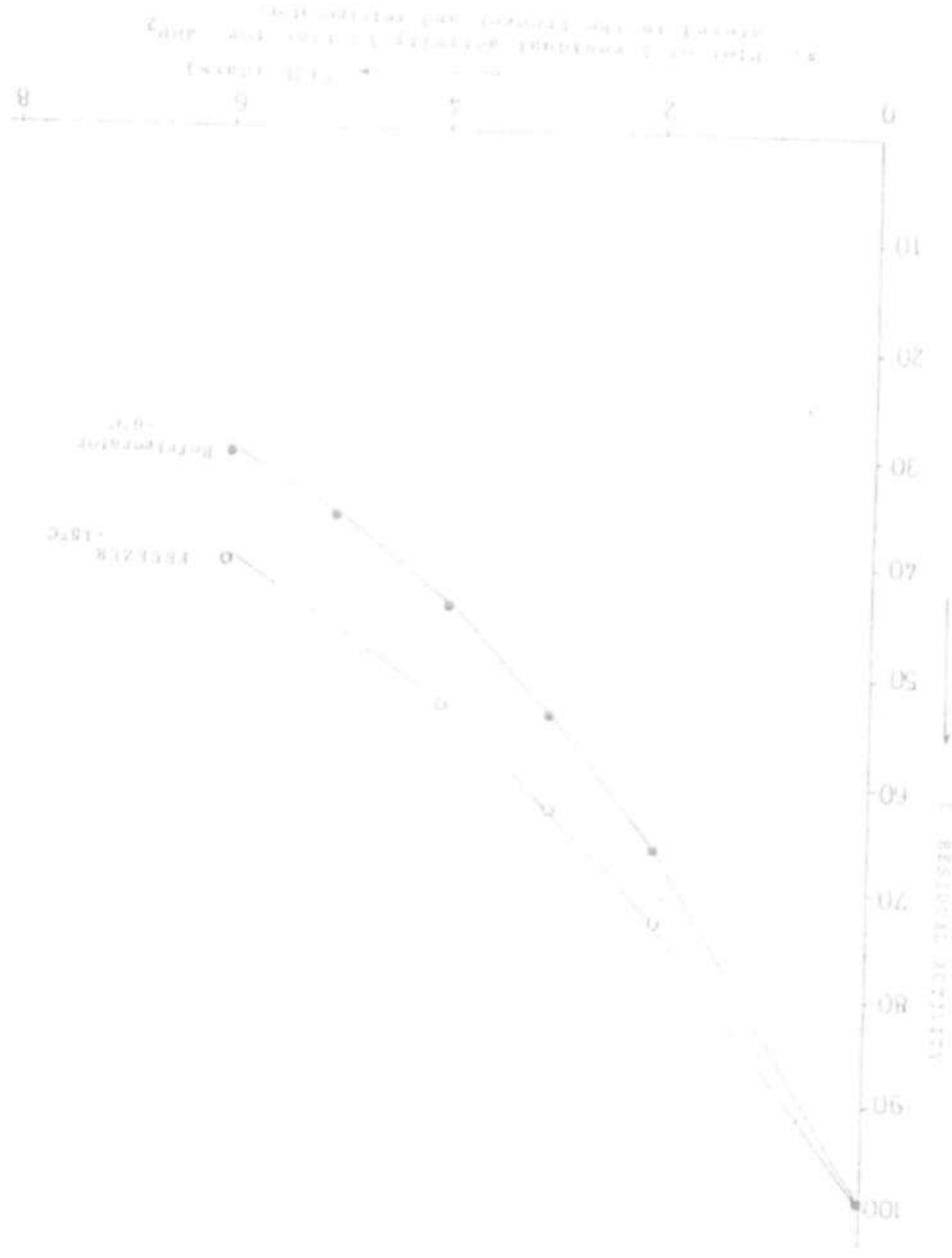
appreciable effect on the rate of reaction. The overall rate therefore is increased with increase in temperature. At high temperatures, inactivation becomes important so that the concentration of the activated enzyme falls during the reaction. At this temperature, the secondary and tertiary structures of the enzyme are destroyed, hence catalytic function, impaired.

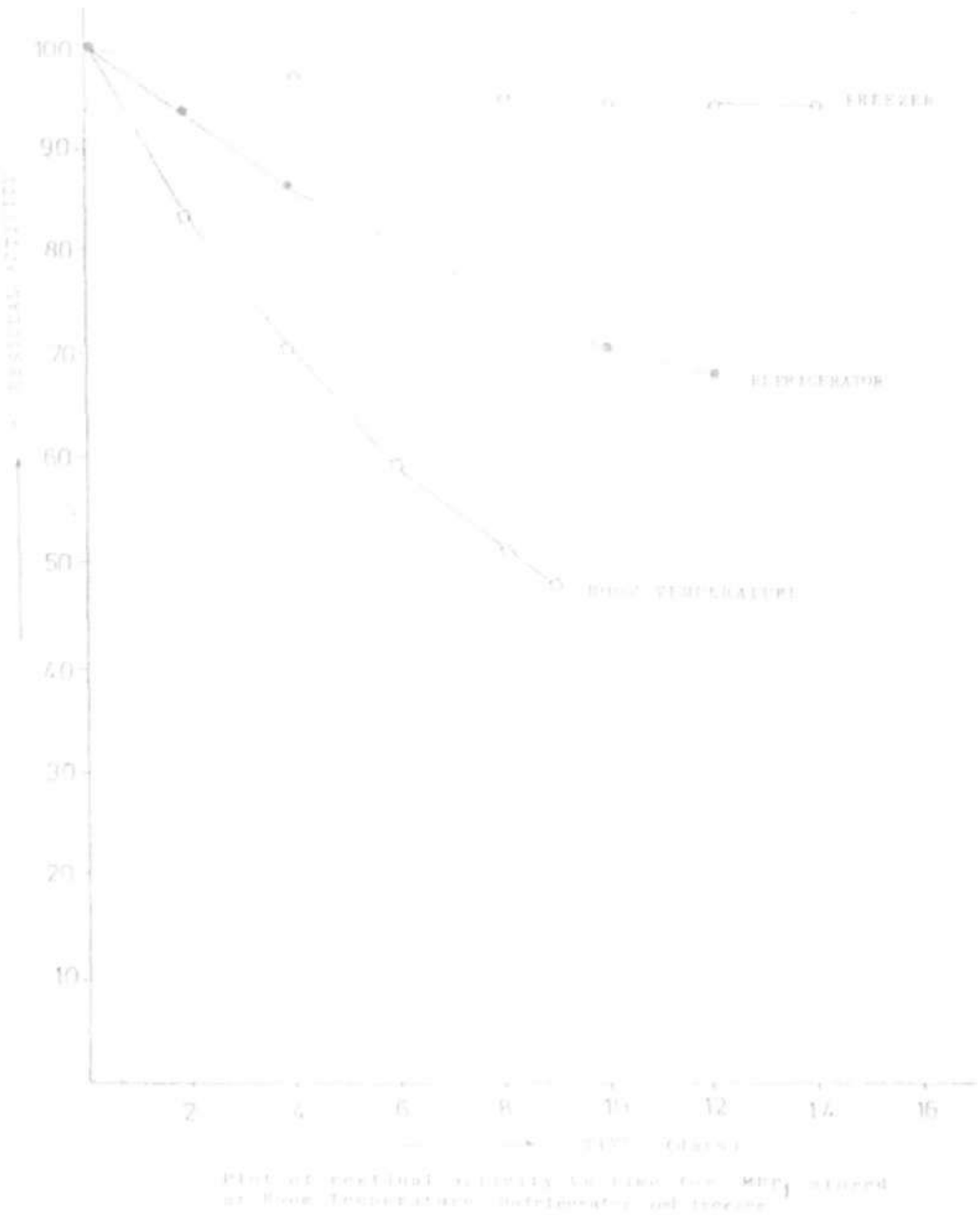
The thermal stabilities of both enzymes at 46°C were significantly different. First order rate constants for inactivation were 0.057 $min^{-1}$ and 0.28 $min^{-1}$ corresponding to $t_{1/2}$ of 12.15 min and 2.5 min for $MDH_1$ and $MDH_2$ respectively (Fig. 8). These differences in stability may be further associated with differences in tertiary structure of both enzymes, and this invariably means differences in primary structure. Differences in their primary structure is consistent with the iso-enzymes being different gene products.

The various storage conditions, viz storage of the enzymes at room temperature (25°C), refrigerator (4°C) and freezer (-15°C) elicited a clear cut difference in their stability (Figs. 9-13). The first order rate constants for $MDH_2$ stored at these conditions were 0.55 $day^{-1}$, 0.19 $day^{-1}$ and 0.15 $day^{-1}$ corresponding to $t_{1/2}$ of 1.25 days, 3.6 days, and 4.6 days respectively. The more stable $MDH_1$ had the following rate constants: 0.084 $day^{-1}$, 0.034 $day^{-1}$, and 0.005 $day^{-1}$ corresponding to $t_{1/2}$ of 8.3 days 20 days and 122 days respectively. These glaring differences substantiates that MDH





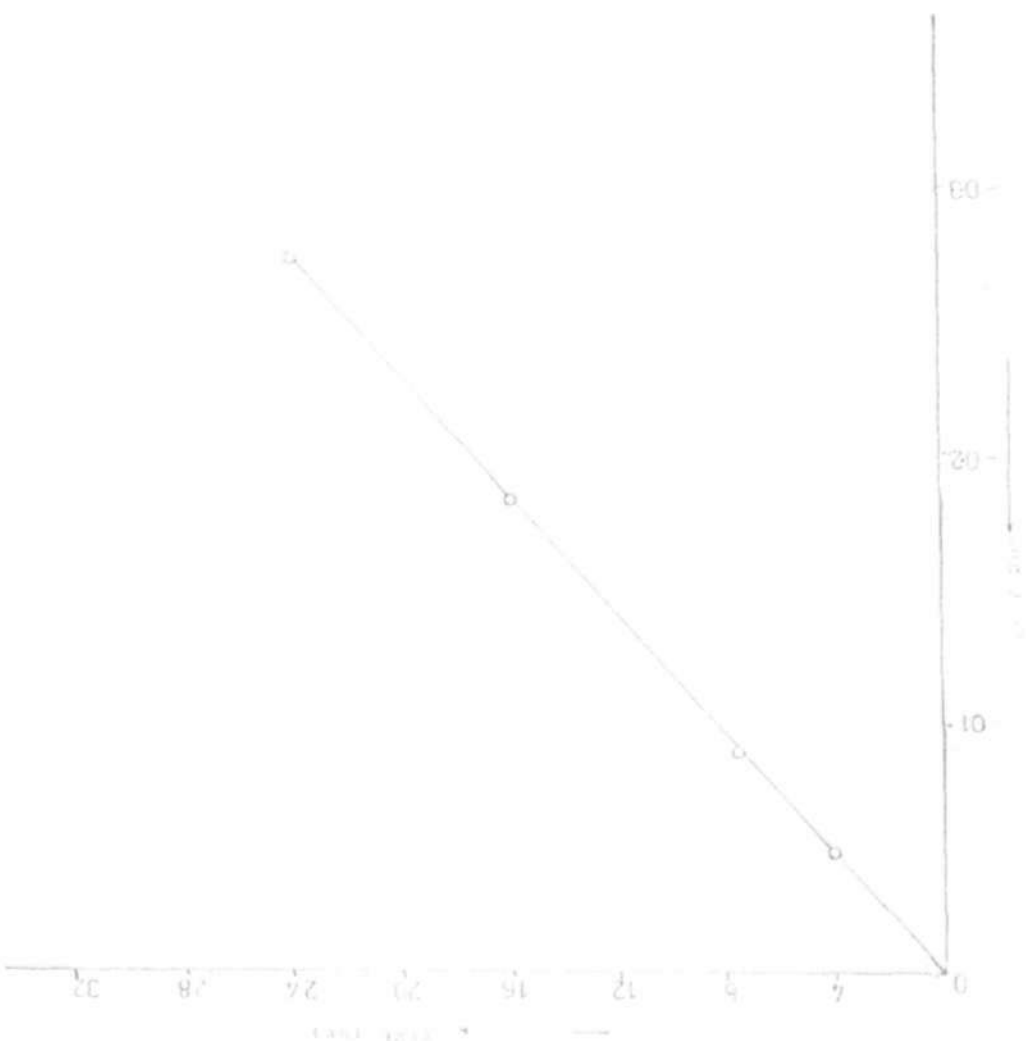


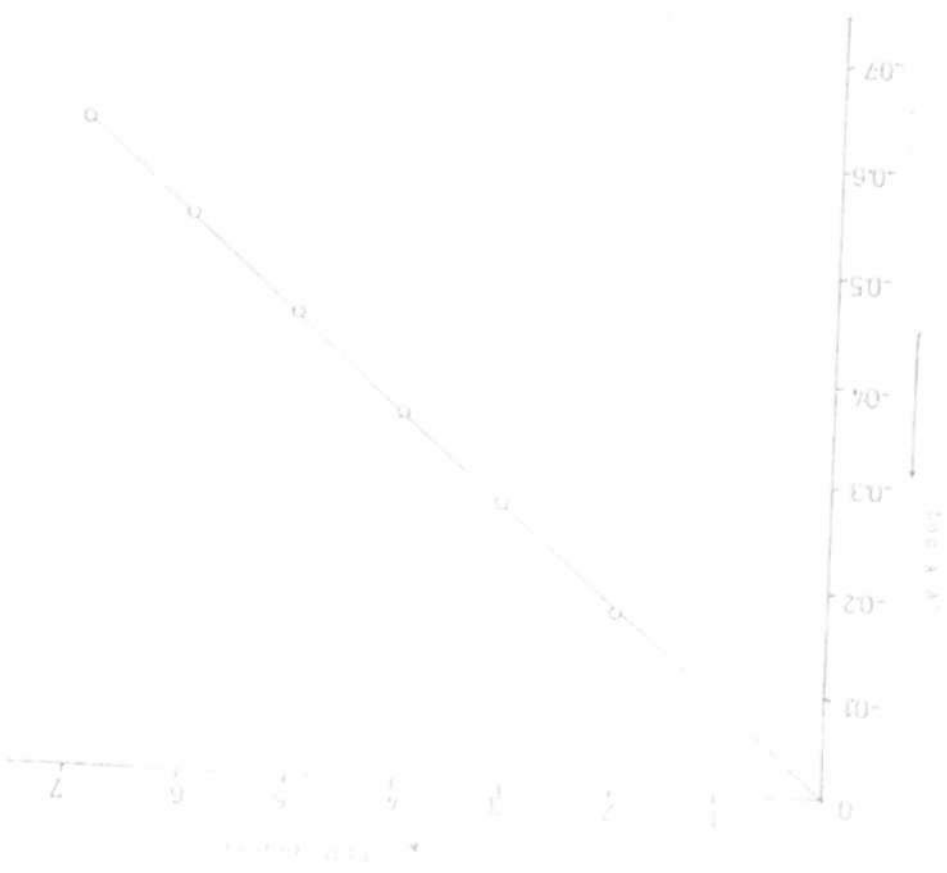

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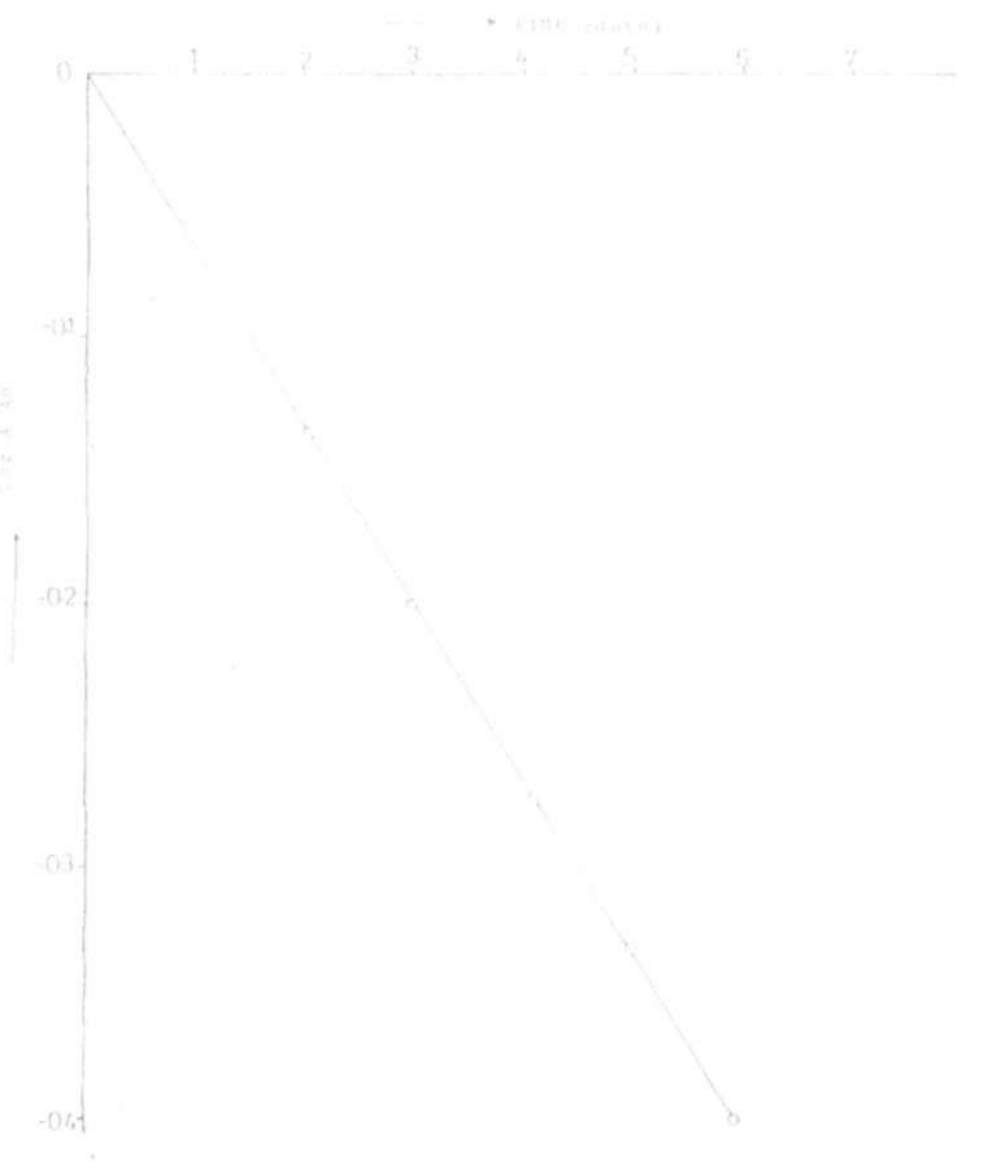
isozymes must have originated from different genetic loci.

Among factors highlighted which cause the several loss of sound yam tubers are deleterious endogenous reactions of the tuber. These reactions include those associated with weight losses, the hydrolysis of proteins, and the disintegration of the membranes of the tuber (Osuji and Umezurike, 1985). Earlier attempts to formulate a technology for long term yam storage centred on temperature control. Rivera et al (1974) did speculate that the yam tissue being a living system with its reactions under enzymic control shows that the storage of yam tubers at low temperature will significantly slow down the tuber's life process thus prolonging its shelf life. In the same vein Olorunda, et al (1974) reported that tubers stored at 15°C experience less dessication than those stored at 10°C, 20°C and 25°C. The weight loss at 10°C was about 4 times and 5 times higher than loss at 20°C and 25°C respectively. This large loss was attributed to chilling injury on membrane system of the tuber.

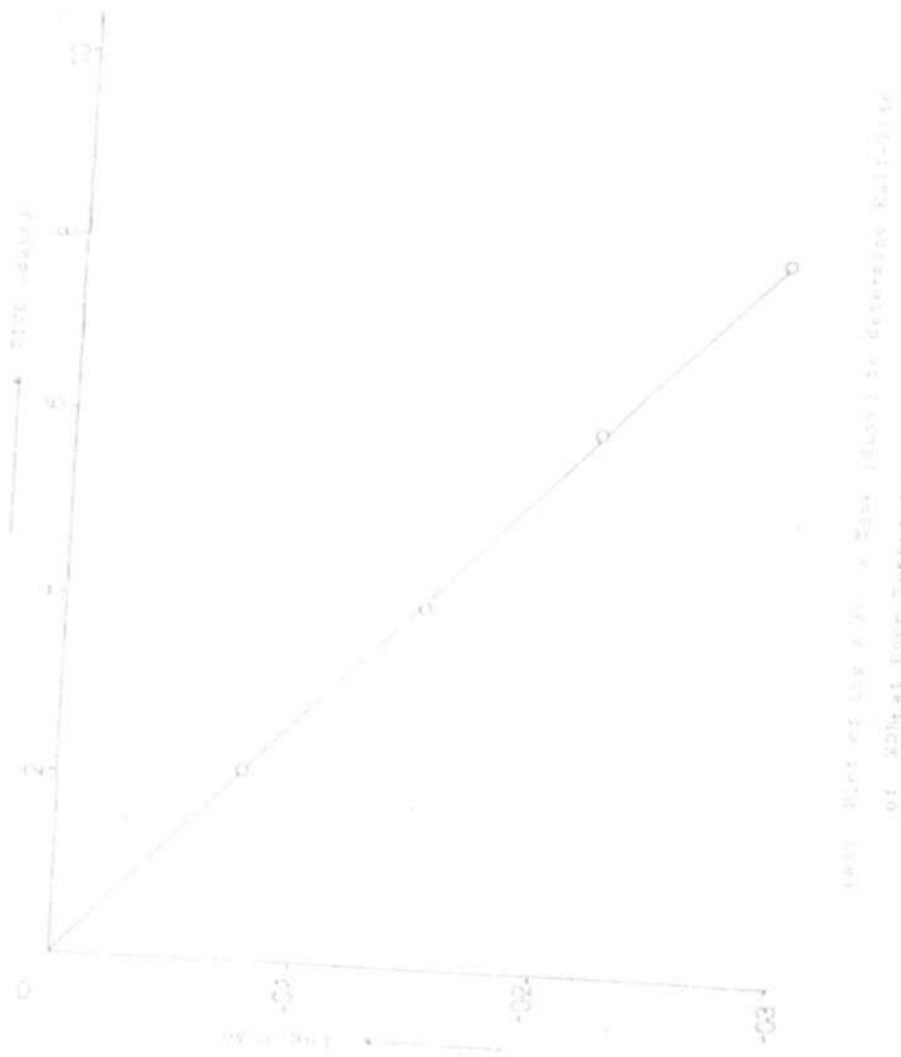
From the Arhenius plots of malate dehydrogenase catalysed reactions, it is evident that chilling temperatures will affect certain thermodynamic parameters, and precisely the Arhenius frequency factor of collision (i.e. entropy of the system) will be reduced and invariably this will reduce the rate of the tubers life process. However, since storage of yam tubers at chilling temperatures is known to cause deterioration of the membrane and

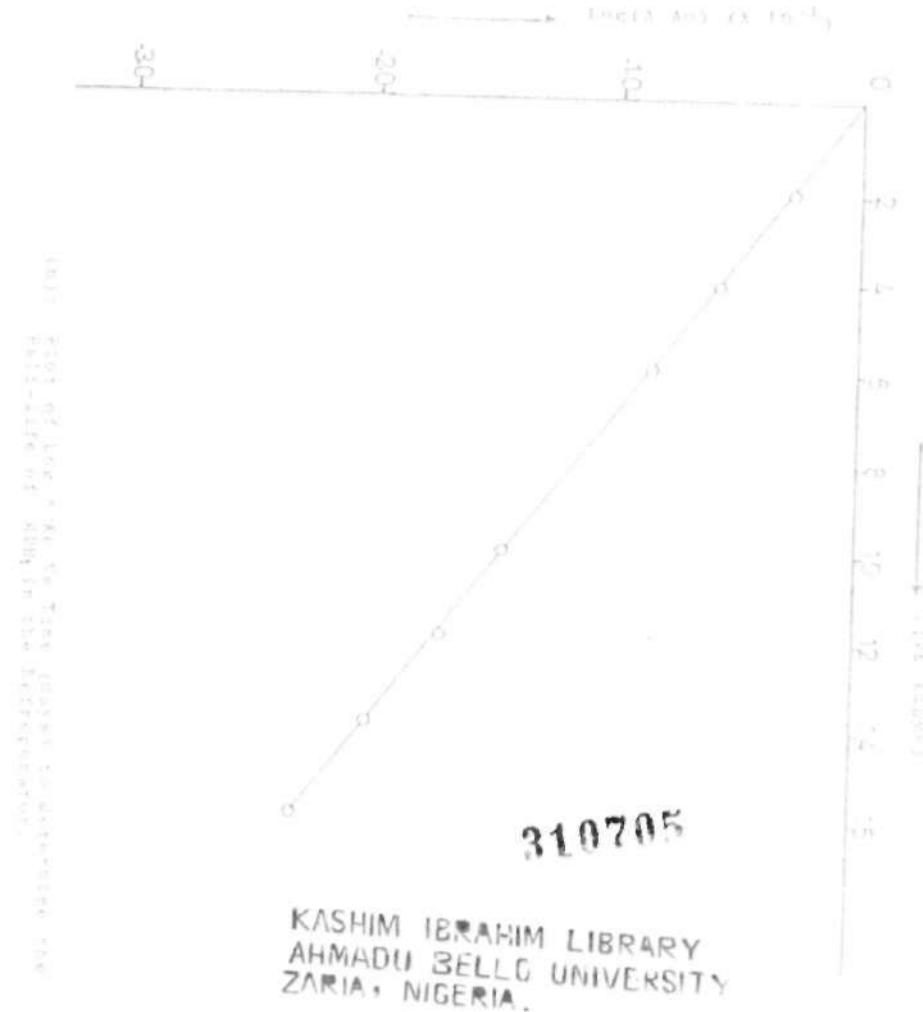






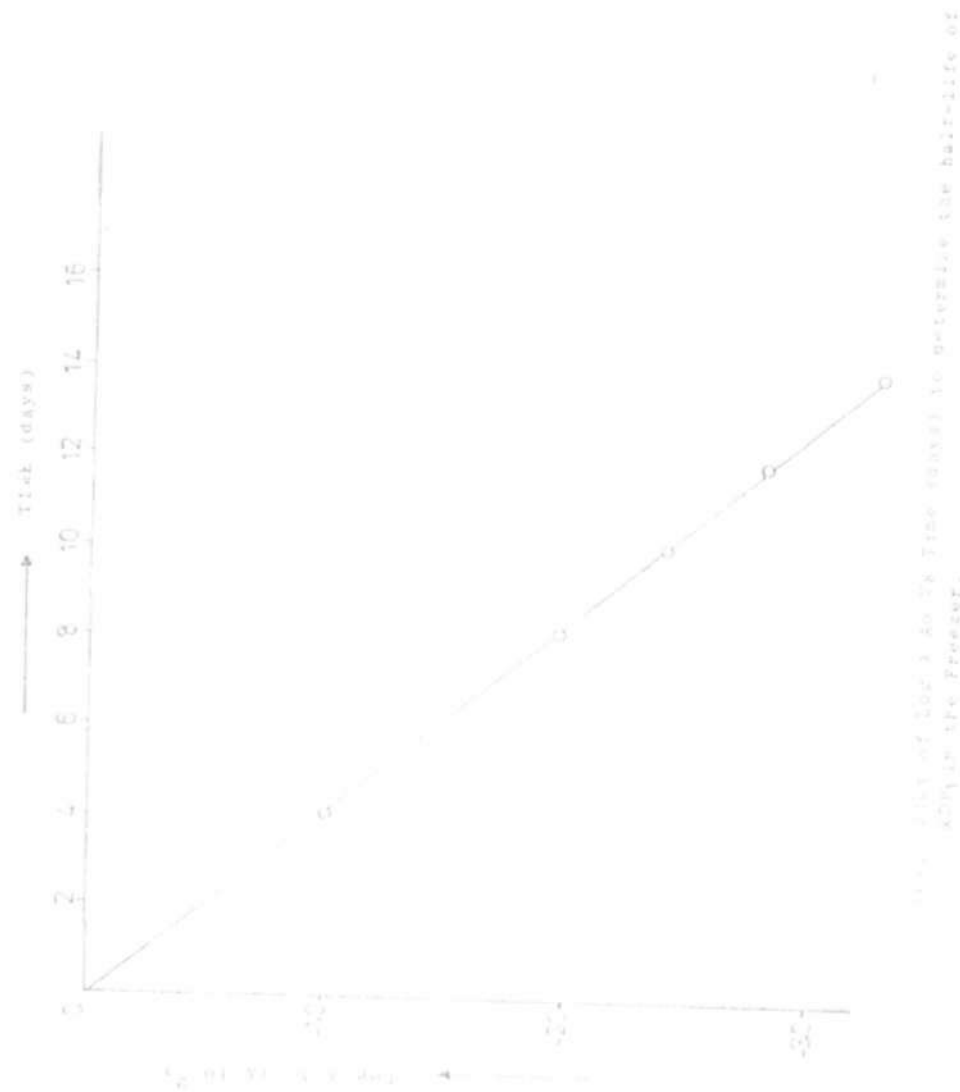

(c) Plot of $\ln A_{340}$ Vs Time (days) to determine (3) $NO_2$ in the Effluent





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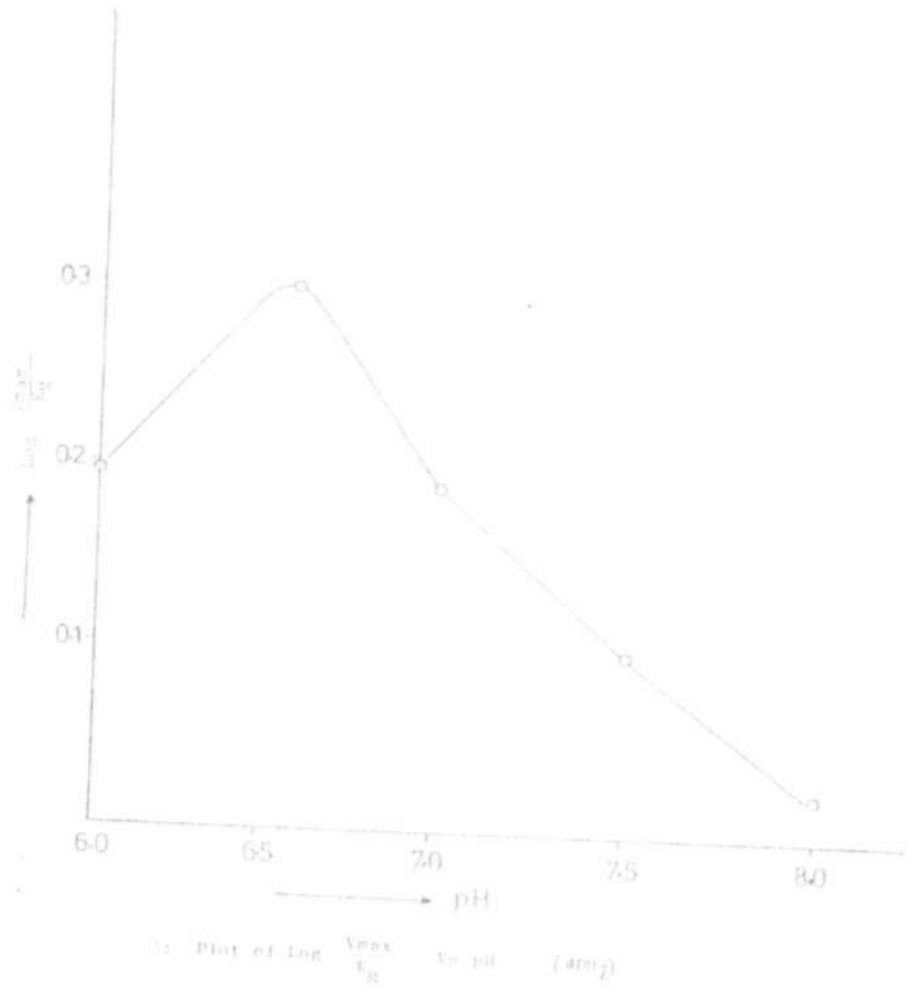

14
12
10
8
6
4
2
0

the entire tuber (Osuji and Umezurike, 1985) its prescription for the extension of the tuber's shelf life may not achieve desired goals. The option left is to explore the possible manipulation of the yam tuber physiological process at room temperature.

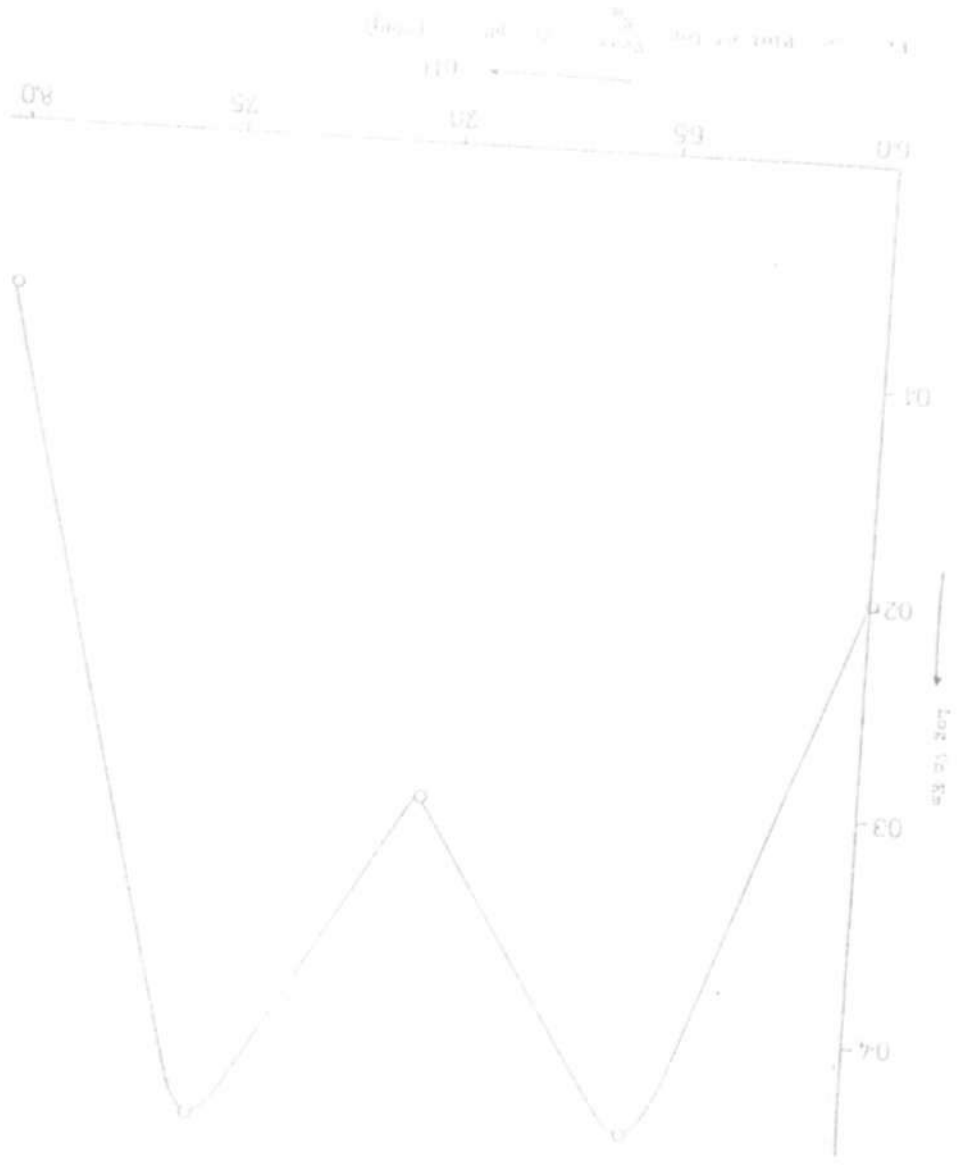
The observed $t_{1/2}$ of $MDH_2$ stored at room temperature was 1.25 days. One may rule out the catalytic potency of this enzyme in yam tuber when stored at room temperature. Unless it behaves otherwise *in vivo*. However, if the former possibility takes precedence, one could wonder how malate shuttle is achieved in yams stored under ambient conditions, since $MDH_2$ appears to be the cytoplasmic form of the enzyme. This may be reconciled with the observed increased level of L-malate in yam tuber stored aerobically as reported by Ugochuku and Anosike (1975).

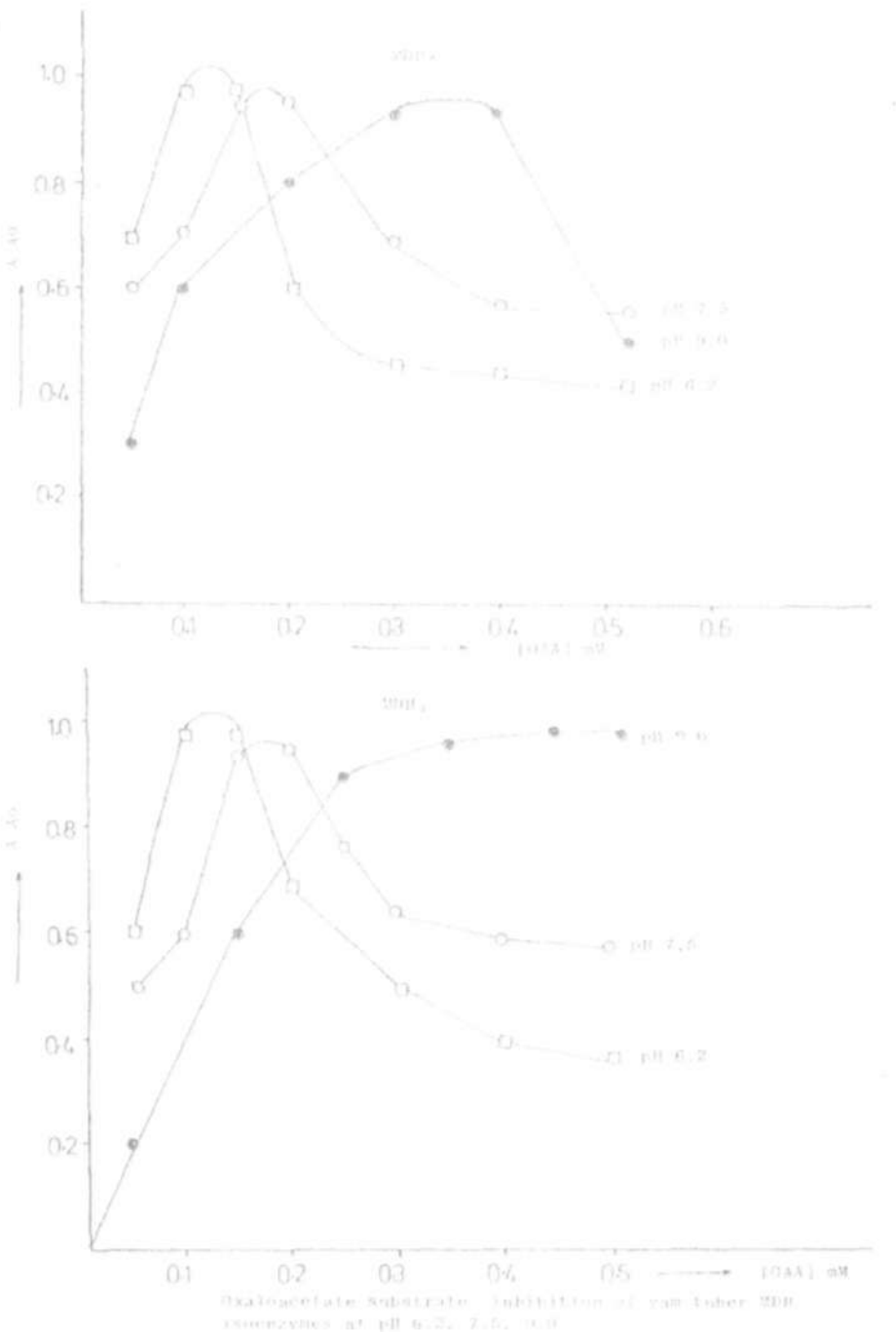
### 3.4 pH and enzyme stability

Initial pH analysis revealed two pH optima for $MDH_1$ at 6.5 and 7.5. A single pH optimum of 6.5 was obtained for $MDH_2$. Kinetic analysis showed a general increase in Km with pH for both enzymes (Fig. 14 and 15). Logarithmic plots of Vm/Km against pH suggests the presence of ionizable groups associated with the catalysis of they enzymes with pKa values of 6.3 and 7.6 for $MDH_1$ and 6.3 for $MDH_2$. Victor and Ting (1971) and Toshihisa and Haruhiko (1985) reported values of pH optimum in the range 6.8-7.2.




1: Plot of Log $\frac{V_{max}}{K_m}$ Vs pH $(Mn^{2})$






Oxaloacetate Substrate Inhibition of rat liver MDH isoenzymes at pH 6.2, 7.5, 9.0

## 3.5 Kinetic studies

Initial velocity and product inhibition analysis for the enzymes were performed to investigate the steady state mechanism and to determine the kinetic parameters of the substrates.

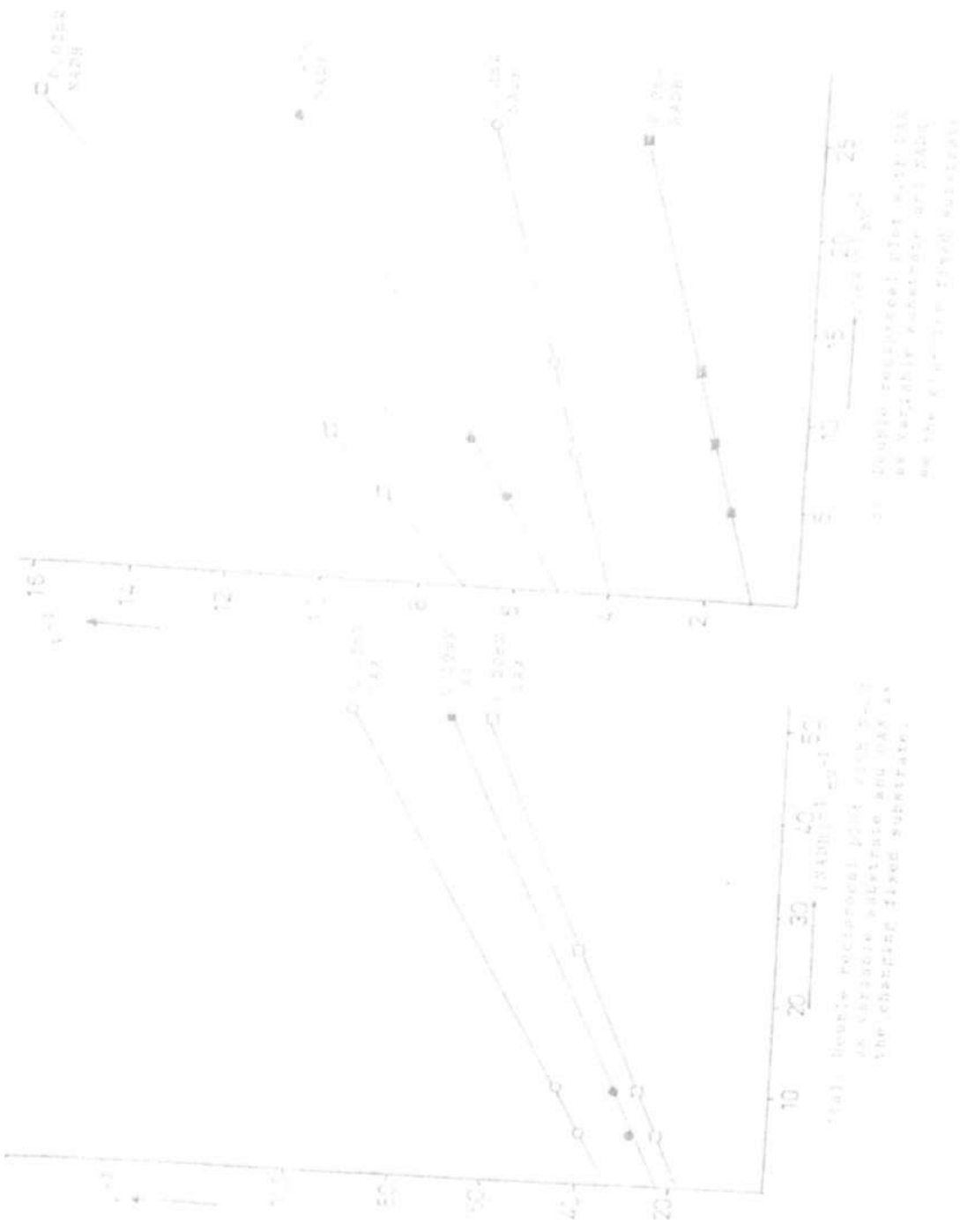
Initial velocity patterns for $MDH_1$ were determined with NADH and OAA in turn as variable substrates (Fig. 17). In the primary plot of reciprocal initial velocity with respect to reciprocal of substrate concentration, the concentration range of NADH was 0.02mM - 0.20mM. The secondary plots (Fig. 18 and 19) of ordinate intercept as a function of fixed substrate concentration of OAA and NADH represents the replots of data from (Fig. 17). These report enable the direct extrapolation of Km values of 0.05mM and 0.08mM for NADH and OAA respectively. In the reverse reaction, primary and secondary analysis performed with $NAD^+$ and L-malate as fixed and variable substrates in turn gave Km of 0.48mM for $NAD^+$ and 2.56mM for L-malate (Fig. 11-13). The kinetic constants determined for $MDH_2$ were found to be 0.25mM 0.83mM, 0.02mM and 0.083mM for $NAD^+$, L-malate NADH, and OAA respectively (Fig. 26-29).

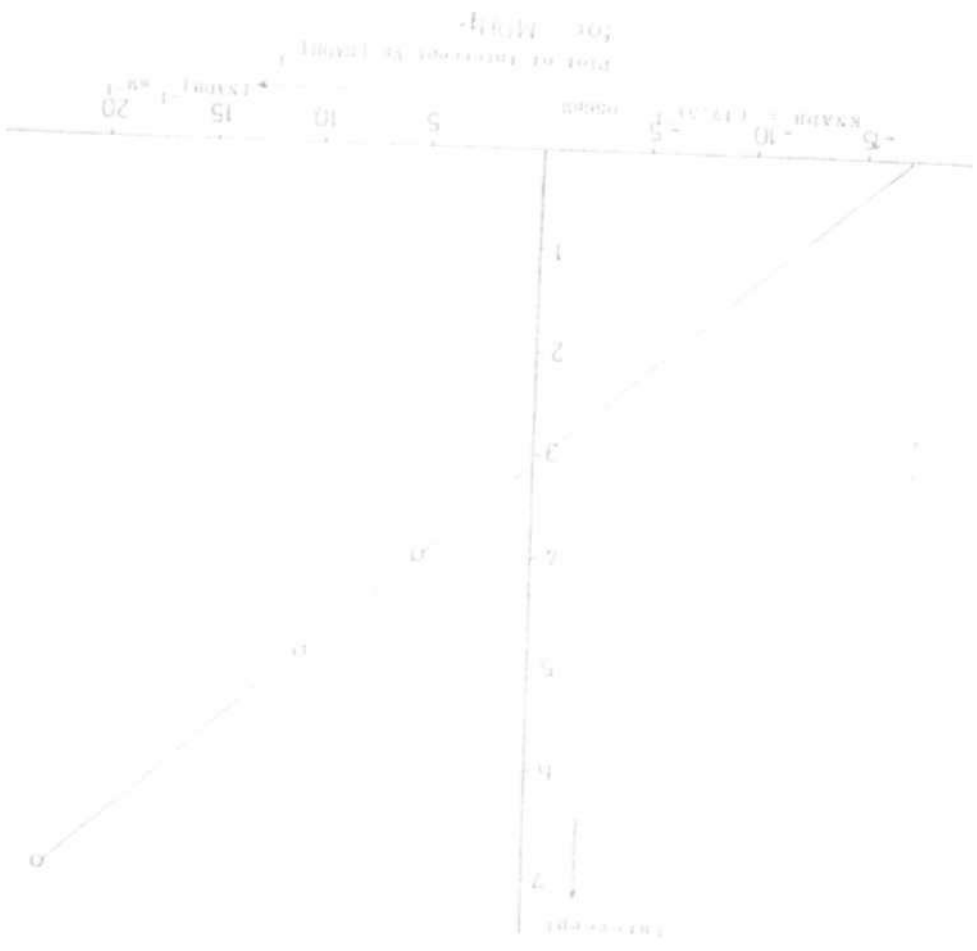
The initial velocity patterns on $MDH_1$ (Fig. 20-22) supported either sequential or Theorell Chance mechanisms, but rules out the possibility of a ping pong scheme.

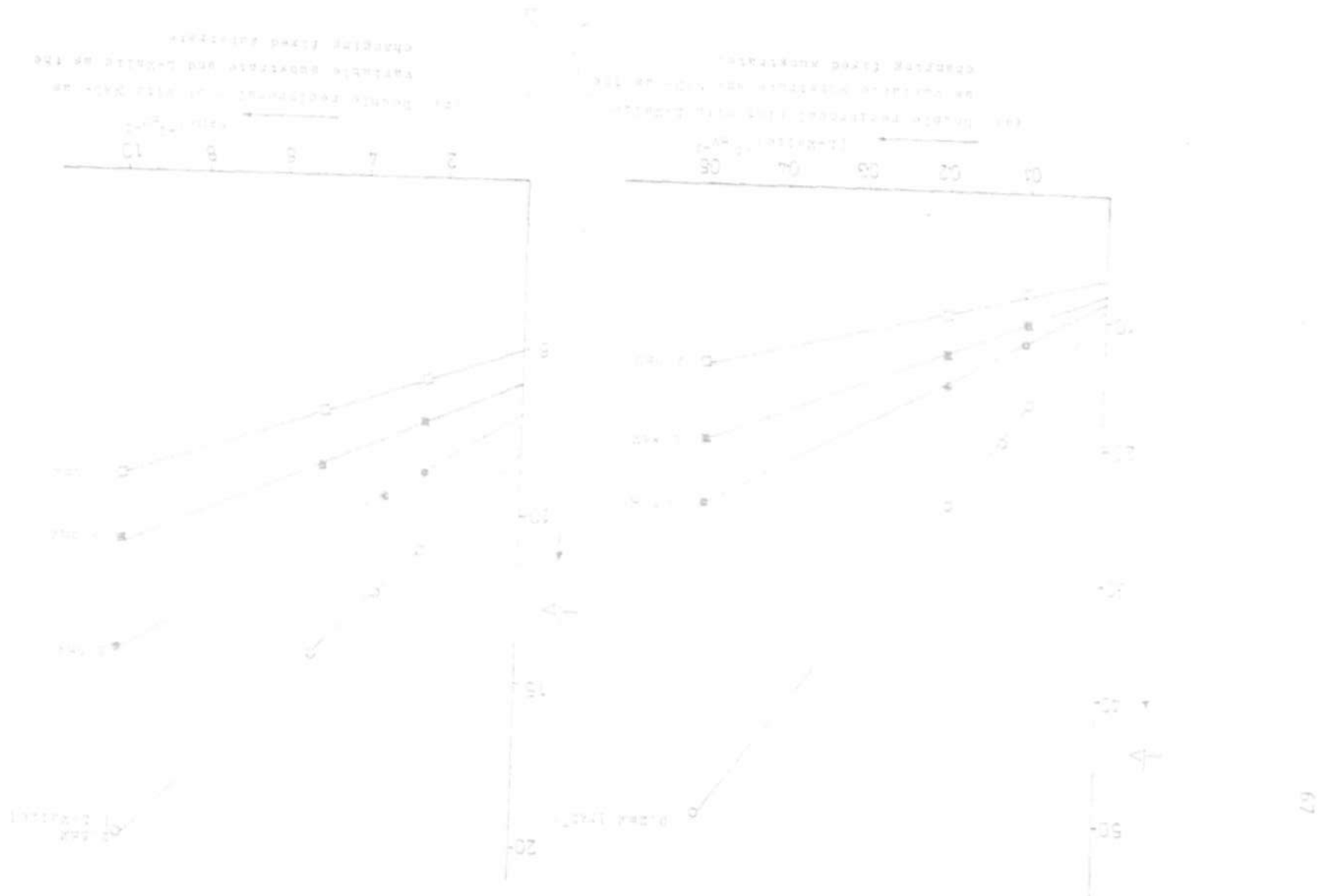
Product inhibition analysis on $MDH_1$ for the oxidation of L-malate performed to determine the order of substrate binding and

product release gave the following results: with OAA as product inhibitor, double reciprocal plot of velocity against L-malate concentration at a fixed saturating level of $NAD^+$ showed a competitive inhibition pattern. A non-competitive inhibition was observed towards $NAD^+$ at a fixed saturating concentration of L-malate (Fig. 27). On the other hand, the second product, NADH, exhibited a competitive inhibition towards L-malate and non-competitive inhibition towards $NAD^+$ (Fig.[28]). In the reverse reaction (i.e. reduction of OAA) similar product inhibition showed L-malate and $NAD^+$ as competitive product inhibitors towards NADH and non-competitive inhibitors towards OAA (Fig. 29-30).

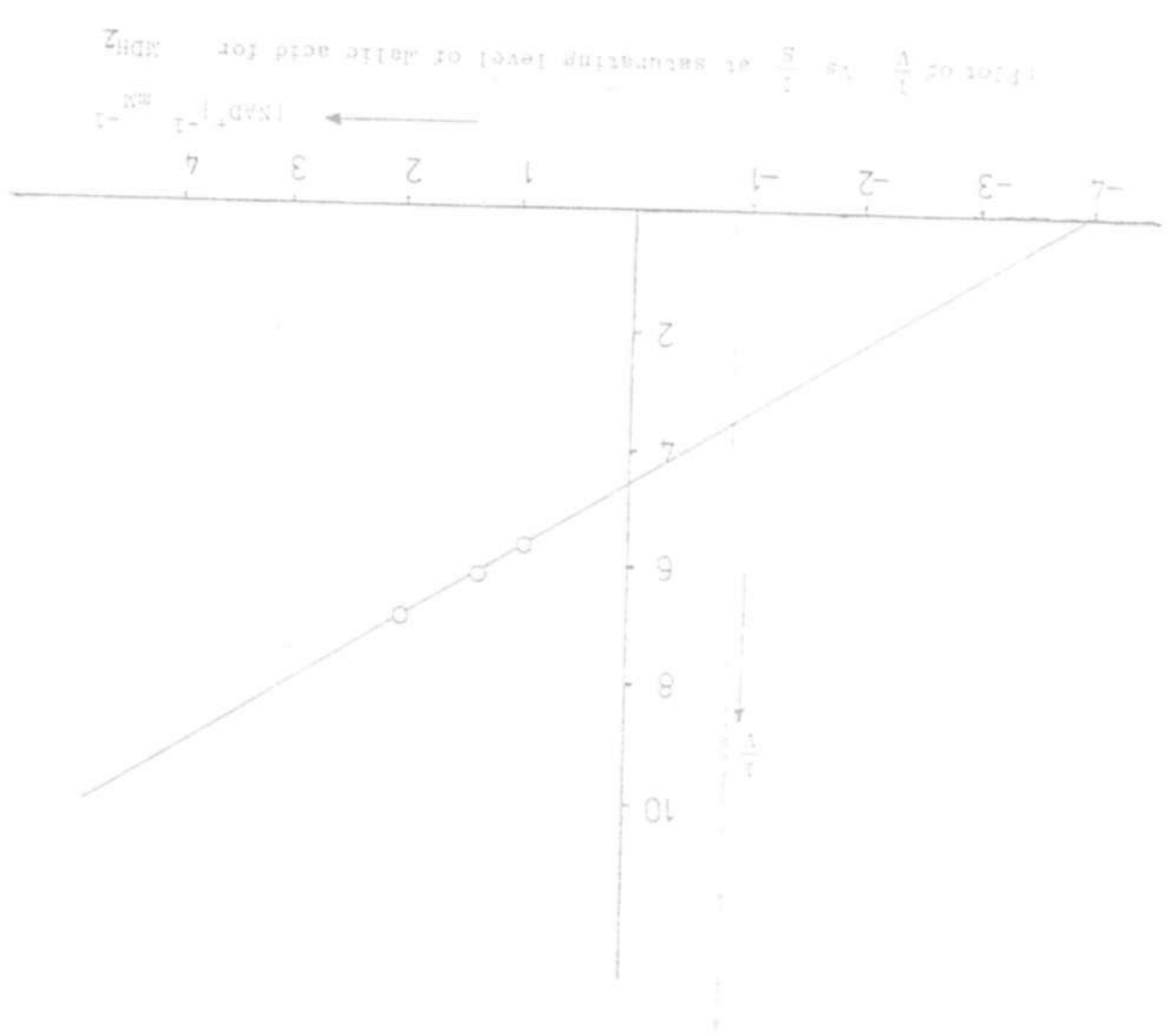
Product inhibition phenomenon takes place when one of the reaction products is initially present thereby causing the initial rate of reaction to be reduced. Such a reduction may arise based on two main factors (i) the product owing to structural similarity, could compete with either one of the substrates to form an inactive binary complex (i.e. between enzyme and product) or an inactive ternery complex i.e. between enzyme, co-enzyme and product inhibitor). If any of these possibilities hold, the inactive complexes will reduce the concentration of free enzyme or active enzyme-substrate complexes. (ii) the presence of the product inhibitor could shift the equilibrium towards the reaction step that consumes it (Le-chatelies principle). Fromm and Nelson (1961) Zewe and Fromm (1962) earlier reported that product inhibition data for Ribitol










Plot of $\frac{1}{V}$ vs $\frac{1}{S}$ at saturating level of malic acid for MDH2
$[NAD^+]^{-1}$ $mM^{-1}$
-4
-3
-2
-1
1
2
3
4
2
6
8
10

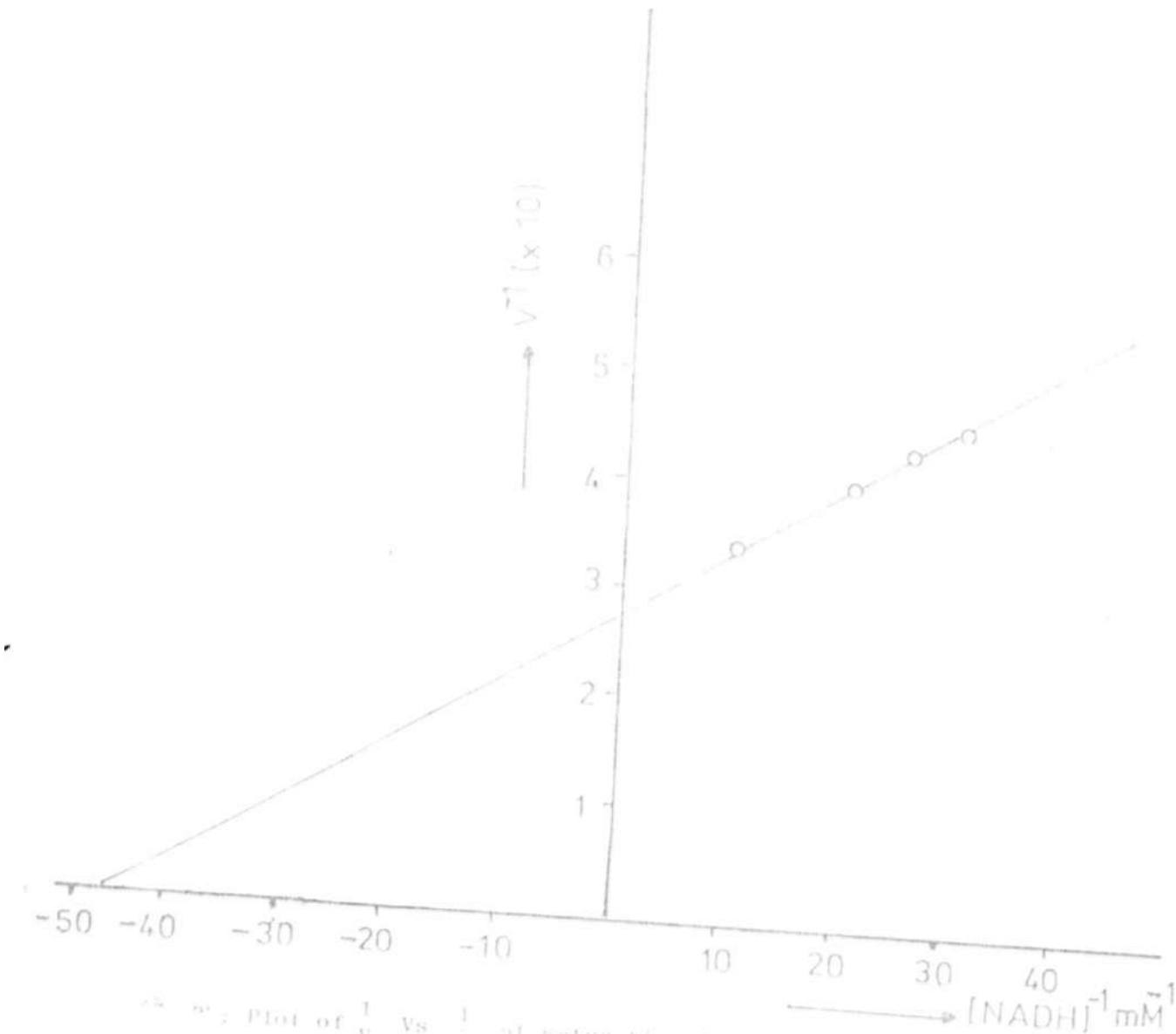
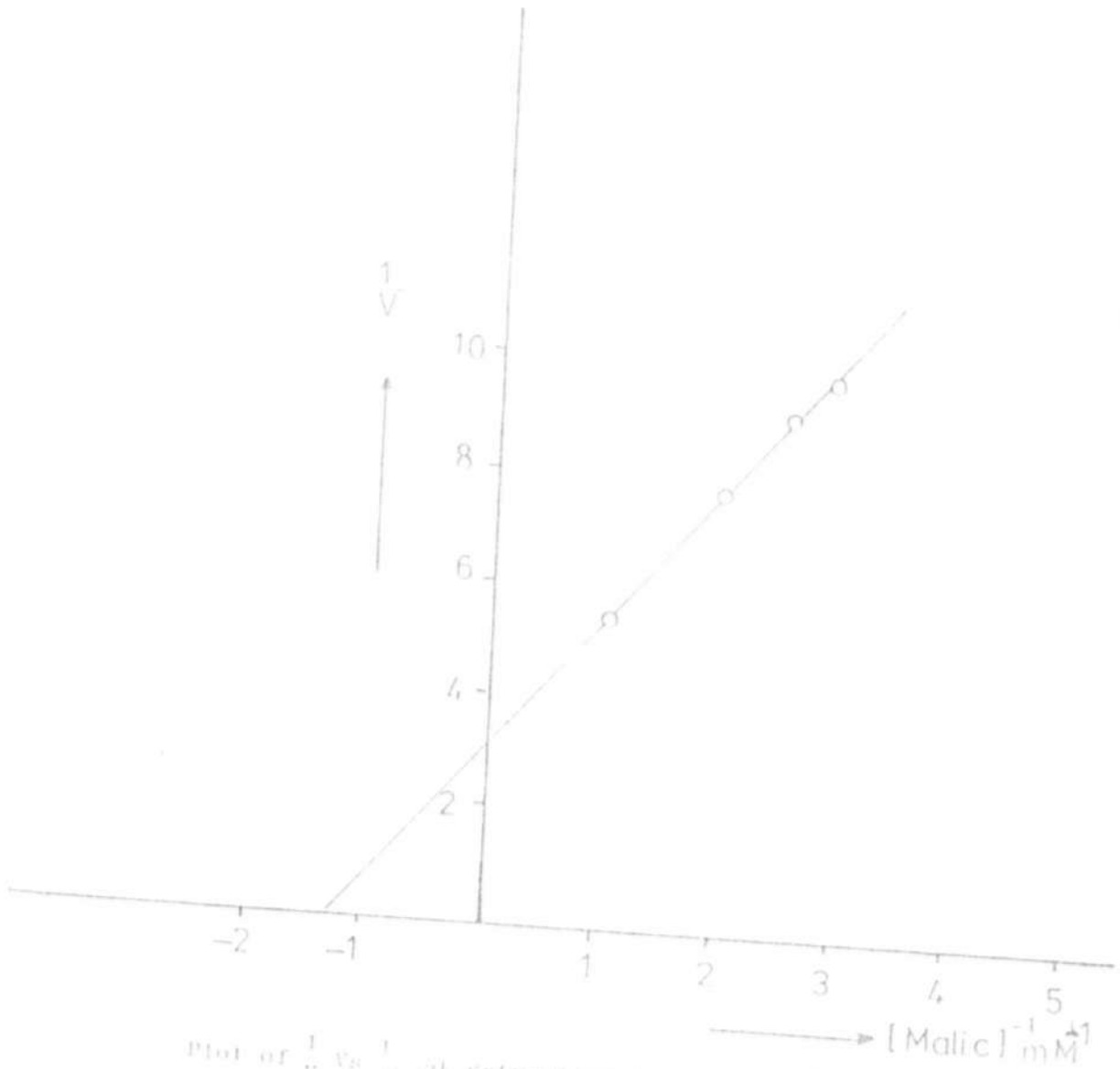
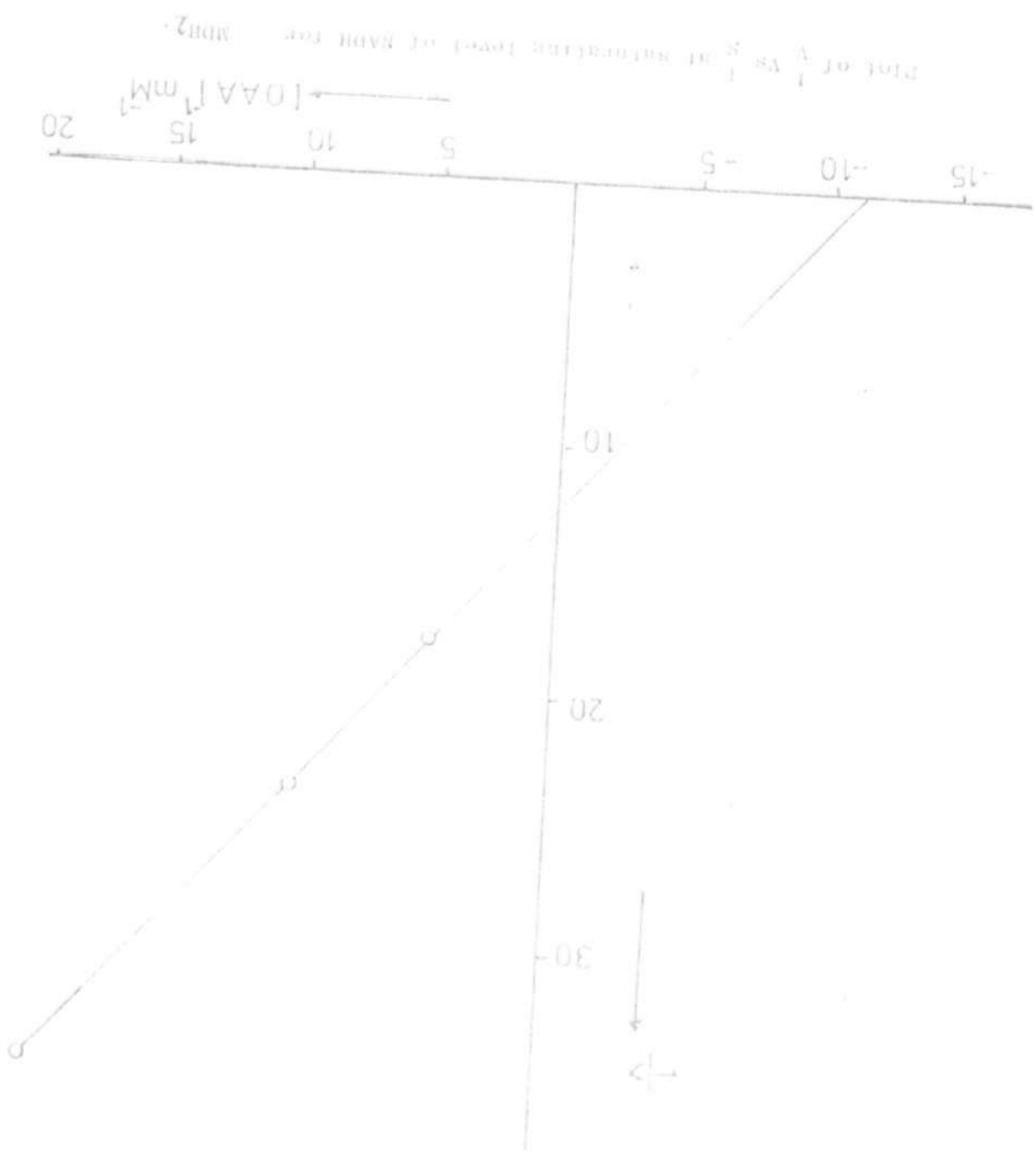



Fig. 9: Plot of $\frac{1}{V}$ Vs $\frac{1}{S}$ at saturating level of oxaloacetic acid for $MDH_2$.

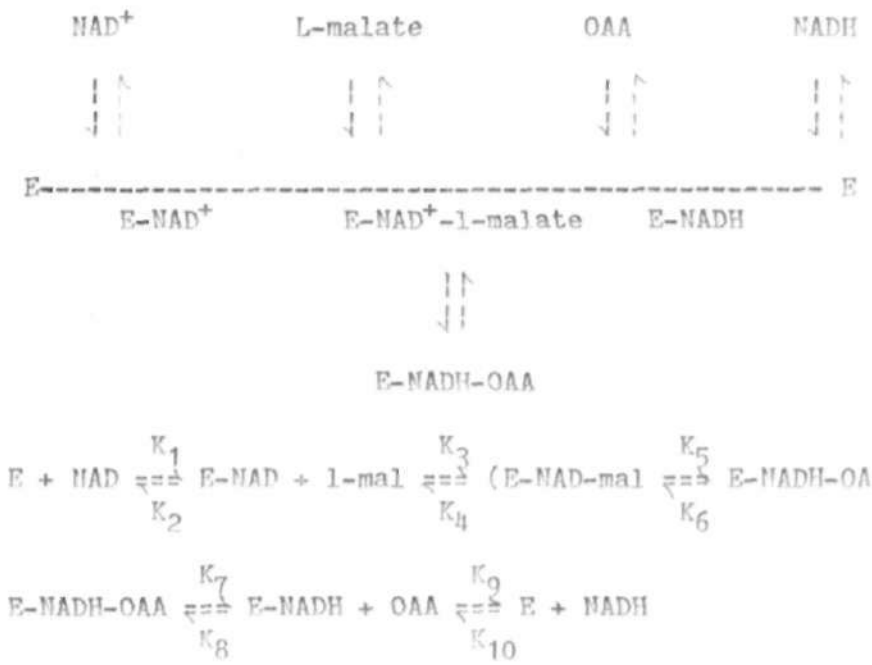



Plot of $\frac{1}{V}$ Vs $\frac{1}{S}$ at saturating level of $NAD^+$ for $MDH_2$.


Plot of $\frac{1}{V}$ Vs $\frac{1}{S}$ at different concentration of NADH for MDH2.
$10^{-1}$ AA $l^{-1}$ $mM^{-1}$
20
15
10
5
-5
-10
-15
10
20
30

dehydrogenase and muscle LDH respectively to be consistent with the formation of inactive binary and ternery complexes as well as mass law effect. They however observed that the formation of an inactive ternery complex between enzyme, co-enzyme and the product was experienced only when high concentrations of the product inhibitor were used.

These product inhibition analysis (Fig. 27-30) suggests an ordered bi-bi sequential mechanism for $MDH_1$ and rules out the possibility of a Theorel Chance mechanism. The mechanism supports an initial binding of $NAD^+$ and a final release of NADH as represented by the scheme below.



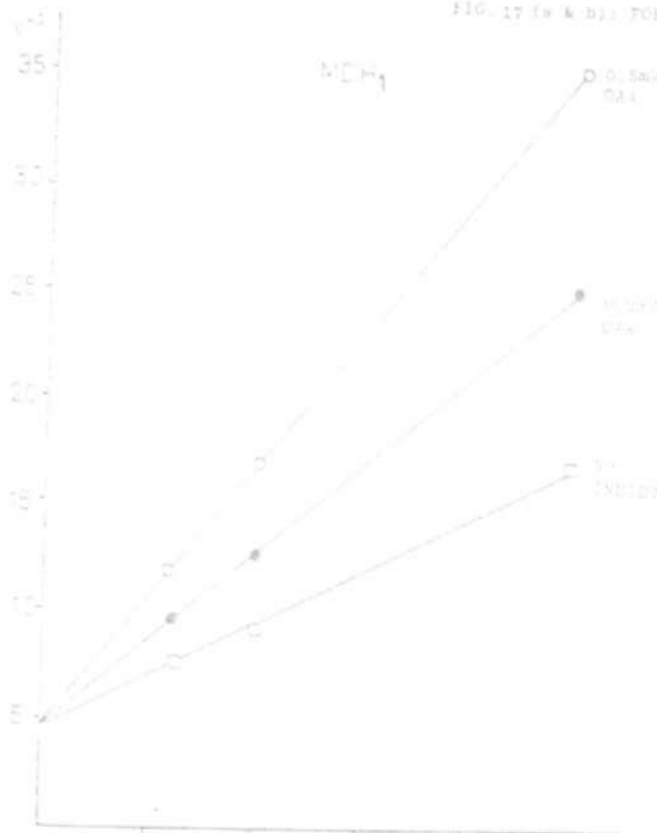
Yoshihisa and Haruhiko (1985) reported a random binding of substrate and compulsory ordered release of products of MDH from R capsulata. An ordered bi-bi mechanism has earlier been reported by Raval and Wolfe (1962a) for the enzyme from Pig liver. Isotopic exchange experiments confirmed this at pH 8.0.

Most reaction mechanisms of dehydrogenases are known to procede via the sequential mechanism. Ikediobi et al (1986) reported a random bi-bi sequantial mechanism for G6PDH from Dioscorea rotundata. LDH, MDH, and ribitol dehydrogenase have been shown to have a compulsory binding order mechanism (Theorell, and Bonnichsen 1951, Nordlie and Fromm, 1959). Yeast and Liver ADH appear to have a compulsory binding order mechanism (Dalziel, 1957). Glutamic dehydrogenase, a three substrate enzyme appears to have an ordered binding mechanism for the three substrates (Frieden, 1959). On account of kinetic analysis it seems that an ordered sequential mechanism may be a rather common feature for most dehydrogenases.

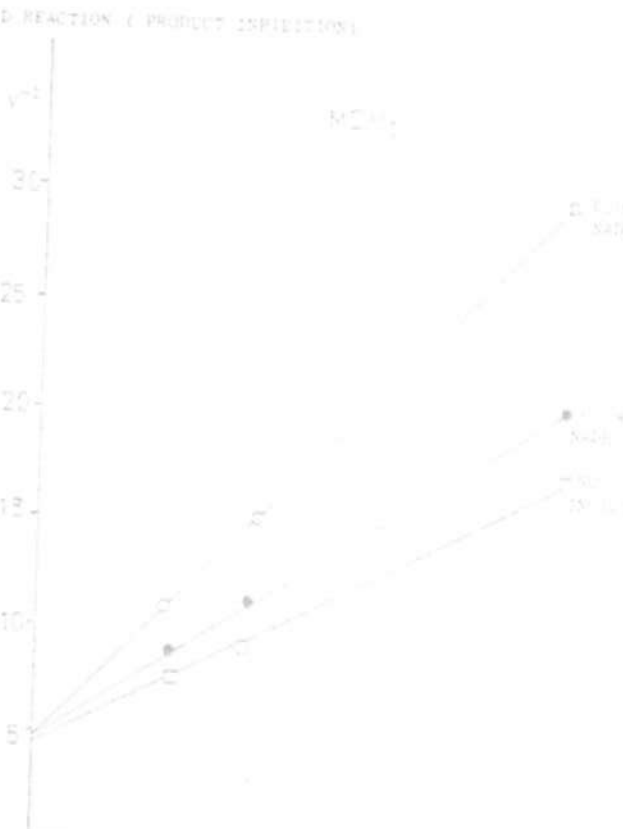
### 3.6 Substrate and co-enzyme specificity

Both enzymes showed marked specificity for L-malate oxidation and OAA reduction. None of these substrates D-malic, DL lactate, and -keloglutarate along with either $NAD^+$ or NADH showed any trace of activity. Likewise L-malate and $NADP^+$ showed no reactivity, this rules out the possibility of malic enzyme acting as an artifact.

FIG. 17 (a & b): FORWARD REACTION ( PRODUCT INHIBITION)

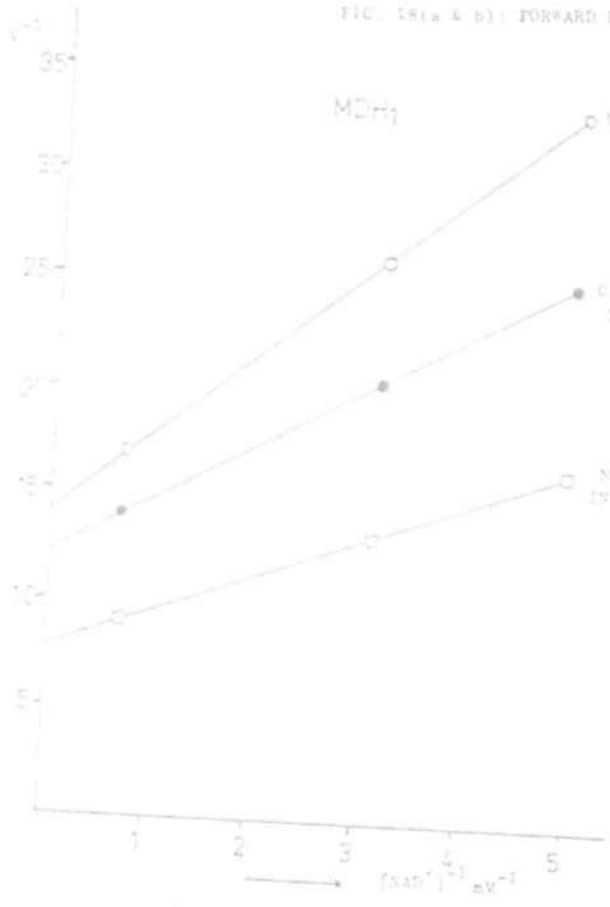



Product inhibition studies with oxaloacetate as product inhibitor

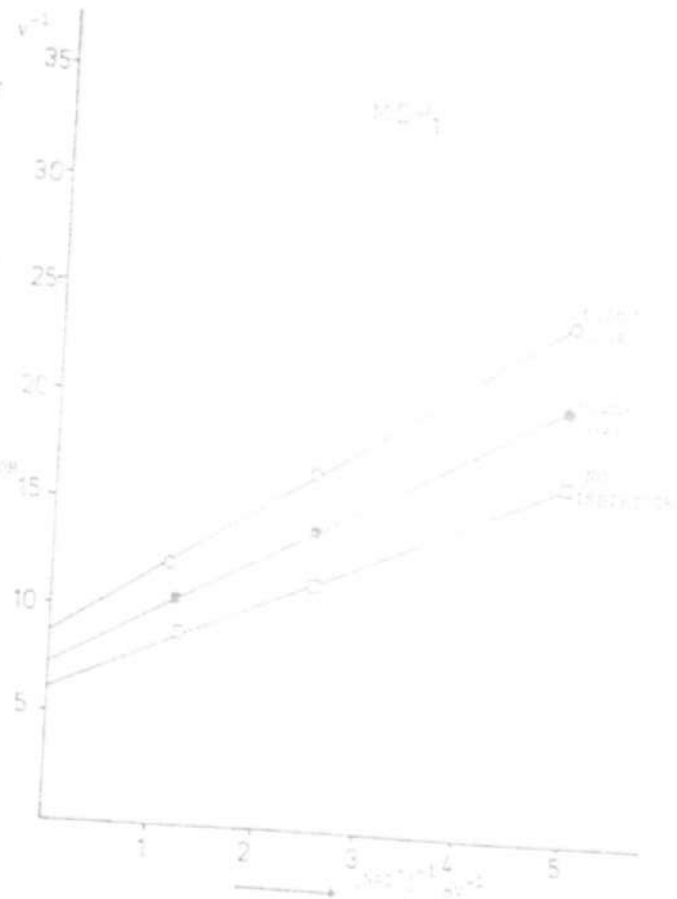



Product inhibition studies with malic acid as product inhibitor

FIG. 18(a & b): FORWARD REACTION ( PRODUCT INHIBITION)




Product inhibition studies with NADH as product inhibitor.




Product inhibition studies with oxaloacetate as product inhibitor

Previously, Grim and Doherty (1961) reported the reduction of derivatives of OAA in the presence of NADH by the enzyme, from rat liver. The enzyme from R capsulata has been shown to require exclusively $NAD^+$ for L-malate oxidation and NADH for OAA reduction.

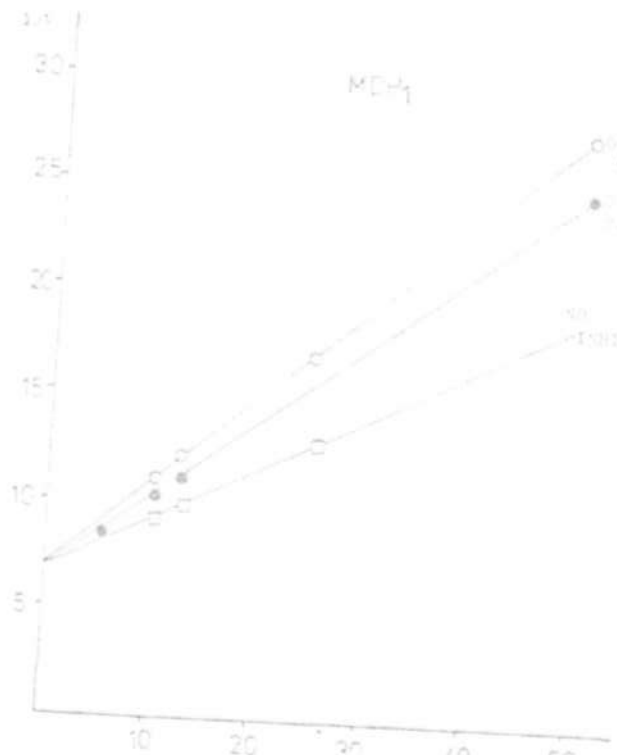
### 3.7 Inhibition by natural metabolites

Both enzyme forms were inhibited to different extents by the following organic acids: pyruvic acid, succinic acid, citric acid, isocitric acid, -keto-glutaric acid, aspartic acid glutamic acid (Table 2) and the nucleotides ATP and ADP. Victor and Irwin (1971) had earlier observed similar inhibition of the organic acids towards MDH isoenzymes from greem stem Opuntia ficus. The higher rate of inhibition of these organic acids towards $MDH_1$ than $MDH_2$ indicates that the former is most likely of mitochondrial origin. Since the organic acids are essentially intermediates of the TCA cycle.

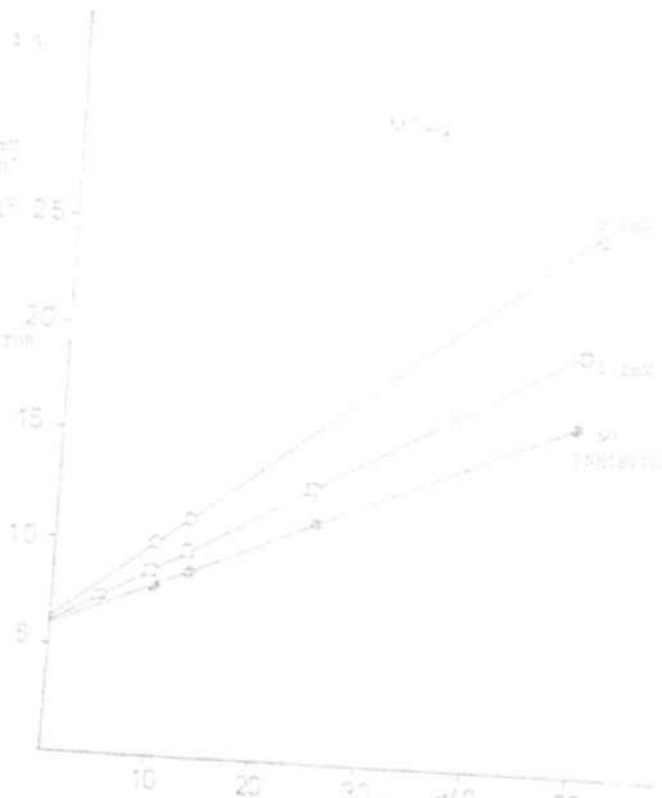
Detailed kinetic analysis on -ketoglutarate and citrate inhibition for $MDH_1$ showed both metabolites as non-competitive inhibitors towards OAA (Ki = 30mM), citric acid was a non-competitive inhibitor towards NADH (Ki = 38mM) while -ketoglutarate behaved as a competitive inhibitor to NADH (Ki = 30mM) (Fig. 31-33). The inhibition of -ketoglutarate on $MDH_2$ was non-competitive in all cases (Fig. 32). Our kinetic data on

inhibition for $MDH_1$ suggest that KG and citrate bind at another site different from the co-enzyme

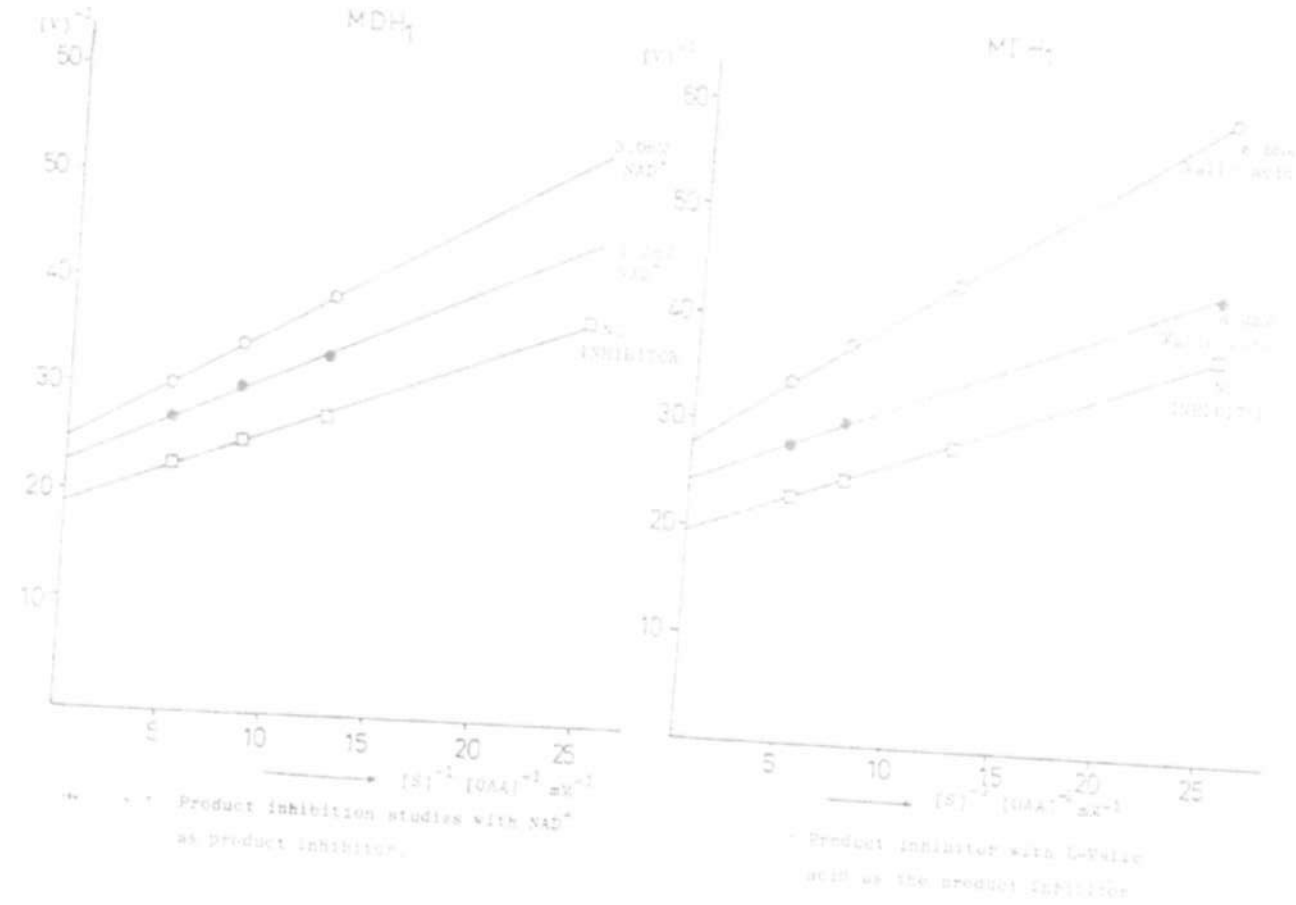
FIG 19a & b: REVERSE REACTION (PRODUCT INHIBITION)




Product inhibition studies with NAD⁺ as product inhibitor.




(b) Product inhibition studies with L-malic acid as product inhibitor.




Product inhibition studies with $NAD^+$ as product inhibitor.

Product inhibitor with L-Malic acid as the product inhibitor



Table 2: Inhibition by naturally occuring metabolites

| Inhibitor | % Inhibition | |
|---|---|---|
| | $MDH_1$ | $MDH_2$ |
| 10mM Citric Acid | 25 | 22 |
| 10mM Isocitric Acid | 15.4 | 14 |
| 10mM -Ketoglutarate | 29 | 16.7 |
| 10mM Succinic Acid | 25 | 44 |
| 10mM Pyrovic Acid | 13.5 | 11 |
| 10mM Aspartic Acid | 16 | 4.4 |
| 10 mM Glutamic Acid | 4.6 | 8.7 |
| 3.3mM ATP | 51.2 | 45.8 |
| 3.3mM ADP | 39.5 | 29.7 |

site. Otherwise, the presence of these metabolites triggers conformational changes on the enzyme thus decreasing the affinity of the enzyme to its substrate. Invariably the potency of the enzyme is affected.

A number of enzymes have been shown to be either inhibited or stimulated by adenine nucleotides. Kurimatsu (1968) reported that pig heart MDH could be stimulated by varying degrees of adenine nucleotides with ATP as the most effective stimulator. This observation cannot be rationalized in terms of known control mechanisms. It thus became imperative to investigate if the yam tuber MDH isoenzymes are affected in a similar manner. In contrast to Pig heart MDH, the yam tuber malate dehydrogenases were strongly inhibited by adenine nucleotides (Table 2) with ATP as the stronger inhibitor. Since the mitochondria serves as the main depot for ATP synthesis, and the inhibition of ATP is more on $MDH_1$ than $MDH_2$, it is suggestive that $MDH_1$ is of mitochondrial origin.

Initial velocity studies on nature of inhibition for $MDH_1$ showed ATP as a non-competitive inhibitor to OAA and a competitive inhibitor to NADH increasing the apparent Km from 0.042mM to 0.125mM in both cases. The Ki values were 0.95mM and 11mM for OAA and NADH respectively. Kinetic analysis on inhibition with $MDH_2$ revealed a non-competitive inhibition pattern of ATP towards NADH (Fig. 31-33). The competitive nature of inhibition alongwith structural similarity of ATP and NADH suggests that the

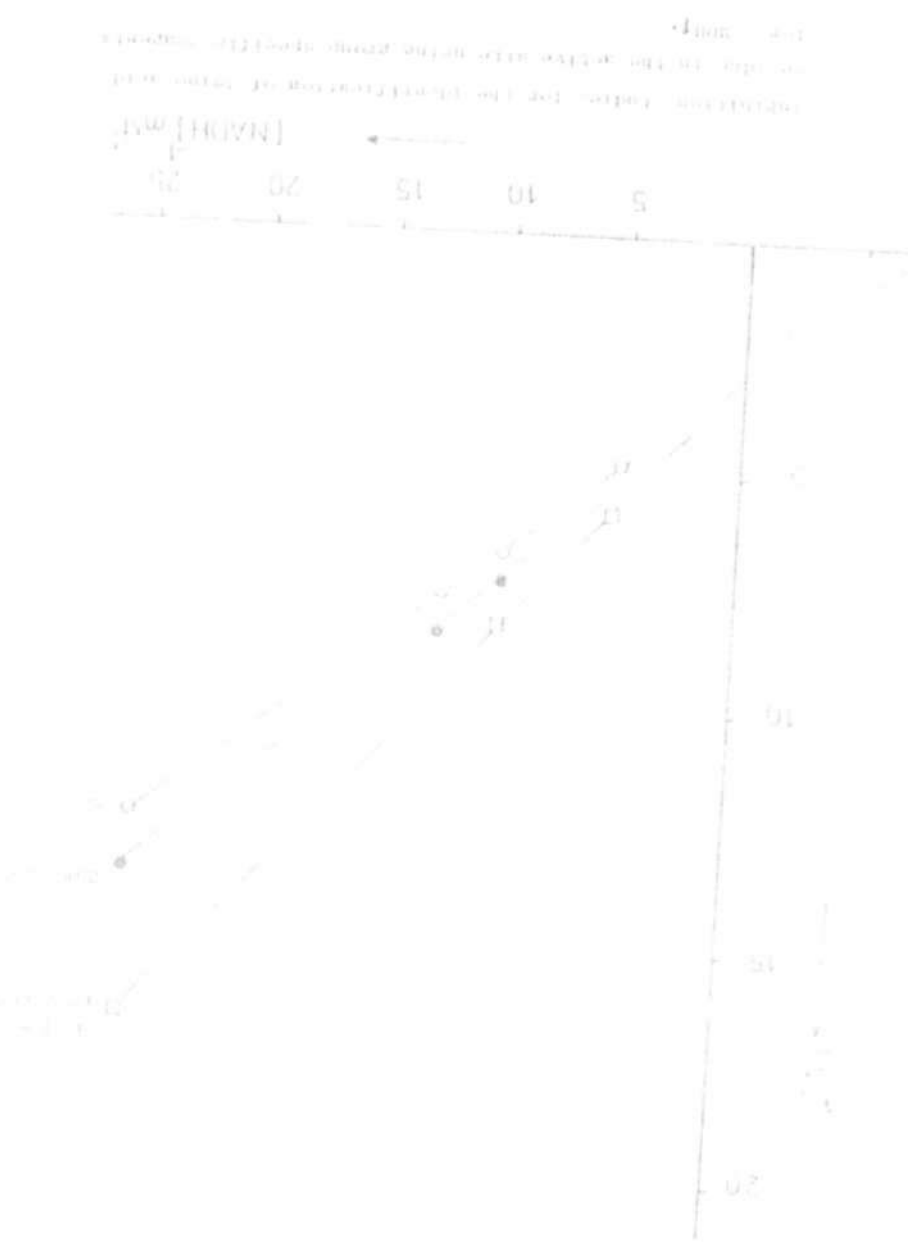
nucleotide binds the enzyme at its co-enzyme site for $MDH_1$.

### 3.8 Inhibition with group specific reagents

Inhibition analysis with group specific reagents have been used for the identification of constituents of the active site responsible for the catalytic potency of an enzyme. Such group specific reagents bind the amino acid component of the active site based on the presence of a particular functional group.

In our analysis of the yam tuber MDH isoenzymes we observed that both enzymes were sensitive to inhibition by p-hydroxymercury benzoate, KCN and $NaN_3$. These observations seem to implicate the thiol group (-SH) as the possible functional entity involved in the catalysis of MDH from the yam tuber. Their mode of inhibition involves covalent binding to the thiol group thereby making it inaccessible to the surrounding substrates. Initial velocity data for inhibition on $MDH_1$ showed KCN and p-hydroxy mercury benzoate as competitive inhibitors to NADH, while $NaN_3$ as a non-competitive inhibitor to the same substrate, their ki were 8mM, 0.5mM, and 0.2mM respectively $NaN_3$ showed a competitive inhibition pattern to OAA. These results indicate that pHMB and KCN bind the thiol group at the co-enzyme site while $NaN_3$ binds the enzyme at the substrate site to inhibit the reaction catalysed by MDH.

From this extensive research conducted, we have been able



to show the existence of malate dehydrogenase isoenzymes from the white yam (Dioscorea rotundata). Further work of this nature should be directed to another enzyme of the TCA cycle, so that when the basic enzymological information of the yam tuber is complete, the problem of the objective can be approached with some precision.

## REFERENCES

**Benveriste,** F. & Munkres (1970). Cytoplasmic and mitochondrial malate dehydrogenase from Neuropspora, regulatory and enzymic properties. Biochim. Biophy. Acta 220: 161-177.

**Coursey,** D. G. (1961). The magnitude and origins of storage in Nigerian yams. J. Ed. Sci. Agc. 21: 574-550.

**Cole,** F. E., Blondin, J. & Temple, L. (1968). Malate dehydrogenase system in chlorella pyrenoi-dosa: Cell tissues Kinet 1:281.

**Cole,** F. E., Pierre, C. & Stromeyer, T. (1971). Purification and properties of malate dehydrogenase from Chlorella pyrenoidosa: Biochem. 10:720-734

**Dalziel,** K. (1957). Liver alcohol dehydrogenase, kinetic studies of reaction mechanism: Acta Chem. Scad. 11:1706.

**Davies,** D. & Kun, A. (1957). Isolation and properties of malate dehydrogenase from Ox-heart mitochondria: Biochem. J. 66:307.

**Davies,** D. Sacher, T. & Tower, C. (1972) in "Davies, D. & Kun, A." Isolation and properties of malate dehydrogenase from Cx heart mitochondria Biochem. J. 66:307.

**Delbruck,** A. Zebe, E. & Bucher, F. (1959). Biochem. Z 331:273: in Russell, A. C. & Irwin, T. Purification and crystallization of three isoenzymes of malate dehydrogenase from zea mays seed Archives Biochem. Biophy 158:213-224 (1973).

**Duntze,** E. (1968). Glucose induced inactivation in intact yeast cells: Europ. J. Bioch. 3:326:331.

**Frieden,** F. (1959). J. Biochem. 334:2891. in "Raval, D. N. & Wolfe, R. G. (1962a)". Malate dehydrogenase, kinetic studies of reaction mechanism: Biochem 1:1112-1117.

**Fromm,** H. J. & Nelson, D. (1961) Fed. Proc. 20:229 in "Raval, D. N. & Wole, R. G. (1962a)". Molate dehydrogenase, kinetic studies of reaction mechanisms: Biochem. 1:1112-1117.

**Grim,** E. & Doherty, D. (1961). Properties of two forms of malate dehydrogenase from beef heart. J. Biol. Chem. 236:1980.

**Hayden,** B. (1972). Malate dehydrogenases in maize endosperm, intracellular location and characterization of two particulate forms. Can. J. Biochem. 50:663-671.

**Ikediobi,** C. O. (1986) in "Advances in yam research" pp 109-137.

**Ikediobi,** C. O. & Oti, E. (1983). Some biochemical changes associated with past-harvest storage of white yam D. rotundata tuber. J. Ssi. Fd. Agc. 34:1123.

**Ikediobi,** C. O. Ationu, A. & Ukoha, A. I. (1987). Kinetic mechanism of G6PDH from white yam D. rotundata accepted for publication in Nig. J. Biochem.

**Irwin,** T. Dugger, W. & Sherman, W. (1966) Plant physiol 41:1083 in "Russel C. & Irwin, T. (1973)". Purification and crystallization of three isozymes of malate dehydrogenase from zea mays seed. Archives Biochim. Biophy. 158:213-224.

**Johnson,** E. (1970). Properties and regulation of $NADP^+$, MDH and malic enzyme in plants with $C_4$, dicarboxylic acid pathway of photosynthesis. Biochem. J. 119:273-280.

**Kaplan,** J. O. (1961) in "Mechanism of action of steroid hormones" Oxford press pp 247.

**Kaplan,** N. O. (1963) Bact. Rev. 27:155 in "Isoenzymes" Chapman & Hall Ltd. pp 207.

**Kun,** E. & Volfin, P. (1966). Tissue specificity of MDH isoenzymes. Kinetic discrimination by mono

and difluoro analogues. Biochim. Biophy. Res. Comm. 22:187.

**Kuramatsu**, A. (1968). Nucleotide inhibition of Pea malate dehydrogenase. Archives. Biochem. Biophy. 125:383-384.

**Laufer**, H. (1961) Ann. N.Y. Acad. Sci. 94:815 in "Isoenzymes" Chapman & Hall Lid. pp 211.

**Longo**, L. (1970). Development of glyoxisomes in peanuts cotyldons and maize scutella. Plant physiol 45:249-254.

**Moore**, R. O. & Villee, C. A. (1963) Sci. 142 389 in "Isoenzymes" Chapman & Hall Ltd. pp 212.

**Murkeji**, S. K. & Irwin, T. (1969). Malate dehydrogenase isozymes from green stem tissue opunia. Archives Biochem. Biophy. 131:336-351.

**Murphey**, W. & Barnaby, F. J. (1962). Purification and properties of MDH from B. subtilis, E. coli. and stereothermophilus. J. Biol. Chem. 242. 1548-1559.

**Nelson**, A. (1970). Glycolate dehydrogenase in green algae: Archives. Biochim. Biophy. 141:102-116.

**Nordlie**, R. C. & Froman, H. J. (1959). J. Biochem. 234:2523: in "Raval, D. N. and Wolfe. Malate dehydrogenase, kinetic studies of reaction mechanism. Biochem. 1:1112-1117.

**Olorunda,** A. McKelvie, A. & Maclon, A. E. (1978). Effect of temperature and chloropham on the storage of the yam. J. Sc. Fd. Agr. 25:1235-1238.

**Oluoha,** C. O. (1982). Bulletin of Science Association of Nigeria pp 25.

**Ornstein,** L. (1964). Disc Electrophoresis Ann. N.Y. Acad. Sci. 121:321.

**Osuji,** G. O. & Umezuruke, G. M. (1985) in "Advances, in yam research" pp 215.

**Passam,** H. & Read, S. (1976). Wound Repair in yam tubers. The dependence of storage procedures on nature of wound repair. Trop Sci. 18:1-11.

**Passam,** H. Read, S. & Hughes, P. A. (1977). Wound repair in yam tubers. The effect inhibitors of respiration and protein synthesis on repair physiology. New Phytol 79:593-603.

**Passam,** H. & Barret, C. (1972). Invertase in yam Dioscorea species tubers. J. root crop 3:1-3.

**Peak,** C. (1972). Isoenzymes of MDH and their regulation in Euglena gracitis Biochim. Biophy. Acta 284: 1-15.

**Raval,** D. N. & Wolfe, R. G. (1962a) MDH, Kinetic studies of reaction mechanism. Biochem. 1:263-209.

**Raval,** D. N. & Wolfe, F. G. (1962b). MDH, Kinetic studies of reaction mechanism by product inhibition. Biochem. 1:1112-1117.

**Rivera,** J. R., Gonzalez, M. A. Collazo de Rivera & Cuevaz-Ruiz, (1979). An improved method for storing yam D. alata. J. Ag. Univ. Peurto Rico 58:456-465.

**Tchen,** E. (1955). The mechanism of $CO_2$ fixation into oxaloacetic acid. J. Biol. Chem. 213:247.

**Theorell,** H. & Bonichsen, R. (1951). Acta Chem. Scand. 5:1105, in a Raval, D. N. and Wolfe, R. G. (1962b) MDH kinetic studies of reaction mechanism by product inhibition: Biochem. 1:1112-1117.

**Thorne,** M. (1960). Characterization of two malate dehydrogenases from rat liver Biochim. Biophy. Acta 42:125.

**Toshihisa,** O. & Haruhiko, S. (1986). Purification and characerization of MDH from phototropic bacteria R. capsulata Biochim. Biophy. ACta 869:171-177.

**Tolbert,** N. E. (1971). Microbiodies, peroxisomes glyoxisomes. Ann Rev Plant Physiol. 22:45-74.

**Ugochukwu,** E. & Anosike, E. O. (1977). Changes in enzyme activity in white yam after prolong storage. Phytochem 76:1159.

**Ugochukwu,** E. & Anosike, E. O. (1979). Effect of storage under nitrogen on ethanol, lactate, malate and their dehyerogenases in yam tubers. Phytochem. 18:1621-1624.

**Ujam,** A. (1979). Studies on alcohol dehydrognase from D. rotundata. Project for B.Sc. degree in Biochemistry University of Nigeria, Nsukka.

**Ujam,** A. (1983). Purification and properties of D. rotundata Hexokinase. A thesis submitted to UNIBEN for the degree M.Sc. Biochemistry.

**Vesell,** E. S. & Bearn, A. G. (1958). J. Clin. Invest. 37:67. in "Isoenzymes" Chapman and Hall Ltd., pp 209.

**Victor,** R. & Irwin, T. (1971). Malate dehydrogenase of leaf tissue from spinacea oleracea properties of three isoenzymes. Archives Biochim. Biophy. 147:114-122.

**Villee,** C. A. (1968) in "Isoenzymes" Chapman & Hall Ltd. pp 207.

**Weber,** K. Pringle, R. & Osborn, M. Measurement of Molecular weights by Electrophoresis on SDS. acrylamide gel. Methods in Enzymology: 26c:3-27.

**Wieland,** T. Pfliederer, G. Haupt, I. & Worner, W. (1959) in "Isoenzymes" Chapman and Hall Ltd., pp 223.