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THE PURIFICATION AND STEADY STATE KINETIC CHARACTERISATION OF RAT LIVER MITOCHONDRIAL MALATE DEHYDROGENASE

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ABSTRACT

Mitochondrial malate dehydrogenase (mMDH) is one of the enzymes in the malate-aspartate shuttle system which facilitates the movement of reducing equivalents across the mitochondrial membrane. The metabolism of ethanol in the cytosol of liver tissue requires large amounts of NAD^+ and produces NADH, which must be oxidised back to NAD^+ in the mitochondria. The transfer into the mitochondria is carried out by the shuttle system which may thereby contribute significantly to the control of ethanol metabolism under certain circumstances. In the shuttle system mMDH acts in the opposite direction to the cytosolic form of the enzyme. Is this due to differences in the functioning of the two isozymes or to differences in their environments?

In this study a new method for the purification of mMDH from rat liver has been developed. This method was developed to be quick and to cause as little disruption to the protein structure of the enzyme as possible. The scheme involves the partial purification of mitochondria by centrifugation followed by elution, by NADH, off carboxymethyl cellulose chromatography resin. The whole process takes two days, because of the need to dialyse the sample to remove NADH, before any kinetic studies can be carried out. The specific activity of the purified enzyme compares favourably with other published values and the enzyme shows up as a single band on an SDS polyacrylamide electrophoresis gel. The major problem with the enzyme produced was its lack of stability during storage.

The second part of the project was to carry out steady state kinetic experiments with the purified enzyme under conditions that simulate the *in vivo* state. These are 37°C, pH 7.4, and 0.1 M phosphate buffer. The dissociation and Michaelis constants, for other tissues, have been published before but not those for rat liver. Very few of these experiments have been carried out under these near physiological conditions. The kinetic parameters produced are (μM): Ka, 72; K_{ia}, 12; K_b, 86; K_{ib}, 18; K_p, 1900; K_{ip}, 15000; K_q, 170; K_{iq}, 1100; where a = NADH, b = oxaloacetate, p = malate, and q = NAD^+. Some of these values have extremely high standard errors and so need further refining.

The values produced in this work can be compared to those for cytosolic malate dehydrogenase (Crow *et al.*, 1982) and because the two sets of constants are quite similar it appears that the different modes of action *in vivo* are mostly due to differences in the environment of the two isozymes.

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TABLE OF CONTENTS

			Page
ABSTRACT			ii
ACKNOWLEDGEMENTS			iv
TABLE OF CONTENTS			v
LIST OF ABBREVIATIONS			viii
LIST OF FIGURES			x
LIST OF TABLES			xii
CHAPTER 1		INTRODUCTION	1
	1.1	The Role of Mitochondrial Malate Dehydrogenase in the Metabolism of ethanol	1
	1.2	Characterisation of malate dehydrogenases	5
		1.21 Physical properties	5
		1.22 Kinetic properties of mMDH	7
		1.23 mMDH *in vivo*	8
	1.3	Aims of this Project	9
CHAPTER 2		MATERIALS AND METHODS	10
	2.1	Materials	10
	2.2	Methods	11
		2.21 Homogenisation buffer	11
		2.22 Other buffers	11
		2.23 Enzyme assays	11
		2.24 Protein assays	12
		2.25 Isoelectric focusing	12
		2.26 CM-cellulose resin	12
		2.27 Dialysis	12
CHAPTER 3		PURIFICATION OF MITOCHONDRIAL MALATE DEHYDROGENASE	13
	3.1	Introduction	13
		3.11 Ion exchange chromatography	15
		3.12 Affinity and dye-binding chromatographies	16
		3.13 Affinity elution chromatography	17
	3.2	Development of a Purification Scheme	18

	3.21	Purification scheme I	18
		3.211 Equipment	20
	3.22	Assay development	20
		3.221 Enzyme assays	21
		3.222 Protein assays	21
	3.23	Differential centrifugation and sonication	22
		3.231 Use of protease inhibitor PMSF	23
	3.24	Ion exchange chromatography	23
	3.25	Affinity chromatography	24
	3.26	Affinity elution from an ion exchange resin	25
		3.261 Temperature stability during affinity elution	26
		3.262 Refinement of affinity elution	26
		3.263 Buffer types used for affinity elution	30
		3.264 Ionic strength	32
	3.27	Ultrafiltration	32
	3.28	Enzyme purity	34
		3.281 Specific activity	34
		3.282 Isoelectric focusing	34
		3.283 SDS gel electrophoresis	39
	3.29	Stability of mMDH on freezing	39
3.3	Conclusion		42
	3.31	Purification scheme II	42
CHAPTER 4	STEADY STATE KINETIC STUDIES		45
4.1	Introduction		45
4.2	Manipulation of Steady State Kinetic Data		48
	4.21	Manual plotting of data	48
	4.22	Computer fitting of data	48
	4.23	Correction of constants for unsaturating substrate concentration	50
	4.24	Design of kinetic experiments	51
4.3	Preliminary Steady State Kinetics		52
	4.31	Determination of approximate Michaelis constants	52
	4.32	Assay linearity	55
	4.33	Dual-beam effects	55
	4.34	pH dependence	55
	4.35	Enzyme concentration effects	56

4.4	The Reactions of mMDH in the Absence of Products	58
	4.41 Enzyme stability during kinetic assays	58
	4.42 Assay inhibition at high substrate concentrations	59
	4.43 6x6 assays: data collection and processing	59
4.5	The reactions of mMDH in the presence of product as an inhibitor	75
	4.51 Manual plotting of data	75
	4.52 Computer analysis of data	79
	4.53 Use of the F-statistic	79
4.6	Conclusion	84

REFERENCE LIST 87

ABBREVIATIONS

GENERAL

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
CM	carboxy methyl
Co A	coenzyme A
DEAE	diethyl aminoethyl
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
H_2O	water
H^+	hydrogen ion
α-KG	alpha-keto glutarate
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NBT	nitroblue tetrazolium
OAA	oxaloacetate
O_2	oxygen gas
PMS	phenazine metho-sulphate
PMSF	phenylmethylsulphonylfluoride
SDS	sodium dodecyl sulphate
TCA	tricarboxylic acid

ENZYMES

ADH	alcohol dehydrogenase
AlDH	aldehyde dehydrogenase
CS	citrate synthase
GDH	glutamate dehydrogenase
gMDH	glyoxysomal malate dehydrogenase
mAAT	mitochondrial aspartate aminotransferase
mMDH	mitochondrial malate dehydrogenase
sAAT	cytosolic (or soluble) aspartate aminotransferase
sMDH	cytosolic (or soluble) malate dehydrogenase

LIST OF FIGURES

Figure		Page
1	Interactions between ethanol metabolism, the malate aspartate shuttle and the electron transport chain showing the transfer of reducing equivalents across the mitochondrial membrane	4
2	Purification scheme I: The initial method for the purification of mitochondrial malate dehydrogenase from rat liver	19
3	Elution profile of affinity elution with citrate and tris/HCl buffers	28
4	Elution profile of affinity elution with phosphate buffers, I = 0.025	31
5	Elution profile of affinity elution with phosphate buffers, I = 0.020	33
6	Broad range (pH 3.5 - 9.5) isoelectric focusing gels. A: Protein stained. B: MDH activity stained	36
7	SDS gel electrophoresis of purified mitochondrial malate dehydrogenase	40
8	Purification scheme II: The final purification method developed in this work	43
9	Preliminary kinetics: Determination of an approximate K_m for malate	53
10	Preliminary kinetics: Determination of an approximate K_m for NAD^+	54
11	Primary Lineweaver Burk plots for the reaction in the absence of products	60
12	Secondary plots of the data for malate oxidation from the primary plots in Figures 11C and D	66

13	Secondary plots of the data for oxaloacetate reduction from the primary plots in Figures 11A and B	67
14	Primary inhibitor plot of inhibition of malate, as a substrate, by oxaloacetate	76
15	Primary inhibitor plot of inhibition of NAD^+, as a substrate, by NADH	77
16	Primary inhibitor plot of inhibition of oxaloacetate, as a substrate, by malate	78
17	Primary inhibitor plot of inhibition of NADH, as a substrate, by NAD^+	79

LIST OF TABLES

Table		Page
I	Ligands for affinity resins	16
II	Effect of PMSF in the purification scheme	24
III	Temperature stability of mMDH during enzyme purification	27
IV	Purification of malate dehydrogenase from rat liver mitochondria	35
V	Sample set of data from a 6x6 assay experiment with the enzyme in the absence of products	68
VI	Sample computer print out of the non-linear least-squares curve-fitting program for the data in Table V	70
VII	Kinetic constants for substrates of the mMDH reaction in the direction of oxaloacetate reduction	72
VIII	Kinetic constants for substrates of the mMDH reaction in the direction of malate oxidation	73
IX	The concentrations of substrates and inhibitors for the product inhibition experiments	75
X	Computer fitted data from product inhibition experiments	80
XI	Kinetic constants produced for substrates of the mMDH reaction by product inhibition experiments	83
XII	Comparisom between constants for mitochondrial MDH determined by experiments with and without product inhibitors and those for cytosolic MDH	85

CHAPTER 1

INTRODUCTION

1.1 THE ROLE OF MITOCHONDRIAL MALATE DEHYDROGENASE IN THE METABOLISM OF ETHANOL

Ethanol is oxidised in the cytosol of liver cells by the enzyme alcohol dehydrogenase (ADH). The acetaldehyde produced is further oxidised to acetate by the enzyme aldehyde dehydrogenase (AlDH). Acetate is a normal metabolic product which can be used by the body.

$$\text{Ethanol} + NAD^+ \xleftrightarrow{\text{Alcohol Dehydrogenase}} \text{Acetaldehyde} + NADH + H^+ \quad \text{(Equation 1)}$$

$$\text{Acetaldehyde} + NAD^+ \xrightarrow{\text{Aldehyde Dehydrogenase}} \text{Acetate} + NADH + H^+ \quad \text{(Equation 2)}$$

All the detectable ADH activity is found in the cytosol of the liver cells. AlDH activity is found in both the cytosol and the mitochondrial matrix although it is not clear whether the oxidation of acetaldehyde during ethanol metabolism takes place in the cytosol, the mitochondrial matrix, or in both. As shown in the Equations 1 and 2, the oxidition of ethanol to acetate requires NAD^+ and produces NADH. As neither NAD^+ and NADH can cross the inner mitochondrial membrane the cell contains two distinct pools of these cofactors, one in the cytosol and the other in the mitochondrial matrix. During the metabolism of ethanol NADH is produced in both the cytosol and the mitochondria, but almost all of the reoxidation to NAD^+ is carried out by the electron transport system in the mitochondrial matrix. Because of this, an indirect method is required to reoxidise the cytosolic NADH. The chief process by which this is achieved is the malate-aspartate shuttle (Lardy *et al.*, 1965; Haynes, 1965; Dawson, 1979). Only the reducing equivalent from the cytosolic NADH is transferred to the mitochondrial NAD^+ (Equation 3).

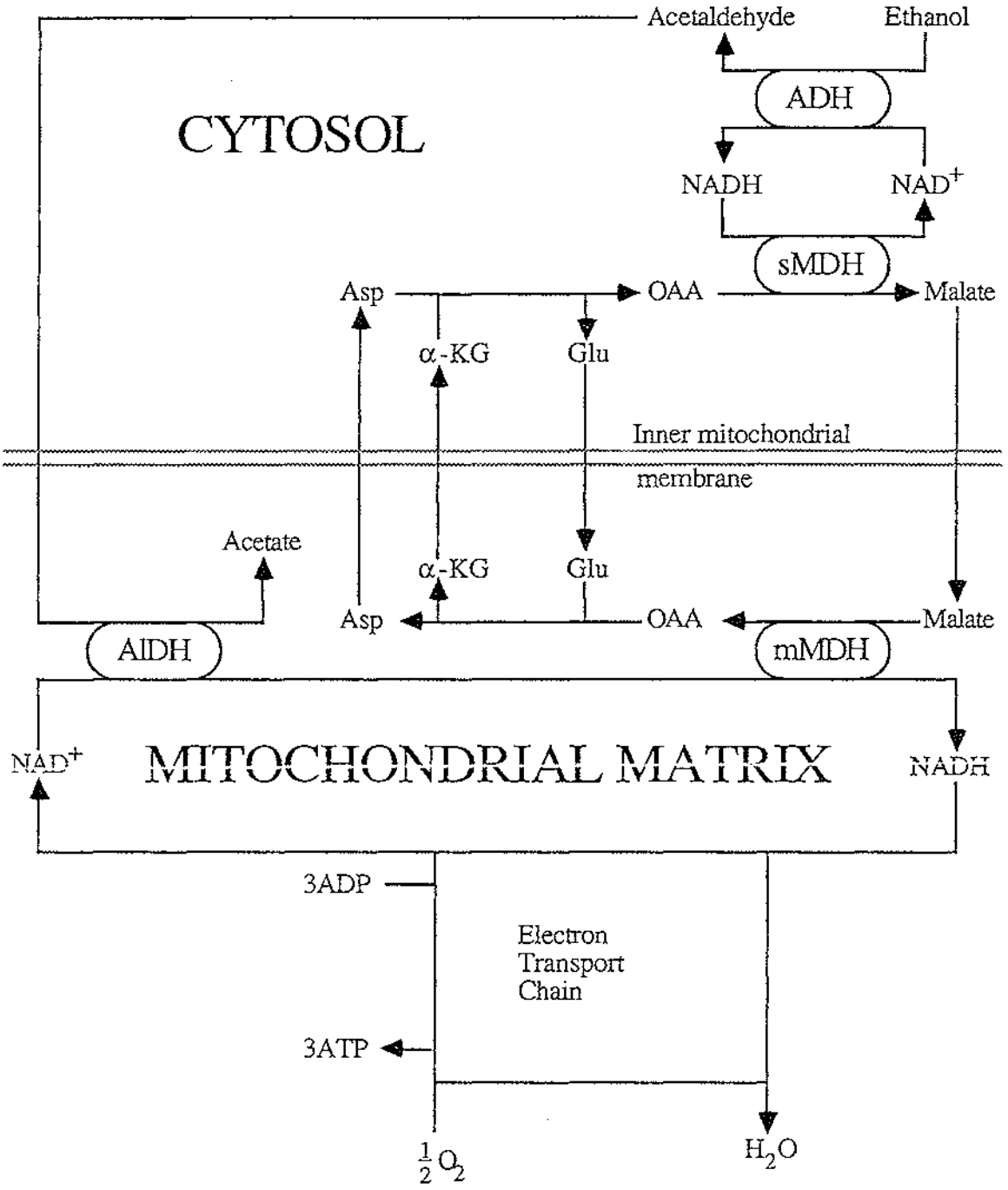
$$NADH_{cyt} + NAD^{+}_{mito} \xrightarrow[\text{Shuttle}]{\text{Malate Aspartate}} NAD^{+}_{cyt} + NADH_{mito} \qquad \text{(Equation 3)}$$

Figure 1 shows the malate-aspartate shuttle system and how it relates to the metabolism of ethanol. NADH in the cytosol is oxidised to NAD^+ by cytosolic, or soluble, malate dehydrogenase (sMDH), with the conversion of oxaloacetate to malate. The malate produced can pass across the mitochondrial inner membrane and is then used by mitochondrial malate dehydrogenase (mMDH) to reduce mitochondrial NAD^+ to NADH. The NADH can then be reoxidised, by the electron transport system, back to NAD^+ with the production of energy in the form of ATP. As oxaloacetate cannot cross the inner mitochondrial membrane the shuttle system is completed by mitochondrial aspartate aminotransferase (mAAT) converting oxaloacetate and glutamate into aspartate and alpha-ketoglutarate, which can both cross the membrane to the cytosol, where they are converted back into glutamate and oxaloacetate by cytosolic aspartate amino-transferase (sAAT).

The oxidation of ethanol is normally controlled by ADH with the V_{max} of the enzyme being the limiting factor (Crow, 1985). The rate at which NADH can be reoxidised to NAD^+ may also play some role in the regulation when rates are lower than those set by the V_{max} of ADH (Crow, 1985). It is of interest to know the extent of the control that the malate-aspartate shuttle system, and in particular the cytosolic and mitochondrial malate dehydrogenases, may have on the rate of NADH reoxidation and therefore on the rate of ethanol metabolism in liver tissue. A significant increase in liver mMDH activity, in drinking alcoholics, has been reported (Jenkins and Peters, 1978). The same study also suggested an increased level of sMDH in alcoholics, but this could not be proven to be statistically significant. Steady state kinetic data for these enzymes can be used in computer simulations to show their action under the conditions of ethanol oxidation. This has been done for rat liver and human liver sMDH in previous studies from this laboratory (Crow *et al.*, 1982 and 1983). The present study aims to produce the steady state kinetic constants for rat liver mMDH. Rat liver is used as the source of mMDH because it is much easier to obtain than human liver tissue samples and comparisoms can still be made with the earlier work.

Cytosolic and mitochondrial MDH are enzymes that carry out the same reaction, but normally they must work in opposite directions *in vivo* to allow the shuttle system to carry out its function. One of the main questions we hoped to answer in this study was

whether the kinetic parameters for the two enzymes are different enough to account for their opposite direction of activity, or whether this is due to differences in the environment of the two enzymes.



ADH = Alcohol Dehydrogenase
AlDH = Aldehyde Dehydrogenase
sMDH = cytosolic Malate Dehydrogenase
mMDH = mitochondrial Malate Dehydrogenase

Figure 1

Interactions between ethanol metabolism, the malate-aspartate shuttle, and the electron transport chain, showing how the reducing equivalents, produced during ethanol metabolism in the cytosol, are transfered across the inner mitochondrial membrane into the mitochondrial matrix for reoxidation by the electron transport chain.

1.2 CHARACTERISATION OF MALATE DEHYDROGENASES

1.21 Physical properties

Malate dehydrogenase is a relatively small (molecular weight ≈ 70,000 daltons) globula protein, first discovered independently by Batelli and Stern in 1910 and by Thunberg in 1911. The existence of two distinct isozymes, located in different subcellular fractions, was first shown in 1959 (Delbrück *et al.*, 1959a and 1959b). These were named cytosolic or soluble MDH (sMDH) and mitochondrial MDH (mMDH) after the fractions in which they were found. More recently a third form has been found in the microbodies of several plant tissues (Breidenbach, 1969; Yamazaki and Tolbert, 1969), and this was named glyoxysomal MDH (gMDH). In animal tissues only the first two are found, and most of the discussion here will be about these two. They are very similar in molecular weight with sMDH being about 70,000 daltons and mMDH about 68,000 daltons depending on the species. Rat liver mMDH has a molecular weight of 66,300 daltons (Mann and Vestling, 1969). Both molecules are made up of two identical subunits (Dévényi *et al.*, 1966) each of which binds one molecule of coenzyme, NAD^+ or NADH (Kitto and Kaplan, 1966). The three dimensional crystallographic structures of both sMDH and mMDH have been published (Hill *et al.*, 1972; Roderick and Banaszak, 1983). Comparisons between these structures, and the structures of other dehydrogenases, show similarity in overall conformation, quarternary structure, location of the NAD^+ binding site, and several catalytically active residues (Roderick and Banaszak, 1983 and 1986), although the amino acid compositions are quite different.

Dissociation of the dimer into two identical monomers was shown to occur at pH 2, in 5 or 7 M guanidine hydrochloride, and in 8 M urea (Mann and Vestling, 1969). Under these conditions the monomers were inactive, but the enzymes could be reactivated by neutralisation of the acid or removal of the denaturant. The amount of activity recovered decreased the longer the enzyme was inactive. Experiments with mMDH, using fluorescent labels, showed concentration dependant dissociation with the $K_D = 2 \times 10^{-7}$ M at pH 8.0 (Shore and Chakrabarti, 1976). Coenzyme binding affected this equilibrium, with NADH causing a shift toward the monomer and NAD^+ binding preventing dissociation even at below 10^{-8} M. Experiments using gel filtration chromatography and sedimentation velocity ultracentrifugation (Bliele *et al.*, 1977) showed that porcine heart mMDH dissociated at concentrations less than 0.2 μM or when the pH was less than 7.0. Bovine mMDH also dissociated at less than pH 7.0 but

did not exhibit a concentration dependant dissociation and porcine heart sMDH did not dissociate under either of the conditions examined. The presence of either coenzyme caused dimer formation to be favoured. The increase in specific activity and the decrease in protein fluoresence during subunit reassociation both followed first order kinetics (Wood *et al.*, 1981). A model of subunit reassociation was proposed in which the rate limiting step was the cis/trans isomerisation about proline imino bonds during protein refolding. Immobilised protomers have been made that exhibit normal mMDH activity and kinetics (DuVal *et al.*, 1985) and kinetic studies with mMDH molecules that have one subunit inactivated showed normal kinetics (McEvily *et al.*, 1985a). The conclusion from this is that catalytic activity and regulation by effectors is independant of the dimeric structure of the native enzyme. However the intramitochondrial MDH concentration is estimated to be high enough to ensure that mMDH is a dimer *in vivo* (Shore and Chakrabarti, 1976).

Upon electrophoresis of mMDH several catalytically active bands are observed (Kitto *et al.*, 1966). These were suggested to be different conformational forms of the enzyme as they could not be separated on the basis of catalytic activity or amino acid composition (Kitto *et al.*, 1966). Mann and Vestling (1968 and 1970) suggested that the different bands were due to the combinations of two different subunits, but no further evidence has been produced to support this.

One of the major differences between sMDH and mMDH is in the amino acid compositions. mMDH has no tryptophan residues, whereas sMDH has 5 - 15 depending upon the species (Kitto and Kaplan, 1966). sMDH also has a much greater number of charged residues than the mitochondrial form, and in mMDH a high level of asparagine and glutamine residues are found with about 50% amidation of the aspartate and glutamate residues (Jones and Vestling, 1979). The mitochondrial enzyme has no disulphide linkages, but does have a number of free sulphydryl groups, 14 in porcine heart (Thorne and Kaplan, 1963).

A number of amino acid types have been examined as possibilities for active site residues in mMDH. Histidine has been implicated as a catalytically active residue (Banaszak and Bradshaw, 1975). Modification of cysteine residues causes loss of activity because of a thiol group located near the coenzyme binding site which appears to be necessary for enzymatic activity (Gregory *et al.*, 1971). Lysine has also been implicated as involved in enzyme activity (Yost and Harrison, 1971).

The two isozymes have very different isoelectric points, due to the differences in amino

acid composition. On an isoelectric focusing gel each enzyme shows a range of catalytically active bands (Kitto *et al.*, 1966) covering about half a pH unit. The different bands have most recently been explained as being caused by the binding of varying amounts of phospholipid to the mMDH molecule (Jones and Vestling, 1979). The phospholipid covers up charged groups on the protein's surface and therefore affects the isoelectric point.The isoelectric point of mMDH ranges from pH 8.9 - 9.6 and sMDH has an isoelectric point that ranges from pH 5.2 - 5.6. Calculation of the isoelectric point of mMDH using normal pKa values for the ionisable groups and 50% amidation of glutamate and aspartate gives a value in the range of pH 9 - 10 (Jones and Vestling, 1979). The difference between the isoelectric points is of great use when separating the two isozymes as this is the reason for the difference in the binding properties on ion exchange resins (see Chapter 3).

1.22 Kinetic properties of mMDH (also see Chapter 4)

A lot of work has been carried out on the kinetic properties of mMDH from various sources, most commonly porcine heart. This enzyme has a number of interesting kinetic properties, including substrate inhibition by oxaloacetate, substrate activation by malate and allosteric control by a variety of compounds, including citrate. The mechanism of action of mMDH has been described as a compulsory ordered bi-bi system (Raval and Wolfe, 1962). Harada and Wolfe (1968b) proposed a reciprocating compulsory ordered mechanism involving a switch from subunit to subunit, but this was later discounted when immobilised monomers were shown to have normal catalytic activity (DuVal *et al.*, 1985).

Most of the kinetic studies that have been carried out previously have used conditions that could not be described as physiological. As mMDH has an activity optimum at about pH 10 in the direction of malate oxidation and pH 7.4 in the reverse direction most investigators have used pH 8.0 and above for their experimental conditions so as to strike a balance between the two directions. Because of this it is often hard to distinguish between those results which are physiologically relevant and those which may be artifacts of the nonphysiological conditions. Since for the present work, kinetic data was required for the examination of the physiological activity of mMDH, conditions similar to those *in vivo* were used.

1.23 mMDH *in vivo*

All the isozymes of MDH are synthesised on ribosomes in the cytoplasm. mMDH and gMDH are both then transported into the appropriate organelle. These two isozymes appear to have a common higher molecular weight precursor (Hock, 1984). Mitochondrial MDH is synthesised with an N-terminal transit peptide which contains a large number of basic residues, although there is little conservation of sequence between species. There are only 12 differences, out of 314 residues, between the mature subunits of rat heart and porcine heart mMDH's (Grant *et al.*, 1987). Purified mature mMDH can also be transfered into the mitochondria whereas sMDH can not (Passerella *et al.*, 1980). It appears that for this process there is a common receptor for mMDH and mAAT (Marra *et al.*, 1985) although only one molecule is transfered at a time.

The location of mMDH in the mitochondria is not completely certain. It is not found in the inner or outer membranes, but there is evidence which indicates that lipids do affect the properties of mMDH in very dilute solutions (Callahan and Kosicki, 1967). Lysolecithin reduces the rate of thermal inactivation of mMDH (Dodd, 1973), which is normally rapidly inactivated at elevated temperatures (Müller and Klein, 1982). It seems probable that mMDH is located in the matrix of the mitochondria but that under certain conditions it is able to interact with the inner membrane.

The low level of free oxaloacetate believed to exist in the mitochondria has led to the proposal that enzymes in the mitochondria interact to allow the formation of localised high substrate concentrations (Srere *et al.*, 1973). Interactions between mMDH and mAAT have been measured using counter-current distribution (Backman and Johansson, 1976). sMDH and sAAT were also shown to interact, but no interaction was found between cytosolic and mitochondrial species. An effective increase in the rate of the reaction of coupled sMDH and sAAT has been shown (Arrio-Dupont *et al.*, 1985). The mitochondrial enzymes probably show a similar enhancement of activity when coupled together. mMDH also interacts with NADH: ubiquinone reductase, complex I of the electron transport chain, which could use the NADH produced by the mMDH (Sumegi and Srere, 1984). A bienzyme complex is also formed between mMDH and citrate synthase (CS). The dissociation constant for this reaction is 10^{-6} M (Tompa *et al.*, 1987). Each of these interactions cause one of the products of the mMDH reaction to be removed and therefore assist an otherwise energetically unfavourable reaction. The interactions of mMDH with other enzymes were not investigated in this work as it was first considered necessary to obtain the kinetic parameters of pure mMDH, under physiological conditions.

1.3 AIMS OF THIS PROJECT

The first aim of this project was to develop a cheap, gentle, and quick method of purification for mitochondrial malate dehydrogenase from rat liver. The second aim was to use this purified enzyme to carry out steady-state kinetic studies under simulated physiological conditions so that the kinetic constants determined can be compared to data determined for cytosolic malate dehydrogenase and information gained on their differing modes of action.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Adult male Sprague-Dawley rats were obtained from the Massey University Small Animal Production Unit.
The following chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri, USA);

> Malic acid, oxaloacetate, glutamic acid, EDTA, dithiothreitol (DTT), NADH, NAD^+ (Grade III), NAD^+ (AA grade), phenazine metho-sulphate (PMS), phenylmethylsulphonylfluoride (PMSF), coomassie brilliant blue G-250.

CM-32 Carboxy methyl cellulose, was obtained from Whatman Ltd. (Springfield hill Maidstone, Kent, England), and nitroblue tetrazolium (NBT) from Calbiochem.

All other chemicals were reagent grade products.

2.2 METHODS

2.21 Homogenisation buffer

This solution is 5 mM phosphate buffer, pH 7.3 with 0.25 M sucrose, 1 mM PMSF and 1 mM DTT. 10 mM standard Na_2HPO_4 and NaH_2PO_4 solutions were made. Na_2HPO_4 was titrated with NaH_2PO_4 to pH 7.3. Half the final volume of buffer required was measured out and sucrose, to make the final concentration 0.25 M, was added. Next PMSF and DTT, to give a final concentration of 1mM each, were added and the pH was checked to see that it was still 7.3. Finally the solution was made up to the required volume with glass distilled water.

2.22 Other buffers

These were made up with reagent grade chemicals and with glass distilled water. Constant ionic strength buffers were made using the method in the Biochemists Handbook (Long, 1961). Other buffers were titrated to the correct pH.

2.23 Enzyme assays

During the purification process malate dehydrogenase was assayed at 25°C, in a 3 ml reaction volume using 0.12 M L-malate and 2 mM NAD^+ (AA grade) in 0.1 M glycine / NaOH buffer, pH 10.0.

Glutamate dehydrogenase was assayed, at 25°C, in a 3 ml reaction volume. The assay mixture contained 1.5 mM NAD^+ (AA grade), 13 µM glutamate, and 2.6 mM EDTA in 0.1 M phosphate buffer, pH 7.3. For both the above assays the increase in absorbance at 340 nm was measured using a CECIL CE 293 Digital Ultraviolet Spectrophotometer. For the kinetic studies malate dehydrogenase was assayed, at 37°C, in a 3 ml reaction volume. In the direction of malate oxidation the assay mixture contained varying amounts of NAD^+ (Grade III) and malate in 0.1 M phosphate buffer, pH 7.4. For the direction of oxaloacetate reduction NADH and oxaloacetate in 0.1 M phosphate buffer, pH 7.4, were used. The change in absorbance at 340 nm was measured on an Aminco DW 2a UV / Visible Spectrophotometer.

2.24 Protein assays

Measurement of the absorbance at 280 nm was used as a crude indication of the amount of protein present in each fraction collected off the chromatography columns. For more accurate measurements the Lowry method (Lowry *et al.*, 1951) or the Coomassie blue method (Bradford, 1976) were used. For both colourimetric methods a BSA standard curve was used, 0 - 1 mg protein for the Lowry method and 0 - 100 µg for the coomassie blue method. The BSA concentration was calculated using E^{1cm} 1% BSA at 280 nm = 6.67 (Foster and Sterman, 1956). Measurements were made on a Hitachi 101 Spectrophotometer.

2.25 Isoelectric focusing

The LKB multiphor system, with thin layer polyacrylamide gels, pH range 3.5 - 9.5, was used. Gels were stained for protein with coomassie blue G250 (1g/litre) in 7% perchloric acid. Activity staining was with phenazine metho-sulphate (PMS), 0.0875 mg/ml; nitroblue tetrazolium (NBT), 0.375 mg/ml; NAD^+, 0.5 mg/ml and 0.1 M L-malate in 0.1 M glycine / NaOH buffer pH 10.0 (as in Crow *et al.*, 1982).

2.26 CM-cellulose resin

The resin was prepared, and the columns packed, using the method described in the manufacturers information leaflet, except the resin was also degassed in 0.5 M solution of the acid component of the desired buffer.

2.27 Dialysis

All samples to be dialysed (normally approximately 10 mls) were put in 3 litres of the dialysis buffer. This buffer was changed once and the sample was left in each buffer for at least 4 hours. The dialysis tubing was prepared by washing 3 times in distilled water at 80°C.

CHAPTER 3

PURIFICATION OF MITOCHONDRIAL MALATE DEHYDROGENASE

3.1 INTRODUCTION

The 'purification' of MDH was first reported in the late 1940s and early 1950s, but until the existance of two distinct isozymes, located in different subcellular fractions, was first shown in 1959 (Delbrück *et al.*, 1959a and 1959b), the enzyme could not be considered truly purified. These two isozymes, cytosolic and mitochondrial MDHs, are still considered to be the only ones to be found in animal tissues, although other isozymes have been identified in plant tissues. For the purification of rat liver mitochondrial malate dehydrogenase (mMDH) advantage can be taken of the differences between the two isozymes (see Chapter 1). In particular the difference between the isoelectric points, with sMDH having an isoelectric point about pH 5.5 and mMDH about pH 9.5, can be used as this difference means that the two forms have very different binding properties on CM-cellulose resin and this plays an important part in the purification scheme developed in this work.

In the past mMDH has been purified by a variety of methods from a large number of different sources, including pig (porcine) heart (Ochea, 1955; Wolfe and Neilands, 1956; Ravel and Wolfe, 1962; Gregory *et al.*, 1971; Smith and Sundaram, 1983; McEvily *et al.*, 1985a), beef (bovine) heart (Davies and Kun, 1957; Grimm and Doherty, 1961; Davies and Scopes, 1981), chicken heart (Kitto and Kaplan, 1966), chicken liver (Casadó *et al.*, 1980), rat heart (Mavrides and Nadeau, 1987), rat liver (Sophianopoulos and Vestling, 1962; Kuan *et al.*, 1979; Passarella *et al.*, 1980; Cremel *et al.*, 1985) and, watermelon cotyledons (Walk and Hock; 1976). Pig heart is the most common source of tissue used for the purification of mMDH.

Since MDH was first purified, in the early fifties, a wide variety of purification techniques have been used. The first step in most of the early procedures was the making of an acetone powder. This is prepared by making a crude homogenate of the tissue being used and washing this in acetone. The acetone is then dried off to leave a powder. This treatment is harsh on enzymes as they are taken out of the normal aqueous environment and placed in one of nonaqueous acetone. An acetone powder is a convienient way to store a tissue in a crude state. Other purification procedures have

used a crude homogenate as the starting point.

The most commonly used purification technique is ammonium sulphate fractionation. In fact three quarters of the methods cited above use this technique, although in recent years it seems to have become less popular. Proteins are precipitated by the high salt concentration of the ammonium sulphate and the technique is based on different proteins precipitating at different concentrations, usually measured as percent saturation. Normally proteins are separated between two different concentrations, or cuts, of ammonium sulphate. The lower amount is added, the unwanted precipitated protein removed and then the higher concentration precipitates the desired protein. mMDH is precipitated between 40 and 80 percent saturated ammonium sulphate. A large amount of other, unwanted, protein is also precipitated within this range. Therefore to get maximum effectiveness from this step it is necessary to limit the concentrations of ammonium sulphate used so that the maximum amount of the unwanted protein is excluded and as much of the MDH activity as possible is retained. The ammonium sulphate cuts that have been used have varied from a maximum of 40 - 80% saturation as the widest range used down to 45 - 60% or 50 - 65% saturation as the narrowest ranges used. Normally the more effective the other techniques used in the purification scheme the wider the ammonium sulphate cuts are. This technique again puts the enzyme in an unnatural and therefore possibly damaging environment. This is probably one of the reasons the technique is becoming less popular. Another is the time the whole process takes, as the ammonium sulphate must be dissolved and the precipitate redissolved very slowly to avoid mechanical disruption to the protein structure.

Another harsh technique that has been used in the past is that of ethanol fractionation (Ochea, 1956; Wolfe and Neilands, 1956; Davies and Kun, 1957; Grimm and Doherty, 1961). This is similar to the ammonium sulphate process with protein being precipitated between varying concentrations of ethanol. The normal range used for the fractionation of mMDH has been between 45 and 70% ethanol. This step does not appear to have been used in any scheme for purifying mMDH since 1962.

The use of heat as a purification step has been tried, originally by Sophianopoulos and Vestling (1962), when they used one minute at 58°C, and much more recently by Mavrides and Nadeau (1987) who used 55°C for five minutes. The basis behind this step is that the less heat stable proteins will be denatured by the higher temperatures and so can be removed by centrifugation. Again it is hard to evaluate the effects that this step may have on the desired protein whether in terms of denaturation or in small changes to

the three dimensional structure. Work on the thermal stability of dehydrogenases (Müller and Klein, 1982) shows pig heart mMDH to become irreversibly denatured at 43°C with a half life of 20 minutes. These experiments were carried out using purified enzyme and in a crude homogenate the enzyme may be better protected.

One of the largest groups of techniques remaining, that have been used for the purification of mMDH, is the chromatographic procedures. Included are ion exchange, affinity and affinity elution chromatographies. All these procedures are much less likely to affect protein structure and are therefore preferable to the techniques discussed above. (Further discussion on these chromatography techniques is given in sections 3.11, 3.12, and 3.13).

The last techniques availiable for the purification of enzymes which have been applied to mMDH are gel filtration (Kitto and Kaplan, 1966; Davies and Scopes, 1981; Mavrides and Nadeau, 1986) and starch gel electrophoresis (Davies and Kun, 1957; Grimm and Doherty, 1961). Gel filtration separates molecules on the basis of size by passing them down a column containing a porous resin which has varying sized holes making a different column volume availiable to different sized molecules. Gel filtration can be used to remove mitochondrial aspartate amino transferase which can be a major contaminant of mMDH, in some purification schemes (Kitto and Kaplan, 1966). Starch gel electrophoresis separates molecules on the basis of overall charge, but this also is a very slow process with the electric current having to be applied for 18 - 20 hours. Because of this the technique was only used in methods from prior to 1965.

3.11 Ion exchange chromatography

Ion exchange chromatography separates proteins on the basis of their net charge. Resins are made having either positively or negatively charged groups and proteins of the opposite charge should bind to these at neutral pH's. The bound protein can be eluted off the column by changing the pH to affect the binding or by using added salt to increase the ionic strength. Using an increasing salt gradient will elute the bound proteins with those having the lowest charge density eluting first. The two most common types of ion exchange resins are diethyl aminoethyl (DEAE) cellulose, which has a positive charge at neutral pH's and carboxy methyl (CM) cellulose, which has negatively charged groups. Both of these types of resin have been used for the purification of mMDH. At pH 7.4 with 5 mM phosphate buffer, sMDH from rat liver will bind to DEAE-cellulose (Crow *et al.*, 1982) wheras mMDH will pass straight

through the column and is found in the void volume. The reverse is true with the CM-cellulose resin, mMDH will bind to this resin, even at pH 5.0. sMDH does not bind to CM-cellulose under any normal conditions. Because of these properties ion exchange resins are often used as a method to separate the two isozymes. When purifying mMDH, CM-cellulose is the most commonly used resin. mMDH is easily eluted by using a salt gradient (Kitto and Kaplan, 1966; McEvily *et al.*, 1985a; Mavrides and Nadeau, 1987).

3.12 Affinity and dye-binding chromatographies

Affinity chromatography is a most powerful technique in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance (ligand) immobilised on an insoluble support (matrix). Though only widely used in the last fifteen to twenty years affinity chromatography was first used in 1910 for the selective adsorbtion of amylase onto starch. A wide variety of compounds can theoretically be isolated this way (Table I).

TABLE I

Ligands for affinity resins

An enormous number of compounds can be isolated by affinity resins. A range of possible ligands for various types of compound to be isolated is shown.

Compound to be Isolated	Possible ligands
Enzyme	Substrate analogue, Inhibitor, Cofactor
Antibody	Antigen, Virus, Cell
Lectin	Polysaccharide, Glycoprotein, Cell surface receptor, Cell
Nucleic acid	Complementary base sequence, Histone, Binding protein
Hormone, Vitamin	Receptor, Carrier protein
Cell	Cell surface specific protein, Lectin

The most commonly used affinity resins for the purification of mMDH are 5'AMP sepharose and blue sepharose. 5'AMP sepharose is a group specific adsorbant that will bind enzymes which require NAD^+. mMDH will bind to this resin and can be eluted by the addition of NADH to the buffer (Walk and Hock, 1976).

The blue sepharose matrix contains a dye which has an affinity for enzymes requiring adenylyl-containing cofactors (including NAD^+ or $NADP^+$) with the interaction occuring at the nucleotide binding site. It is therefore not a true affinity system in the biospecific sense (Scopes, 1987). Adsorbed proteins can be eluted by low concentrations of free cofactor, affinity elution, while less specifically bound proteins require the use of much higher concentrations of cofactor, or salt. mMDH has been eluted both with salt (Mavrides and Nadeau, 1987; McEvily *et al.*, 1985) andwith NADH (Crémel *et al.*, 1985; Passarella *et al.*, 1980; Kuan *et al.*, 1979). Because the blue sepharose does not specifically bind MDH the inclusion of other steps is required.

The major problem with affinity resins is that they can be difficult to make and are usually expensive to buy. The resins used for mMDH purification are also not specific to one enzyme and so require additional purification steps.

3.13 Affinity elution chromatography

Affinity elution chromatography combines both ion exchange and affinity chromatographies. Protein is bound to an ion exchange resin and eluted by the inclusion of a substrate or specific ligand in the eluting buffer (Scopes, 1977 and 1981). The binding of an oppositely charged ligand to a protein molecule reduces the overall charge and so decreases the strength of the interaction with the ion exchange resin. Since most proteins are negatively charged affinity elution is virtually restricted to cation exchangers. mMDH fits this category quite adequately. The binding of the ligand also causes a change in the conformation of the protein which affects binding. The technique is somewhat more limited in its uses than affinity chromatography, but in the right circumstances can be just as powerful and it uses a resin that is more easily prepared, more readily availiable and cheaper. It has been used previously as a step to help purify mMDH (Davies and Scopes, 1981).

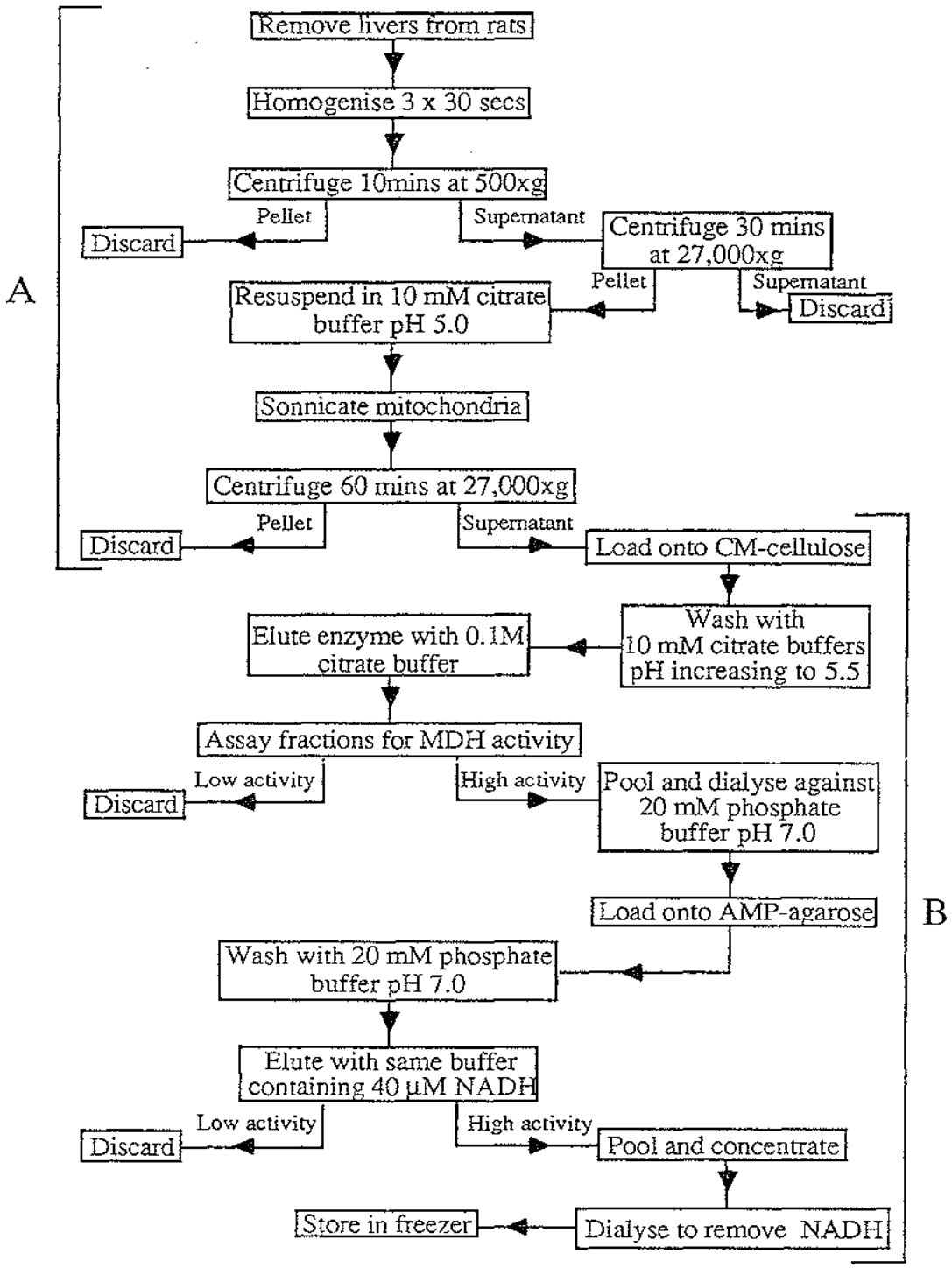
3.2 DEVELOPMENT OF A PURIFICATION SCHEME

In looking for a cheap, gentle, and quick method for the purification of mMDH we decided to discard ammonium sulphate and ethanol fractionation steps as too likely to cause disruption to the protein's structure. Starting with this idea, and the fact that the shorter the procedure the better, Purification Scheme I (see Section 3.21) was drawn up. The rest of the chapter follows the development of this scheme into the final workable method.

3.21 Purification Scheme I (see Figure 2)

The livers were removed from freshly killed rats and immediately placed on ice. They were cut into smaller pieces with scissors and then homogenised in four volumes of chilled homogenisation buffer (see Section 2..21) for three 30 second bursts. A sample of the extract was taken for assaying. The total homogenate was then centrifuged at 500xg and 4°C for 10 minutes and samples for assaying were taken from both the supernatant and the resuspended pellet. The pellet was then discarded. The supernatant was centrifuged at 27,000xg and 4°C for 30 minutes. A sample was removed from the supernatant for assaying, and the supernatant was then discarded. The pellet was resuspended in chilled 10 mM citrate buffer, pH 5.0, containing 1 mM PMSF. This solution was then sonicated twice for 45 seconds at maximum power. A sample of this solution was taken for assaying, then the extract was centrifuged at 27,000xg and 4°C for 60 minutes. Samples of both the supernatant and the pellet were taken for assaying.

CM - cellulose resin was equilibrated with 10 mM citrate buffer, pH 5.0, in the cold room at 4°C. All remaining steps were carried out at 4°C in the cold room. The final supernatant from the above steps was loaded onto the column and the column washed with 10 mM citrate buffer, pH 5.0, until the A_{280} droped to below 0.01 absorbance units. The eluate was collected in 10 ml fractions and these were all assayed for activity and protein concentration. The buffer was changed to 10 mM citrate, pH 5.1 and the column washed with this until the A_{280} had again droped to below 0.01 absorbance units. After this 10 mM citrate, pH 5.3, and then 10 mM citrate buffer, pH 5.5 were used to wash the column. Finally the buffer was changed to 0.1M citrate buffer, pH 6.0. For each buffer change the column was washed until the A_{280} droped to below 0.01 absorbance units. All fractions from these elutions were assayed for MDH activity and those with the highest activity were pooled and if necessary concentrated by



A Carried out at room temperature with sample kept on ice

B All steps carried out in cold room at 4°C

Figure 2

Purification Scheme I: The original method for the purification of mitochondrial malate dehydrogenase from rat liver.

ultrafiltration. The sample was dialysed against 20 mM sodium phosphate buffer, pH 7.0. The dialysed sample was loaded onto a column containing AMP sephrose 4B resin which had been equilibrated with 20 mM sodium phosphate buffer, pH 7.0, and the column was then washed with the same buffer. The adsorbed protein was eluted by the same buffer to which 40 μM NADH had been added. The fractions were assayed and the active fractions were pooled then concentrated by ultrafiltration. Finally the enzyme sample was dialysed to remove NADH and then stored in the freezer. All buffers contained 1 mM DTT.

3.211 Equipment

Homogenisation was carried out with an Ultraturrax homogeniser using the medium sized probe.
Centrifuging was done in a Sorval superspeed RC2-B automatic refrigerated centrifuge.
Sonication was carried out using an M.S.E. Sonicator.
Fractions were collected using a LKB 7000 ultrorac fraction collector.
Ultrafiltration was carried out in an Amicon 8050 stirred ultrafiltration cell with a Diaflow PM 30 ultrafilter.

3.22 Assay Development

Scheme I consisted of a partial separation of the mitochondrial contents by centrifugation, followed by the use of a CM-cellulose ion-exchange resin which binds mMDH and not sMDH. The eluate was loaded onto a 5'AMP-sepharose 4B affinity chromatography resin. The 'purified' enzyme eluted off the column could then be concentrated and stored.

The first thing it was necessary to do was to develop the assay methods so that it was possible to detect the appropriate enzymes and follow progress through the purification method. Assays for MDH and GDH (Section 2.23) were carried out on each fraction from the centrifugation steps and MDH assays were carried out on all the other fractions. Protein levels were also measured (see Section 2.24) so that a specific activity could be determined. For some of the early samples (those in the centrifugation, particularly the pellet fractions) there was a large amount of solid material included in the suspension and then in the assay mixture. This made it difficult to measure the correct amount of sample and gave the assays a wide varience. As it was necessary to carry out these assays relatively quickly they were only taken as a guide to the

measurement of each enzyme or protein level during the preparative procedure.

3.221 Enzyme assays

A trial set of assays were carried out with pure mMDH and GDH, purchased from the Sigma Chemical Company and these showed good linearity. The first enzyme assays carried out on the early centrifugation steps showed very little linearity, in fact the line seemed to wander up and down with no particular direction. This was found to be due to the extract containing intact mitochondria which were being placed in an environment in which osmotic pressure would disrupt their membrane structure, but this disruption was taking up to five minutes to occur. This was resolved by adding the enzyme sample to the assay mixture and incubating for five minutes before starting the reaction with one of the substrates.

The MDH assay was carried out in the direction of malate oxidation and at pH 10.0. This direction was chosen because the substrates are more stable and cheaper than those for the other direction. pH 10.0 was chosen because this is approximately the pH for which mMDH has maximum activity in the direction in which the assays were run. These assays were straight forward as mMDH is a very active enzyme under these conditions.

The GDH assay on the other hand was a little more difficult. The assay was to be used to follow the mitochondrial contents through the centrifugation part of the preparative method. Trouble was found with low levels of activity and the disappearance of most of this activity through the course of these steps. These problems were not really adequately resolved, but were not too important as the assay was mainly to look at mitochondrial breakage in the second centrifugation step and most of the activity loss occured after this stage.

3.222 Protein assays

During the affinity elution steps and earlier chromatography methods protein levels were initially measured using direct spectrophotometric determination at 280 nm. This is a quick and easy method but is not extremely accurate. Colourimetric analysis was carried out on all samples with high MDH activity. Initially the Lowry method (Lowry *et al.*, 1953) for protein determination was used and what appeared to be consistent results were obtained. The Coomassie blue method (Bradford, 1976) gave comparable results

through the preparative method but the final 'purified' enzyme sample showed a much higher specific activity. It is unknown whether this is due to a lack of reaction between the Coomassie reagent and the purified protein or whether there are matrix effects with the Lowry complex. The Lowry reaction is subject to interference from various componds, including potassium ions, magnesium ions, EDTA, tris, and thiol reagents (Vallejo and Lagunasi, 1970).

3.23 Differential centrifugation and sonication

The livers from four rats (approximately 50 gms) were prepared as in Scheme I. The amount of liver, to the nearest 0.1 gm, was determined. Homogenisation was used to break up the liver cells but leave the smaller subcellular particles, such as the mitochondria, intact. The first slow spin separated the homogenate so that any large cell debris, unbroken cells and the nuclei were in the pellet. The supernatant contained all the other cellular contents. When this spin was first carried out a large proportion of the mitochondria were observed to be in the pellet. The mitochondria are visible as a fluffy pinkish layer on the top of the pellet. A large proportion of the MDH activity, up to 50%, was found in the pellet and although there is no distinction between the isozymes most of the MDH should be in the supernatant. To reduce the proportion of MDH activity in the pellet the spin was reduced in length from 10 to 5 minutes, which resulted in 70 and 90% of the MDH activity being found in the supernatant.

The first supernatant was recentrifuged at 27,000xg for 30 minutes. The pellet contains all the mitochondria but also contains large numbers of lysozomes. Because of the proteolytic enzymes that the lysozomes contain, PMSF (see Section 3.231) was added as a protease inhibitor. The supernatant contains 30 - 50% of the MDH activity. This activity should be mostly sMDH as the cytosolic enzymes should be in the supernatant. The mMDH from any broken mitochondria will also be in the supernatant, but the MDH assay can not distinguish between the two isozymes. The number of broken mitochondria can be followed by assaying for glutamate dehydrogenase (GDH) as GDH is an enzyme found solely in the mitochondria. Data from these assays suggested that about 30% of the mitochondria were broken. GDH assays on the first centrifugation step suggest about 15% of the mitochondria are in the first precipitate.

The pellet from the second spin was resuspended in the same buffer that was used to equilibrate the CM-cellulose resin with 1 mM PMSF added. This pellet was very difficult to resuspend. The suspension was sonicated for three 30 second bursts at full

power. Sonication was designed to break up the mitochondrial membranes and release their contents into the solution, and any unsuspended lumps of the pellet were also broken up. The solution of broken mitochondria was centrifuged at 27,000xg for 60 minutes. All the membraneous debris and unbroken mitochondria were in the pellet. The supernatant should therefore contain all the soluble mitochondrial enzymes and be ready to be used for the following chromatography steps. When this spin was first carried out it was found that about 50% of the MDH activity was in the pellet. I decided that the pellet should be resuspended, resonicated and recentrifuged. The two supernatants were added together and the level of activity in this combined supernatant was about 50 - 70% of the original. This was higher than expected, but throughout this part of the purification scheme the total MDH activity increased at each step as if some inhibitor was being removed.

3.231 Use of protease inhibitor PMSF.

To prevent proteolysis of the MDH a protease inhibitor, phenylmethylsulphonylfluoride (PMSF), was added to the homogenisation buffer and to the first suspension buffer. This was considered necessary because the lysozomes would be separated in the same fraction as the mitochondria. Because PMSF is a highly toxic compound an experiment was carried out to try and determine if the addition of it was really required.

Samples of rat liver were homogenised and put through the first spin with and without PMSF. The results (Table II) showed that the samples without PMSF were about 25% less active than those with PMSF. It was therefore decided to include PMSF in all further purifications.

3.24 Ion exchange chromatography

Ion exchange chromatography as a technique is explained in more detail in section 3.11. The sonnicated mitochondrial supernatant was loaded onto a column containing CM-32 carboxymethyl cellulose resin. This was originally equilibrated at pH 5.0 with 10 mM citrate buffer. Under these conditions mMDH bound to the column resin. sMDH if present, should not bind and therefore should pass straight through the column and be found in the wash. Most of the protein in the samples loaded onto the column passed straight through. To elute the enzyme increasing the pH was tried. Firstly, pH 5.1, then pH 5.3 and pH 5.5, citrate buffers (10 mM) were passed through the column, but these eluted very little protein and no MDH activity. The next tried was

TABLE II

Effect of PMSF in purification scheme

An experiment to determine whether the protease inhibitor PMSF should be included in the initial steps of the purification procedure. Samples of rat liver were put through the first centrifugation step with and without PMSF added and then the total activity was measured.
(units are µmols / min / g wet weight of liver)

Sample	with PMSF	without PMSF	without PMSF as % of with PMSF
1	41.6	29.9	72%
2	56.3	43.5	77%

a pH 6.0, citrate buffer, with the ionic strength increased to 0.1M. This buffer was able to elute some MDH activity off the column, but no more was eluted at higher pHs or with stronger ionic strengths. As very little protein was eluted by the pH 5.1, 5.3, and 5.5 buffers it was decided that these would be left out and the pH 6.0 buffer should follow the loading buffer. A large proportion of the activity recovered was found in the pH 5.0 wash and only a small amount in the pH 6.0 buffer spread out over 2 - 3 column volumes. The total enzyme activity recovered was only about 30% and nearly 100% would be expected from this method. Whether the enzyme was still bound to the column or if a large proportion had been inactivated was unknown. All the MDH activity eluted off this column should be mMDH. The eluted enzyme was collected and concentrated by ultrafiltration. It was necessary to dialyse the sample against 20 mM phosphate buffer, pH 7.0 so that it could be loaded onto an AMP sepharose resin.

3.25 Affinity chromatography

The sample from the CM-cellulose column was dialysed and loaded onto an 5' AMP-sepharose 4B column equilibrated with 20 mM sodium phosphate buffer pH 7.0. The

technique of affinity chromatography is explained earlier (see Section 3.12). In this case AMP acts as an analogue for NADH and should bind dehydrogenase enzymes. mMDH was eluted by the addition of 40 μM NADH to the phosphate buffer after unbound protein has been washed off the column. Problems occured with the binding of the mMDH to the column as about 60% of the activity loaded was found in the unbound protein wash. The NADH eluted mMDH was found spread across a large volume (about 4x the column volume), so again the sample needed to be concentrated and then the NADH dialysed out. The specific activity of the sample that had been loaded onto the column was about 4.5 μmol / min / mg and that of the NADH eluted sample was 3 μmol / min / mg therefore this step did not provide any further purification.

This preparative method took about three days with the enzyme sample being dialysed twice before the 'purified' enzyme was produced. The combination of the above two techniques posed a number of problems, there was a large amount of activity lost and the remainder did not always separate into the expected fractions. These methods would have required a lot of refinement but this turned out to be unnecessary as a new technique was tried out and it proved to be much more satisfactory.

3.26 Affinity elution from an ion exchange resin

This technique is a combination of the two previously used and is described in more detail earlier (see Section 3.13). The combined sonicated mitochondrial supernatants (see Section 3.23) were loaded onto CM-cellulose resin equilibrated with 10 mM citrate buffer, pH 6.0. The column, loaded with sample, was washed with the same 10 mM citrate buffer, pH 6.0, until all the unbound proteins were removed (A_{280} dropped below 0.01 absorbance units). About 90% of the total protein passed through the column at this stage and only a small fraction of the MDH activity also passed through.

It has been reported that mMDH becomes extremely unstable above pH 8.0 (Davies and Scopes, 1981). Because of this it was decided to elute the mMDH from the column at pH 8.0. This was carried out by the addition of 200 μM NADH to the buffer at pH 8.0. Because citrate will not give an effective buffer at pH 8.0 it was decided to use a 0.01 M tris buffer with 0.01 M KCl added to maintain the ionic strength. These conditions are very similar to those used by Davies and Scopes (1981) for partial purification of beef heart mMDH.

The column was washed with the 0.01 M tris buffer, pH 8.0, + 0.01 M KCl and a large

amount of the bound protein was eluted, including a large red-brown band (presumably haemoglobin). The eluate appeared to take a long time to change from pH 6.0 to pH 8.0 probably because of the need to titrate some of the groups on the resin. The pH change could be followed down the column by measuring the pH of the eluate or alternately by watching the progress of the red-brown band down the column. After the A_{280} had dropped to below 0.01 absorbance units the mMDH was eluted off the column by the addition of 200 μM NADH to the 0.01 M tris buffer, pH 8.0, containing 0.01 M KCl.

3.261 Temperature stability during affinity elution

Was it necessary to carry out the chromatography steps at 4°C? Affinity elution allowed the whole chromatographic procedure to be carried out in less than 5 hours, so the temperature stability of the enzyme both at 4°C and at room temperature was tested. The purification procedure was followed to the end of the centrifugation steps and the sample divided into two parts. The volume of each was measured and one was left at room temperature and the other in the cold room at 4°C. Assays were carried out at various intervals on both samples (Table III).

Up to 20 hours there appeared to be no difference between the two samples but after three days the sample at room temperature had lost nearly 50% of its activity whereas the sample in the cold room still retained about 90% of its activity (Table III). From these results it was decided that the enzyme was stable enough for the chromatography to be carried out at room temperature. No difference in the effectiveness of the method was noticed when this was done.

3.262 Refinement of affinity elution

For these experiments it was decided to use small 10 ml columns and only one rat liver (≈ 12.5 gm), as this allowed the experiments to be completed much more rapidly.A column was prepared and 5 ml of an MDH sample, about 50 units of enzyme, was loaded.A total of just over 40 units was recovered, about a 20% loss of activity on the column. The elution profile of this column (Figure 3) shows both A_{280} measurements, for protein concentration, and MDH activity. There are three A_{280} peaks and three MDH activity peaks. The first protein peak was in the pH 6.0 wash and the second much

TABLE III

Temperature stability of mMDH during enzyme purification

To test the temperature stability of mMDH a sample was put through the centrifugation steps of the purification scheme and then part of this was left at room temperature and part in the cold room at 4°C. Both samples were assayed, at regular intervals, for MDH activity.

Time	Activity at room temperature (µmol/min/ml)	Activity in cold room (µmol/min/ml)
0	7.03	7.03
1 hr	7.03	6.21
2 hr	6.67	6.45
3 hr	7.44	7.92
19 hr	6.99	6.53
21 hr	6.75	6.13
3 days	3.75	6.33

smaller peak was found in the pH 8.0 wash. The third A_{280} peak is not due to protein, but represents the NADH that was added to the pH 8.0 buffer to elute the enzyme. These three peaks are found throughout the development of the method.

There were also three MDH activity peaks, two of which are in the pH 6.0 wash, the first in the major protein peak and the second, retarded peak, following after the protein. The third is in the NADH-containing elution buffer and it is quite sharp and right at the point in which the NADH comes through the column. The activity collected off the column was split, with 16 units in the first pH 6.0 wash peak, 22 units in the retarded pH 6.0 wash peak and only 4 units of MDH activity in the NADH elution peak. The ideal elution profile would have only one or two activity peaks, one of purified mMDH

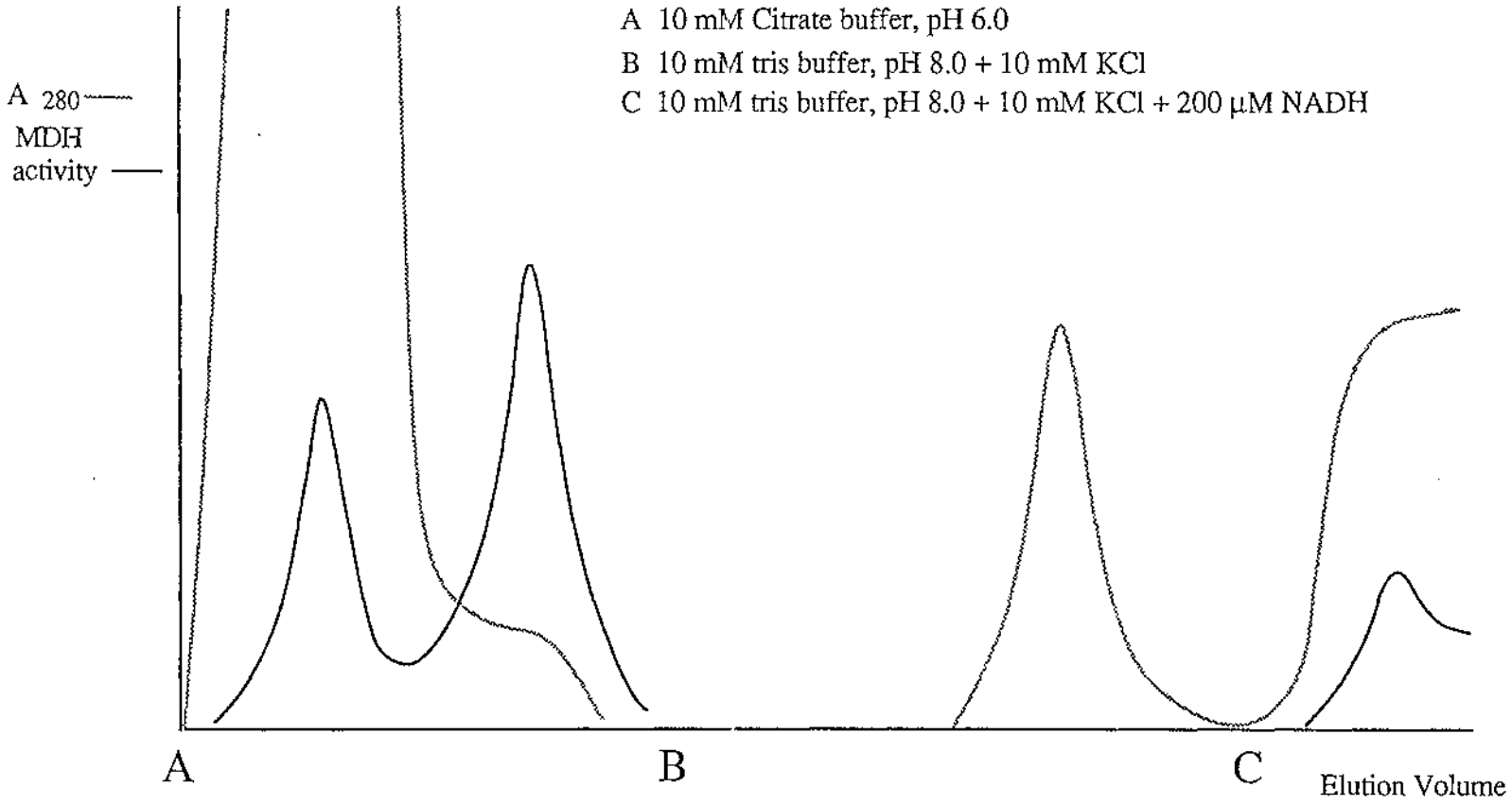


Figure 3

Elution profile of affinity elution with citrate and tris/HCl buffers showing three protein peaks, one in each buffer and three MDH activity peaks, two in the pH 6.0 wash and one in the NADH elution buffer. The point at which new buffer is first loaded is marked A, B, or C.

in the NADH containing elution buffer and possibly a second peak of sMDH, which does not bind to the column and is therefore found in the pH 6.0 wash, with the first protein peak. The NADH elution peak was not large enough to account for all the expected mMDH activity and the first pH 6.0 peak could be identified as sMDH by isoelectric focusing (see Section 3.282). The problem was with the retarded pH 6.0 peak, what was it and why was it there? Possible answers were:

1 The resin had limited capacity and was therefore being overloaded. mMDH is the retarded pH 6.0 peak.
2 The binding conditions (pH, ionic strength etc.) were not quite correct causing overloading.
3 The sample was too crude, with the mMDH binding to compounds in the solution rather than to the resin, by either specific or nonspecific binding.
4 The retarded peak was a third unknown isozyme.

A series of experiments were designed to answer these questions. Firstly I assumed that there was no enzymatic or structural difference between the enzyme in the retarded and the NADH elution peaks (i.e. they were not different isozymes). The first thing looked at to try and identify the peaks was the loading of the sample. A smaller volume, 2 ml of solution, was loaded onto a column and the affinity elution process carried out. The elution profile showed an increased proportion of activity in the NADH elution peak and a corresponding decrease in the pH 6.0 retarded peak, while the pH 6.0 wash peak was unchanged. This suggested that, maybe the column was being over loaded. In this case 100% of the activity loaded was recovered. Another column was run with the smaller volume of sample loaded but this time it was diluted to 2 in 5 before loading. This showed a further increase in the proportion of the NADH elution peak with a corresponding decrease in the pH 6.0 retarded peak. Once again 100% of the activity loaded was recovered.

The next set of experiments carried out involved taking the enzymatically active fractions for each of the three peaks and rerunning each through the affinity elution method. The pH 6.0 wash peak passed straight through the column and all the activity was regained. The NADH elution peak (after dialysis to remove NADH) all bound to the column and was eluted in the pH 8.0 buffer, containing NADH. Lastly, the pH 6.0 retarded peak when put back through the affinity elution technique gave two activity peaks, one in the pH 6.0 retarded position and the other in the NADH elution peak position. Because of the much lower protein concentrations in this solution the chances

of nonspecific binding preventing binding of mMDH to the column are reduced. From these experiments it seems that the pH 6.0 retarded peak was mMDH that had not bound to the column because of a limited capacity for that particular enzyme but was retarded because of slight interactions with the column. By adjusting the conditions it seemed that more mMDH could be made to bind to the column.

3.263 Buffer types used for affinity elution

Citrate buffers were used at first because they work effectively in the range pH 5.0 - 6.0 which was the range required for the ion exchange chromatography method. Phosphate at pH 7.0 was used for the affinity chromatography step. When the method was changed to affinity elution chromatography the pH range was also changed, it was now pH 6.0 - 8.0. A new buffer was required for pH 8.0 and tris (plus KCl to keep up the ionic strength) was chosen as this was similar to the buffer used in a similar preparative method (Davies and Scopes, 1981). I later decided to use phosphate buffers for all pH's, as both pH 6.0 and pH 8.0 are within the effective buffering range of phosphate buffers. This change was supported by data published (Mullinax *et al.*, 1982) which suggested that citrate binds to an allosteric site on mMDH and affects the monomer-dimer equilibrium, the protein's conformation and enzymatic activity. When the change was made to phosphate buffers the effects were immediately obvious on looking at the elution profiles.

The change appeared to be extremely effective as the elution profile (Figure 4) showed the pH 6.0 retarded peak had completely disappeared. A small peak (less than 5% of activity through the column) had appeared in the pH 8.0 wash. At least 90% of the bound activity was in the NADH elution peak. Also with the phosphate buffer the capacity of the column for mMDH appeared to increase enormously.

It seems therefore that the citrate was inhibiting the binding of the enzyme to the column. This was possibly due to the extra negative charges of the citrate changing the overall ionic charge on the enzyme. This explains the lower apparent binding capacity and the reason for the pH 6.0, retarded peak with the citrate buffer. The small pH 8.0 wash peak found with the phosphate buffers is due to the conditions being very close to those at which mMDH would be eluted by ionic strength or pH. To try to remove this small peak it was necessary too look at the ionic strength of the buffers.

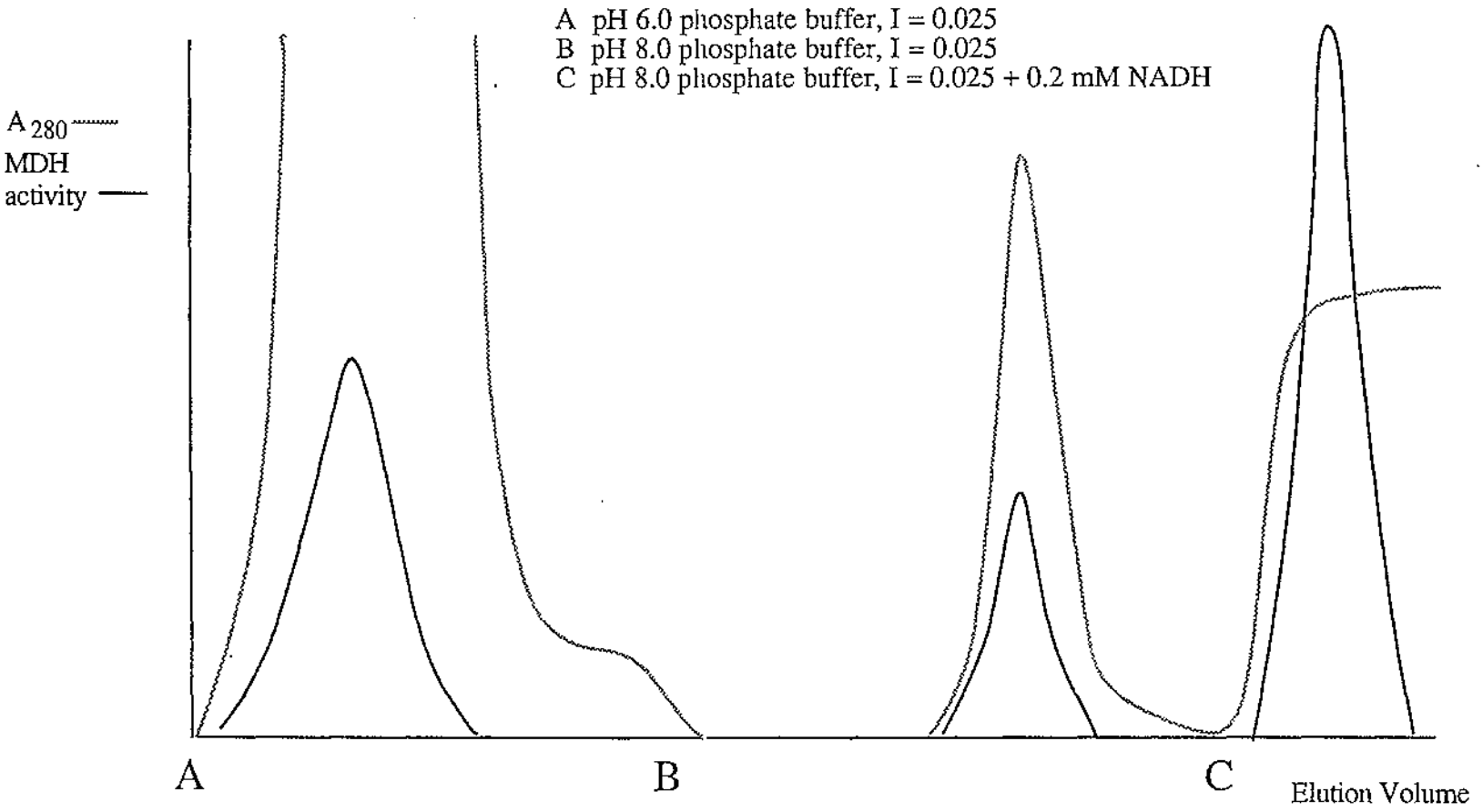


Figure 4

Elution profile of affinity elution with phosphate buffers, I = 0.025: Showing three protein peaks and three MDH activity peaks, one in each buffer. The point at which a buffer is first loaded onto the column is marked A, B, or C

3.264 Ionic strength

With the change to phosphate buffers the change was also made to constant ionic strength buffers (Long, 1961).With normal constant molarity buffers the ionic strength of di or tri protic buffers is difficult to calculate and for chromatography ionic strength is more important than molarity. The first phosphate buffers used were of ionic strength (I) 0.025 (Figure 4). This appeared to be adequate until I tried to scale up the method to four rat livers (≈ 50 gm) and a 70 ml column. The results were similar to the smaller columns except that the pH 8.0 wash peak had increased to about 25% of the bound MDH. A preparation with a lower ionic strength buffer (I = 0.020) was tried. This was more effective and reduced the pH 8.0 wash peak down to less than 5% of the bound enzyme (Figure 5). The reduction in ionic strength also had an effect on the NADH elution peak. This peak had divided into two new peaks and was spread out over a much larger volume. Both peaks had a similar specific activity, and the differences could be caused by phospholipid binding (Jones and Vestling, 1979). The duration of this project did not provide enough time to look into the differences between these two peaks and so the activity was pooled for further study (kinetics). However, it is clear that ionic strength does have an effect on the elution of mMDH from a CM-cellulose resin by NADH.

3.27 Ultrafiltration

Ultrafiltration was used to reduce the volume of the enzyme sample by forcing the small solvent molecules through a membrane and retaining the protein molecules in a smaller volume of solvent. The first efforts with this process had a loss of up to 40% of the enzyme activity. The membrane (PB 30) was designed to pass globular proteins of molecular weight less than 30,000 daltons. Rat liver mMDH has a molecular weight of about 67,000 daltons, but is made up of two identical subunits with a molecular weight of just over 33,000 daltons each. These may be small enough to pass through the membrane if the enzyme has dissociated, but very little activity was found in the wash. This was possibly because the monomer has very little activity or because the enzyme loss was due to protein adsorbing to the membrane or the ultrafiltration cell. In later trials with previously used membranes it was found that recovery approached 100%. It seemed as though the enzyme had filled the possible adsorbtion sites on the membrane in the earlier run. After the enzyme has been concentrated it must next be dialysed (see Section 2.27) to remove the NADH, because this would interfere with any kinetic studies.

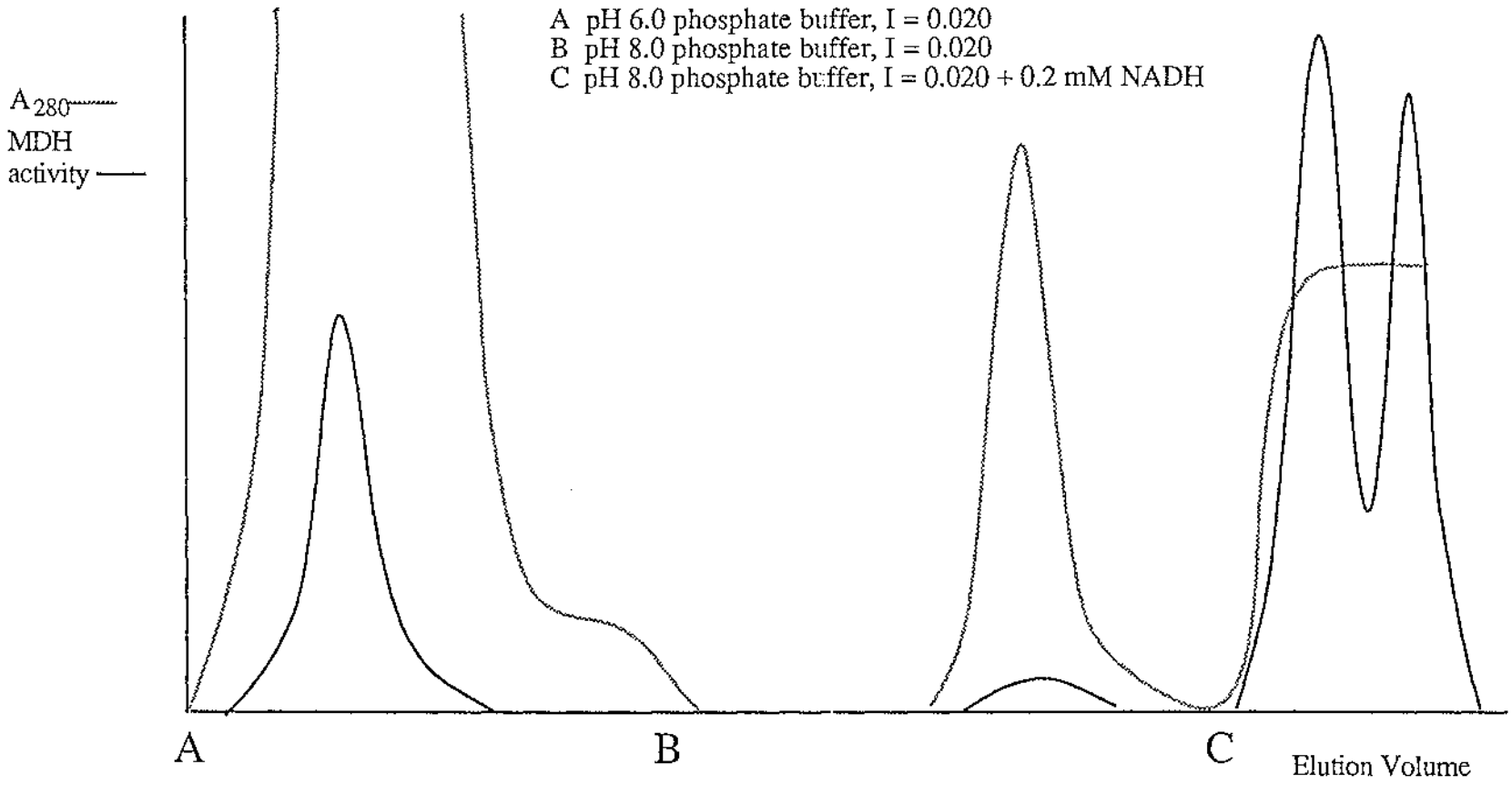


Figure 5

Elution profile of affinity elution with phosphate buffers, I = 0.020: Showing three protein peaks and four MDH activity peaks, one in each buffer, but the pH 8.0 wash peak has almost disappeared and the NADH elution peak has split into two. A, B, and C are the points at which each buffer is first loaded onto the column.

3.28 Enzyme purity

3.281 Specific activity

Specific activity can be used as a measure of the purity of an enzyme, but only when compared to values published, by other people, for the same enzyme. This is not always possible because of the wide variety of conditions, units, and methods used in determining these values. In about fifteen papers reporting methods used to purify mMDH from various sources only two have identical methods for determining specific activity and none used the same method as the one in this work. The conditions used in this work were the same as used in the purification of rat liver sMDH (Crow *et al.*, 1982).

Specific activity is best used to look at the increase in purity throughout the purification method itself. These values are shown in Table IV. A large increase in specific activity is observed during the affinity elution step, approximately 250 fold. The increase in specific activity throughout the purification method can be used as another way to compare different published methods, though it is hard to say how easily comparable the levels of enzyme will be *in vivo* from different tissue sources. When looked at in both these ways the enzyme produced by this purification method appears quite pure. Only one method reported an increase in specific activity that was greater than the 570x (Table IV) shown by this method and most were of the order of 40x. A better idea of enzyme purity can be gained by looking at electrophoresis gels.

3.282 Isoelectric focusing

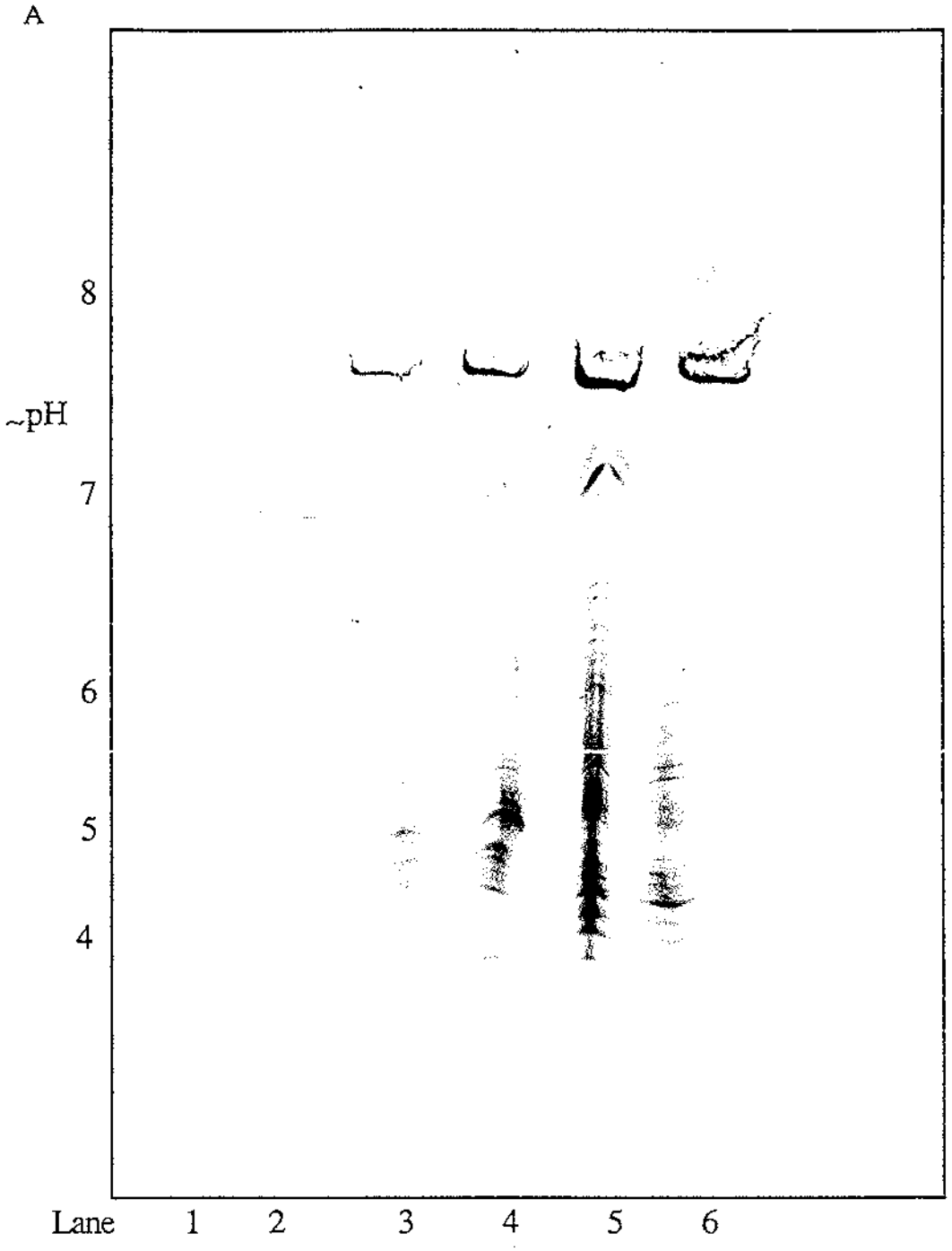
One of the major differences between cytosolic and mitochondrial MDH is the isoelectric point. This can be visualised using an isoelectric focusing system, in which proteins are separated across a pH gradient according to their isoelectric points. sMDH has an isoelectric point of about 5.5 and mMDH has an isoelectric point of between 8.9 and 9.6 (Jones and Vestling, 1978). This system can also be used to follow the separation of proteins throughout the purification scheme, as the gels can be stained for either MDH activity or proteins (Figures 6A and B). If the protein is pure there should be a single protein band visible that should match with an identical activity band on another gel of the same sample. In Figure 6A protein staining of fractions from the purification method can be seen. Lanes two and three show a separation of the protein bands with those of lower isoelectric points being in the pH 6.0 wash (lane 3) and those

TABLE IV

Purification of mitochondrial malate dehydrogenase from rat liver

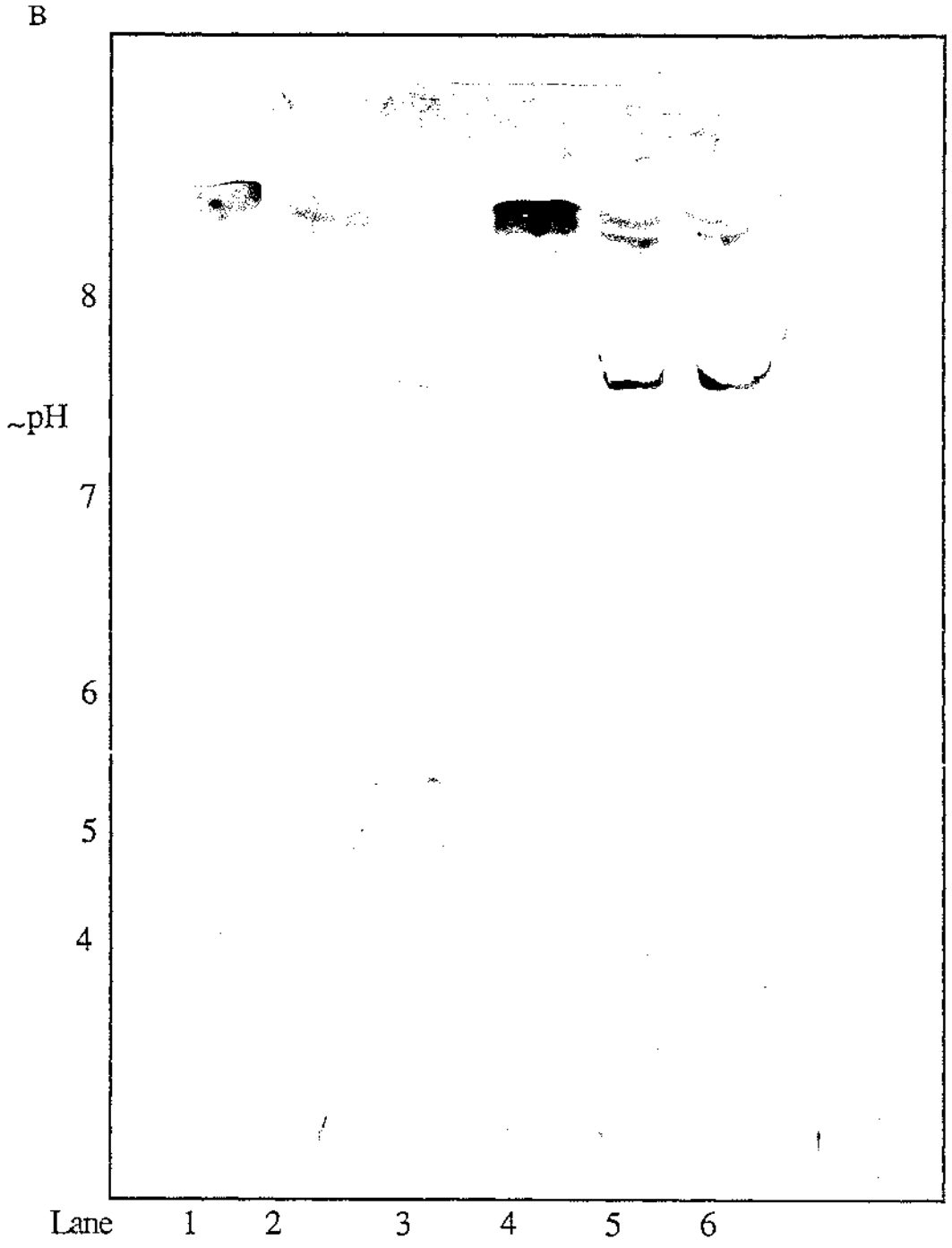
Mitochondrial malate dehydrogenase was purified from rat liver. The total activity was determined and the total protein was determined by the Coomassie blue method. 1 unit of activity = 1 μM NADH produced per minute.

Fraction	Total Activity (units)	Recovery (%)	Total Protein (mg)	Specific Activity (units/mg)	X Purification
Total Homogenate	1316.0	100	11020	0.12	1
500xg Spin Precipitate	205.3	15.6	1780	0.12	1
500xg Spin Supernatant	1210.2	92.0	9600	0.13	1.1
30 min Spin Precipitate	656.8	49.9	2790	0.24	2
30 min Spin Supernatant	508.5	38.3	5900	0.09	0.75
60 min Spin Precipitate	439.0	33.4	1270	0.35	2.9
60 min Spin Supernatant	321.5	24.4	970	0.33	2.8
pH 6.0 Wash Peak Fraction	15.6	-	247	0.06	0.5
NADH Elution Peak	427.0	32.4	6.3	67.78	565



Lane

1 NADH elution peak
2 pH 8.0 wash peak
3 pH 6.0 wash peak
4 sample loaded onto CM- cellulose
5 30 min spin supernatant
6 Total homogenate



Lane

1 NADH elution peak
2 pH 8.0 wash peak
3 pH 6.0 wash peak
4 sample loaded onto CM- cellulose
5 30 min spin supernatant
6 Total homogenate

Figure 6

Broad range (pH 3.5 - 9.5)
isoelectric focusing gels

A Coomassie blue stained protein stain of gel. The separation of unwanted proteins by affinity elution shows up clearly in lanes 2, 3, and 4 with lane 1, having no visible protein band, being the purified enzyme sample.

B MDH activity stain of gel. mMDH shows up clearly at the top of the gel but appears to be split into more than one band. sMDH is visible in lanes 3, 5, and 6 but is much less clear

with higher isoelectric points being in the pH 8.0 wash (lane 2). Between them these two lanes contain most of the protein bands found in the sample loaded onto the column (lane 4). A broad range, pH 3.5 - 9.5, system was used to cover both isozymes, but this system has limitations when reaching the higher pH's and so an accurate value for the isoelectric point could not be determined. Protein staining of the NADH eluted enzyme (lane 1) did not show up any bands. Almost all of the protein bands loaded onto the column are found in lanes 2 and 3 but enough protein was loaded so that any bands should show up. This suggests the sample was quite pure.

Figure 6B shows activity staining of a gel that had the same samples loaded as the gel in Figure 6A. In lane 1 the MDH shows up clearly as a single wide, or possibly double, band. mMDH stains right at the top of these gels around pH 9 and sMDH shows up faintly in lanes 3, 5 and 6, at about pH 5.5. A number of different isoelectric bands for mMDH have been reported, between pH 8.9 - 9.6 (Jones and Vestling, 1978), probably caused by phospholipid binding. The mMDH shows clearly as two bands in lanes 5 and 6. A high pH range (maybe pH 8 -10) isoelectric focusing system would probably have given better separation of these bands, but this was not availiable.

3.283 SDS gel electrophoresis

As no protein band was visible for the pure mMDH, on the isoelectric focusing gels, another method was required to show purity. A sodium dodecyl sulphate (SDS) poly acrylamide gel electrophoresis showed a single protein band (Figure 7) when stained with Coomassie blue. A good match is observed between this band and the mMDH molecular weight standard from pig heart. An activity stain can not be shown, as SDS is a protein denaturing reagent. This, with the evidence from the specific activity and isoelectric focusing, suggests that the enzyme is a single protein species.

3.29 Stability of mMDH on freezing

The purified sample obtained now needed to be stored so that it could be used when required. One of the most common ways of storing enzymes is by freezing and this was used as mMDH is not stable for long periods in aqueous solution. In test samples about 30% of activity was lost when frozen for one week at pH 8.0. mMDH stored for assays appeared to be more stable and retained most of their activity for over two months. The stability was unaffected by the addition of glycerol or the presence or absence of NADH. The process of thawing and refreezing caused a loss of over 70% of remaining

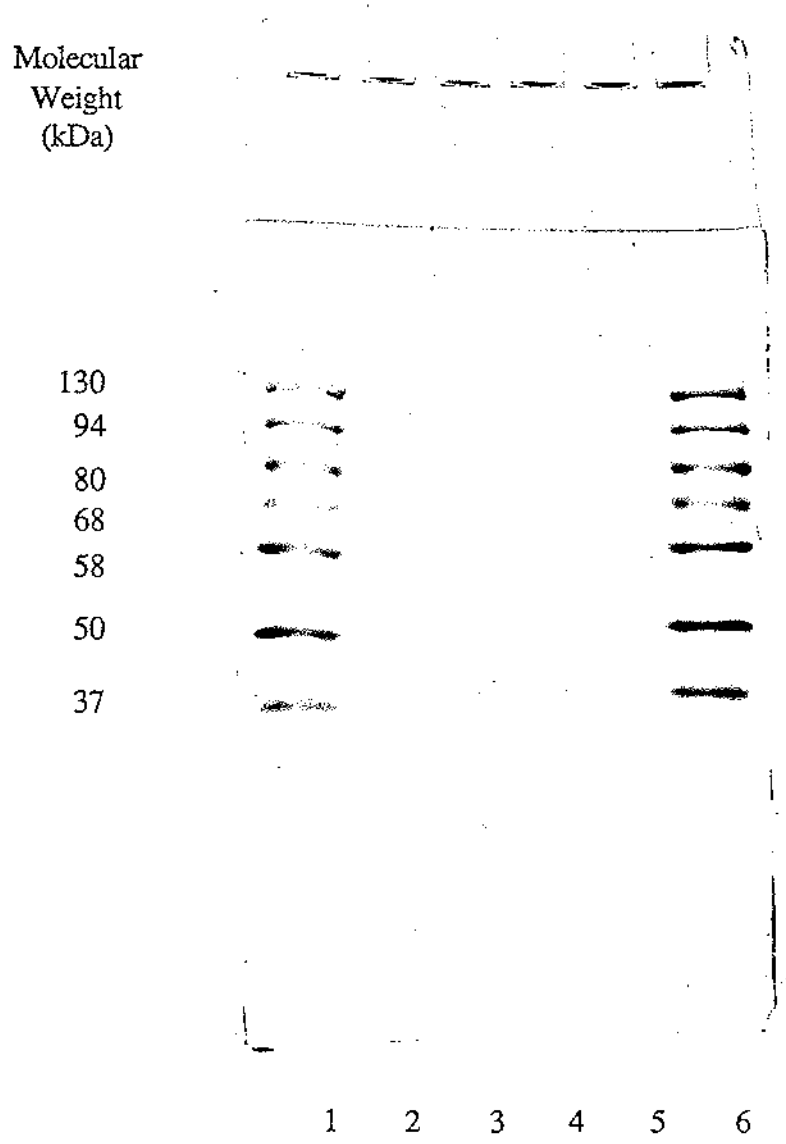


Figure 7

SDS gel electrophoresis of purified mitochondrial malate dehydrogenase.

Wells 1,6: molecular weight markers;
β- galactosidase, 130 kDa; Phosphorylase b, 94 kDa;
Transferrin, 80 kDa; Bovine serum albumin, 68 kDa;
Catalase, 58 kDa; Citrate synthase, 50 kDa, Malate dehydrogenase, 37 kDa.
Wells 2-5: purified rat liver malate dehydrogenase at increasing concentrations.

I would like to thank Ms Kathryn Stowell for running this gel

enzyme activity and so it was therefore necessary to freeze the enzyme in large numbers of individual small fractions of which a new one could be used as required. The effect of pH on the enzyme stability should be looked at and more work could be put into finding conditions at which the enzyme is stable for storage.

3.3 CONCLUSION

The method of purification developed, in this work, for rat liver mitochondrial malate dehydrogenase is one that accomplishes the aims of this section of the project. It is quick and can be completed in less than two days. There is no use of large quantities of expensive chemicals, and the techniques used should cause as little disruption as possible to the tertiary and quarternary structures of the protein. The initial idea, purification scheme I (Figure 2) was developed into the final product, purification scheme II (see Section 3.31 and Figure 8). Problems have been encountered with the storage of the enzyme and with measurements of enzyme purity in the final method, but the enzyme does appear to be of high specific activity and free of contaminating proteins.

Affinity elution chromatography is a technique which in this case is extremely effective. It has advantages over the combination of ion exchange and affinity chromatographies.

1 One dialysis step and one column step have been removed giving a significant time saving and reducing the number of times the enzyme must change environments.

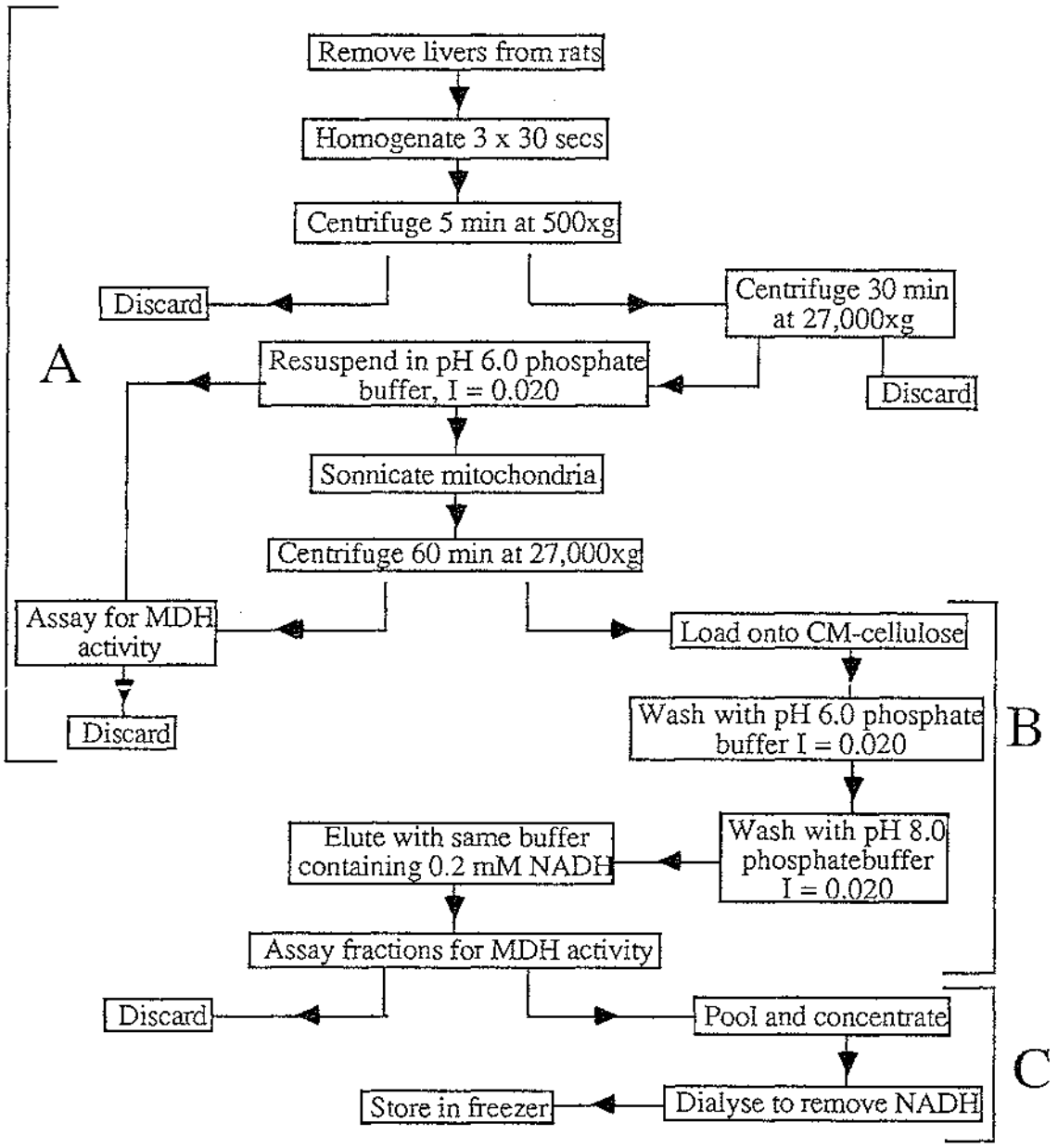
2 The enzyme is eluted in a smaller volume meaning less concentration is required.

3 A significantly greater factor of purification is achieved.

This last is partly due to the high isoelectric point of the enzyme which means the conditions can be adjusted so that very little else is binding to the column. With a more neutral enzyme there would be more chance of having contaminating enzymes co-eluting, meaning extra purification steps would be required. A more specific substrate may have allowed simplier elution from the column, but all in all this is an extremely effective technique.

3.31 Purification scheme II (see Figure 8)

The livers are removed from freshly killed rats and immediately placed on ice. They are cut into smaller pieces with scissors and then homogenised in four volumes of chilled homogenisation buffer (see methods) for three 30 second bursts. A sample of the total homogenate is taken for assaying. It is then centrifuged at 500xg and 4°C for 5 minutes. A sample of both the supernatant and the resuspended pellet are kept for assaying. The resuspended pellet can be discarded. The supernatant is centrifuged at 27,000xg and 4°C for 30 minutes, then a sample of the supernatant is removed for assaying and the rest is discarded. The pellet is resuspended in chilled pH 6.0 phosphate buffer, I = 0.020, + 1mM PMSF. This suspension is sonicated three times



A All steps were carried out with the sample kept on ice
B All steps carried out at room temperature
C Carried out in cold room at 4°C

Figure 8

Purification Scheme II: The final purification method developed in this work

for 30 seconds at maximum power, and kept on ice for 1 minute between each sonication. A sample of this extract is retained for assaying. The extract is centrifuged at 27,000xg and 4°C for 60 minutes and samples of both the supernatant and the resuspended pellet are taken for assaying. The resuspended pellet is then resonicated and recentrifuged. A sample of both the supernatant and the pellet is taken for assaying and then the pellet is discarded. The two supernatants are now pooled.

CM-cellulose resin is equilibrated at pH 6.0 with phosphate buffer, I = 0.020. This is carried out at room temperature (approximately 25°C) and all the subsequent chromatography occurs at room temperature. The pooled supernatants from above are loaded onto the column. The column is then washed with pH 6.0, phosphate buffer, I = 0.020 until the A_{280} drops below 0.01 absorbance units. 20 ml fractions are collected off the column. The buffer is changed to pH 8.0, phosphate buffer, I = 0.020 and the column is washed until the A_{280} drops below 0.01 absorbance units. When this has occured 200 μM NADH is added to the buffer and mMDH is eluted. All the fractions are assayed and those fractions from the NADH eluate with the highest activity are pooled. These fractions are concentrated by ultrafiltration and then dialysed to remove the NADH. The final product is stored in the freezer. All buffers contain 1mM DTT, except that during ultrafiltration the concentration is increased to 10 mM to account for the increase in protein concentration and this larger amount is added to the dialysis buffers.

CHAPTER 4

STEADY STATE KINETIC STUDIES

4.1 INTRODUCTION

Kinetic studies have been carried out on 'purified' mMDH since the early 1960's. These studies have used various tissues, with porcine heart being the most common. Generally heart tissues have the highest levels of mMDH activity and so are prefered as a source of enzyme, but in this work rat liver is used as the source of mMDH for reasons given earlier (Chapter 1). In the past steady state kinetic studies have been carried out at varying pH's and temperatures. This is because mMDH has its maximum activity for malate oxidation at around pH 10, and for oxaloacetate reduction at about pH 7. Various studies have been carried out at these pH values and at intermediate pH values, which simplify the experimental methods, but it is unlikely that the higher pH values have physiological significance and so in this work the studies are all carried out at pH 7.4. Other studies have used 25°C, 37°C and a number of intermediate temperatures at which to carry out their work, but in this work 37°C has been used, as this temperature is the closest to physiological.

From early studies the mechanism for mMDH has been described as a compulsory ordered mechanism (Raval and Wolfe, 1962; Raval and Wolfe, 1963; Harada and Wolfe, 1968a; Fahien and Strmecki, 1969; Silverstein and Sulebele, 1969) in which the coenzyme, NAD^+ or NADH depending on the direction of activity, binds first followed by the substrate, malate or oxaloacetate respectively, with the products being released in the reversed order. This is a common mechanism for NAD^+ linked dehydrogenases. When refering to these substrates it is often useful to give each of the substrates a letter for the order in which they bind to the enzyme. A and B for the substrates in one direction, with A binding first, and P and Q for the other direction, with Q binding first. The reaction scheme is shown in equation 4.

$$E + A \underset{k_{-1}}{\overset{k_1}{<==>}} EA + B \underset{k_{-2}}{\overset{k_2}{<==>}} (EAB \underset{k_{-p}}{\overset{k_p}{<==>}} EPQ) \underset{k_{-3}}{\overset{k_3}{<==>}} EQ + P \underset{k_{-4}}{\overset{k_4}{<==>}} E + Q \quad \text{(equation 4)}$$

Where k_1, k_2, k_p, k_3, and k_4 are the rate constants in the forward direction and k_{-1}, k_{-2}, k_{-p}, k_{-3}, and k_{-4} are the rate constants in the reverse direction.

In this work, A = NADH, B = oxaloacetate, P = malate, and Q = NAD^+, have been used. The kinetic data are really only useful if there is only one form of the enzyme in the pure sample. Because mMDH has to carry out more than one function in the mitochondria, as part of the malate-aspartate shuttle and also as part of the TCA cycle, more than one isozyme is possible, but only one form has so far been identified.

Each enzyme dimer binds two molecules of coenzyme, one per subunit (Kitto and Kaplan, 1966; Holbrook and Wolfe, 1972) and though individual subunits are largely inactive, under certain circumstances they can be made active (DuVal *et al.*, 1985), showing that each subunit contains an active site at whch the reation can occur. It has been suggested that there is an active and an inactive conformation and although the active conformation favours dimer formation and the inactive favours monomer formation, the interaction between subunits appears to have little affect on catalytic activity (McEvily *et al.*, 1985b).

Kinetic studies of mMDH show a range of behaviour not normally associated with normal Michaelis-Menten kinetics.Substrate inhibition, when enzyme activity is lower than expected at higher substrate concentrations, has been shown to occur with oxaloacetate (Raval and Wolfe, 1963; Bernstein *et al.*, 1978). The mechanism proposed to account for this inhibition is the formation of an abortive binary complex between the enzyme and the *enol* form of oxaloacetate (Bernstein *et al.*, 1978). Earlier an abortive enzyme-NAD^+-oxaloacetate complex was suggested as the cause of the substrate inhibition (Harada and Wolfe, 1968b), but this has never been isolated. Evidence for an unreactive complex of enzyme-NADH-malate has been reported (Silverstein and Sulebele, 1969) but it is unknown if this has any physiological significance. The inhibition constant for oxaloacetate is about 2.0 mM (Bernstein *et al.*, 1978) and the free oxaloacetate concentration in the mitochondria is very low, of the order of 1 μM (Siess *et al.*, 1976), although it has been reported even as low as 40 nM (Srere, 1972). This suggests that the substrate inhibition may not be of any significance *in vivo*.

Substrate activation, when enzyme activity is higher than expected, is found with malate (Telegdi *et al.*, 1973) at concentrations between 0.03 and 0.2 M. Similar activation has been shown to occur with citrate. Citrate inhibits the reaction in the NADH ------> NAD^+ direction (Cennamo *et al.*, 1967; Mullinax *et al.*, 1982). It is proposed that citrate binds at a regulatory site, that is distinct from the catalytic site, on each subunit. If malate and oxaloacetate also bind at this site independently, even if much less tightly, this could explain the substrate activation and even the substrate inhibiton. The model suggested has the regulatory site availiable only on an enzyme conformation that can

bind NAD^+. Other divalent ions, such as phosphate and sulphate, also activate the enzyme when NAD^+ and malate are the substrates (Telegdi *et al.*, 1973).

The enzyme is also inhibited by ATP, ADP, and AMP with enzyme-inhibitor and enzyme-NAD^+-inhibitor abortive complexes being formed (Casadó *et al.*, 1979; Bush, 1985). The last major group of compounds that affect mMDH are the fatty acyl CoA salts. Acetate and volatile fatty acyl CoA salts inhibited NAD^+ reduction by bovine mMDH's (Bush, 1985). Palmitoyl CoA and longer chain fatty acyl CoAs inhibit oxaloacetate reduction in mMDH from porcine heart (Kawaguchi and Bloch, 1976). The reverse direction is much less sensitive but cytosolic enzyme from the same tissue is inhibited in both directions.

The cationic surfactant, cetyl trimethyl ammonium bromide, does not affect the activity of mMDH at concentrations that completely inhibit sMDH (Smith and Sundarum, 1986). The reverse shows for the anionic surfactant, sodium dodecyl sulphate, with mMDH being more sensitive than sMDH. NADH makes the enzymes more resistant, but increasing the ionic strength makes mMDH more resistant and sMDH less. Both enzymes show considerable resistance to the non-ionic detergent Triton X-100.

The present work was designed to give steady state kinetic data so that the two isozymes of MDH could be compared, and so that the differences in the direction of operation of sMDH and mMDH required for the functioning of the malate-aspartate shuttle *in vivo* could be explained.

4.2 MANIPULATION OF STEADY STATE KINETIC DATA

4.21 Manual plotting of data

Data from a 6x6 set of assays, six concentrations of each substrate, for the reaction in the absence of products, was plotted as the reciprocal of the reaction rates against the reciprocal of the concentration of one of the substrates (Lineweaver and Burk, 1934; Segel, 1975), for each substrate. This gave a primary plot with six lines, one for each of the second substrate's concentrations, that all intersected at the same point. The slope and the y-intercepts of each of these lines were measured, and these were both plotted against the reciprocal of the second substrate's concentrations. If the primary plot was 1 / v versus 1 / [A] then the secondary plots were made against 1 / [B]. The initial estimates for the parameters required were calculated from these secondary plots (Equations 5 and 6). From the plot of y-intercept versus 1 / [B], the x-intercept = $-1 / K_b$, the y-intercept = $1 / V_{max}$ and the slope = K_b / V_{max}. From the plot of slope versus 1 / [B] the x-intercept = $-K_a / K_{ia}K_b$, the y-intercept = K_a / V_{max} and the slope = $K_{ia}K_b / V_{max}$. Plotting the other substrate first gives gives a secondary plot of y-intercept versus 1 / [A] for which the slope = K_a / V_{max}, the x-intercept = $-1 / K_a$, and the y-intercept = $1 / V_{max}$. The secondary plot of slope versus 1 / [A] has a slope = K_bK_{ia} / V_{max}, the x-intercept = $-1 / K_{ia}$, and the y-intercept = K_b / V_{max}. The values gained from these plots are used as initial estimates for the computer fitting programs. After the first constants were found to be similar to those for sMDH (Crow *et al.*, 1982), the sMDH constants were used as initial estimates for the non-linear least-squares curve-fitting program.

The data from the product inhibition assays could be treated in the same way, with the plotting of primary and secondary plots. The slopes of the lines in the primary inhibition plots can give an indication as to the type of inhibition. All the lines should in principle cross at the same point; if this point is on the y-axis then the inhibition is competitive, if they cross to the left of the y-axis then it is noncompetitive, using the nomenclature of Cleland (1970) or mixed type competitive using the nomenclature of Segel (1975) and if the lines are parallel then the inhibition is uncompetitive.

4.22 Computer fitting of data (Crow *et al.*, 1982)

Initial velocities for the mMDH reation in both directions were determined in the

presence of varying concentrations of substrates and products. Data were fitted to velocity equations (Cleland, 1970; Plapp, 1970) for an ordered bi-bi system (Segel, 1975) using a non-linear least-squares curve-fitting program (Daniel and Wood, 1971). Initial estimates of parameters were obtained from manual reciprocal plots and replots (see Section 4.21).

The velocity equations were as follows:

(1) Oxaloacetate reduction in the absence of products;

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \qquad \text{(Equation 5)}$$

where v = observed velocity, V = maximum velocity, A = NADH, B = oxaloacetate, K_a = Michaelis constant for A, K_b = Michaelis constant for B, and K_{ia} = dissociation constant for A.

(2) Malate oxidation in the absence of products;

$$v = \frac{VPQ}{K_{iq}K_p + K_pQ + K_qP + QP} \qquad \text{(Equation 6)}$$

where P = malate, Q = NAD^+, K_p = Michaelis constant for P, K_q = Michaelis constant for Q, and K_{iq} = dissociation constant for Q.

(3) Linear competitive inhibition of oxaloacetate reduction by NAD^+, or malate oxidation by NADH.

$$v = \frac{VS}{K(1 + I/K_{is}) + S} \qquad \text{(Equation 7)}$$

where $K = K_{a\ (apparent)}$ and $K_{is} = K_{iq}$ for S = NADH and I = NAD^+,
or $K = K_{q\ (apparent)}$ and $K_{is} = K_{ia}$ for S = NAD^+ and I = NADH.

(4) Linear noncompetitive inhibition, according to Cleland and mixed according to Segel, of oxaloacetate reduction by malate, or malate oxidation by oxaloacetate.

$$v = \frac{VS}{K\,(1 + I / K_{is}) + S\,(1 + I / K_{ii})} \qquad \text{(Equation 8)}$$

where $K = K_{b\,(apparent)}$, $K_{is} = K_{iq}K_p / K_q$ and $K_{ii} = K_{ip\,(apparent)}$ for S = oxaloacetate and I = malate;

or $K = K_{p\,(apparent)}$, $K_{is} = K_{ia}K_b / K_a$ and $K_{ii} = K_{ib\,(apparent)}$ for S = malate and I = oxaloacetate.

This equation could also be considered the basic inhibition equation of which the other equations are only specialised subsets. The competitive equation is only missing the 1 / Kii term which has been reduced to zero.

4.23 Correction of constants for unsaturating substrates

Since the nonvaried substrates were not saturating the apparent values were corrected with the following equations (Plapp, 1970);

$$K_a = K_{a(apparent)}\,(1 + K_b / B) - K_{ia}K_b / B; \qquad \text{(Equation 9)}$$

$$K_b = K_{b(apparent)}\,(1 + K_a / A) / (1 + K_{ia} / A); \qquad \text{(Equation 10)}$$

$$K_q = K_{q(apparent)}\,(1 + K_p / P) - K_{iq}K_p / P; \qquad \text{(Equation 11)}$$

$$K_p = K_{p(apparent)}\,(1 + K_q / Q) / (1 + K_{iq} / Q); \qquad \text{(Equation 12)}$$

$$K_{ib} = K_{ii} / (1 + K_q / Q); \qquad \text{(Equation 13)}$$

$$K_{ip} = K_{ii} / (1 + K_a / A); \qquad \text{(Equation 14)}$$

using the values determined in the absence of inhibitors.

4.24 Design of kinetic experiments

For the reaction in the absence of products, data were gained from a 6x6 set of assays. Six concentrations of each substrate were chosen and the rate of reaction at each concentration of one substrate was assayed at each of the concentrations of the other substrate, giving a total of 36 assays for one experiment. The initial slope of each assay was measured and and this was converted into a reaction rate (v). From these values the desired kinetic constants could be determined. The optimum set of concentrations for each substrate was one that included both the K_m and K_i values for that particular substrate within its range. If not, the concentrations should be changed so that the K_m and K_i are within the range. This is not always possible and in any such case values as close as can be achieved should be used. These experiments were carried out for both directions in which the enzyme works. It was important that all these assays were carried out in at least duplicate, or if possible triplicate, and that the whole experiment was repeated enough times to give statistically accurate values for the constants.

Values for the constants K_{ib} and K_{ip} can not be obtained from the data produced by the reaction in the absence of products, so further experiments were carried out in which the reaction proceeded in the presence of one of the products, which acted as an inhibitor. During these experiments only one of the substrates was varied and the reaction rate was measured at varying concentrations of the product inhibitor. Four of these experiments were necessary to gain the required data;

1 Variable [NAD^+] as substrate, with NADH as inhibitor and [malate] constant and unsaturating.
2 Variable [malate] as substrate, with oxaloacetate as inhibitor and [NAD^+] constant and unsaturating.
3 Variable [NADH] as substrate, with NAD^+ as inhibitor and [oxaloacetate] constant and unsaturating.
4 Variable [oxaloacetate] as substrate, with malate as inhibitor and [NADH] constant and unsaturating.

Only three inhibitor concentrations were used, but with a set of assays without inhibitor these experiments entailed 24 different assays and was expected to give enough data to determine the values required.

4.3 PRELIMINARY STEADY STATE KINETICS

4.31 Determination of approximate Michaelis constants

From steady state initial rate kinetic experiments values for the K_m (Michaelis constant) and K_i (substrate dissociation constant) for any substrate can be determined. These are the values that this work has attempted to determine for rat liver malate dehydrogenase.

The first step in the kinetic analysis was to find an approximate value for the K_m of each of the substrates. These values were used as a starting point for the choosing of the concentrations for the 6x6 assays (Section 4.21). We decided to try to calculate approximate values for malate and NAD^+, as substrates, first, as this was the direction in which all the preliminary assays had been carried out. Concentration ranges chosen for malate were from 5 mM down to 50 μM, and for NAD^+ were from 1 mM down to 10 μM. The assay was carried out for each range of concentrations with a saturating concentration of the other substrate; for malate this was 60 mM and for NAD^+ it was 2 mM. The reaction rates for the lower malate concentrations were very hard to measure, because of the low activity. The K_m appeared to be near the top end of the range and therefore a new set of concentrations was used, between 20 mM and 200 μM (Figures 9 and 10). When reciprocal values of the initial rates were plotted versus the reciprocal of the variable substrate concentration the straight line produced would cross the x-axis at $-1 / K_m$. This approximate K_m is also only an apparent K_m because variation in the concentration of the other substrate can affect its value. If it follows true Michaelis-Menten kinetics the curve of a velocity versus substrate concentration plot should show an initial steep, apparently linear slope that later curves off until it is horizontal with the x-axis. This level is equivalent to V_{max}, the maximum rate of activity for this particular enzyme. A decrease in activity at higher substrate concentrations (Figures 9A and 10A) was observed in all these plots from the present study. This was probably due to some sort of product inhibition. It was therefore important to have concentration values from the middle part of this curve because these values, when plotted inversely, gave a straight line that crossed the x-axis at $-1 / K_m$. From the reciprocal plots (Figures 9B and 10B) the values were calculated for the approximate K_m's. For malate the approximate K_m = 1.7 mM, and for NAD^+ the approximate K_m = 0.13 mM.

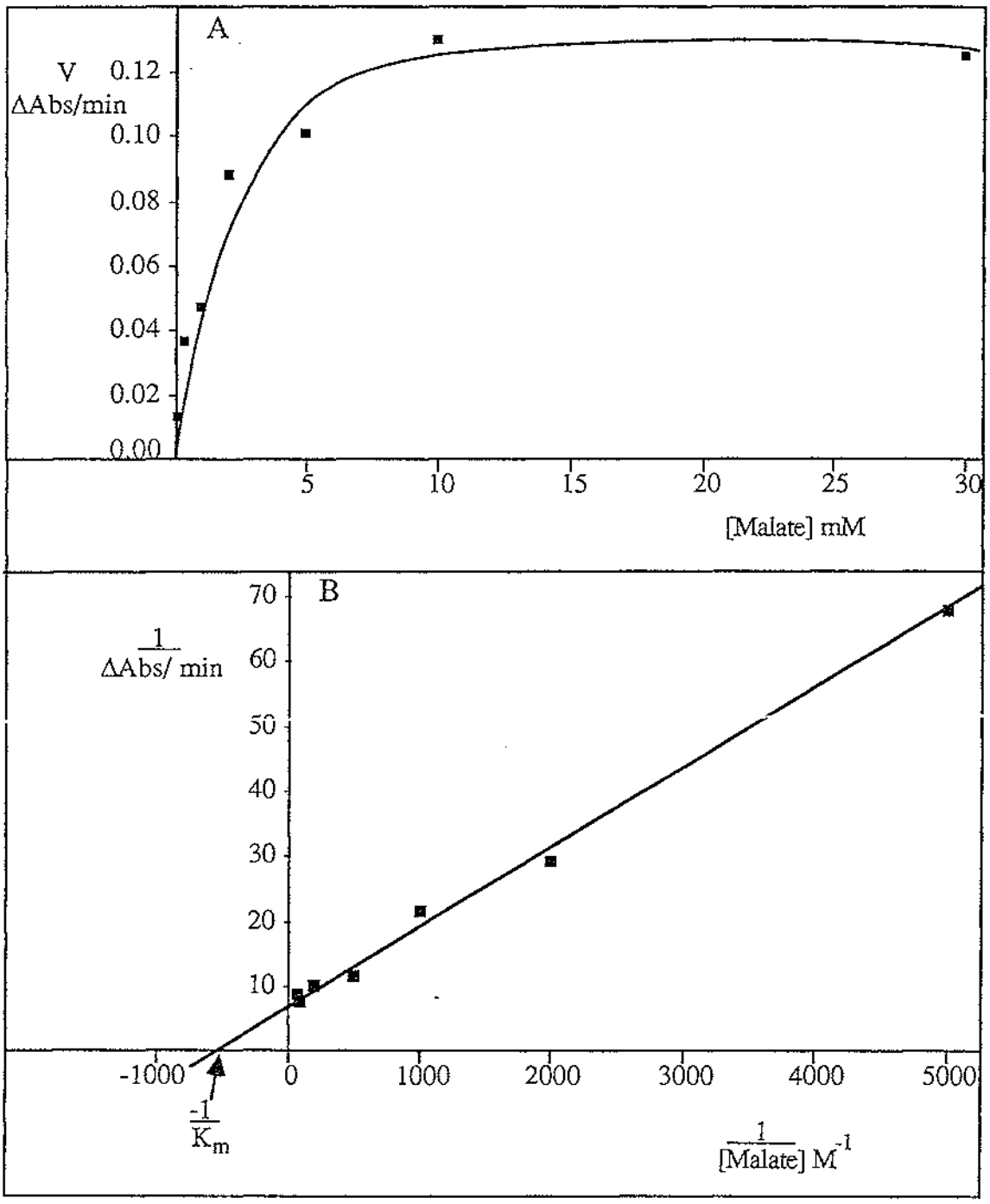


Figure 9

Preliminary kinetics: Determination of approximate K_m for malate

A Plot of velocity versus [malate] showing normal enzyme saturation curve

B Lineweaver Burk plot of the same data showing how to determine approximate K_m. The approximate K_m for malate is 1.7 mM.

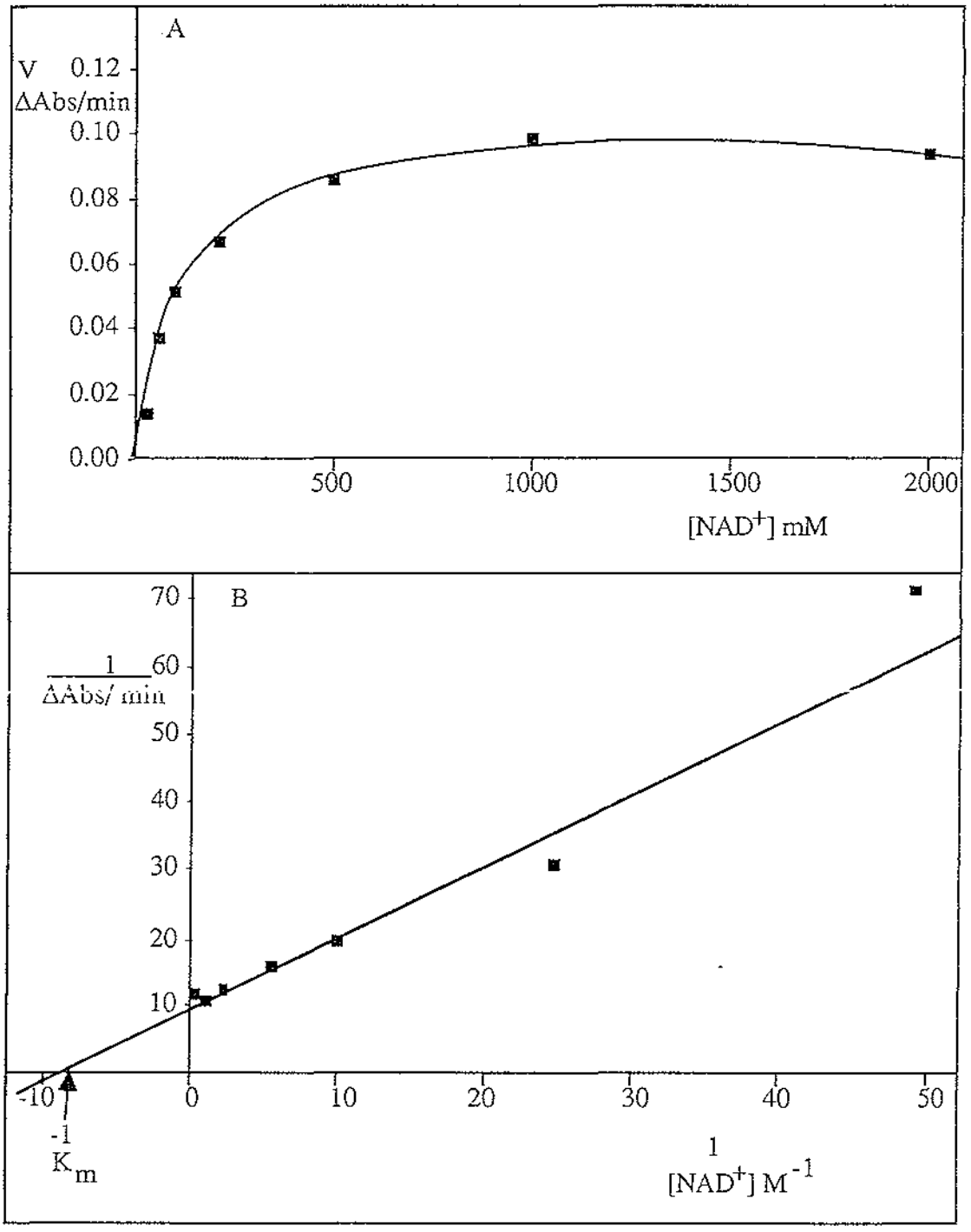


Figure 10

Preliminary kinetics; Determination of approximate K_m for NAD^+

A Plot of velocity versus $[NAD^+]$ showing normal saturation curve

B Lineweaver Burk plot of the same data showing how to determine approximate K_m. The approximate K_m for NAD^+ is 0.13 mM.

4.32 Assay linearity

As all previous assays for MDH, in this work, were carried out in the direction of malate oxidation and at pH 10, and the kinetic experiments were to be carried out at pH 7.4, it was necessary to conduct preliminary tests on the linearity and sensitivity of the assay under the new conditions. For measuring initial rates it is important to have assays that are linear, if possible, as this makes calculation of the initial rates simpler. When MDH assays were first carried out for kinetic studies they were not very linear. The cause of this was unknown.

As the kinetic assays were carried out using a different spectrophotometer it was possible that the change to a dual beam system was affecting the assays. A dual-beam spectrophotometer has two cuvettes in which the rate of absorbance change is measured and the difference between the two is what is actually recorded. A blank is placed in one of the positions and so only the effects of the desired reaction should be seen. The other obvious possibility was that the change in pH, from 10 to 7.4 had affected the assay method. Therefore it was necessary to look at both these possibilities in greater depth.

4.33 Dual-beam effects

The effects of using a dual-beam spectrophotometer were looked at by making up a series of assay mixtures which had one or more components left out. These were run against a blank that contained only water. When the sample tube contained water no change in absorbance was detected. This was also true if the sample tube contained the phosphate buffer. If this assay then had NAD^+ added there was a jump in the total absorbance due to the NAD^+ and then a small decrease was observed during the next few minutes. If enzyme was added to a mixture containing only NAD^+ then a linear drop in the absorbance was noted for the next few minutes. However these effects appeared to be minor and were only visible at high sensitivities.

4.34 pH dependence

A series of assays were carried out at different pH values to to see if decreasing the pH affected the linearity. These assays were quite curved; they started out steeply and quite rapidly tailed off to a much lower rate. It was impossible to measure an accurate initial slope from these curves so that it was necessary to try to spread out the initial part of the curve. This could be done in three different ways, either by increasing the sensitivity of the recording, by increasing the speed of the recording, or lastly by decreasing the

amount of enzyme in the assay. Increasing the sensitivity of the recording would spread out the recorded line, but would also make it steeper thereby making it harder to measure the initial slope. An increase in the sensitivity also increases the background effects. If the speed of the recording was increased this would increase the width and so make the line less steep and therefore easier to measure in this case. The optimum slope for ease of measuring is one that has an angle of about 45° and increasing the speed past this point will only make measurement harder. Increasing the speed of the recorder tends to reduce the clarity of the recording and so an optimum speed must be determined. Decreasing the amount of enzyme present in the assay would reduce the maximum velocity and so the reaction would take longer and therefore the slope of the recording would be more spread out, including any early linear portion, making it easier to measure the initial slope.

Even after adjusting the sensitivity and the speed of the assays it was found necessary to reduce the amount of enzyme in the assay mixture. The assays were found to have much better linearity at the higher pH values, where the activity was higher. From this we decided that the reaction could have been approaching a state of equilibrium. This could be tested by measuring the absorbance change that took place for the complete reaction and calculating an equilibrium constant. The equilibrium constant for the enzyme reaction in the direction of malate oxidation was calculated to be about 7.3×10^{-13} M. This was then compared to the published equilibrium constant for the reaction, of 6.4×10^{-13} M (Barman, 1969). Because of the similarity between these two values it appeared that the reaction was reaching its equilibrium point and so we decided it was necessary to reduce the amount of enzyme in the reaction mixture before further experiments were carried out. This was achieved by diluting the enzyme sample before it was added to the assay mixture.

4.35 Enzyme concentration effects

To see what effects diluting the enzyme would have on the assay, an experiment was carried out with different enzyme concentrations. Assays were run with 2 mM NAD^+ and 60 mM malate plus varying dilutions of the enzyme sample. The enzyme was diluted, prior to being added to the assay mixture with the buffer the enzyme was presently in. This was normally the pH 8.0 phosphate buffer, I = 0.020. It was found that by increasing the dilution the linearity of the assays improved. The level of activity in the assays was also higher, due to the initial slope of the linear assays being easier to measure. When the assay is very curved measuring the initial slope by eye becomes almost a matter of guess work. Because of the better assays achieved with 1/50 and

1/100 dilutions of the enzyme, these were the conditions used for future experiments, although for each new enzyme preparation it was necessary to re-evaluate the appropriate dilution factor, because of the difference in the enzyme concentration between different preparations.

4.4 THE REACTIONS OF mMDH IN THE ABSENCE OF PRODUCTS

The first 6x6 assays were carried out using six NAD^+ concentrations, 1.0, 0.25, 0.15, 0.1, 0.75, 0.06 mM and six malate concentrations, 10.0, 2.5, 1.5, 1.0, 0.75, 0.6 mM. Running the whole 36 assays in duplicate took approximately an hour and a half. For experiments in the direction of oxaloacetate reduction, the substrate concentrations used were, for NADH, 200, 50, 25, 15, 10, and 7.5 μM, and for oxaloacetate, 200, 100, 50, 25, 15, and 10 μM.

4.41 Enzyme stability during kinetic assays

When the 6x6 experiments were first tried out in the direction of malate oxidation the assays were found to be very curved and so the other direction was tried. In these experiments the linearity was better but it was noticed that the enzyme did not appear to be as active at the end of the experiment as it was at the begining. When this was further checked out as a timed experiment in which samples were taken every few minutes it appeared that about 20% of the enzyme activity was being lost in 20 minutes and over the period of time needed for an experiment about 50% of the activity was being lost. The loss appeared to occur much more rapidly at the start of the experiment and to then tail off. Enzyme activity also appeared to be lost more quickly during a 6x6 experiment when samples were being taken out every minute or so, than when samples were only taken out every fifteen minutes for the stability tests. This may have been due to mechanical disruption of the enzyme by the pipette tip.

Because the level of enzyme activity was much higher, at pH 7.4, for oxaloacetate reduction than for malate oxidation it was necessary to use higher dilutions of the enzyme solution. This may lead to one possibility for explaining the activity loss, that the enzyme was dissociating into inactive subunits. This could happen at low enzyme concentrations.In the assay mixture the enzyme concentration of a 1/200 dilution of the sample was estimated to be about 0.02 μM which is lower than the level at which pig heart mMDH will dissociate, K_D of 2×10^{-7} M at pH 8.0 (Shore and Chakrabarti, 1976) and pH 7.0 (Bleile *et al.*, 1977).

However even at lower dilutions the enzyme was showing some loss of activity. It was possible the enzyme was being inactivated through the oxidation of thiol groups and to try to prevent this, extra DTT was added to the solution. This did not appear to have a signifcant effect on the reaction, as a sample with extra DTT showed very similar loss

of activity over time as did a sample without DTT. Adding extra protein, in the form of bovine serum albumin (BSA), to the enzyme solution to see if a higher protein concentration would prevent the inactivation of the enzyme was also tried. 1 mg / ml BSA gave a small reduction in the rate of activity loss, but this was not considered extremely useful and BSA was not added to further experiments as the possibility of it binding to the substrates and therefore changing the concentrations of free substrate in the assay mixture outweighed the decrease in activity loss. The best increase in stability was gained when the ionic strength of the diluted enzyme was kept at 0.1 M. A significant loss was still observed over a short period time straight after mixing but it was found that if the diluted enzyme was left to stand for five minutes after mixing then no significant further loss was recorded through the period of an experiment.

4.42 Assay inhibition at high substrate concentrations

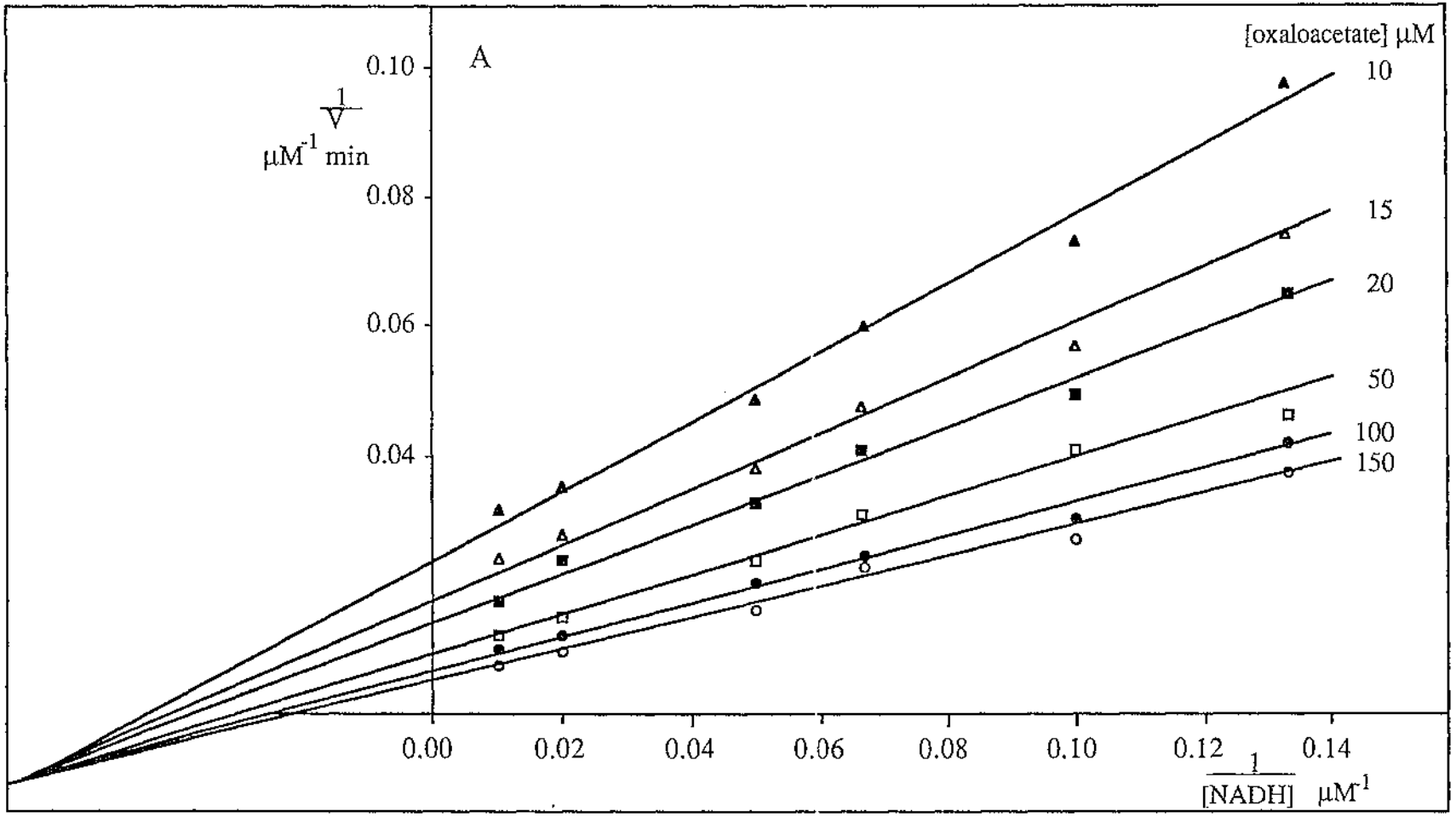
Substrate inhibition was observed at the highest substrate concentrations used for the reaction in the diection of oxaloacetate reduction. To try to reduce any effects this would have on the computer analysis of the results the high concentrations were lowered until these effects were not visible to the eye. For NADH, the highest concentration was decreased to 100 μM and for oxaloacetate, the highest concentration was decreased to 150 μM. In the other direction these effects were not as noticeable and so the substrate concentrations were not changed.

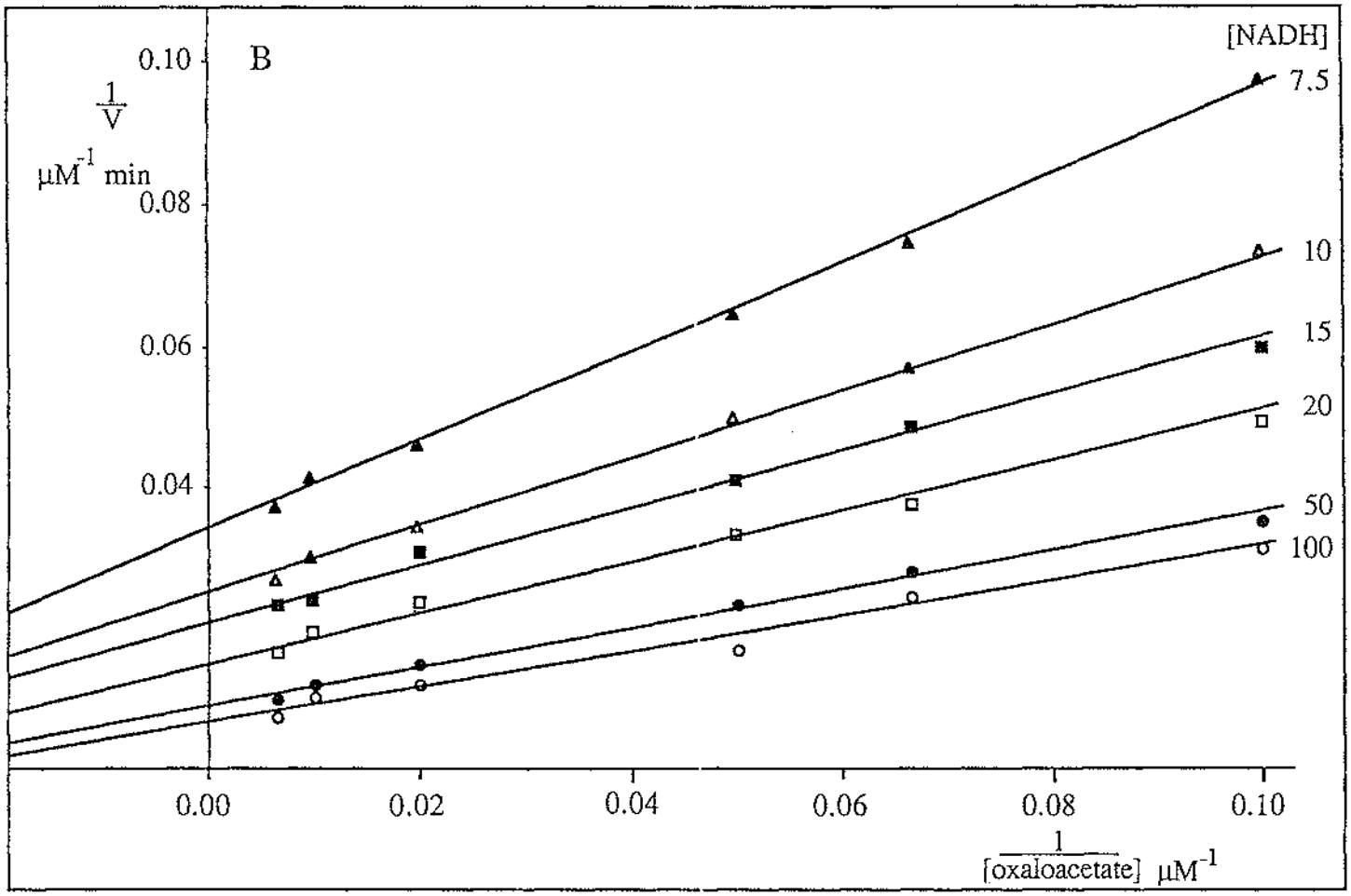
4.43 6x6 assays: data collection and processing

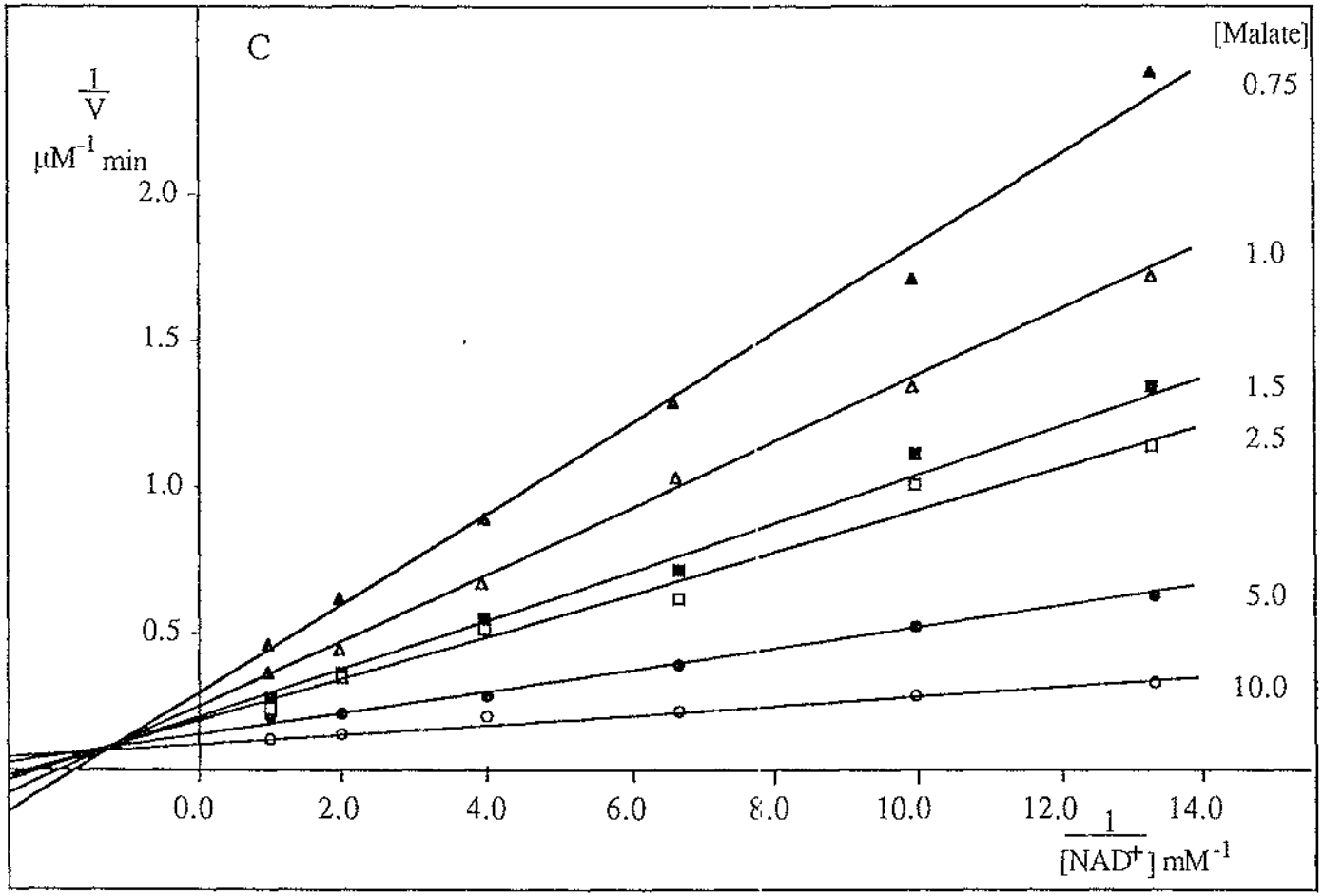
The data collected from the 6x6 experiment was in the form of 36 duplicate pairs of reaction rates, that were averaged, and for each of these there were two sets of substrate concentrations. These were then plotted by the method of Lineweaver and Burk (1934) see section 4.22. Theoretically all the lines on each plot should intersect at a single point on the left hand side of the y-axis. Although it is improbable that all the lines to cut at exactly the same point in a real case it can be seen in figures 11A to D that this is almost true of the data from this work. From the point of intersection information can be obtained on the K_i and K_m for the first binding substrate for each direction. If the point of intersection is below the x-axis (as in Figures 11A and B) then $K_i < K_m$ and if it is above the x-axis (as in Figures 11C and D) then $K_i > K_m$, so just by looking at the primary plots it can determined that $K_{iNADH} < K_{NADH}$ and that $K_{iNAD} > K_{NAD}$. The slopes and y-intercepts of all the six lines for each primary plot should be determined.

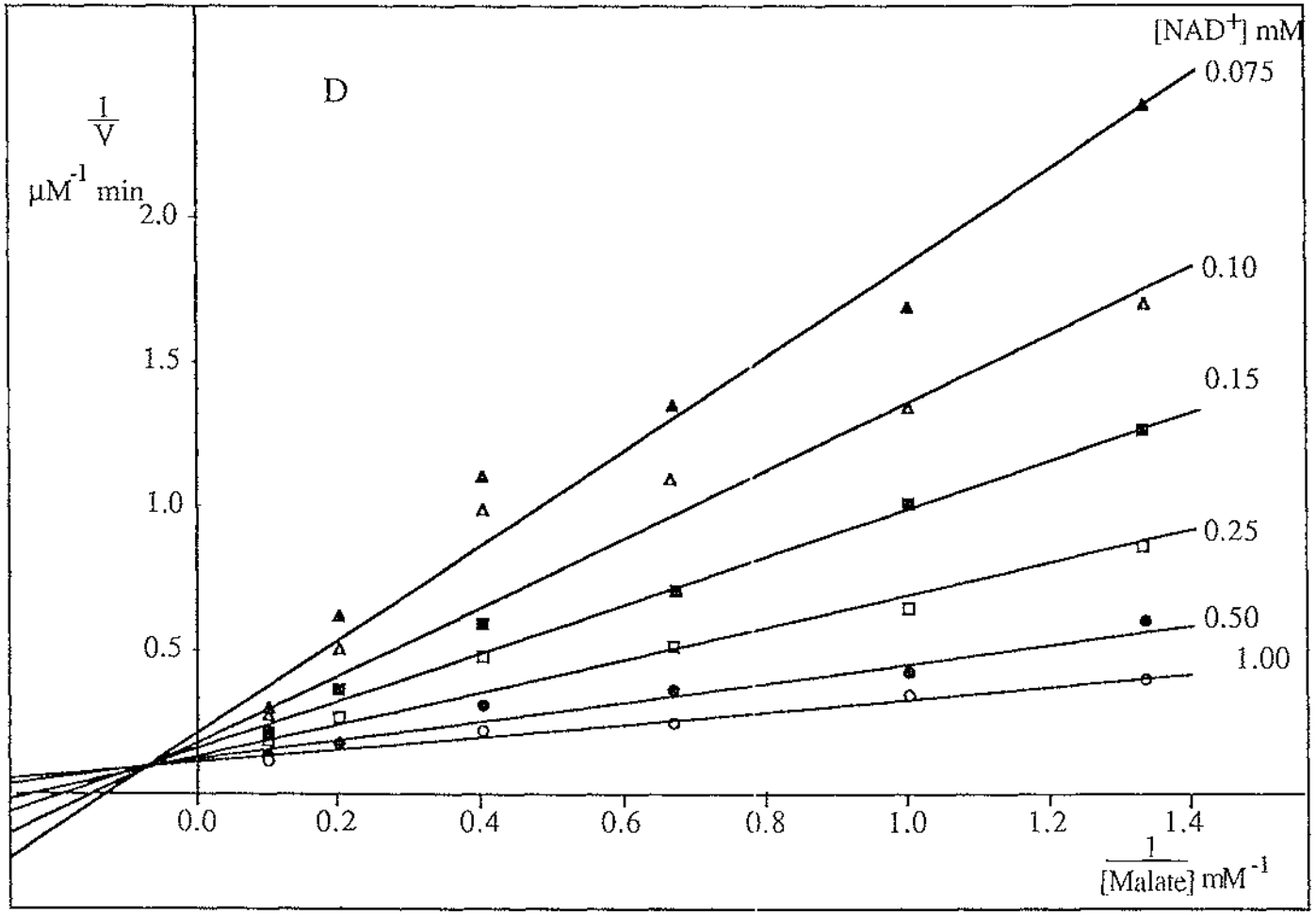
Figure 11

Primary kinetic plots for the mMDH reaction in the absence of products, in the direction of oxaloacetate reduction (A and B) and malate oxidation (C and D).


A
[oxaloacetate] µM
10
15
20
50
100
150
0.10
0.08
0.06
0.04
1/V
µM-1 min
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
1/[NADH] µM-1


B
0.10
1/V
μM-1 min
0.08
0.06
0.04
[NADH]
7.5
10
15
20
50
100
0.00
0.02
0.04
0.06
0.08
0.10
1/[oxaloacetate] μM-1


C
[Malate]
0.75
1.0
1.5
2.5
5.0
10.0
1/V
μM⁻¹ min
2.0
1.5
1.0
0.5
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
1/[NAD+] mM⁻¹


D
[NAD+] mM
0.075
0.10
0.15
0.25
0.50
1.00
1/V
μM-1 min
2.0
1.5
1.0
0.5
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1/[Malate] mM-1

Secondary plots were made by plotting these values against the inverse of the other substrate concentration (Figures 12 and 13). From these plots the required constants can be determined (see Section 4.22).

These values are then used as starting estimates for the non-linear least-squares curve-fitting program. This program takes the data as two substrate concentrations and a reaction rate and returns values for V_{max}, K_a, K_b, K_{ia}, and a fitted reaction rate for each point. A sample set of data is presented in Table V and this has been plotted manually (see Figure 11A and B for the primary plots and Figures 12A and B for the secondary plots). The constants obtained from these plots have been used as initial estimates for the computer analysis which is shown in Table VI. This process was repeated enough times in both directions for the results to be considered statistically significant. The values determined from the reactions in the absence of products are shown in Tables VII and VIII.

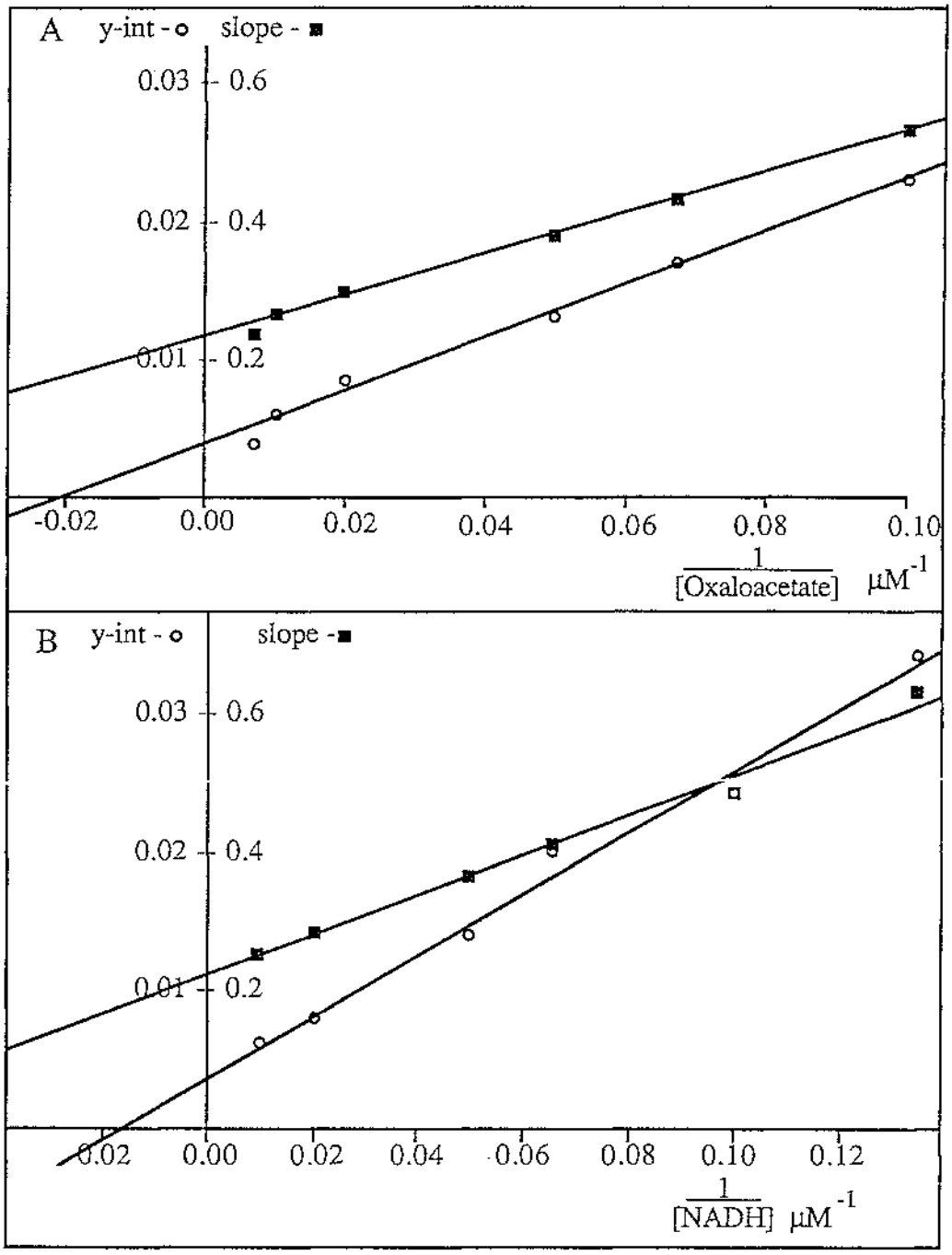


Figure 12

Secondary plots for the sample set of data (Table V). From these can be calculated values for the constants that were used as initial estimates for the computer analysis. (A) Secondary plots of the slope and y-int versus 1/ [mal] from Figure 11A. (B) Secondary plots of the slope and y-int versus 1 / [NAD^+] from Figure 11B. The values obtained from these plots were V_{max} , 300 μmol/min; K_a , 62.5 μM; K_b, 50 μM; K_{ia}, 11.25 μM.

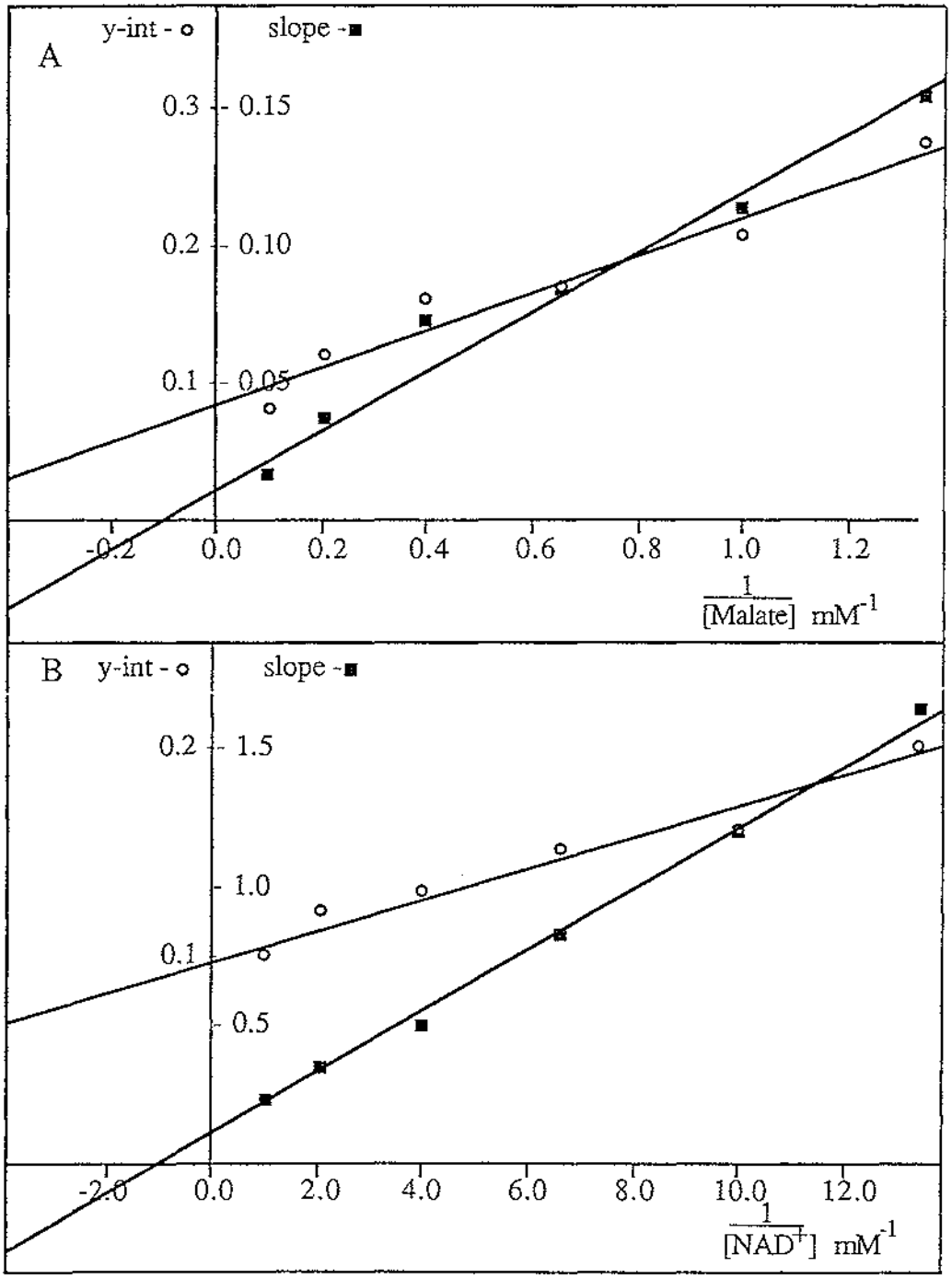


Figure 13

Secondary plots for oxaloacetate reduction, from the primary plots in Figure 11C and D.

(A) Secondary plots of the slope and y-int versus 1/ [OAA] from Fig. 11C.

(B) Secondary plots of the slope and y-int versus 1 / [NADH] from Fig. 11D.

Table V

Sample set of data from a 6x6 experiment with the enzyme in the absence of products

The data are shown as the two substrates' concentrations, the reaction rates, and the inverse of each.

Assay	[NADH] µM	1/[NADH]	[OAA] µM	1/[OAA]	V µmol/min	1/V
1	100	0.01	150	0.0067	137.7	0.0073
2	100	0.01	100	0.01	103.4	0.0097
3	100	0.01	50	0.02	84.6	0.0118
4	100	0.01	20	0.05	59.1	0.0169
5	100	0.01	15	0.067	43.1	0.0232
6	100	0.01	10	0.10	32.6	0.0307
7	50	0.02	150	0.0067	106.3	0.0094
8	50	0.02	100	0.01	84.6	0.0118
9	50	0.02	50	0.02	69.4	0.0144
10	50	0.02	20	0.05	44.6	0.0224
11	50	0.02	15	0.067	36.6	0.0273
12	50	0.02	10	0.10	29.4	0.0340
13	20	0.05	150	0.0067	61.3	0.0158
14	20	0.05	100	0.01	50.4	0.0198
15	20	0.05	50	0.02	44.0	0.0227
16	20	0.05	20	0.05	31.1	0.0322
17	20	0.05	15	0.067	27.5	0.0369
18	20	0.05	10	0.10	20.7	0.0483
19	15	0.067	150	0.0067	45.4	0.0220
20	15	0.067	100	0.01	42.8	0.0234
21	15	0.067	50	0.02	33.0	0.0303
22	15	0.067	20	0.05	24.9	0.0402
23	15	0.067	15	0.067	21.1	0.0474
24	15	0.067	10	0.10	16.9	0.0592
25	10	0.10	150	0.0067	37.7	0.0265
26	10	0.10	100	0.01	33.7	0.0297
27	10	0.10	50	0.02	30.1	0.0332
28	10	0.10	20	0.05	20.8	0.0481
29	10	0.10	15	0.067	17.8	0.0562
30	10	0.10	10	0.10	13.8	0.0725
31	7.5	0.13	150	0.0067	27.0	0.0370
32	7.5	0.13	100	0.01	24.4	0.0410
33	7.5	0.13	50	0.02	22.2	0.0450
34	7.5	0.13	20	0.05	15.7	0.0637
35	7.5	0.13	15	0.067	13.6	0.0735
36	7.5	0.13	10	0.10	10.4	0.0962

Table VI

Sample computer print out of the non-linear least-squares curve-fitting program for the data in Table V

The 36 rates measured are shown along with the values that the program has fitted them to. These are also shown in the order of the highest residual to the lowest. At the top are the values that have been calculated for the required constants. These are accompanied by a standard error coefficient, a T-value and 95% confidence limits for each constant.

NON-LINEAR LEAST-SQUARES CURVE-FITTING PROGRAM

MDH KINETICS 3/2/C DEP. VAR. MIN. Y=1.040E+01 MAX. Y=1.377E+02
RANGE Y=1.273E+02

Ind. Var (I)	Name	Coef. B (I)	S. E. Coef.	T-Value	95% Confidence Limits Lower	Upper
1	Vmax	2.57648E+02	2.24E+01	11.5	2.12E+02	3.03E+02
2	Kia NADH	1.15915E+01	4.49E+00	2.6	2.45E+00	2.07E+01
3	Ka NADH	5.56715E+01	8.38E+00	6.6	3.86E+01	7.27E+01
4	Kb OAA	6.05145E+01	1.03E+01	5.9	3.94E+01	8.16E+01

No. of observations	36
No. of coefficients	4
Residual degrees of freedom	32
Residual root mean square	3.92143321
Residual mean square	15.37763786
Residual sum of squares	492.08441162

--ORDERED BY COMPUTER INPUT--				----------ORDERED BY RESIDUALS--------				
Obs. No.	Obs. Y	Fitted Y	Residual	Obs. No.	Obs. Y	Fitted Y	Residual	Seq.
0001	137.700	128.380	9.320	0001	137.700	128.380	9.320	1
0002	103.400	115.433	-12.033	0007	106.300	98.701	7.599	2
0003	84.600	88.621	-4.021	0004	59.100	52.228	6.872	3
0004	59.100	52.228	6.872	0013	63.100	58.281	4.819	4
0005	43.100	42.526	.574	0025	37.700	34.638	3.062	5
0006	32.600	31.006	1.594	0012	29.400	26.929	2.471	6
0007	106.300	98.701	7.599	0027	30.100	28.065	2.035	7
0008	84.600	90.122	-5.522	0017	27.100	25.369	1.731	8
0009	69.400	71.483	-2.083	0006	32.600	31.006	1.594	9
0010	44.600	44.113	.487	0018	20.700	19.311	1.389	10
0011	36.600	36.376	.224	0028	20.800	19.668	1.132	11
0012	29.400	26.929	2.471	0016	31.100	30.089	1.011	12
0013	63.100	58.281	4.819	0026	33.700	32.722	.978	13
0014	50.400	54.362	-3.962	0029	17.800	16.864	.936	14
0015	44.000	45.239	-1.239	0030	13.800	13.123	.677	15
0016	31.100	30.089	1.011	0005	43.100	42.526	.574	16
0017	27.100	25.369	1.731	0010	44.600	44.113	.487	17
0018	20.700	19.311	1.389	0011	36.600	36.376	.224	18
0019	45.400	47.478	-2.078	0024	16.900	16.688	.212	19
0020	42.800	44.543	-1.743	0035	13.600	13.784	-.184	20
0021	33.000	37.574	-4.574	0033	22.200	22.397	-.197	21
0022	24.900	25.572	-.672	0031	27.000	27.265	-.265	22
0023	21.100	21.718	-.618	0034	15.700	15.978	-.278	23
0024	16.900	16.688	.212	0036	10.400	10.813	-.413	24
0025	37.700	34.638	3.062	0023	21.100	21.718	-.618	25
0026	33.700	32.722	.978	0022	24.900	25.572	-.672	26
0027	30.100	28.065	2.035	0015	44.000	45.239	-1.239	27
0028	20.800	19.668	1.132	0032	24.400	25.860	-1.460	28
0029	17.800	16.864	.936	0020	42.800	44.543	-1.743	29
0030	13.800	13.123	.677	0019	45.400	47.478	-2.078	30
0031	27.000	27.265	-.265	0009	69.400	71.483	-2.083	31
0032	24.400	25.860	-1.460	0014	50.400	54.362	-3.962	32
0033	22.200	22.397	-.197	0003	84.600	88.621	-4.021	33
0034	15.700	15.978	-.278	0021	33.000	37.574	-4.574	34
0035	13.600	13.784	-.184	0008	84.600	90.122	-5.522	35
0036	10.400	10.813	-.413	0002	103.400	115.433	-12.033	36

End of problem
MDH kinetics 3/2/C

TABLE VII

Kinetic constants for substrates of the mMDH reaction in the direction of oxaloacetate reduction

Comparison of constants from ten experiments in the direction of oxaloacetate reduction, showing standard errors for each experiment calculated by the computer program and average values with standard errors.

Date	V_{max}	K_{NADH}	K_{OAA}	$K_{i\ NADH}$
26/11	850 ± 210	130 ± 40	89 ± 30	12 ± 5
2/12	450 ± 90	81 ± 24	95 ± 31	4 ± 5
3/12	540 ± 100	78 ± 21	144 ± 42	9 ± 5
10/12	1200 ± 200	81 ± 19	91 ± 24	12 ± 6
12/12	580 ± 60	56 ± 9	80 ± 14	11 ± 4
26/1	460 ± 80	64 ± 16	84 ± 24	14 ± 6
30/1	410 ± 60	85 ± 17	138 ± 31	12 ± 4
3/2A	190 ± 20	40 ± 7	32 ± 7	26 ± 9
3/2B	190 ± 10	35 ± 4	39 ± 5	13 ± 4
3/2C	290 ± 20	66 ± 8	68 ± 9	11 ± 3
average	520 ± 100	72 ± 8	86 ± 11	12 ± 2

TABLE VIII

Kinetic constants for substrates of the mMDH reaction in the direction of malate oxidation

Comparison of constants from six experiments in the direction of malate oxidation, showing standard errors for each experiment calculated by the computer program and average values with standard errors.

Date	V_{max}	K_{NAD^+}	K_{malate}	$K_{i\,NAD^+}$
10/2	34.6 ± 1.4	0.10 ± 0.02	3.4 ± 0.4	1.01 ± 0.13
12/2	33.9 ± 1.1	0.09 ± 0.02	2.6 ± 0.3	1.00 ± 0.12
24/2	16.1 ± 0.6	0.24 ± 0.03	1.4 ± 0.2	1.14 ± 0.22
25/2	11.3 ± 0.7	0.18 ± 0.04	1.3 ± 0.3	0.89 ± 0.24
10/3	15.2 ± 1.9	0.29 ± 0.08	1.6 ± 0.7	1.63 ± 0.57
16/3	7.3 ± 0.4	0.17 ± 0.02	1.3 ± 0.3	0.75 ± 0.18
average	20 ± 5	0.17 ± 0.03	1.9 ± 0.4	1.07 ± 0.12

4.5 REACTIONS OF mMDH IN THE PRESENCE OF PRODUCT AS AN INHIBITOR

Because some of the required constants cannot be obtained from the reactions in the absence of products, a further set of experiments that have varying concentrations of product added to cause inhibition, were carried out (see section 4.31). As these experiments required products, for inhibition, instead of substrates a new set of concentrations were required for each product (Table IX). We decided that only three concentrations and a zero inhibitor set of assays needed to be run for each substrate. This meant a total of 24 different assays in each experiment.

TABLE IX

The concentrations of substrates and inhibitors for the product inhibition experiments.

Inhib.	Conc.	Var. sub.	Conc.	Fixed sub.	Conc.
P	0, 5, 10, 20 mM	B	150, 100, 50, 20, 15, 10 μM	A	25 μM
B	0, 50, 100, 200 μM	P	5, 2.5, 1.5, 1.0, 0.75, 0.6 mM	Q	0.5 mM
Q	0, 1.25, 2.5, 5 mM	A	100, 50, 25, 15, 10, 7.5 μM	B	50 μM
A	0, 5, 10, 20 μM	Q	0.5, 0.25, 0.15, 0.1, 0.075, 0.06 mM	P	5 mM

Where A = NADH, B = oxaloacetate, P = malate, and Q = NAD^+.

4.51 Manual plotting of data

The four different sets of assays were graphed manually in a similar way to the earlier data by plotting 1 / v versus 1 / [substrate] (see Figures 14 to 17). These plots showed competitive inhibition between NAD^+ and NADH and noncompetitive inhibition (Cleland, 1975) between malate and oxaloacetate. Replots of slope and y-intercept versus 1 / [inhibitor] could also have been drawn, but since the equations for the constants from these graphs are quite complex it was simpler to use the values for sMDH as the initial guesses in the computer analysis of the data.

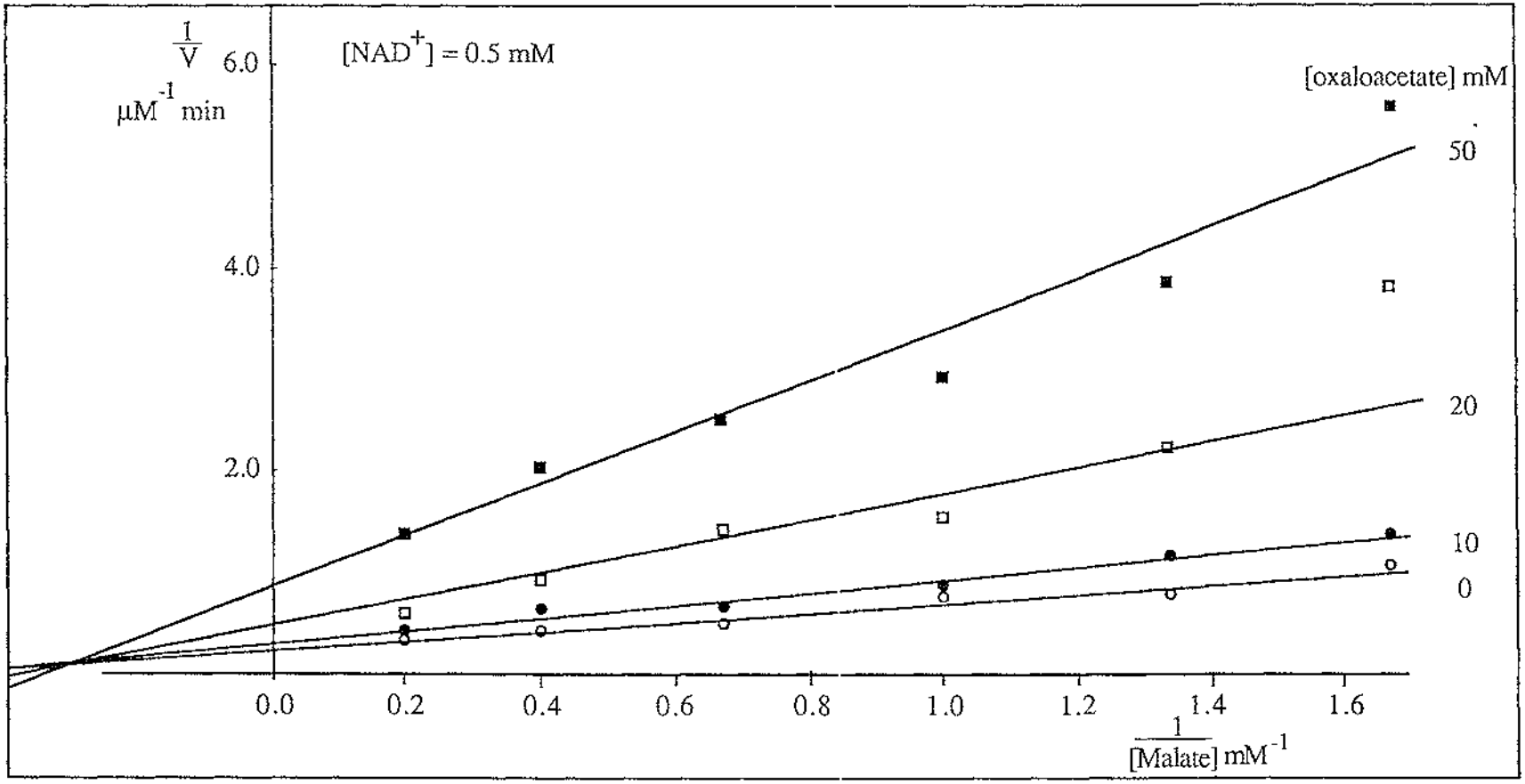


Figure 14 Inhibitor plot of oxaloacetate inhibiting malate oxidation, with malate being the variable substrate. The lines on this plot clearly all intersect at a point to the left of the y-axis. This suggests the inhibition is noncompetitive, which is what would be expected from an ordered mechanism.

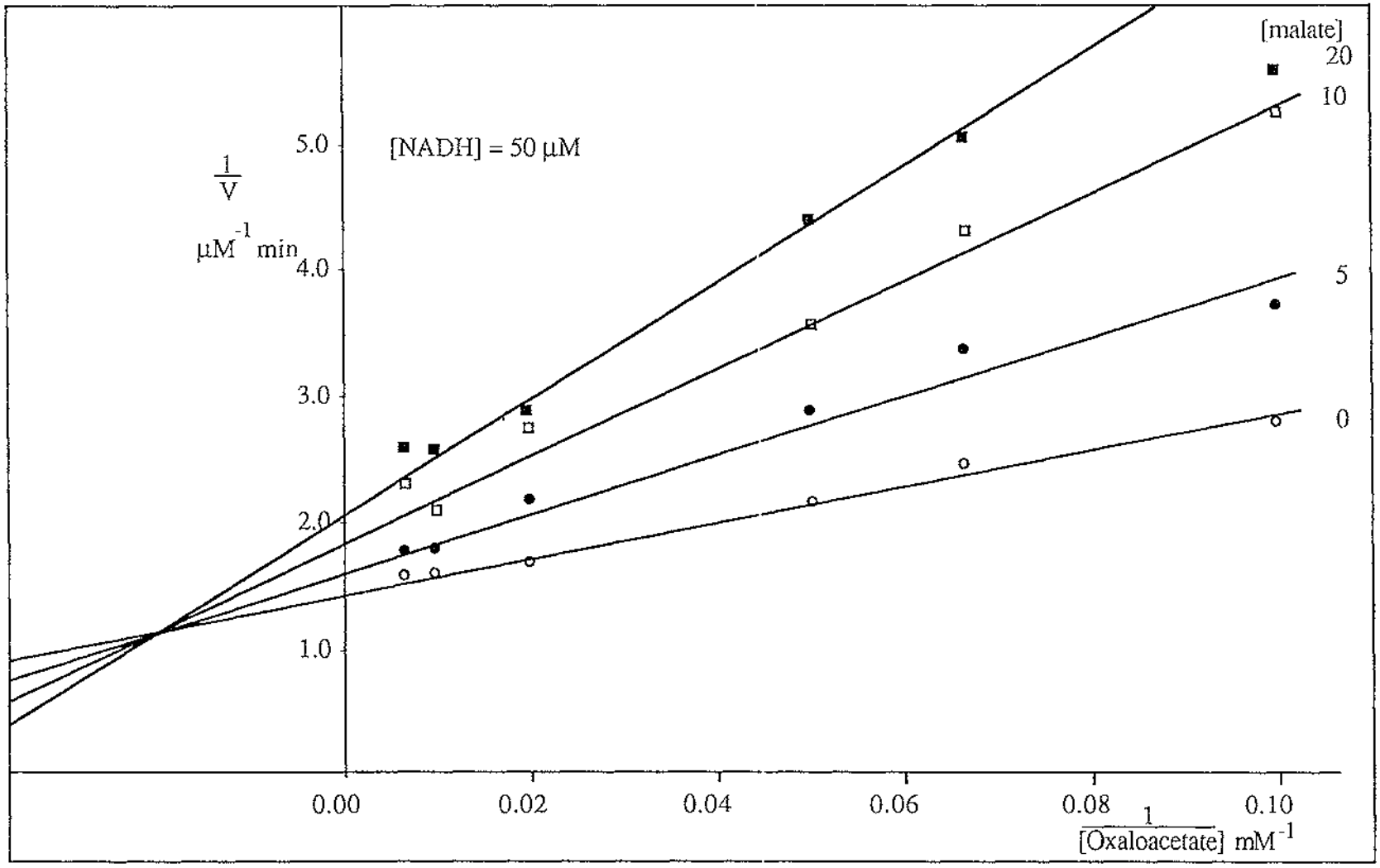


Figure 15 Inhibitor plot of malate inhibiting oxaloacetate reduction, with oxaloacetate being the variable substrate. The lines all intersect at a point to the left of the y-axis. This suggests the inhibition is noncompetitive.

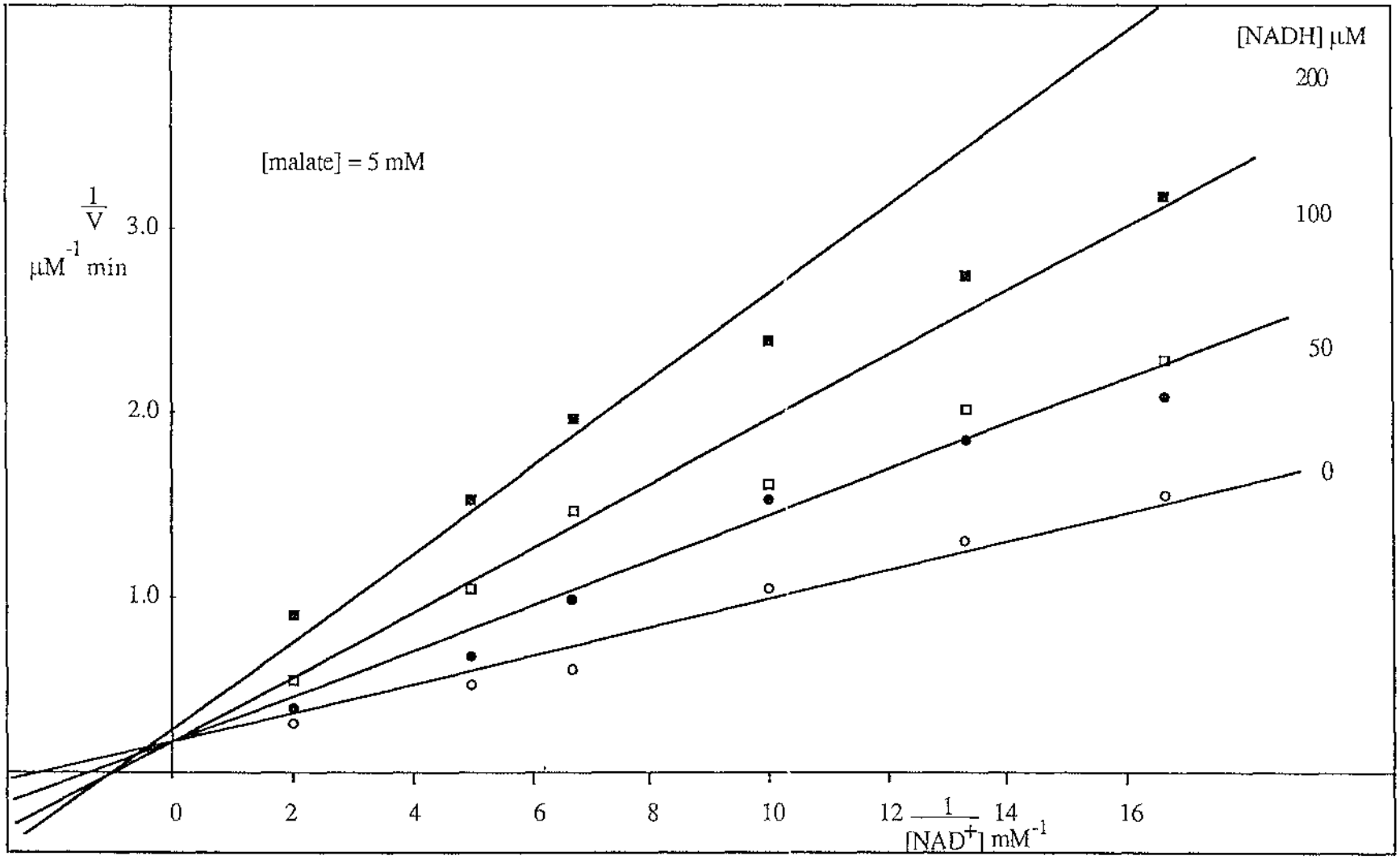


Figure 16 Inhibitor plot of NADH inhibiting malate oxidation, with NAD^+ being the variable substrate. The lines on this plot all appear to intersect at a point on or near the y-axis. This suggests the inhibition is probably competitive.

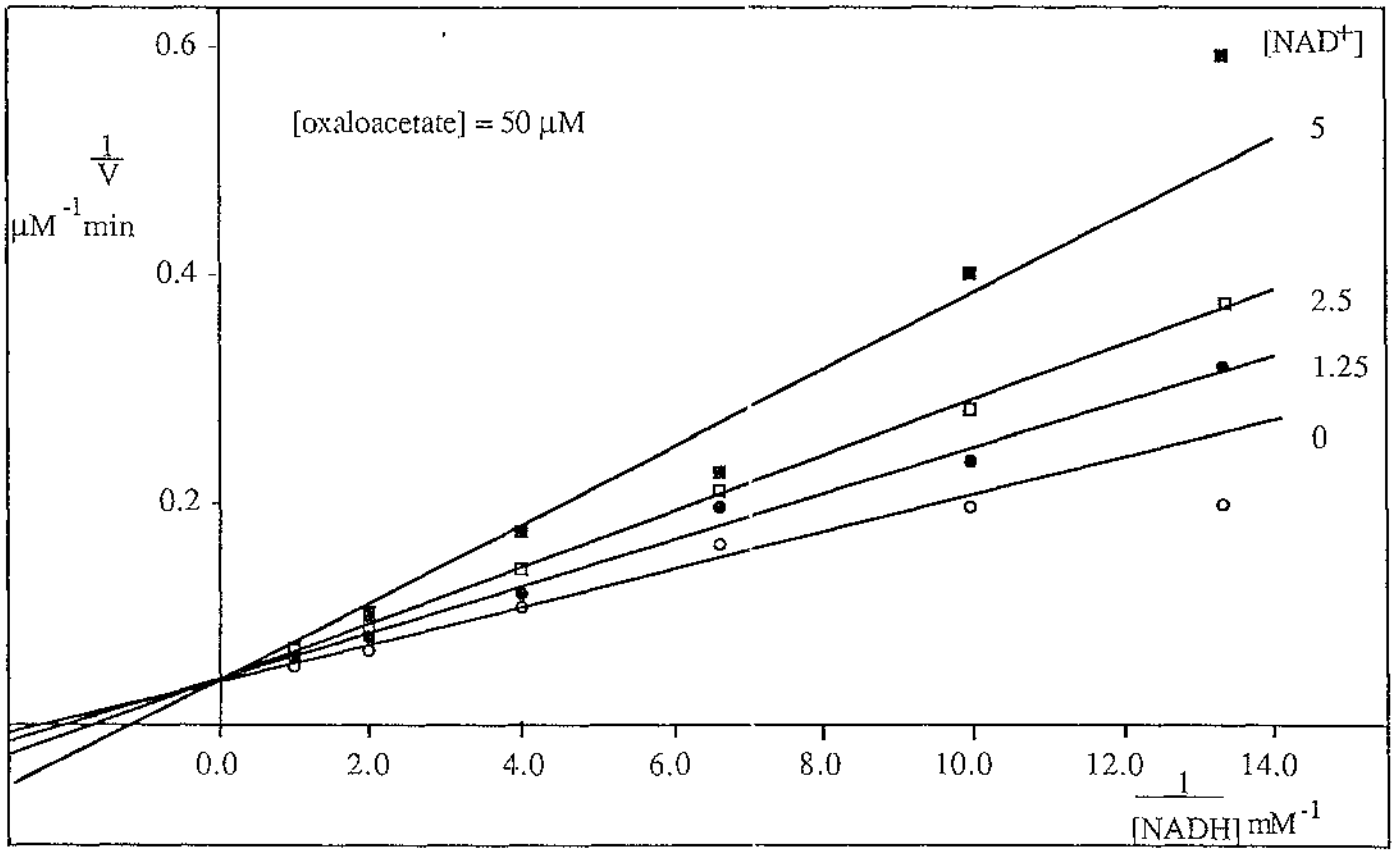


Figure 17 Inhibitor plot of NAD^+ inhibiting oxaloacetate reduction, with NADH being the variable substrate. The lines on this plot all intersect on the y-axis. This suggests the inhibition is competitive.

4.52 Computer analysis of data

Values for all the required constants can be determined by computer analysis of these four sets of data. Further confirmation of the type of inhibition can also be obtained. The results produced are in the form of K_{ii} and K_{is} (intercept and slope) values (Table X) which must be corrected, because of the nonsaturating concentration of the fixed substrate, to give the constants (see Equations 9 - 14). The data were fitted to both a competitive and a noncompetitve inhibition program and the results were then compared, to distinguish between the two types of inhibition. For an enzyme with a compulsory ordered reaction the inhibition patterns should be noncompetitive for the substrates and competitive for the co-factors; for mMDH this means malate and oxaloacetate should be non-competitive and NAD^+ and NADH should be competitive. The competitive fitting program only gives three parameters, V_{max}, K_m for the substrate, and K_{is}, a value determined from the secondary plot of the primary slopes versus inhibitor concentration. The noncompetitive fitting program will give four, the extra one being K_{ii}, a value determined from the secondary plot of the y-intercepts, from the primary plots, versus inhibitor concentration. A set of data that is competitive will give a meaningless K_{ii} value, a value that has a 95% confidence interval that is extremely large and extends well into the negative numbers. This is because the competitive equation (Equation 7) is a special case of the general equation (Equation 8) in which the K_{ii} term is nonexistent as all the primary lines have the same y-intercept. A non-competitive set of numbers will show a lower residual sum of squares and will more clearly define all the constants when fitted to the non-competitive program than when fitted to the competitive program.

4.53 Use of the F-statistic

The significance of the reduction in the residual sum of squares can be tested by the forming of the F-statistic (Brook and Arnold, 1985). Only two or three experiments, using at least two different preparations of enzyme, were carried out for each inhibitor study (Table X). Each experiment was fitted to both programs and the residual sum of squares tested with Equation 14.

$$F_{r,n} = [(RSS_{(comp)} - RSS_{(noncomp)}) / r] / [RSS_{(noncomp)} / n] \qquad \text{(Equation 14)}$$

TABLE X

Computer fitted data from product inhibition experiments

Data from the product inhibition experiments has been fitted to both competitive and noncompetitive equations. The results are shown with standard errors produced by the computer programs. The F-statistic for comparing the competitive and noncompetitive fitting for each set of data can be calculated from the residual sum of squares (RSS) and the residual mean square (RMS) using equation 14. The values produced are: malate inhibition, A: 19.2,B: 13.4; NAD inhibition, B: 6.5; NADH inhibition, A: 2.7; oxaloacetate inhibition, A: 18.1, B: 9.2, C: 1.8. To have greater than 95% significance these F-statistics must be greater than 9.12

malate inhibition of oxaloacetate reduction				
constant	A		B	
	competitive	noncomp	competitive	noncomp
V_{max}	0.65 ± 0.03	0.71 ± 0.03	6.3 ± 0.2	6.8 ± 0.2
K_m (OAA)	8 ± 1	11 ± 1	16 ± 2	20 ± 2
K_{is}	4.1 ± 0.8	8 ± 2	14 ± 3	30 ± 10
K_{ii}	—	40 ± 10	—	70 ± 20
RSS	0.0182	0.00828	1.779	1.065
RMS	0.0011	0.00052	0.085	0.053

NAD^+ inhibition of oxaloacetate reduction				
constant	A[a]		B	
	competitive	noncomp	competitive	noncomp
V_{max}	27.9 ± 0.5	27.6 ± 0.7	1.52 ± 0.07	1.65 ± 0.09
K_m (NADH)	48.0 ± 2	47 ± 3	31 ± 3	37 ± 4
K_{is}	5.6 ± 0.4	5.3 ± 0.6	2.4 ± 0.3	3.6 ± 0.8
K_{ii}	—	$1x10^5 \pm 3x10^5$	—	11 ± 4
RSS	1.332	1.4406	0.0417	0.03142
RMS	0.067	0.0758	0.0020	0.00157

NADH inhibition of malate oxidation				
constant	A		B[a]	
	competitive	noncomp	competitive	noncomp
V_{max}	8 ± 1	9 ± 1	0.31 ± 0.06	0.18 ± 0.03
K_m (NAD^+)	0.6 ± 0.1	0.7 ± 0.2	0.9 ± 0.2	0.4 ± 0.1
K_{is}	72 ± 8	100 ± 20	150 ± 30	130 ± 70
K_{ii}	—	150 ± 100	—	$4x10^4 \pm 1x10^5$
RSS	0.3113	0.2745	$6.26x10^4$	$1.12x10^3$
RMS	0.0148	0.0137	$3.13x10^5$	$5.91x10^4$

Oxaloacetate inhibition of malate oxidation						
constant	A		B		C	
	competitive	noncomp	competitive	noncomp	competitive	noncomp
V_{max}	16 ± 3	20 ± 4	5.2 ± 0.5	5.7 ± 0.5	11 ± 1	11 ± 2
K_m (malate)	5 ± 2	8 ± 2	2.4 ± 0.5	2.8 ± 0.5	1.8 ± 0.5	2.1 ± 0.6
K_{is}	9 ± 1	25 ± 9	8 ± 1	14 ± 4	9 ± 2	13 ± 6
K_{ii}	—	8 ± 2	—	23 ± 9	—	40 ± 40
RSS	3.2069	1.6846	0.6722	0.4599	7.9413	7.2729
RMS	0.1527	0.0842	0.0320	0.0230	0.3971	0.3828

[a] Initially the computer would not fit these sets of data to the noncompetitive program as a divide by zero error was produced for the K_{ii} value which is meaningless in a competitive set of data fitted to a noncompetitive program. The data shown in the table were produced by cutting the program off early so the divide by zero does not occur. This means that the fit has not been completed and so an F-statistic was not determined.

where RSS = residual sum of squares

r = number of restrictions of the model: In this case r = 1

n = degree of freedom associated with RSS: For these experiments RSS / n is given on the computer print out (Table VI) as the residual mean square.

The residual sum of squares for the competitive computer fitting should always be larger than the noncompetitive because the noncompetitive equation has an extra parameter, K_{ii}. In the cases NAD^+ inhibition, A, and NADH inhibition, B, no F-statistic was calculated. This was because the noncompetitive fitting program was not able to run to completion as after a few iterations it became necessary to effectively divide by zero to try to calculate Kii and when this happened the program would switch off. This did support both inhibitions being competitive.

From these values it appeared that malate inhibition of oxaloacetate reduction, with varying oxaloacetate, was noncompetitive, with the F-statistic showing greater than 95% significance in both cases and the K_{ii} value having positive 95% confidence intervals. NAD^+ inhibition of oxaloacetate reduction, with varying NADH, clearly appeared to be competitive with the F-statistic calculated above the 95% significance and the K_{ii} values being very high and having very wide 95% confidence intervals. NADH inhibition of malate oxidation, with varying NAD^+, also appeared to be competitive for similar reasons. Oxaloacetate inhibition of malate oxidation, with varying malate, was a little more ambiguous with two of the three experiments giving an F-statistic that was better than 95% significance though one was only just above the required value. The K_{ii} values were also quite variable but the data tend to favour noncompetitive inhibition.

So with the information gained from manual plotting and the computer printouts it appeared that the inhibition pattern was normal for an ordered reaction mechanism with the coenzymes, NAD^+ and NADH, inhibiting each other competitively and the substrates, malate and oxaloacetate, inhibiting each other noncompetitively. The required constants can be obtained from the data on the computer print out by correction for the nonsaturating substrate using the Equations 9 - 14. These values are shown in Table XI.

TABLE XI

Kinetic constants for the substrates of the mMDH reaction by product inhibition experiments

These constants are produced by correction for the unsaturating substrate on the values direct from the computer programs (see table X). The corrections are by equations 9 - 14. A, B, and C are the values from three different experiments. The standard errors shown are those produced by the computer program and the average values are shown without standard errors.

Constants corrected for unsaturated substrate (μM)				
	A	B	C	average
K_a	110 ± 5	63 ± 7	—	87
K_{ia}	72 ± 8	150 ± 30	—	110
K_b	28 ± 4	52 ± 5	—	40
K_{ib}	6 ± 1	17 ± 3	30 ± 10	18
K_p	3000 ± 1000	1100 ± 500	900 ± 700	1700
K_{ip}	11000 ± 3000	19000 ± 6000	—	15000
K_q	450 ± 90	800 ± 200	—	630
K_{iq}	5600 ± 400	2400 ± 300	—	4000

Where A = NADH, B = oxaloacetate, P = malate, Q = NAD^+.

4.6 CONCLUSION

The steady-state kinetic characterisation of rat liver mitochondrial malate dehydrogenase that has been carried out in this work gives values for the Michaelis constants and the dissociation constants for each substrate (Table XII). The standard errors on some of these constants are very large and for some the agreement between the values produced from the studies without inhibitors and those produced from the product inhibition studies is not very good in some cases. This is particularly true for K_{ia}, K_q and K_{iq}. The apparent differences may not be real when the standard errors are taken into account, especially those for K_q and K_{iq}. The calculation of K_{ia} and K_{iq} can be done a third way using the K_{is} values from the noncompetitive plots of malate and oxaloacetate inhibitions is also shown in Table XII and both of these values are much closer to the values for the noninhibitor plots.

Large standard errors are produced because of the low number of times the inhibitor experiments were carried out, the problems with enzyme stability and other difficulties with the experimental methods, but because of time limitations work on improving these was unable to be done. Some of the constants also show values that are towards the extremes or even out of the range of the concentrations used for the experiments; this could make it difficult to accurately narrow down the constants. Changing the values of the concentrations used is the answer to this, but practically this could be difficult. At the lower levels the measurable rates are very low or there is little change of rate between the different concentrations, as in the inhibition by oxaloacetate, and at the other end higher concentrations of substrates can cause substrate inhibition and higher levels of inhibitor also have very low rates. Therefore it may prove very difficult to further narrow down the constants but more work should be carried out.

It appears from the values determined that the mitochondrial MDH is similar kinetically to the cytosolic form (Table XII), with there being no more than a five fold difference between any of the constants. From this it seems that most of the difference in direction of catalytic action *in vivo* for these two enzymes is due to differences in the environment in which each exists. For futher estimations on the rate of mMDH *in vivo*, values for the *in vivo* V_{max} of the enzyme in both directions are required. Because of the existence of the different isozymes in the cell the measurement of these values provide some further problems although these measurements could be attempted on isolated mitochondria. This may be an approach to try in the future.

TABLE XII

Comparison between constants for mitochondrial MDH determined by experiments with and without inhibitors and with those for cytosolic MDH

Constant	Mitochondrial MDH (μM)		Cytosolic MDH[a] (μM)
	Abscence of inhibitor	Presence of inhibitor	
K_a	72 ± 8	87 [b]	26 ± 3
K_{ia}	12 ± 2	110[b], 14.5 [c]	4.9 ± 0.6
K_b	86 ± 11	40 [b]	88 ± 4
K_{ib}	—	18 [b]	63 ± 5
K_p	1900 ± 400	1700 [b]	1100 ± 100
K_{ip}	....	15000 [b]	7100 ± 400
K_q	170 ± 30	630 [b]	114 ± 7
K_{iq}	1100 ± 120	4000[b], 1900[d]	940 ± 100

[a] Data from Crow *et al.* (1982).

[b] Average values from Table XI

[c] Value determined from K_{is} for linear non competitive inhibition by oxaloacetate using equation $K_{ia} = K_{is} K_a / K_b$, values determined in the abscence of inhibitors were used for K_a and K_b.

[d] Value determined from K_{is} for linear noncompetitive inhibition by malate using the equation $K_{iq} = K_{is} K_q / K_p$, values determined in the abscence of inhibitors were used for K_p and K_q.

Possibly the most important environmental factor affecting mMDH is the low levels of oxaloacetate found in the mitochondria (Srere, 1972; Seiss *et al.*, 1976). The likely build up of higher localised concentrations of oxaloacetate produced by mMDH, which would cause the reaction to go in the wrong direction, can be solved by the formation of enzyme-enzyme complexes between mMDH and other enzymes such as citrate synthase (Tompa *et al.*, 1987), mitochondrial aspartate amino transferase (Backman and Johansson, 1976), and NADH:ubiquinone reductase (Sumegi and Srere, 1984), complex I of the electron transport chain, which funnel off the products of the reaction and move the equilibrium to favour malate oxidation.

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