KINETIC STUDIES OF NAD-LINKED MALIC ENZYME FROM FLIGHT MUSCLE OF THE TSETSE FLY GLOSSINA AND THE BEETLE CATHARSIUS

By

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D E C L A R A T I O N

I, Mabel Opanda Imbuga, hereby declare that this thesis is my original work and it has not been presented to any other University.

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A C K N O W L E D G E M E N T S

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ABBREVIATIONS

AMP	Adenosine 5′ monophosphate
ADP	Adenosine 5′ diphosphate
ATP	Adenosine 5′ triphosphate
ADH	Alcohol dehydrogenase
Ac.CoA	Acetyl CoA
app	Apparent
Bis	NN-methylenebisacrylamide
CME	*Catharsius* malic enzyme
$CaCl_2$	Calcium chloride
C_3	Three carbon
C_4	Four carbon
CO_2	Carbon dioxide
Cit	Citrate
cyt. C	Cytochrome C
CHO	Carbohydrate
Cu^{2+}	Copper
DTT	Dithiothreital
E	Enzyme
EDTA	Ethylene diaminetetra acetic acid
EMD	Ethylmaleimide
ETS	Electron transport system
Fum	Fumarate
G6PD	Glucose-6-phosphate dehydrogenase
GME	*Glossina* malic enzyme
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate

HMB	Hydroxymercuribenzoate
Hg	Mercury
IEF	Isoelectrofocusing
IBA	Iodobenzoic acid
KCl	Potassium Chloride
$KClO_4$	Potassium perchlorate
K_i	Inhibitor binding constant
K_m	Michaelis constant
KOH	Potassium hydroxide
LDH	Lactate dehydrogenase
Me^{2+}	Metal ion
Mb	Myoglobin
Mal	Malate
Mn^{2+}	Manganese
Mg^{2+}	Magnesium
MDH	Malate dehydrogenase
ME	Malic enzyme
NAD	Nicotinamide-adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide-adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide-adenine dinucleotide phosphate (reduced)
NaN_3	Sodium azide
nH	the Hill coeficient
$(NH_4)_2SO_4$	Ammonium sulphate

NBT	Nitro blue tetrazolium
OAA	Oxaloacetic acid
PAGE	Polyacrylamide gel electrophoresis
PEP	Phosphoenol pyruvate
Pyr	Pyruvate
PCA	Perchloric acid
PK	Pyruvate kinase
PMS	Phenazine methosulphate
SDS	Sodium duodecyl sulphate
TCA	Trichloroacetic acid
TEA	Triethanolamine
Temed	N, N, N', N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
F-1:6-dip	Fructose 1,6,diphosphate
v/v	Volume : volume
w/v	Weight : volume
V_{max}	Maximum velocity (f = forward, (b = backward)
V_t	Enzyme activity left at time t
V_o	Initial velocity at time o
S	Substrate
$ZnCl_2$	Zinc chloride
MWC - Monod Wayman Changeux	

UNIT ABBREVIATION

g	Gram
mg	Milligram

µg	Microgram
min	Minute
sec	Second
ml	Millilitre
µl	Microlitre
M	Molar
mM	Millimolar
µM	Micromolar
nM	Nanomolar
mm	Millimeter
cm	Centimeter
nm	Nanometer

SUMMARY

1. Introduction

NAD malic enzyme (L-malate: NAD^+ oxidoreductase (decarboxylating) E.C.1.1.1.38 has been reported in a variety of organisms. Generally low activities have been found in vertebrate tissues, but high activities have been reported in certain invertebrate tissues, especially those having an operative proline catabolic pathway.

NAD and NADP-malic enzymes from various sources differ in their kinetic properties. For example, some show classical Michaelis kinetics, whilst others have aggregated subunits and strongly sigmoidal velocity-substrate relationships.

Some malic enzymes have been reported to be mitochondrial whilst others have been found to be cytoplasmic or even located in the plant chloroplast. Most of them have been found to be tetrameric with molecular weights in the range 200,000 - 280,000 daltons. Some malic enzymes however, especially from plant sources have been found to exist in a higher state of aggregation, at least under certain conditions of pH and salt concentration, and in the presence of substrates.

Malic enzymes have been implicated in various physiological roles, ranging from the supply of CO_2 for photosynthesis in plants to provision of pyruvate to facilitate oxidation of proline to alanine or to complete oxidation via the tricaboxylic acid cycle.

Malic enzymes appear to display different mechanisms of binding the two substrates. Some have been reported to have a completely random binding mechanism. Others such as the extensively studied pigeon NADP-malic enzyme were found to have a compulsory order of substrate addition, with NADP binding before malate. NAD malic enzyme from the beetle Catharsius was thought to have a compulsory order of substrate addition with malate binding before co-enzyme.

To obtain more information about binding mechanism, co-operativity, pH effects and the functional groups involved at the active site, and about the molecular mechanism of the reaction, the kinetic properties of NAD-malic enzyme from two insect flight muscle sources were studied and compared.

2. Comparison of Basic Properties of Glossina Malic Enzyme with those of Catharsius Enzyme

The K_m values for malate and NAD were determined at pH 7.8 for the Glossina enzyme and pH 7.2 for the Catharsius enzyme, at high (near saturating) concentrations of the other substrate, using Mg^{2+} or Mn^{2+} as metal activator. Values obtained with the Glossina enzyme, 1.17 mM for malate and 0.22 mM for NAD, are similar to those of the Catharsius enzyme (1.16 mM for malate and 0.3 mM for NAD.) at optimum pH 7.2 but much lower than those of the Catharsius enzyme at pH 7.8.

The Glossina enzyme showed little activity with NADP. The V_{max} obtained at pH 7.8 was only 4% of that with NAD and the K_m for NADP (.19 mM) was similar to that for NAD using 10 mM malate. Similar K_m results were obtained with the Catharsius enzyme but the activity with NADP as coenzyme was much higher (40% of that with NAD).

Substitution of Mn^{2+} for Mg^{2+} had little effect on the affinity of the Glossina enzyme for either substrate, whereas with the Catharsius enzyme, substitution of Mn^{2+} greatly reduced K_m values (to 0.66 mM for malate, 0.2 mM for NAD and 0.08 mM for NADP).

With Mg^{2+} as activator, the Glossina enzyme tended to give a sigmoidal velocity substrate curve. Normal Michaelis kinetics were achieved however, by inclusion of 2 mM fumarate in assays. The Catharsius enzyme was more strongly co-operative and needed higher fumarate concentrations (10 mM) to overcome sigmoidicity.

Certain protein properties of the Glossina malic enzyme were compared with those of Catharsius enzyme. Isoelectrofocusing on sucrose gradient revealed pI values of 6.0 for the Glossina enzyme and 5.4 for the Catharsius enzyme. This pI difference parallels a kinetic difference in the pH optima of the two enzymes (7.8 for the Glossina enzyme, 7.2 for the Catharsius enzyme at the same high fixed substrate concentrations).

The molecular weights of the two insect malic enzymes were determined on a sephacryl S-200 superfine column and by SDS gel electrophoresis. They were both found to be tetrameric with native molecular weight determined as 230,000 for the Glossina enzyme and 260,000 daltons for the Catharsius enzyme

3. Mechanism of Substrate Addition of the Glossina Malic Enzyme Reaction

With 2 mM fumarate present and at pH 7.8, the effect on velocity of varying each substrate at a number of fixed concentrations of the other substrate was investigated. Limiting K_m values for malate and NAD were determined as 0.18 mM and 0.20 mM respectively. $\bar{K}_{malate}$ and $\bar{K}_{NAD}$ (limiting K_m values when the other substrate concentration tended to zero) were 1.12 mM and 1.31 mM respectively.

The primary and secondary plot patterns obtained indicated that the reaction proceeded via a ternery complex, either via a random order rapid equilibrium, or fixed order steady state binding mechanism. If the mechanism was indeed random order rapid equilibrium then, since K_m values were much lower than $\bar{K}$ values, the binding of one substrate greatly enhanced the binding of the other.

The three products were each investigated as inhibitors with respect to each of the two substrates. Competitive inhibition was found only in the case of NADH (with respect to NAD) and CO_2 (with respect to malate). Pyruvate gave mixed inhibition with either substrate. This would fit with a random order mechanism in which however, HCO_3^- or CO_2, rather than pyruvate, is released last from the malate binding site. It would be more difficult to interpret in terms of a compulsory order mechanism.

4. Inhibition by Nucleotides and Substrate Analogues

With 2 mM fumarate again present and co-operativity therefore removed, various compounds were tested for their effects on malic enzyme activity. Most compounds that were competitive inhibitors with respect to malate gave mixed inhibition with respect to NAD. Fumarate stimulated the enzyme at low substrate concentrations but caused inhibition at high substrate concentration. Presumably fumarate acted as an analogue of malate and could bind at malate site. Malonate was a very strong inhibitor and was competitive with respect to malate but gave mixed inhibition with respect to NAD. Hydroxy malonate however gave mixed inhibition with respect to both substrates although it was very inhibitory. Ketomalonate caused the strongest inhibition of malic enzyme activity. ATP inhibited presumably as a result of binding at the NAD site. Because of the mixed inhibition obtained, it is assumed that these analogues can bind to the binary complex and dead end complexes can be formed. Generally compounds that were competitive inhibitors with one substrate gave mixed inhibition with the other implying that they could bind either to the free enzyme or to the binary complex. ADP was competitive with respect to both substrates, presumably it binds at the NAD site but only on the free enzyme.

5. pH Effect

The effect of pH on kinetics was studied thoroughly. Two substrate plot experiments were performed as before at a number of pH values between

6.6 - 8.4; $K_{m_{malate}}$, $\bar{K}_{malate}$, $\bar{K}_{NAD}$ and V_{max} were determined at each pH. Log plots of these parameters were made to obtain pK values which might relate to substrate, to free enzyme or to substrate complexed enzyme. Five pK values were identified, at pH 6.7 - 6.8, 7.2, 7.6, 7.8 and 8.2, none of which could correspond to an ionisation on either of the two substrates. These pK values must represent the titration of groups on the enzyme in, close to, or strongly affecting the active centre. The value $\frac{K_m}{\bar{K}}$, designated α, decreased from about unity at acid pH to a value of 0.06 at alkaline pH.

6. Studies of Co-operativity and the Effects of Activators and Inhibitors

The velocity-substrate relationship was determined for the Catharsius enzyme with and without various modifiers present.

With malate as varied substrate, the Hill coefficient obtained was 1.8 and seemed to be independent of NAD concentration. On the other hand, with NAD as varied substrate, the Hill coefficient or sigmoidicity appeared to be greater at lower concentrations of malate and a value of 2.0 was achieved with 2 mM malate.

A number of modifiers stimulated activity at low concentrations of malate by removing co-operativity; values of $S_{0.5}$ were generally not much altered. Substrates such as fumarate, aspartate, ATP tended to show a dual effect, stimulating at low concentration but inhibiting at high concentration. The inhibition is presumably a reflection of the tendency of these compounds to compete with substrates. A similar effect was found with the Glossina enzyme with nH values reduced to less than unity.

It was difficult to explain co-operativity in terms of a simple MWC model, especially the negative co-operativity displayed by the Glossina enzyme.

7. Other Activities of Malic Enzyme.

In the absence of Mn^{2+} or Mg^{2+}, the products obtained at pH 7.4, from incubations of malate and NAD, with the Glossina enzyme included OAA as well

as pyruvate i.e the enzyme displayed some MDH activity. Maximum rate of reduction of NAD observed in the absence of metal was 1.7% of the Mg^{2+} dependent malic enzyme activity at pH 7.8. The K_m of this metal independent activity for malate was very high (greater than 100 mM). Metal independent activity, probably MDH activity with V_{max} similar to that at pH 7.4 persists at pH 10, but K_m was a little lower (22 mM). In absence of metal ion an OAA reductase activity could also be observed.

The enzyme did not appear able to catalyse the reduction of lactate with NAD. It had high OAA decarboxylase activity, however, which appeared to depend on Ca^{2+} and which showed a low pH optimum. The reversal of malic enzyme activity, i.e reductive carboxylation of pyruvate, was not detected even with high concentrations of substrate.

For comparison, a true MDH activity was partially purified from Glossina flight muscle and some kinetic parameters determined at pH 10. It was found to have K_m for malate of 1.23 mM and for NAD 0.2 mM, far lower than values obtained for the metal independent activity of malic enzyme at pH 10.

These side activities of malic enzyme are consistent with a mechanism in which enzyme-bound

OAA is a reaction intermediate, with reduction preceding decarboxylation. The retention of OAA at the active centre and the subsequent decarboxylation requires a divalent metal activator.

8. Inactivation and Protection Studies

Specific amino acid inhibitors and heavy metals were used in inactivation studies of the Glossina enzyme. Of the sulfhydril group inhibitors tested, p-hydroxy-mercuribenzoate had the strongest effect. At about 1 μM, this compound completely abolished activity. Mercuric compounds were also particularly inhibitory, with full inactivation of the enzyme after incubation with 10 mM mercuric chloride.

Malate, fumarate and NADH protected the enzyme slightly against sulfhydril inhibitors, but more effectively against the imidazole reagent, diethyl-pyrocarbonate and against inactivation by 0.6 M urea.

CHAPTER I

INTRODUCTION

THE STUDIES OF CO-OPERATIVITY AND MECHANISM OF ACTION ON INSECT NAD-LINKED MALIC ENZYME

1.1 SUMMARY OF EARLY REPORTS OF PRESENCE OF ENZYME

NAD-linked malic enzyme (L-malate: NAD^+ oxidoreductase (decarboxylating) E.C.1.1.1.38 was first noted in micro-organisms (Korkes et al 1950, Takeo et al 1967) and in plants (Macrea and Moorhouse 1970, Coleman and Palmer 1972). In invertebrates, it has been reported in flight muscle of the Japanese beetle, Popillo japonica (Hansford and Johnson 1975), and the tsetse fly, Glossina (Hoek et al 1976), in Ascaris, (Fodge et al 1972) and in flatworms, Hymenolepis diminuta (Li et al 1972). It was first reported in vertebrates by Mounib and Einsan (1972 a, b) but has now been found in a variety of vertebrate tissues, including rabbit heart, (Lin and Davis 1974) calf adrenal cortex, (Sauer 1973) and rat liver, (Sauer et al 1980, Nagel and Sauer 1982). Levels of NAD-malic enzyme in vertebrate tissues have always been found to be low, whereas quite high activities are sometimes present in invertebrates. In plants, low levels of the enzyme have been found in many species with a C_3 type of photosynthesis; by contrast, high levels have been reported in those using a C_4 strategy

and utilising aspartate as a source of photosynthetic CO_2 (Hatch and Kagawa 1976, Hatch et al 1974) as well as in some plants exhibiting crassulacean acid metabolism (CAM) (Dittrich 1976, Day 1980).

The related NADP-linked malic enzyme was known earlier in animal tissues (Ochoa et al 1947) and has been more extensively studied, especially the enzyme from pigeon liver (Hsu et al 1967, Hsu et al 1976, Schimerlik and Cleland 1977), but also that from other organisms and tissues, as diverse as Drosophila (Geer et al 1980) rat skeletal muscle (Swierzynski et al 1980a) and crustacean tissues (Swierzynski et al 1980b).

1.2 CELLULAR LOCATION

The NAD-malic enzymes so far studied in vertebrates and invertebrates have appeared to be mitochondrial (Mandella and Sauer 1975, Lin and Davis 1974, Hoek et al 1976, Allen and Harris 1980), and no cytoplasmic activity has been conclusively demonstrated. In higher plants too, they have been found to be associated with the mitochondria (Hatch and Kagawa 1976, Macrea 1971, Valenti and Pupillo 1981, Day et al 1984), and no activity has been detected in the chloroplast fraction (Dittrich 1976).

In contrast, NADP-linked malic enzyme was at first thought to be exclusively cytosolic. A mitochondrial localisation was observed however, by Kortzerke (1963) and by Henderson (1966) in mice and dual distribution of NADP-linked malic enzyme has now been established in a number of tissues (Frenkel 1971, Mandella and Sauer 1975).

In vertebrates, particular tissues tend to have a characteristic localisation of the NADP-malic enzyme: Thus, in liver and adipose tissues, it is exclusively cytosolic (Martin and Denton 1970), whereas in kidney and heart, it is found to be mainly mitochondrial (Hendersen 1966, Frenkel 1972, Nagel et al 1982).

1.3 ROLES IN METABOLISM

Malic enzyme has been implicated in a number of functional roles in different tissues studied. In plants it has been implicated in the regulation of the NAD^+/NADH ratio of the cytosol through the combined operation of mitochondrial NADH dehydrogenases and malic enzyme (Coleman and Palmer 1972, Day and Wiskitch 1974). Thus, it is involved in the transhydrogenase across the inner mitochondrial membrane of plants which transfers reducing equivalents from within the matrix to extramitochondrial NAD^+.

It is also known that the malate pool in plants is large and may function as an intermediary 'carbohydrate' store in the cell vacuole (Mazellas and Vennesland 1957). During conditions of low glycolysis when the TCA cycle has slowed down, as is found in dormant tissue, NAD-malic enzyme can oxidise this stored malate via the respiratory chain to provide energy, or alternatively, the pyruvate produced could be utilised in lipid and amino acid biosynthesis (Coleman and Palmer 1972). Therefore, in plants, it seems that NAD-malic enzyme may play a crucial role in regulating energy supply by the TCA-cycle (Canellas and Wedding 1980, Papa et al 1970). In this situation, plant malic enzyme will also play a role in controlling levels of CO_2 and will provide a means of oxidising TCA-cycle compounds to pyruvate via malate without relying entirely on pyruvate kinase.

NAD-malic enzyme may also have an important role in the channelling of four carbon (C_4) acids to acetyl CoA for complete oxidation. This could be achieved by conversion to malate via the TCA cycle, followed by decarboxylation to pyruvate and finally to acetyl CoA for complete oxidation. In addition to providing respiratory substrate, malic enzyme can serve a major role in photosynthetic cells by providing CO_2 for fixation in the calvin cycle. This is seen in some plants exhibiting crassulacean acid

metabolism (CAM) (Dittrich 1976) and some four carbon (C_4) plants. (Hatch and Kagawa 1974, Day 1980).

C_4 plants are subdivided into three classes

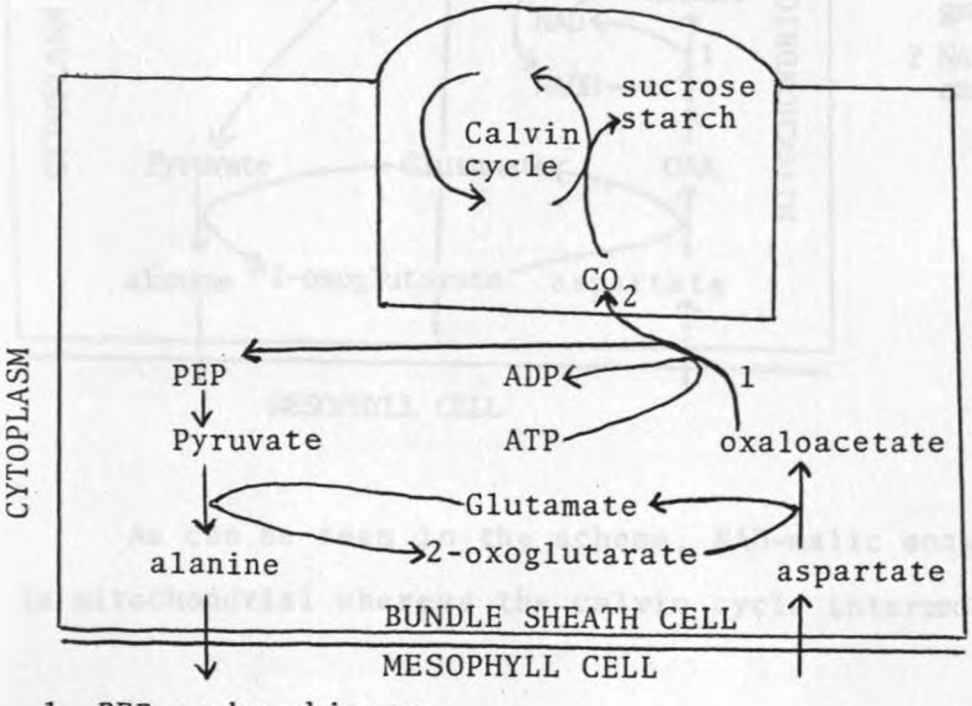
a) The PEP carboxykinase type

b) The NAD-malic enzyme type

c) The NADP-malic enzyme type

In the PEP-carboxykinase type, decarboxylation in bundle sheath cells is brought about by PEP-carboxykinase. CO_2 generation does not involve malic enzyme at all. The PEP formed after decarboxylation of the C_4 acids is converted to pyruvate and finally alanine which then leaves the bundle sheath cell. Aspartate from mesophyll cells is the CO_2 carrier and is converted to OAA for decarboxylation as is depicted in the scheme below.

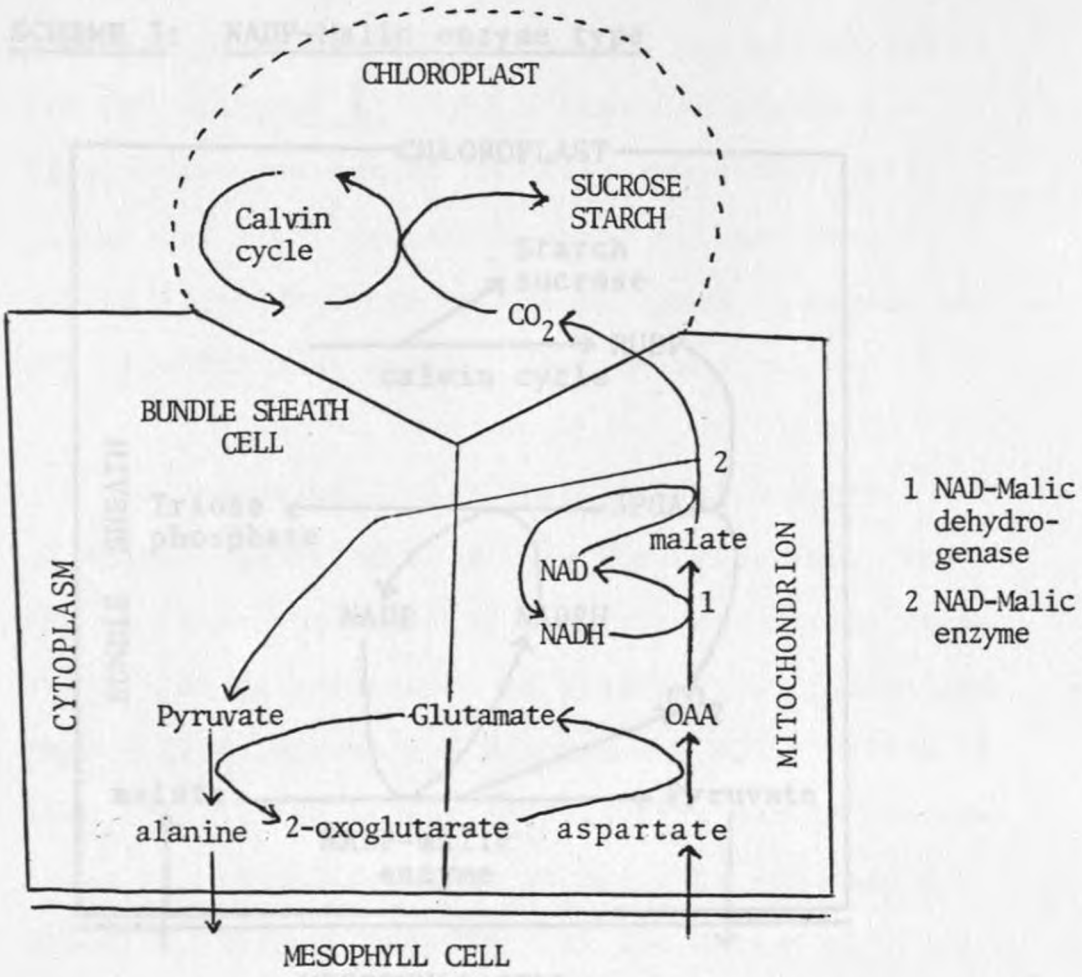
SCHEME 1: PEP carboxykinase type



1 PEP carboxykinase

With the NAD-malic enyzme type, the malic enzyme is located in the mitochondrion and only interacts with the calvin pathway by supplying CO_2. The pyruvate formed may later be converted to alanine, then to aspartate to complete the cycle as shown below.

SCHEME 2:
NAD-malic enzyme type

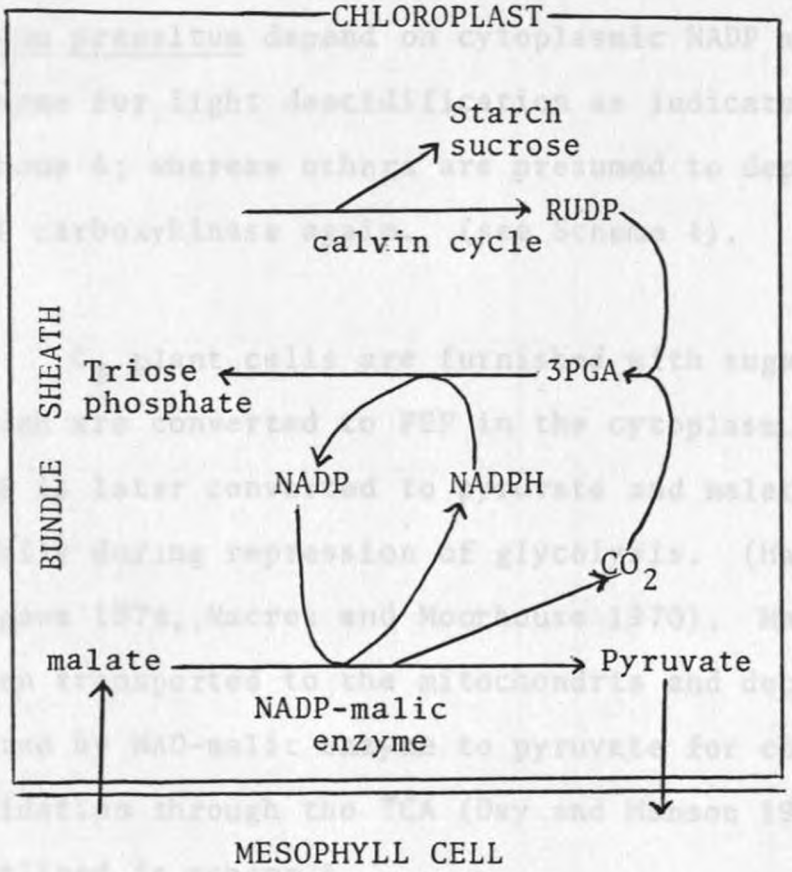


As can be seen in the scheme, NAD-malic enzyme is mitochondrial whereas the calvin cycle intermediates

are in plastids; an interesting example of metabolic interaction between organelles must therefore be in operation.

In the NADP-malic enzyme C_4 type, malic enzyme is localised within the chloroplast and appears to be directly involved in the calvin pathway, supplying both CO_2 and NADPH as outlined below.

SCHEME 3: NADP-Malic enzyme type



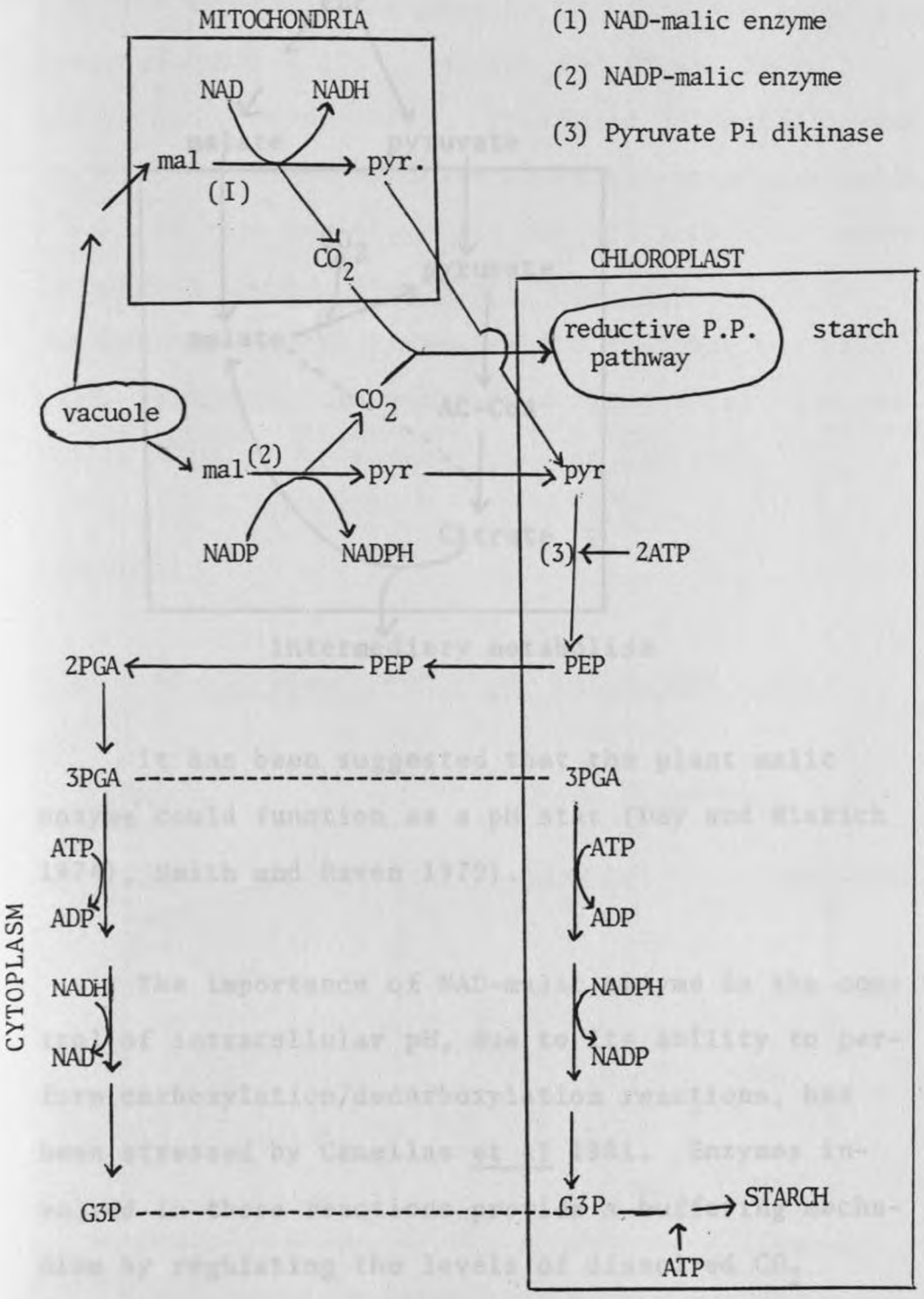
CAM plants fix atmospheric CO_2 into malic acid during the night (dark acidification) and release it

(light deacidification) for photosynthetic utilisation during the day. In some species, the generation of CO_2 in the day time depends on the action of NAD-malic enzyme (Chapman and Hatch 1977, Dittrich 1976). The malate formed in the cytoplasm at night by the activities of PEP carboxylase and MDH is stored in a vacuole. From here, it is transported to the mitochondrion during the day and decarboxylated, thus furnishing the calvin pathway with CO_2, whilst the pyruvate skeleton can be utilised for starch synthesis (Spalding et al 1976). Some CAM plants i.e. Sedum praealtum depend on cytoplasmic NADP malic enzyme for light deacidification as indicated in scheme 4; whereas others are presumed to depend on PEP carboxykinase again. (see Scheme 4).

C_3 plant cells are furnished with sugars, which are converted to PEP in the cytoplasm. The PEP is later converted to pyruvate and malate especially during repression of glycolysis. (Hatch and Kagawa 1976,,Macrea and Moorhouse 1970). Malate is then transported to the mitochondria and decarboxylated by NAD-malic enzyme to pyruvate for complete oxidation through the TCA (Day and Hanson 1977) as outlined in scheme 5.

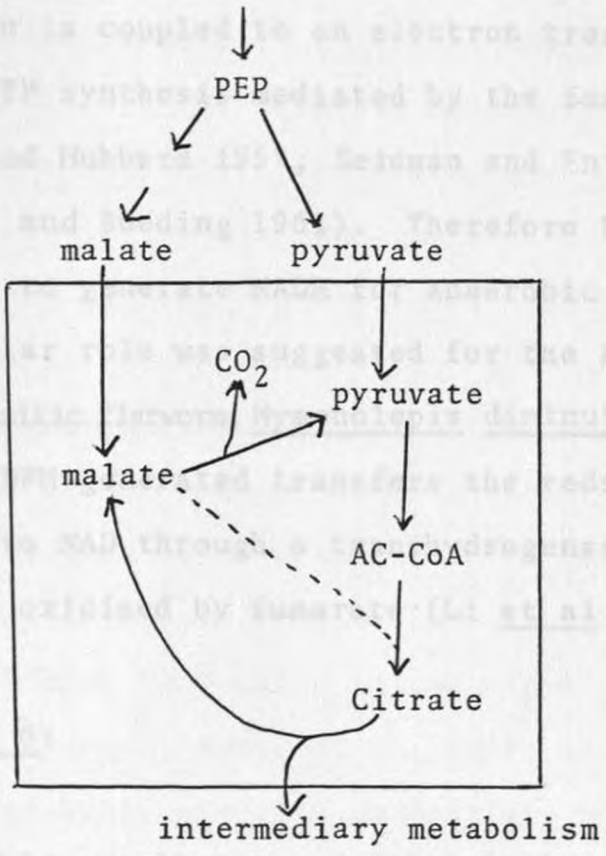
SCHEME 4:

Schemes for carbon flow in CAM plants during light deacidification



SCHEME 5:

Utilisation of 3 carbon sugars by C_3 plants



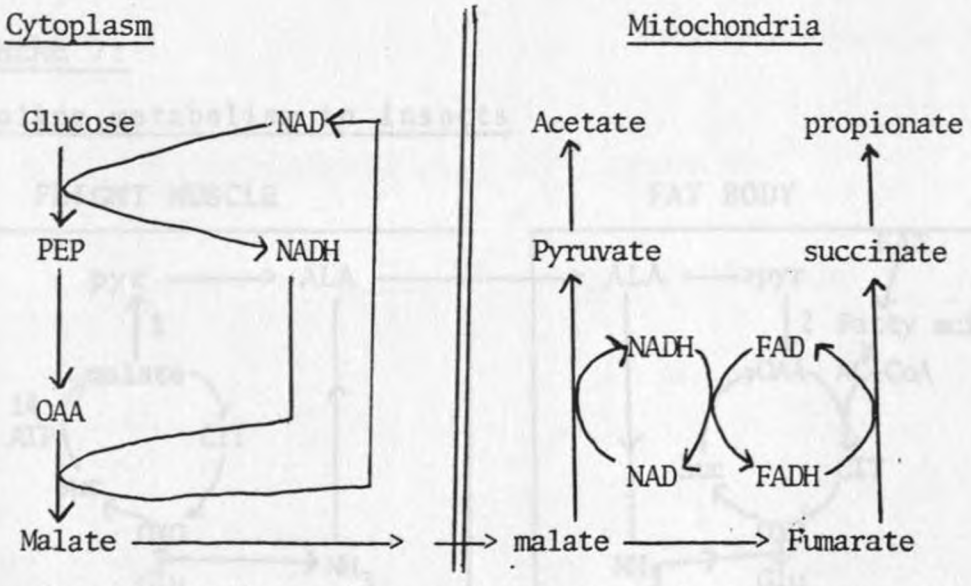
It has been suggested that the plant malic enzyme could function as a pH stat (Day and Wiskich 1974), Smith and Raven 1979).

The importance of NAD-malic enzyme in the control of intracellular pH, due to its ability to perform carboxylation/decarboxylation reactions, has been stressed by Canellas et al 1981. Enzymes involved in these reactions provide a buffering mechanism by regulating the levels of dissolved CO_2 (Davis and Patil 1974).

In Ascaris, NAD-malic enzyme generates reducing equivalents in the form of NADH, used to convert fumarate to succinate (see scheme 6). This reduction is coupled to an electron transport associated ATP synthesis mediated by the fumarate reductase (Saz and Hubbard 1957, Seidman and Entner 1961, Kmetec and Bueding 1961). Therefore NAD-malic enzyme serves to generate NADH for anaerobic phosphorylation. A similar role was suggested for the NADP-malic enzyme in parasitic flatworm, Hymenolepis diminuta, in which the NADPH generated transfers the reducing equivalents to NAD through a transhydrogenase and is eventually oxidised by fumarate (Li et al 1972).

SCHEME 6:

Anaerobic succinate production in Ascaris muscle

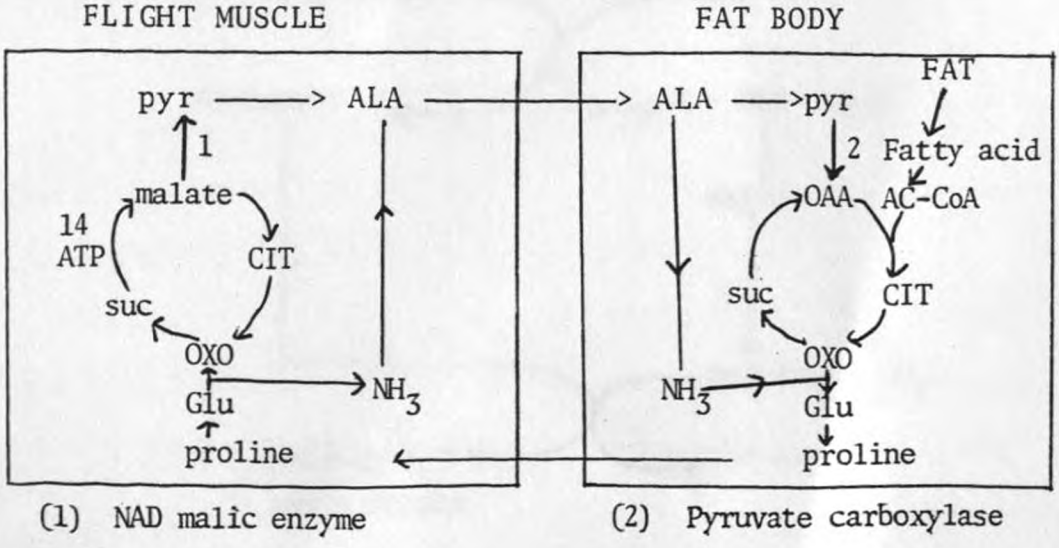


These helminths are known to accumulate large amounts of succinate and propionate.

NAD-malic enzyme has been implicated in several insects in the proline pathway, where energy for flight is supplied by proline catabolism; for example, in the dung beetles, Catharsius and Heliocopris (Imbuga 1979), the colorado beetle, Leptinotarsa (Weeda et al 1980). The tsetse fly Glossina (Bursell and Slack 1976), the Japanese beetle, Popillio (Hansford and Johnson 1975) and even in the blow fly, Sarcophaga (Olembo and Pearson 1982). The pyruvate formed from malate decarboxylation is converted to alanine by alanine aminotransferase. The alanine is then presumed to be transported to the fat body for the resynthesis of proline in which Weeda et al (1981) suggest that NADP-malic enzyme plays an important role. However, Konji et al (1984) have argued that it is probably pyruvate carboxylase which is involved here, converting pyruvate into OAA as outlined in scheme 7.

SCHEME 7:

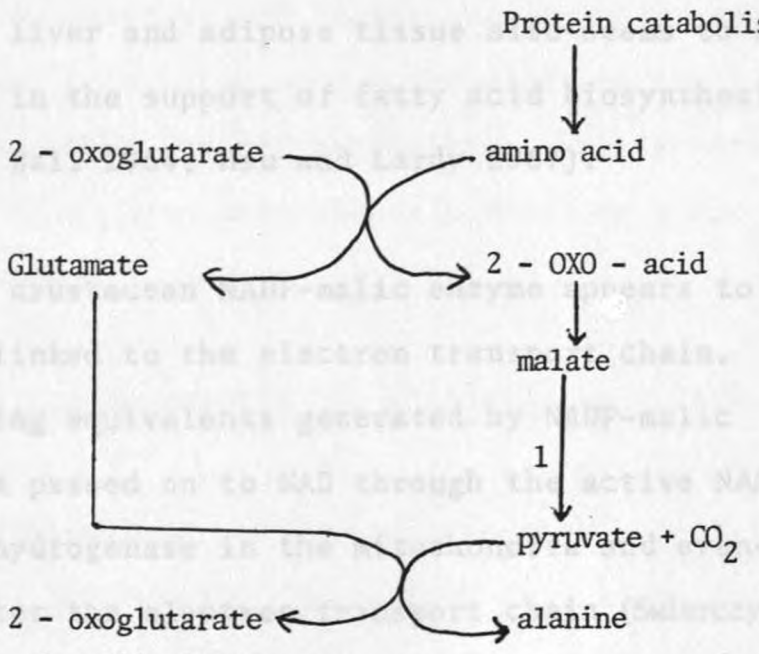
Proline metabolism in insects



(1) NAD malic enzyme (2) Pyruvate carboxylase

In vertebrates, a proposed role for NAD-malic enzyme is that of maintaining flux through part of the TCA cycle, producing pyruvate from non - CHO substrates such as glutamate, glutamine and propionate (Mandella and Sauer 1975, Nagel and Sauer 1982) especially in the small instestinal mucosa whose major respiratory fuels are known to be glutamine and ketone bodies (Windmueller and Spaeth 1978). Malic enzymes in muscle have been implicated in the provision of pyruvate, and finally alanine, from protein catabolism as presented in the following scheme 8 (Davis and Bremer 1973).

SCHEME 8: Suggested pathway for nitrogen disposal by muscle, coupled to intermediate synthesis and breakdown of TCA intermediates derived from amino acids



1 malic enzyme.

The NAD-malic enzyme from undifferentiated hepatomas has been suggested to have a role similar to that outlined for the NADP-malic enzyme above, and appears to be involved in net glutamine oxidation i.e. in facilitating the oxidation of glutamine to pyruvate (Sauer et al 1980, Sauer and Dauchy 1978). Glutamine has been shown to be an important respiratory fuel in these cells (Reitzer et al 1979).

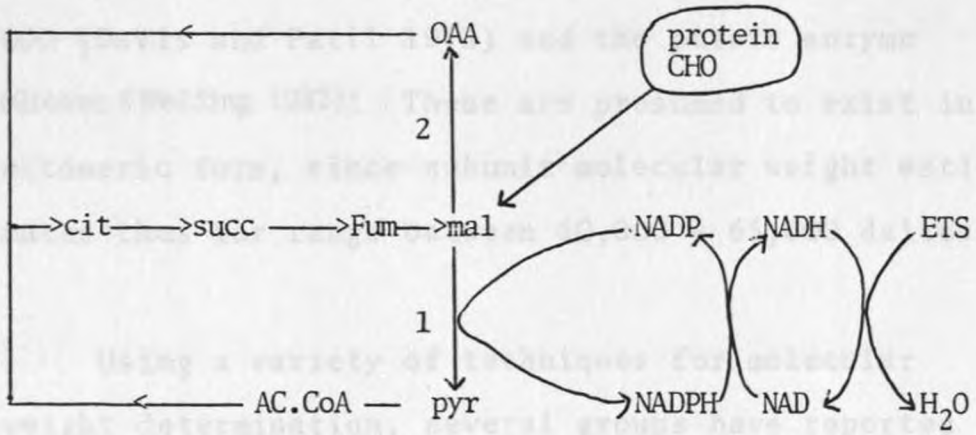
The vertebrate mitochondrial NADP-malic enzyme however seems to function in situations where the generation of NADPH is important; e.g. for β-hydroxylation reactions, as in adrenal steroid biosynthesis (Simpson and Estabrook 1969). A role in NADP reduction has been associated with the bacterial NADP-malic enzyme, which appears to provide reducing powers for lipogenesis (Sanwal and Smando 1969). The cytoplasmic NADP-malic enzyme of avian liver, mammalian liver and adipose tissue also seems to be important in the support of fatty acid biosynthesis (Wise and Ball 1964, Hsu and Lardy 1967).

The crustacean NADP-malic enzyme appears to be directly linked to the electron transport chain. The reducing equivalents generated by NADP-malic enzyme are passed on to NAD through the active NADPH: NAD transhydrogenase in the mitochondria and eventually enter the electron transport chain (Swierczynski

et al 1980) as shown in scheme 9.

SCHEME 9:

Metabolic role of NADP-malic enzyme in crayfish



(1) NADP-malic enzyme

(2) Malate dehydrogenase

Thus the NADPH formed during malate decarboxylation is oxidised by NAD in the transhydrogenase reaction and eventually enters the electron transport system (ETS) for ATP production. Malate formation may take place from either protein or carbohydrate breakdown.

1.4 PHYSICAL PROPERTIES AND MOLECULAR MECHANISM

Investigations so far indicate that NAD-malic enzymes are tetrameric, composed of four identical

subunits. The native enzyme from various sources has a molecular weight ranging from 200,000 - 280, 000 daltons (Fodge et al 1972, Yamaguchi et al 1973, Lin and Davis 1974, Norden and Matanganyidze 1977, Sauer 1982, Weeda et al 1981). Exceptions are the cauliflower enzyme, which had a molecular weight of 400, 000 (Davis and Patil 1975) and the potato enzyme (Grover & Wedding 1982); These are presumed to exist in octomeric form, since subunit molecular weight estimates thus far range between 60,000 - 65,000 daltons.

Using a variety of techniques for molecular weight determination, several groups have reported NADP-malic enzyme to be more variable in its degree of aggregation. Spina et al (1970) found a molecular weight of 550,000 for the E.coli enzyme. After fully dissociating it into its component polypeptide chains with 6 M guanidine hydrochloride, the molecular weight was 61,000. The E. coli enzyme thus appeared to be composed at eight subunits, as compared to various other NADP-malic enzymes that were found to be tetrameric (Sanwal and Smando 1969, Hsu and Lardy 1967, Silpana and Goodridge 1971). A dimeric form of NADP-malic enzyme has also been reported for the tapeworm enzyme (Li et al 1972). Whereas Hendersen (1966) and Shows and Ruddle (1968) have proved the mouse Mus musculus liver NADP-malic enzyme exists as an isologous

tetramer in which a pair of symmetrical dimers are held in the tetrameric conformation by weak forces.

Apart from the oxidative decarboxylation of malate, there are several other enzymatic activities associated with the malic enzyme molecule. Depending on the specificity for the coenzymes, and whether or not the enzyme will decarboxylate oxaloacetate, three different classes of malic enzyme have been proposed.

(1) Those that utilise NADP and decarboxylate oxaloacetate as well E.C.1.1.1.40 (Hsu and Lardy 1967).

(2) Utilise NAD and do not decarboxylate OAA E.C.1.1.1.39 (Fodge et al 1972).

(3) Utilise NAD and decarboxylate OAA E.C.1.1.1.38 (Hoek et al 1976).

Most malic enzymes have been shown to catalyse the decarboxylation of oxaloacetate. Some can catalyse this activity with high efficiency, but its physiological significance is questionable as the pH optimum is typically well below the physiological range (Korkes et al 1950, Hsu and Lardy 1967, Li et al 1972, Hoek et al 1976, Norden and Matanganyidze

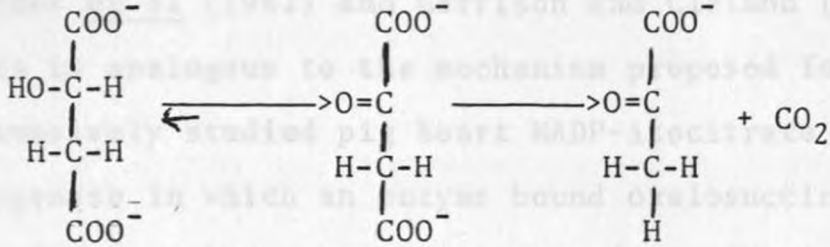
1977). However, some malic enzymes appear to be devoid of this oxaloacetate decarboxylase (Saz and Hubbard 1957, Fodge et al 1972, Lin and Davis 1974, Frenkel 1975, Mandella and Sauer 1975, Kagawa and Hatch 1975).

Also associated with the malic enzyme molecule is the reductive carboxylation of pyruvate (the reverse reaction). Malic enzymes from several sources catalyse the reverse activity but with extremely high K_m values, especially that for pyruvate (Norden and Matanganyidze 1977, Hsu et al 1967, Macrea 1971, Frenkel 1972, Landsperger et al 1978, Swierczynski et al 1980). It is unlikely that pyruvate reduction is a major physiological activity of these enzymes because such high substrate concentrations do not normally occur within the cell. With some malic enzymes, it has not been possible to demonstrate the reverse reaction (eg. Hoek et al 1976).

An OAA reductase reaction has also been found to reside in the malic enzyme molecule. This is typically only a minor activity with a V_{max} in the range 2-15% of the oxidative decarboxylation of malate (e.g Hsu and Lardy 1967, Hsu 1970). Because of this and the high K_m values for OAA, it is unlikely that the OAA reductase reaction plays an important role in the cellular functions of these

enzymes.

A molecular mechanism of catalysis has been proposed for the pigeon liver cytoplasmic NADP-malic enzyme (Hsu 1970, Rutter and Lardy 1958) in which bound OAA acts as an intermediate. Thus, during catalysis, oxidation occurs before decarboxylation.



This was deduced from the ability of the enzyme to react with and thus presumably bind OAA. The enzyme also shows an OAA decarboxylase activity. Both OAA decarboxylase and reductase activities depend on Mn^{2+}.

It is likely that during this OAA reduction activity, the enzyme forms a central

$$E\begin{matrix}\swarrow \text{NADPH} \\ \searrow \text{OAA}\end{matrix}$$

ternary complex which converts to

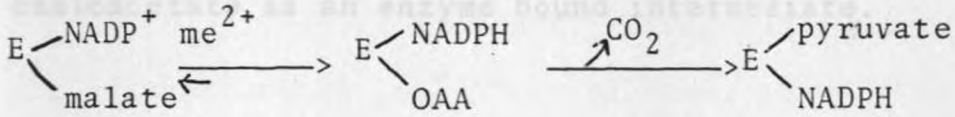
$$E\begin{matrix}\swarrow \text{NADP}^{+} \\ \searrow \text{malate,}\end{matrix}$$

before releasing malate and NADP (Dalziel and Londesborough 1968). The same

$$E\begin{matrix}\swarrow \text{NADPH} \\ \searrow \text{OAA}\end{matrix}$$

ternary complex might well be

involved in the normal conversion of malate to pyruvate.



The above molecular mechanism, with OAA as an intermediate, has been proved to be correct by Hermes et al (1982) and Garrison and Cleland (1985). This is analogous to the mechanism proposed for the extensively studied pig heart NADP-isocitrate dehydrogenase in which an enzyme bound oxalosuccinate was found to be a reaction intermediate (Moyle 1956, Siebert et al 1957).

These various activities tend to have different metal ion requirements, e.g. oxidative decarboxylation of malate invariably needs Mg^{2+} or Mn^{2+}, whereas OAA decarboxylase may need Ca^{2+} and the OAA reductase reaction may proceed without metal activator.

The similarities between isocitrate dehydrogenase and NADP-malic enzyme were first pointed out by Ochoa (1951). In the case of NAD-malic enzyme, both OAA decarboxylase and OAA reductase activities could be demonstrated with the enzyme from the dung beetle (Imbuga 1979) and the tsetse fly (Hoek et al 1976). It seems probable that NAD-malic enzyme from both

these insects has a molecular mechanism similar to that of the pigeon liver NADP-linked enzyme, with oxaloacetate as an enzyme bound intermediate.

A chemical mechanism has also been postulated for pigeon liver NADP-malic enzyme (Schimerlik and Cleland 1977) in which the first step involves a hydride transfer; and again OAA is postulated as an enzyme bound intermediate.

1.5 K_m VALUES AND SUBSTRATE BINDING MECHANISM

Kinetic studies have been made of several NADP-linked as well as NAD-linked malic enzymes from various sources in order to gain insight into the mechanism and order of substrate binding and product release.

The cytoplasmic NADP-malic enzyme has been reported to have rather low K_m values for both NADP and malate (1.4 - 45 µM and 86 - 670 µM respectively) (Hsu and Lardy 1967, Davis and Patil 1974). However, the mitochondrial NADP-linked as well as NAD-malic enzymes appear to have higher K_m values for both substrates (not less than 1 mM for malate and 55 µM for NAD(P)) (Hoek *et al* 1976, Landsperger *et al* 1978, Hatch *et al* 1974). It appears that, *in vivo*, the mitochondrial malic enzymes may frequently

be substrate limited.

All malic enzymes have a general requirement for divalent metal ion. Most of these can utilise either Mg^{2+} or Mn^{2+} efficiently (Spalding et al 1950, Dittrich 1976, Landsperger et al 1978). However NAD-malic enzyme from photosynthetic tissues, of some CAM plants (e.g crassulaceae family), and some C_4 plants (e.g those that utilise aspartate for a source of CO_2), are reported to be quite specific for Mn^{2+} which cannot be replaced by Mg^{2+}. (Hatch et al 1974, Dittrich 1976, Rathman 1978). On the other hand, the enzyme from non-photosynthetic tissues of some C_3 plant sources (e.g. Helianthus sp., cauliflower), prefers Mg^{2+} to Mn^{2+} (Coleman and Palmer 1972, Macrea 1971, Hirai 1978).

Some malic enzymes are entirely NADP-dependent. This is typical of cytoplasmic enzymes such as that from pigeon liver (Hsu et al 1967), rat liver and skeletal muscle (Zahiten and Nastek 1979). Others are highly specific for NAD (e.g. the enzyme from rabbit heart (Lin and Davis 1974), Kalanchoe sp (Dittrich 1976)). Others show an ability to work with NAD and also to a limited extent with NADP (e.g. the enzyme from tsetse fly flight muscle, Hoek et al 1976 ; citrus fruit, Hirai 1978 ; potato, Grover et al 1981).

Malic enzymes of course have two substrates, the pyridine nucleotide coenzyme and malate. For a two substrate enzyme catalysed reaction, there are several possible systems of substrate binding that can be envisaged.

a) A double displacement mechanism

b) Mechanisms with a ternary complex:

(i) Steady state random ordered substrate binding

(ii) Steady state compulsory ordered substrate binding

(iii) Rapid equilibrium random ordered substrate binding

(iv) Rapid equilibrium compulsory ordered substrate binding

These mechanisms can be differentiated on the basis of primary plot characteristics as well as the patterns of product inhibition obtained.

1.5.1 The double displacement (Ping Pong) mechanism

In this mechanism, the first product is released before the second substrate binds, and a stable modified form of the enzyme is formed, without the occurrence of kinetically significant amounts of ternary complex (Eccleston *et al*. 1979, Burger

and Gorish 1981).

The initial rate equation relating to this mechanism is

$$v = \frac{V[A][B]}{K_a[B] + K_b[A] + [A][B]} \quad (1)$$

Where A and B represent the concentration of the first substrate A and second substrate to bind B. and K_a and K_b represent their corresponding Michaelis constants; V is the maximum initial velocity; or in reciprocal representation:-

$$\frac{1}{v} = \frac{K_b}{V}\left(\frac{1}{[B]}\right) + \frac{1}{V}\left(1 + \frac{K_a}{[A]}\right) \quad (2)$$

The mechanism is characterised by families of parallel double reciprocal plots.

No malic enzyme studied thus far has shown kinetics conforming to this mechanism.

1.5.2 The Steady State Random Ordered System

Such a system has a rate equation of the form:

$$v = \frac{V\left(K_a[A][B] + K_b[A]^2[B] + [A][B]^2\right)}{K_c + K_d[A] + K_e[B] + K_f[A]^2 + K_g[B]^2 + K_h[A][B] + K_i[A]^2[B] + [A][B]^2} \quad (3)$$

where K values are various constants and V is the maximum initial velocity.

This mechanism is characterised by non-linear reciprocal plots of $\frac{1}{v}$ versus $\frac{1}{[A]}$ or $\frac{1}{[B]}$ and can easily be distinguished from the others. The sigmoid kinetic behaviour displayed by malic enzymes studied thus far, can be overcome by addition of activators and appears to be due to co-operative binding (see chapter V) rather than to kinetic effects.

1.5.3 The Steady State Compulsory Ordered System

The initial rate equation for this mechanism is:-

$$v = \frac{V[A][B]}{\bar{K}_a K_b + K_a[B] + K_b[A] + [A][B]} \qquad (4)$$

K_a and K_b have the same meaning as defined before

$\bar{K}_a$ is the dissociation constant for the E-A complex

The primary double reciprocal plots intersect behind the $\frac{1}{v}$ - axis, with either A or B as variable substrate. The binding of one substrate (i.e A) on the enzyme is obligatory before the other substrate (i.e B) can bind. The last product released

(modified A) should inhibit competitively with respect to A but show mixed behaviour with respect to B. The first product released will not on the other hand inhibit competitively with respect to either substrate.

1.5.4 The Rapid Equilibrium Random Ordered Mechanism

The rate equation, primary and secondary plot patterns for this mechanism are same as for the steady state compulsory ordered system discussed above. The product inhibition patterns however, should be different. If no dead end complex formed, each product should inhibit competitively with respect to both substrates overcome however by saturating with second substrate. If dead end complex is formed, competitive inhibition is obtained with respect to the corresponding substrate, and is not overcome by saturation with the second substrate. Moreover inhibition with respect to 2nd substrate is mixed rather than competitive.

For a BI-TER reaction in which an initial bound product Q splits to form two final products R and S as represented below

$$A + B \longrightarrow P + Q \longrightarrow P + R + S$$

then random equilibrium binding would still be expected to lead to a situation where at least two of the products should inhibit competitively with respect to A and B respectively.

If binding of A and B are completely random and independent of each other, then we have a special case which can be recognised. The double reciprocal plots then intersect on the x-axis and K_a values are independent of the concentration of second substrate.

As stated earlier, it is possible to get dead end E-malate-NADH complex forming and this case NADH will give mixed inhibition with malate as the variable substrate as is the case in steady state compulsory BI-TER system. In such a case, alternate and substrate analogues, as well as the reverse reaction have to be employed to differentiate the two mechanisms.

The Ascarid NAD-malic enzyme appears to follow a random rapid equilibrium system. Landsperger et al (1978), using a variety of techniques, including isotope partitioning, concluded that either NAD

or malate could bind to the enzyme first in the absence of the other. With this enzyme, NADH inhibited competitively with respect to malate as the variable substrate. This combined with the primary plot patterns supported a random order mechanism and implied that either NADH or malate could bind to the free enzyme. It is also possible that a Theorell-Chance mechanism, in which the ternary complex does not accumulate, and the only rate limiting step is the release of first product, was being followed. In this mechanism, the second substrate added and the first product released show competitive inhibition (Theorell and Chance 1951). However the Theorell-Chance mechanism can only be accepted if it's proved that no kinetically significant steady state concentration of the ternary complex are in the system. The fact that NAD could displace the Ascarid enzyme from an NAD-agarose column went further to prove the ability of NAD to bind to free enzyme (Allen and Harris 1981).

The random rapid equilibrium BI-TER mechanism appears to be followed by other malic enzymes, including the cauliflower enzyme (Valenti and Pupillo 1981, Macrea 1971). A random rapid equilibrium mechanism has been postulated for the similar NADP-isocitrate dehydrogenase of Mytilus edulis (Head 1980).

1.5.5 The Rapid Equilibrium Compulsory Ordered Mechanism

This mechanism involves the obligatory binding of one substrate before the other, and the step involving the addition of the first substrate to the enzyme maintains equilibrium. With this mechanism, the first substrate to bind (A) can be identified, because double reciprocal plots of $\frac{1}{v}$ versus $\frac{1}{[B]}$ intersect on the $\frac{1}{v}$-axis, implying that apparent V_{max} values are independent of the concentration of A.

The rate equation simplifies to

$$v = \frac{V\,[A][B]}{\bar{K}_a K_b + K_b[A] + [A][B]} \qquad (5)$$

where $\bar{K}_a$, K_b have same meaning as defined earlier.

K_a is equal to zero, meaning that the apparent K_m for A approaches zero as the concentration of B becomes larger. This can be seen from the fact that slope replots against $\frac{1}{[B]}$ intersect the origin.

A rapid equilibrium ordered mechanism is characterised by the same product inhibition patterns as the steady state ordered mechanism.

Such a mechanism appears to be followed by some malic enzymes. The NADP-malic enzyme from pigeon liver has been extensively studied in this respect (Hsu et al 1967, Hsu 1970). The initial velocity and product inhibition data obtained, as well as the coenzyme binding studies, indicate a sequential ordered mechanism with NADP binding first. This was confirmed by further studies utilising inhibitors and alternate substrates (Schimerlik and Cleland 1977). The finding that coenzyme binds to the enzyme first is consistent with most pyridine nucleotide linked dehydrogenases e.g Lactate dehydrogenase (Zewe and Fromm 1962) MDH (Raval and Wolfe 1962) ADH (Wratten and Cleland 1965) GDH (Freiden 1959) Glyceraldehyde 3 phosphate dehydrogenase (Orsi and Cleland 1972) as well as for Horse liver aldehyde dehydrogenase (Feldman and Weiner 1972).

In the case of the pigeon liver enzyme, release of products occurs in the order bicarbonate, pyruvate and finally NADPH. This was deduced from the fact that NADPH was the only competitive inhibitor with respect to $NADP^+$.

Other malic enzymes that seem to follow this mechanism include that from the plant, Helanthus tuberosus (Coleman and Palmer 1972), the E. coli enzyme (Sanwal and Smando 1969), the potato NADP-malic enz-

yme (Nascimento et al 1975) and the mitochondrial enzyme from Bovine adrenal cortex (Simpson and Estabrook 1969).

NAD-malic enzyme from the dung beetle Catharsius sp apparently also had an ordered mechanism but with malate as the first binding substrate. This was deduced from initial velocity studies, which suggested that the K_m for malate tended towards zero as the coenzyme concentrations were increased. This was also supported by product inhibition studies in which inhibition by NADH was non-competitive with respect to NAD^+ (Imbuga 1979). It is not a unique phenomenon with dehydrogenases, for substrate to bind on the enzyme before coenzyme. A similar situation was reported for Yeast ADH (Bradbury and Jackoby (1971) UDP-Glucose dehydrogenase (Ordmann and Kirkwood 1977) and for Histidinal dehydrogenase (Burger and Gorish 1981) as well as for NADP-isocitrate dehydrogenase (Buzdygon et al 1973).

Unlike the pigeon liver enzyme, the Catharsius enzyme did not show any uncompetitive product inhibition results. It has been assumed that uncompetitive product inhibition normally indicates an ordered mechanism in which the second product combines with the complex of enzyme and first substrate to form a dead end complex E-A-Q, from which the second product

cannot dissociate, or that the inhibitor binds to a different form of enzyme than does the substrate (Cleland 1963). If this is the case then the kinetics of the Catharsius enzyme have to be investigated and interpreted further.

1.6 CO-OPERATIVITY

Many NADP-linked malic enzymes display true Michaelis kinetics, especially those with a cytoplasmic location (Frenkel 1972, Hsu and Lardy 1967). Although these enzymes are oligomeric proteins, it is supposed that each individual active site binds the substrate, and catalyses the malate decarboxylation reaction, just as if the subunit on which it is located were not aggregated with others. There is no evident interaction between the different substrate binding sites.

A few NAD-linked malic enzymes behave similarly and give classical hyperbolic velocity-substrate plots (e.g Valenti and Pupillo 1981), but most of these have so far yielded sigmoid plots in the absence of allosteric ligands (Sauer 1973, Landsperger and Harris 1976, Milne and Cook 1979).

Sigmoid velocity substrate plots are characteristic for a large number of regulatory enzymes, and

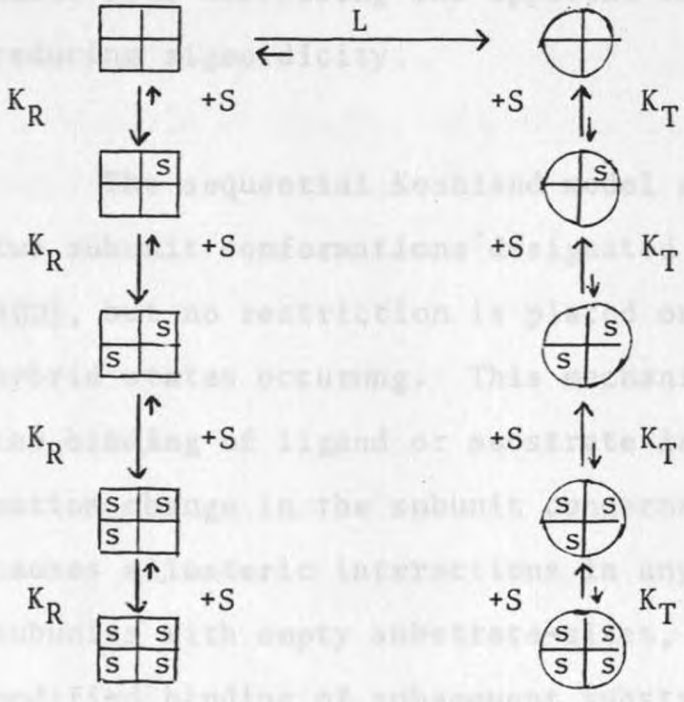
usually indicate co-operative binding of more than one substrate molecule to the enzyme. Such plots can however arise, with non saturation concentrations of the second substrate in some non-rapid equilibrium enzyme systems for kinetic rather than binding reasons. The enzyme concerned need not be oligomeric (Sweeny and Fischer 1968).

Different model explanations of this phenomenon were proposed by Monod et al. (1965) and Koshland et al (1966). Both models assume that sigmoidicity is due to subunit interactions.

The model of Monod et al, assumes that an allosteric enzyme is composed of two or more subunits and that there are symmetry requirements, such that a conformational change in one subunit is accompanied by an equivalent change in all other identical subunits. The model assumes that each subunit possesses a single catalytic site specific for substrate and a separate allosteric site specific for effectors. Two conformational states, R_n (⊞) and T_n (⊕), (n is the number of subunits) thus exist in equilibrium. In the absence of substrate (or ligand) the equilibrium greatly favours the (low affinity) T_n state. Substrate (or activator) bind preferentially to or only to, the high affinity R_n state; their introduction thus stabilises this state and modifies

the $R_n \rightleftharpoons T_n$ equilibrium, facilitating subsequent substrate binding by making available more empty high affinity R-subunits. Sigmoidicity is thus caused by the increased number of high affinity binding sites which become available as the substrate concentration begins to rise.

The situation may be represented as below:



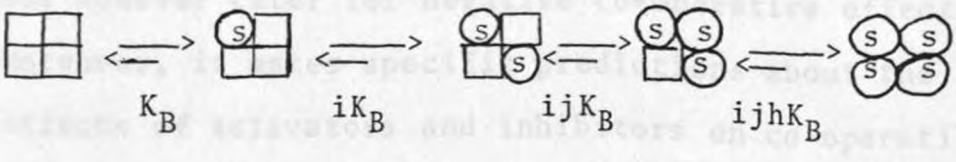
The equilibrium constant $L\left(=\frac{T_n}{R_n}\right)$ is much greater than unity. C, the ratio K_R/K_T (K_R and K_T are the substrate site dissociation constants for the R and T subunits respectively) must be less than unity, and could be zero.

The greater the value of L and the lower the

value of C, the more sigmoid will be the substrate-velocity curve.

An inhibitor according to this model, binds preferentially (or only) to T subunit thus stabilising the T_n form, increasing the apparent value of L, and thus increasing sigmoidicity. An activator on the other hand, binds preferentially or only to R subunits thus decreasing the apparent value of L and reducing sigmoidicity.

The sequential Koshland model again assumes two subunit conformations designated i.e A(O) and B(□), but no restriction is placed on the number of hybrid states occurring. This mechanism assumes that the binding of ligand or substrate induces a conformation change in the subunit concerned which then causes allosteric interactions in any neighbouring subunits with empty substrate-sites, which leads to modified binding of subsequent substrate molecules. Model can be represented as follows:-



If the subunit dissociation constant for the first substrate molecule was K_B, due to new interactions between subunits caused by the binding of

the first substrate, the dissociation constant of the second substrate is altered by a factor i and becomes iK_B. The binding of the second substrate in turn further alters the binding of the third substrate by another factor j, this dissociation constant thus becomes ijK_B and so on. The subunits thus undergo ligand induced conformational changes in a sequential manner, if i, j and h are each less than unity, it will become progressively easier for subsequent substrate molecules to bind. One or more of these factors could be greater than unity in which case it would be more difficult to bind substrate. The first case is positive co-operativity.

It should be noted that apparent negative co-operativity can also be caused where an enzyme exists as a mixture of two kinetically distinct forms i.e having different K_m values (Dixon and Webb 1979).

The model of Monod et al explains quite well the regulatory properties of many proteins. It does not however cater for negative co-operative effects. Moreover, it makes specific predictions about the effects of activators and inhibitors on co-operativity while the Koshland model allows a greater range of possibilities. In the presence of an activator according to the Monod model, a lowering of $[S]_{0.5}$ is automatically associated with a decrease in

sigmoidicity, a Monod model inhibitor will automatically increase both $[S]_{0.5}$ and sigmoidicity.

The most fundamental characteristic of regulatory enzymes is their susceptibility to activation or inhibition by metabolites other than the substrate. Malic enzymes that have allosteric characteristics have been shown to respond variously to different modulators. Fumarate is known to stimulate some enzymes by as much as about 3-fold (Frenkel 1973, Sauer 1973, Davis and Patil 1975, Hirai 1978, Swierczynski et al 1980, Garisson et al 1983. Other modulators which activate malic enzymes from various sources include aspartate (Takeo et al 1967, Hirai 1978, Sanwal 1970), coenzyme A (Day et al 1983, Neuburger and Douce 1980), acetyl CoA (Sanwal and Smando 1969, Hirai 1978, Valenti and Pupillo 1981, Grisson et al 1983), F-1,6-diP (Chapman and Hatch 1977, Day 1980) SO_4^{2-} (Macrea 1971, Carnellas et al 1982) and succinate (Davis and Patil 1975, Mandella and Sauer 1975, Landsperger and Harris 1976). These modulators usually activate by simultaneously removing co-operativity and decreasing the $[S]_{0.5}$, and have no effect on V_{max}. Inhibitors, which include malonate, ATP and acetyl CoA, usually increase the $[S]_{0.5}$; some however also have an effect on the V_{max} (Norden and Matanganyidze 1977). Generally their effects on sigmoidicity have not been thoroughly in-

vestigated.

Some malic enzymes that do not display sigmoidal substrate kinetics respond to activators differently. Thus in malic enzyme from Panicum miliaceum, F-1:6-diP and CoA activate by increasing V_{max} (Hatch et al 1974, Chapman and Hatch 1977).

In some enzymes displaying co-operativity, substrate analogues have been found to activate, presumably at same time removing co-operativity, when added in presence of low concentration of substrate. In this case, it is presumed that the analogue is mimicking the substrate and binding to unoccupied substrate sites, perhaps at the active centre.

A different form of regulation has been suggested for the potato and cauliflower NAD-malic enzymes. These enzymes show substrate and ligand induced changes in oligomeric state that have major kinetic consequences (Grover et al 1981, Grover and Wedding 1984). Sigmoidicity can supposedly result from malate induced oligomerisations, the substrate promoting the conversion of a less active dimeric to a more active tetrameric enzyme form. The enzyme could thus be strongly regulated by malate concentration physiologically (Grover and Wedding 1984). This finding was used to explain the sigmoid velocity

versus malate curves in the crassula NAD-malic enzyme (Wedding 1982). Activation as a result of oligomerisation has been observed in other allosteric enzymes, e.g in threonine dehydrase (Dunne and Wood 1975), in isocitrate dehydrogenase (Kelly and Plaut 1981) aspartate glutamate transaminase, (Kurganov 1967) cystine synthase (Cook and Wedding 1978) and crassula NAD-malic enzyme (Grover and Wedding 1982, 1984), and also in animal enzymes, bovine liver glutamate dehydrogenase (Freiden and Colman 1967) rabbit muscle phosphorylase a (Metzger et al 1967) and acetyl CoA carboxylase (Vagelos et al 1963).

Oligomerisation can affect enzyme activity by altering either K_m or V_{max}. In the case of threonine dehydrase, aspartate glutamate transaminase, and crassula NAD-malic enzyme, its the K_m that is altered not V_{max}.

High salt concentration and high pH promote disaggregation with consequent increase in both K_m and sigmoidicity with respect to malate (Grover and Wedding 1984). Thus pH, ionic strength and substrate concentration could all serve to regulate the activity physiologically by modulating the state of aggregation of this plant enzyme.

Malate, Mn^{2+} or Mg^{2+} and DTT have been found

to stabilise various malic enzymes during storage, an effect also associated with aggregation. In certain cases i.e the potato and cauliflower enzymes, DTT causes the enzyme to aggregate to octomeric form of molecular weight 400,000 which is more active than the tetrameric or dimeric forms. (Grover and Wedding 1984, Grover et al 1982).

1.7 SCOPE AND AIMS OF THE PRESENT STUDY

Previous studies of malic enzyme in this laboratory were centred specifically on the occurrence and localisation of NAD-malic enzymes in different insects, and on the kinetic properties of the enzyme from muscle of the dung beetle, Catharsius sp. The dung beetle enzyme was partially purified from both the flight and coxal muscle and basic properties examined to establish the similarities of the two types of muscle. An attempt was made to relate these kinetic properties to the control of proline metabolism in this insect.

The localisation and basic properties of the tsetse fly NAD-malic enzyme had been briefly investigated in this laboratory (Hoek et al 1976, Olembo pers. comm.) and also by Norden and Matanganyidze (1977).

The present work was aimed at studying the Glossina NAD-malic enzyme in more detail. The basic properties of both Glossina and Catharsius enzymes were compared. At the same time, the substrate kinetics were thoroughly investigated in order to gain insight into mechanism of substrate binding. pH effects were also investigated in detail.

Enzymes from the two insect sources were directly compared as regards kinetic and protein properties, and further purification of both enzymes was attempted.

Co-operative behaviour was investigated in detail including the effects of activators, inhibitors and substrate analogues.

CHAPTER II

MATERIALS AND METHODS

2.1 CHEMICALS

All chemicals used in this study were of analytical grade obtained from either BDH chemicals Ltd (Poole England) or Sigma Chemical Company (USA) Diethylaminoethylcellulose (DEAE 52) was from Whatman (England) sepharose 6B and sephacryl S-200 superfine, from Pharmacia Fine Chemical (Sweden) and Hydroxylapatite Bio-Gel HTP was kindly donated by Prof. O. Ole Moiyoi of International Laboratory for Research on Animal Diseases (ILRAD) Nairobi, Kenya. All enzymes used in the metabolite assays were from Sigma Chemical Company (USA).

2.2 INSECTS

Newly emerged female Catharsius sp were collected at night around lights at Ngulia Lodge, Tsavo National Park (West) Kenya, during rainy periods in November - January and April and were frozen immediately in dry ice at -20°C. Glossina morsitans, 3-7 day old males bred and reared under identical laboratory conditions, were kindly provided by the International Centre of Insect Physiology and

Ecology (ICIPE) and International Laboratory for Research on Animal Diseases (ILRAD) Kenya.

2.3 ENZYME ASSAYS

Except where otherwise indicated, all enzyme assays were carried out at 30°C using a Pye Unicam SP 800 (UNICAM Instruments Ltd. Cambridge England) or SP 1800 (UNICAM Instruments Ltd. Cambridge England) spectrophotometer, fitted to a Pye Unicam recorder. The appearance or disappearance of NADH or NADPH was monitored at 340 nm. In these assays 1 cm. light path cuvettes (R 100 England) were used and in all cases initial rates of reaction were determined. The molar extinction coefficient at 340 nm was taken as 6.22×10^3 litre $moles^{-1} cm^{-1}$.

A unit of enzyme activity was defined as the amount of enzyme that catalysed the conversion of 1 µmole of substrate to products per minute at 30°C under the specified conditions of the assay system.

2.3.1 Oxidative Decarboxylation of Malate

NAD-malic enzyme was assayed in a 2 ml system that contained 10 mM malate, 0.76 mM NAD, 5 mM $MgCl_2$ in 50 mM Triethanolamine-HCl buffer at pH 7.2 for the Catharsius sp enzyme (Catharsius enzyme) and at pH 7.8 for the Glossina morsitans enzyme (Glossina

enzyme). The reaction was initiated by the addition of the enzyme, appropriately diluted in the homogenisation buffer (10 mM Tris -HCl, 1 mM EDTA, (pH 7.6).

2.3.2 Reductive Carboxylation of Pyruvate

The reverse reaction catalysed by NAD-malic enzyme, was studied in a 2 ml system, containing 10 mM pyruvate, 10 mM bicarbonate, 0.1 mM NADH and 5 mM $MgCl_2$ or $MnCl_2$ in 50 mM triethanolamine-HCl buffer (pH 7.0). The reaction was initiated by addition of the enzyme.

2.3.3 Oxaloacetate Decarboxylase Activity

This activity was determined (at pH 5.9) by incubation at 30°C of a 2 ml system containing 5 mM $CaCl_2$, 2.5 mM tris-oxaloacetate and 5 µg of the enzyme, in 20 mM imidazole buffer. The enzymic reaction was stopped at different times with perchloric acid (3% v/v final concentration) which caused deproteinisation. The deproteinised extracts were neutralised with KOH and the $KClO_4$ precipitate removed by centrifugation. The final extracts were assayed for oxaloacetate and pyruvate by enzymatic methods as outlined later (2.4.4). For every incubation, a corresponding control was carried out without the addition of the enzyme, to allow for non-enzymatic decarboxyla-

tion of oxaloacetate.

2.3.4 Malate Dehydrogenase (MDH) Activity

The assay was carried out in 0.1 M glycine -KOH (pH 10) containing 0.76 mM NAD and 50 mM malate. The reaction was started by addition of the enzyme.

In specified cases, products of the MDH reaction were also analysed from samples removed at specific intervals after the initiation of the enzymatic reaction. The enzyme activity was stopped by addition of 3% v/v perchloric acid and the system deproteinised and neutralised as described above (see section 2.3.3). The supernatant was then assayed for OAA and pyruvate as described below (2.4.4).

MDH activity at pH 7.4 was determined in an assay that utilised citrate synthase activity to trap OAA in the presence of acetyl CoA. The assay system contained 0.76 mM NAD, 10 mM malate, 0.1 mM acetyl CoA and 0.5 units citrate synthase, in 50 mM triethanolamine - HCl buffer (pH 7.4). The reaction was initiated by addition of 5 µg of enzyme (MDH) and was followed at 340 nm for appearance of NADH.

In some specified assays, the concentration of the products in the assay system at specific time

intervals were determined after deproteinisation and neutralisation as described above (2.3.3). Enzymatic assay for citrate (2.4.5), pyruvate and OAA (2.4.4) were also performed.

2.3.5 OAA Reductase Activity

The reverse of the MDH activity described above (2.3.4) was assayed at pH 7.4 in a system containing 2 mM OAA, 0.1 mM NADH in 0.1 M tris-HCl (pH 7.4), the reaction was initiated with addition of enzyme and followed at 340 nm for the disappearance of NADH.

2.3.6 Lactate Dehydrogenase (LDH) Activity

This enzyme activity was assayed in a system adopted from Bergmeyer and Bernt (1974) in 0.1 M phosphate buffer (pH 7.4) containing 0.1 mM NADH and 2 mM pyruvate. An appropriate concentration of LDH diluted in phosphate buffer was added to the assay system to start the reaction.

2.3.7 Alcohol Dehydrogenase (ADH) Activity

ADH activity was assayed in 2 mls of 50 mM tris -HCl buffer (pH 8.4) containing 5% ethanol and 0.8 mM NAD. The reaction was initiated by addition

of the enzyme.

2.3.8 Pyruvate Kinase Activity

This activity was assayed in 2 mls of 0.2 M Tris - HCl (pH 7.4) containing 5 mM $mgCl_2$, 0.1 mM NADH, 1 mM ADP, 5 mM PEP, 7 mM KCl and 5 µg of LDH. The reaction was started by addition of the enzyme.

2.3.9 Glucose-6-Phosphate Dehydrogenase (G6PD) activity

G6PD activity was assayed in 0.2 M Tris - HCl buffer pH (7.6) containing 0.2 mM NADP, 5 mM $mgCl_2$ and 5 mM Glucose 6-phosphate. The reaction was initiated with the addition of the enzyme.

2.4 ASSAY OF SUBSTRATES AND PRODUCTS

The substrates used in kinetic studies were standardised in NAD or NADP-linked enzymatic buffer systems, using the SP 800 or 1800 spectrophotometer at 340 nm and 1 cm light path cuvettes, and following the appearance or disappearance of NADH. The products formed in certain reactions were also assayed enzymatically. The concentrations of substrates or products were adjusted appropriately so that in all cases they were at limiting concentrations in the

specific assay system.

2.4.1 Malate

Malate as substrate was determined enzymatically in 0.1 M glycine-hydrazine buffer (pH 10) containing 0.8 mM NAD and approximately 0.5 units/ml of commercial MDH (Sigma). The reaction was started by addition of malate.

2.4.2 NAD

This was standardised in the ADH system outlined above (2.3.7) using crystalline ADH (0.5 units/ml) and reaction started with NAD.

2.4.3 NADH

NADH was standardised using the LDH assay outlined earlier (2.3.6). Reaction was initiated by addition of crystalline LDH (0.5 units/ml).

2.4.4 OAA and Pyruvate

As products, these two metabolites were assayed in the same sample; in an assay system that contained, 0.1 mM NADH in 0.1 M phosphate buffer (pH 7.4) and an appropriate amount of sample. Reaction

was started with addition of 5 units/ml of commercial MDH (Sigma) for assay of OAA. After the reaction had stopped completely (when all OAA is used up), 5 units/ml of LDH was added for the assay of pyruvate.

2.4.5 Citrate

This was determined in a system containing 0.2 mM $ZnCl_2$, 0.1 mM NADH, 5 units/ml MDH in 0.1 M triethanolamine buffer (pH 7.4) and an appropriate quantity of the extract. The reaction was started with the addition of about 10 units/ml citrate lyase (Sigma).

2.5 PROTEIN

The protein concentration was determined by the method of Lowry et al (1951) using crystalline bovine serum albumin (BDH) as standard. In some cases, particularly in the chromatography procedures, the absorption at 280 nm was used to estimate the protein concentration as outlined by Warburg and Christian (1942).

2.6 NAD-MALIC ENZYME PURIFICATION

The enzyme was purified from whole Glossina

thoraces or abdominal muscles of Catharsius. The purification was carried out at 4°C. .

a) Ammonium Sulphate Fractionation

Whole thoraces of Glossina were mechanically pounded and homogenised in 10 ml volumes of 0.1 M Tris - HCl buffer (pH 7.6) containing 1 mM EDTA. The homogenate was frozen and thawed three times at room temperature before centrifugation at 10,000 RPM for 5 minutes. The supernatant was brought to 80% (w/v) ammonium sulphate saturation. After about 30 minutes at 4°C, the suspension was centrifuged at 10,000 RPM for 5 minutes. The supernatant was discarded and precipitate was taken up in homogenisation buffer.

b) Sepharose -CL 6B Chromatography

The above fraction was loaded onto a (2 cm by 60 cm) column of sepharose - CL 6B column or onto a (2 cm by 60 cm) sephacryl column. These columns had been pre-equilibrated with homogenisation buffer which was also used to elute the enzyme.

The active fractions as determined by the respective assay systems were pooled, and brought

to 80% (w/v) ammonium sulphate saturation and centrifuged at 10,000 RPM for 5 minutes. The precipitated enzyme was dissolved in a minimum volume of homogenisation buffer and dialysed for 8 hours against 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT and 0.02% NaN_3 in 10 mM TES (pH 7.4) (see Nagel and Sauer 1982). Malic enzyme was eluted from sepharose - 6B column ahead of MDH with only a little overlap in the two activities.

c) DEAE 52 Chromatography

The dialysed enzyme above was then loaded onto a (2 cm by 16 cm) DEAE 52 column pre-equilibrated with the above TES buffer system. The column was then washed with 3 column volumes of TES buffer (pH 7.4) containing 170 mM KCl. The enzyme was then eluted with 2 column volumes of the TES buffer containing 220 mM KCl. This buffer completely separated any remaining MDH activity from malic enzyme activity. The former enzyme was completely eluted with the equilibration buffer. Column fractions containing the malic enzyme activity were pooled and again concentrated by ammonium sulphate precipitation and taken up in a minimum volume of homogenisation buffer, and dialysed against 1 mM phosphate buffer (pH 6.5) for 8 hrs.

d) Hydroxylapatite Column

The DEAE 52 purified enzyme was added onto a column (2 cm by 6 cm) of hydroxyapatite pre-equilibrated with 1 mM potassium phosphate buffer (pH 6.5) and the column was washed with two column volumes of 0.3 M phosphate buffer (pH 6.5). The enzyme was then eluted with three column volumes of 0.5 M phosphate buffer (pH 6.5).

A major contaminating protein component was eluted at the 0.3 M phosphate step. All column fractions with the active enzyme were pooled as above, concentrated by ammonium sulphate and stored as an ammonium sulphate precipitate at -20°C. These enzyme preparations were used for kinetic studies.

The Catharsius malic enzyme was prepared as described above with the following modifications: dissected abdominal muscles rather than whole thoraces were homogenised and the first ammonium sulphate fractionation was carried out in two stages. Firstly the supernatant obtained from the centrifuged homogenate, was brought to 40% (w/v) ammonium sulphate saturation. After a 30 minutes incubation at 4°C, the ammonium sulphate suspension was centrifuged at 10,000 RPM

for 5 minutes and the precipitate discarded. The supernatant was then taken upto 55% (w/v) ammonium sulphate saturation before centrifuging at 10,000 RPM for 5 minutes. The precipitate obtained was then processed as described above for Glossina enzyme.

The Glossina enzyme purified from the final step had a specific activity of about 50 units/mg protein whereas the final Catharsius enzyme had a specific activity of between 40 - 45 units/mg protein. The purification table for Glossina enzyme is presented in Table 1.

2.7 ISOELECTROFOCUSING (IEF)

Electrofocusing was carried out according to Vesterberg and Svenson (1966) and the LKB instruction mannual. An LKB Bromma 8100 ampholine type 110 ml column was used. The stock solutions were as follows:-

a) Dense gradient solution:-

27 gms sucrose

3.0 mls ampholine (pH 3.5-10 or 4 - 6)

In 35 mls distilled water

Test sample (2 mg in 0.5 mls homogenate buffer)

column was maintained at 4°C by a temperature controlled water jacket.

At end of electrofocusing period, the anode solution was sucked off separately by syringe and tubing and the rest of the contents of the column were then collected using a fraction collector, and a constant flow rate of about 2 ml/min. The flow rate was maintained with a peristaltic pump. The absorbance of each fraction at 280 nm was monitored using a Carl Zeiss (Germany) M4 Q11 17928 spectrophotometer. The pH of each fraction was also recorded using a radiometer M62 pH standard pH meter. Fractions were also checked for NAD-malic enzyme activity as described earlier (2.3.1).

Samples with NAD-malic enzyme activity were pooled and stored at -20°C. Although the electrofocusing procedure effectively removed any contaminating proteins in the purified enzyme samples, it was not used for routine purification, because the Glossina and Catharsius malic enzymes lost 60% and 80% activity respectively during the process.

Isoelectrofocusing was also carried out in the same way on small amount of the NAD-malic enzyme which was labelled with I^{125} iodine using the chloramine T method of Greenwood et al (1963).

b) Light gradient solution:-

2.90 gms sucrose

0.7 mls ampholine (pH 3.5 - 10 or 4 - 6)

In 52.3 mls distilled water

c) Anode solution:-

15.0 g sucrose

12.0 mls distilled water

4.0 mls 1 M H_3PO_4

d) Cathode solution (light electrode solution)

2.5 mls 1 M NaOH

7.5 mls distilled water

All solutions were degassed immediately before use. The anode solution, being heavier than the dense gradient solution, was placed at the bottom of the IEF column. A sucrose gradient of 5 - 50% was set up using the dense and light gradient solutions and a gradient mixer. The final concentration of carrier ampholytes on the column was 1%. The protein sample, which had been thoroughly dialysed in homogenisation buffer (10 mM Tris - HCl (pH 7.6) containing 1 mM EDTA) was included in the dense gradient solution.

The lighter cathode solution was finally applied at the top of the column. Electrofocusing was carried out at 800 v for 20 hrs during which the

column was maintained at 4°C by a temperature controlled water jacket.

At end of electrofocusing period, the anode solution was sucked off separately by syringe and tubing and the rest of the contents of the column were then collected using a fraction collector, and a constant flow rate of about 2 ml/min. The flow rate was maintained with a peristaltic pump. The absorbance of each fraction at 280 nm was monitored using a Carl Zeiss (Germany) M4 Q11 17928 spectrophotometer. The pH of each fraction was also recorded using a radiometer M62 pH standard pH meter. Fractions were also checked for NAD-malic enzyme activity as described earlier (2.3.1).

Samples with NAD-malic enzyme activity were pooled and stored at -20°C. Although the electrofocusing procedure effectively removed any contaminating proteins in the purified enzyme samples, it was not used for routine purification, because the Glossina and Catharsius malic enzymes lost 60% and 80% activity respectively during the process.

Isoelectrofocusing was also carried out in the same way on small amount of the NAD-malic enzyme which was labelled with I^{125} iodine using the chloramine T method of Greenwood et al (1963).

Samples of enzyme from before and after electrofocusing were examined by SDS polyacrylamide slab and tube gel electrophoresis.

2.8 ELECTROPHORESIS

2.8.1 SDS - PAGE

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemli (1970) on vertical slab gels.

The glass plates to be used were washed with detergent and rinsed extensively with distilled water and 70% (v/v) methanol. In most cases gradient gels of 7.5 - 15% acrylamide were used and were prepared according to the method of Laemli (1970). The separatory gel contained 0.4 M Tris (pH 8.8) 0.2% SDS and 10 µl Temed (N, N, N´, N´ Tetramethylenediamine) and 10 µl ammonium persulphate for polymerisation. The stacking gel contained 0.1% SDS, 0.125 M Tris (pH 6.8) Temed and ammonium persulphate for polymerisation.

The wells of the stacking gel were rinsed with the running buffer (0.025 M Tris - HCl .192 M glycine, 0.01% SDS pH 8.3)before samples were applied at the cathode end. All samples for electrophoresis

were solubilised by the addition of an equal volume of solubilising buffer (4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2 - mercaptoethanol and 0.01% bromophenol blue (as tracking dye) in 20 mM Tris - HCl (pH 6.8)) and then heated in boiling water for 10 minutes. 2 - mercaptoethanol, a strong reducing agent, cleaves disulphide bonds in proteins. Hence heating in the presence of SDS and mercaptoethanol dissociates all polypeptide chains from each other and produces denatured and negatively charged SDS coated polypeptide chain complexes. For estimation of molecular weight of the polypeptides on SDS-PAGE gels, marker proteins of known molecular weights were processed as described above for samples and electrophoresed coincidentally on the same gel.

Electrophoresis was performed at 150 v for 8 hrs at room temperature in a buffer containing .01% SDS, .192 M glycine and 0.025 M Tris - HCl (pH 8.3). At the end of electrophoresis, the gels were stained for proteins, by immersion in 0.13% w/v coomassie brillant blue R 250 (BDH England) in 50% (v/v) methanol and 10% (v/v) acetic and for 4 - 6 hrs. The gels were destained in 50% (v/v) methanol, 10% (v/v) acetic acid in distilled water for one hour, and further destaining was continued in 7% (v/v) acetic acid, 5% (v/v) methanol in distilled water overnight.

Gels that had very little protein, expecially those containing fractions from electrofocussing purification were stained for protein using the more sensitive silver staining as described by Tsai and Fraschy (1982) using clean glass tray and non-sterile, non-powdered gloves.

The gel was fixed in 100 mls of 50% (v/v) methanol 10% (v/v) acetic acid 40% (v/v) distilled water for ½ - 3 hrs, followed by immersion in 10% (v/v) methanol, 5% v/v acetic acid and 85% (v/v) distilled water, for at least 30 minutes. The fixed gel was washed for 5 minutes in a solution of potassium dichromate and nitric acid (0.0034 M potassium dichromate + 0.0032 M nitric acid - the solution is stable and can be used upto 1 month).

The gel was then washed for 20 minutes in 100 mls of distilled water to remove all silver nitrate, and quickly rinsed twice in 50 ml aliquot of a freshly prepared developer solution containing 15 g Na_2CO_3 and 250 µl formaldehyde (37%) in 500 mls of distilled water. The rest of the developer solution was then poured over the gel and left to develop until the required staining was obtained. The stained gel was then transferred to 100 mls of 5% v/v acetic acid, and could be stored indefinately.

2.8.2 Non-denaturing PAGE

Electrophoresis in non-denaturing conditions was carried out as described by Neville (1971) and Neville and Glossmar (1974).

The solutions were:

a) Resolving gel buffer

0.424 M Tris-HCl (pH 9.8)

b) Stacking gel buffer

0.054 M Tris-HCl (pH 6.14)

c) Lower reservor buffer

0.4 M Tris-HCl (pH 9.18)

d) Upper reservor buffer

0.4 M Tris + 0.4 M Boric acid

(pH 8.64) freshly made.

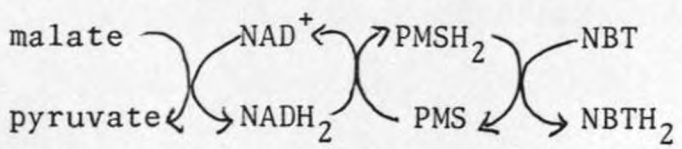
The separating gel acrylamide contained 30% (w/v) acrylamide and 0.7 % (w/v) Bis acrylamide (NN´- methylenebisacrylamide) in resolving gel buffer and stored at $4^{o}C$ in a dark bottle.

The stacking gel acrylamide contained 5% (w/v) acrylamide plus 0.08% Bis acrylamide in stacking gel buffer and also stored at $4^{o}C$ in a dark bottle.

For slab gel preparation, 5% acrylamide was used in the separating gel and 3.5% acrylamide for the stacking gel. The gels were allowed to polymerise and kept at $0^{o}C$ before samples were applied. The samples were mixed in 1:1 ratio (v:v) with 0.0625 M Tris-HCl buffer (pH 6.8) containing 20% (v/v) glycerol and 0.01% (v/v) bromophenol blue as marker dye.

Electrophoresis was performed at 5 mA first to allow the proteins to enter the stacking gel. The current was then increased to 12-15 mA for 8 hrs at $4^{o}C$. The electrophoresed gels were cut into two halves and one half stained for protein by coomassie blue and silver staining methods described earlier (section 2.8.1) and the other identical half gel was stained for enzyme activity as described below.

To stain for enzyme activity in the gel, the entire half gel was incubated for 1 hour at $37^{o}C$ in 50 mM Tris-HCl (pH 7.6) containing 2 mM Mn^{2+}, 20 mM L-malate, 1 mM NAD^{+}, 1 mg/ml PMS (N-methylphenazonium methosulphate) and 0.2 mg/ml NBT (Nitroblue tetrazolium). Longer incubation periods resulted in unspecific staining. Areas of activity showed dark brown bands of insoluble formazan ($NBTH_2$) deposits. The reaction can be represented as follows



To stop further reaction, the gel was kept in 10 mM Tris-HCl buffer pH 7.6 and stored in this buffer at $4^{o}C$.

CHAPTER III

3.0 BASIC PROPERTIES OF NAD-MALIC ENZYME FROM FLIGHT MUSCLE OF THE TSETSE FLY COMPARED WITH THOSE OF THE ENZYME FROM THE BEETLE CATHARSIUS

Some basic properties of NAD-malic enzyme from tsetse fly flight muscle were reported by Hoek et al (1976) and by Norden and Matanganyidze (1977) Olembo (1980), Those of the enzyme from the beetle Catharsius were described by Imbuga (1979) and Imbuga and Pearson (1982).

In order to compare the enzymes from two insect sources, their kinetic properties were re-examined in more detail side by side

3.1 BASIC KINETIC PARAMETERS OF THE TSETSE FLY (GLOSSINA) ENZYME

Unless specified otherwise, assays were performed with a fixed concentration of 5 mM Mg^{2+} as metal activator. Michaelis constants and activation constants were determined using either a Unicam SP 1800 or SP 800 spectrophotometer, fitted with a thermostat control set at 30°C and attached to a recorder.

3.1.1 Apparent K_m and K_a values with Mg^{2+} as Activator

In the presence of 50 mM Triethanolamine buffer pH 7.8, the apparent K_m for malate was determined at a fixed near saturating level of 0.76 mM NAD. At least six different malate concentration points were used. Apparent V_{max} and K_m values were obtained by calculating the linear regression of experimentally determined values of $\frac{1}{v}$ against chosen values of $\frac{1}{[S]}$.

The apparent K_m for NAD was determined with the L-malate concentration fixed at a near saturating concentration of 10 mM. Six different NAD concentration points were used and the results were again analysed by linear regression of $\frac{1}{v}$ against $\frac{1}{[S]}$ values.

The K_a for Mg^{2+} was obtained in 50 mM triethanolamine buffer, pH 7.8 with L-malate and NAD concentrations fixed at 10 mM and 0.76 mM respectively and the concentration of metal ion varied. Results were again plotted in double reciprocal form, and treated by linear regression analysis. Results for basic kinetic parameters are presented in Table 2. Although values for malate and NAD are tabulated as K_{ms}, they are actually $[S]_{0.5}$ values (see Chapter V).

3.1.2 Activity with NADP as Substrate

The basic kinetics of the Glossina enzyme were re-examined again by substituting NADP for NAD,

and using 50 mM Triethanolamine buffer pH 7.8 and 5 mM Mg^{2+}. Results are included in Table 2.

Generally, the activity with NADP was extremely low, the maximum velocity being only 4% of that obtained with NAD.

At a fixed NADP concentration of 0.76 mM, the apparent K_m for malate was slightly lower than that with 0.76 mM NAD. In the presence of 10 mM L-malate, the K_m for NADP was 0.19 mM, substantially lower than that for NAD under the same conditions.

The K_a for Mg^{2+} using 0.76 mM NADP was also substantially lower than that with 0.76 mM NAD.

3.1.3 Activity with Mn^{2+} as Metal Activator

0.5 mM Mn^{2+} was substituted for 5 mM Mg^{2+} and the basic properties of the enzyme were re-investigated. Results are included in Table 2.

The apparent maximum velocity was similar to that found with Mg^{2+}, as was the apparent K_m for malate with 0.76 mM NAD at pH 7.8.

Substitution of Mn^{2+} for Mg^{2+} substantially lowered the apparent K_m for NAD in the presence of

TABLE 2: Kinetic Parameters of Glossina NAD - Malic Enzyme

Enzyme	V_{app} µM/min/mg protein	K_m			K_a	
		Malate (mM)	NAD (mM)	NADP (mM)	Mg^{2+} (mM)	Mn^{2+} (µM)
with Mg^{2+}						
NAD	4.592	1.167±.33(6)	.27±.072(12)		.328±.09(6)	
NADP	0.22	.66±.02(4)		.195±.023(4)	.12±014(4)	
with Mn^{2+}						
NAD	4.81	1.137±.12(5)	0.3±014(6)			14.9±.14(5)
NADP	0.32	.68±.11(5)		.202±014(4)		17.5±.24(6)
in presense of 2mM fumarate						
with Mg^{2+}						
NAD	4.41	.69±.036(5)	0.31±03(7)		0.24±012(4)	-
NADP	.21	.34±07(4)		0.23±01(4)	ND	-
with Mn^{2+}						
NAD	4.26	1.21±.1(5)	.39±021(4)		-	17.3±1.6(5)
NADP	0.36	0.23±003(5)		0.22±015(4)	-	ND

Values given are means for number of experiments shown in parenthesis

10 mM malate. With NADP as substrate, the substitution of Mn^{2+} for Mg^{2+} again had no effect on the K_m for malate nor, in contrast to results with NAD did it appear to affect the K_m for NADP appreciably.

The K_a for Mn^{2+} was determined in a system containing 10 mM malate, 0.76 NAD and 50 mM Triethanolamine buffer pH 7.8. A value of 14.5 µM was obtained, far lower than the K_a for Mg^{2+}. A similar value 17.5 µM, was obtained using NADP as substrate.

3.2 BASIC KINETIC PARAMETERS FOR THE CATHARSIUS ENZYME

Basic parameters of the Catharsius NAD-malic enzyme were determined under similar conditions, in order to provide a full comparison with the Glossina enzyme, and results are summarised in Table 3 ; some of the values presented are taken from an earlier investigation (Imbuga 1979).

The basic medium contained 50 mM Triethanolamine buffer pH 7.2 with 5 mM Mg^{2+}. It was known from earlier work (Imbuga 1979) that the pH optimum (maximum rate with near saturating concentrations of both substrates) for the Catharsius enzyme was 0.6 units lower than that of the Glossina enzyme.

The apparent K_m for malate was again investigated with NAD concentration fixed at 0.76 mM and 5 mM Mg^{2+} and malate varied from 1-15 mM.

With malate concentration fixed at 10 mM, NAD was varied from 0.085 - 1.3 mM to obtain the apparent K_m for NAD. For values see Table 3.

The K_a for Mg^{2+} was again determined using 10 mM malate and 0.76 mM NAD. Results obtained from linear regression analysis of reciprocal values are summarised in Table 3. Also included here are results obtained with the Catharsius enzyme on substituting Mn^{2+} for Mg^{2+} and/or NADP for NAD.

The K_m values for malate and NAD and the K_a values for Mn^{2+} and Mg^{2+} were similar with enzymes from the two insects, but the K_m for NADP with Mg^{2+} as metal activator was far lower for the Glossina enzyme. With the Catharsius enzyme, substitution of Mn^{2+} for Mg^{2+} markedly decreased the K_m of the enzyme for both malate and coenzyme.

In contrast to the Glossina enzyme that from Catharsius showed similar K_m values for NAD and NADP with Mg^{2+} as activator. Also K_m values for malate in the presence of either coenzyme were similar. Maximum activities with NADP were 30% of the NAD

TABLE 3: Kinetic Parameters of Catharsius NAD - Malic Enzyme

Cofactor	$V_{app.}$ µM/min	K_m				
		Malate (mM)	NAD (mM)	NADH (mM)	Mg^{2+} mM	Mn^{2+} mM
With Mg^{2+}						
NAD	4.83	1.16±0.2(3)	.3±.05(4)	-	.24±.003(4)	
NADP	1.60	1.25*		.05±027(3)	ND	
with Mn^{2+}						
NAD	4.96	.66±045(4)	.21±.13(6)			.017
NADP	2.13	.43±.13(3)		.08±007(3)		ND
+10 mM Fumarate with Mg^{2+}						
NAD	4.97	.51±.13(4)	.306±01(4)		ND	
NADP	1.61	.56±.2(4)		.15±077(3)	ND	
with Mn^{2+}						
NAD	4.72	.311±084(5)	.12±042(4)			
NADP	2.04	.49±.053(3)		069±.012(3)		ND
						ND

* values obtained from previous work.

activity as compared to 4% with the Glossina enzyme.

3.3 EFFECTS OF FUMARATE AND SUCCINATE CONCENTRATIONS ON ENZYME ACTIVITY

The effects of increasing concentrations of fumarate were investigated on the Glossina enzyme. The system contained 50 mM Triethanolamine, pH 7.8, 2.5 mM malate, 0.3 mM NAD in the presence of 5 mM Mg^{2+} or 0.5 mM Mn^{2+}. Rates expressed as percentages of rates without fumarate, are plotted against the concentration of fumarate in Fig. 1.

Effects were more marked with low levels of substrates. Maximum stimulation (375%) in the presence of Mg^{2+} was obtained with 2 - 10 mM fumarate. Rates decreased at higher fumarate concentrations (see Fig. 1a).

With Mn^{2+} as activator, fumarate upto 2 mM caused slight stimulation. Higher concentrations were distinctly inhibitory (Fig. 1b).

An even more marked effect of fumarate was observed with the Catharsius enzyme. Further studies of fumarate activation were therefore carried out using the enzyme from this insect. Higher concentrations of fumarate (10 - 40 mM) were needed to

achieve maximum stimulation with Mg^{2+} as activator, and no apparent inhibition was observed with higher concentrations (Fig. 2a).

With Mn^{2+}, fumarate upto 10 mM caused slight stimulation but inhibited at higher concentrations (Fig. 2b) as found with the Glossina enzyme.

With both enzymes, succinate showed similar effects to fumarate, i.e 2 mM and 10 mM succinate was needed for maximum stimulation for the Glossina and the Catharsius enzymes respectively and inhibition was again obtained at higher succinate concentrations.

3.3.1 Dependence of Fumarate and Succinate Activation on L-Malate Concentration

Using Glossina enzyme, the effect of malate concentration on fumarate activation was investigated in a system containing 50 mM Triethanolamine buffer pH 7.8, 5 mM Mg^{2+} and 0.3 mM NAD. L-malate was varied from 0.095 - 3.31 mM and results are plotted as velocity against malate concentration with and without 2 mM fumarate and presented in Fig. 3. Malate concentration had a similar effect when succinate was the activating metabolite.

FIGURE 1: ACTIVATION OF GLOSSINA NAD-MALIC ENZYME BY FUMARATE

The system contained

50 mM Triethanolamine (TEA) buffer pH 7.8

2.5 mM malate

0.3 mM NAD

Fumarate was varied 0 - 20 mM

a) x——x with 5 mM Mg^{2+}

b) o——o with 5 mM Mn^{2+}

FIGURE 2: ACTIVATION OF CATHARSIUS NAD-MALIC ENYZME BY FUMARATE

The assay system contained

50 mM TEA-buffer pH 7.8

2.5 mM malate

0.3 mM NAD

Fumarate was varied 0 - 60 mM

a) x——x with 5 mM Mg^{2+}

b) o——o with 5 mM Mn^{2+}

Fig. 1

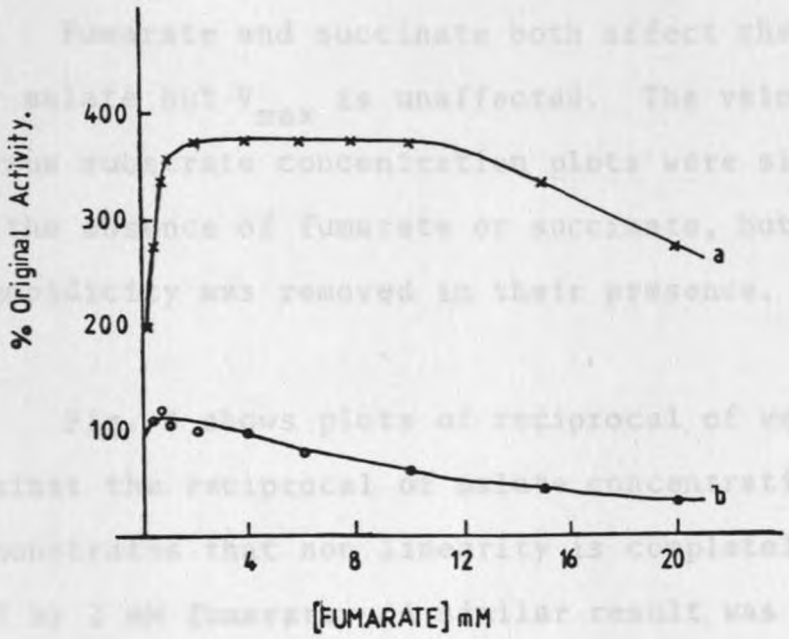
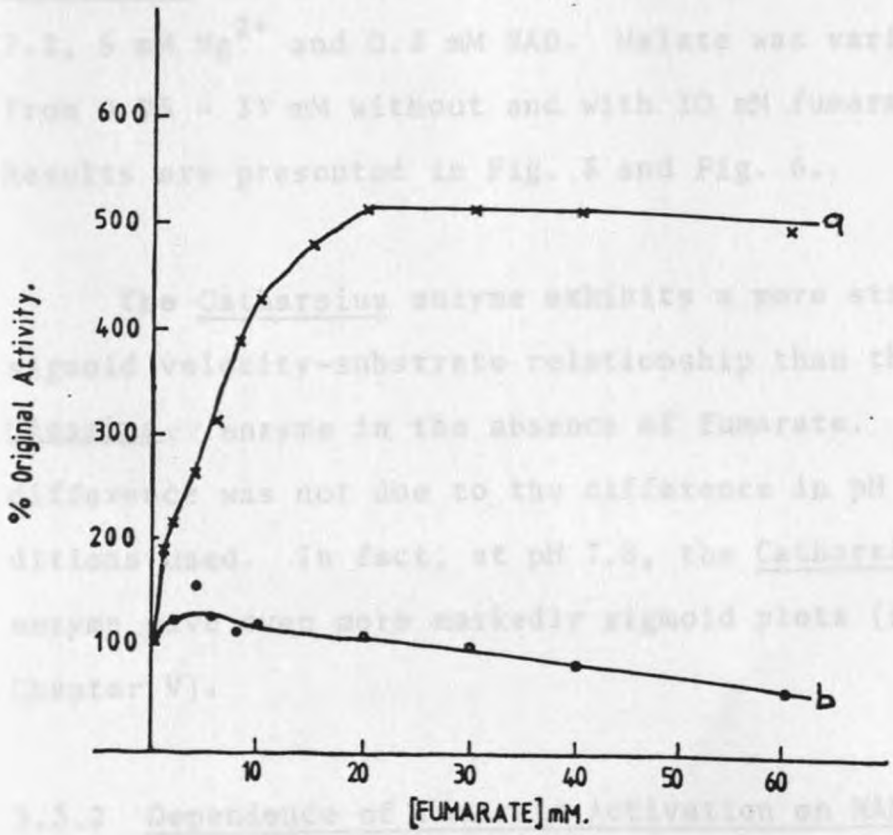

% Original Activity.
400
300
200
100
a
b
4
8
12
16
20
[FUMARATE] mM

Fig. 2


% Original Activity.
600
500
400
300
200
100
a
b
10
20
30
40
50
60
[FUMARATE] mM.

Fumarate and succinate both affect the $[S]_{0.5}$ for malate but V_{max} is unaffected. The velocity versus substrate concentration plots were sigmoidal in the absence of fumarate or succinate, but this sigmoidicity was removed in their presence.

Fig. 4 shows plots of reciprocal of velocity against the reciprocal of malate concentration and demonstrates that non linearity is completely removed by 2 mM fumarate. A similar result was obtained using 2 mM succinate.

A similar experiment was carried out with the Catharsius enzyme using 50 mM Triethanolamine pH 7.2, 5 mM Mg^{2+} and 0.3 mM NAD. Malate was varied from 0.95 - 33 mM without and with 10 mM fumarate. Results are presented in Fig. 5 and Fig. 6.

The Catharsius enzyme exhibits a more strongly sigmoid velocity-substrate relationship than the Glossina enzyme in the absence of fumarate. This difference was not due to the difference in pH conditions used. In fact, at pH 7.8, the Catharsius enzyme gave even more markedly sigmoid plots (see Chapter V).

3.3.2 Dependence of Fumarate Activation on NAD Concentration

FIGURE 3: EFFECT OF MALATE CONCENTRATION ON FUMARATE ACTIVATION OF THE GLOSSINA ENZYME

The assay system included

50 mM TEA-buffer pH 7.8

5 mM Mg^{2+}

0.3 mM NAD

Malate varied 0.1 - 3 mM

o——o without fumarate

x——x with 2 mM fumarate

FIGURE 4: EFFECT OF MALATE CONCENTRATION OF FUMARATE STIMULATION OF THE GLOSSINA ENZYME

The system contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

0.3 mM NAD

Malate varied 1 - 3 mM

o——o without fumarate

x——x with 2 mM fumarate

FIGURE 5: EFFECT OF MALATE CONCENTRATION ON FUMARATE ACTIVATION ON CATHARSIUS ENZYME

The assay system consisted of

50 mM TEA buffer pH 7.2

5 mM Mg^{2+}

0.3 mM NAD

Malate varied 1 - 33 mM

o——o without fumarate

x——x with 10 mM fumarate

FIGURE 6: RECIPROCAL PRESENTATION OF THE EFFECT OF MALATE CONCENTRATION ON FUMARATE ACTIVATION OF CATHARSIUS ENZYME

The system is the same as in Fig. 5

x——x without fumarate

o——o with 10 mM fumarate

Fig. 5

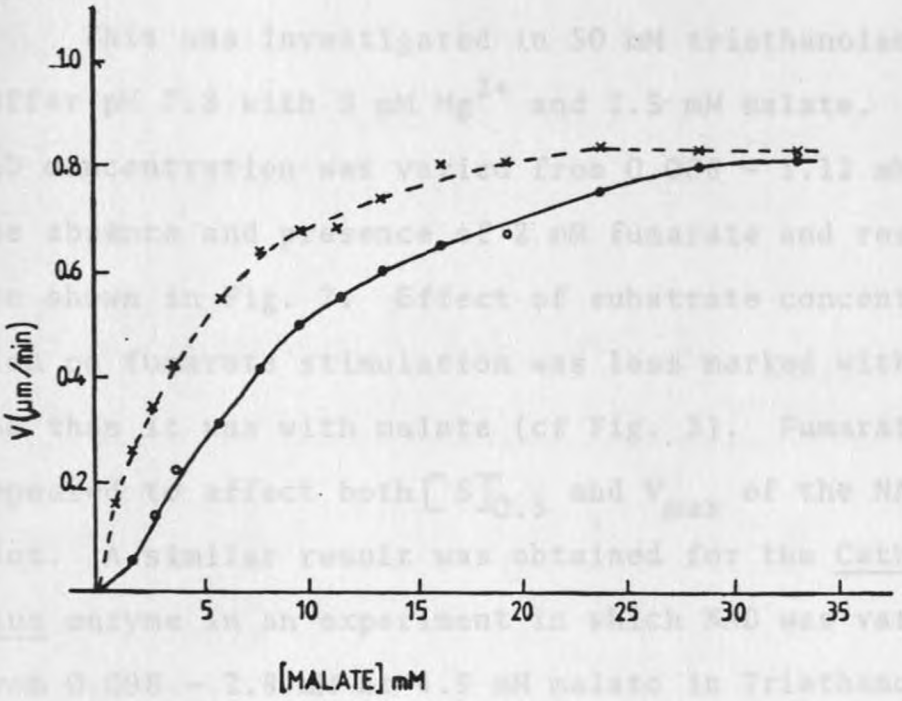
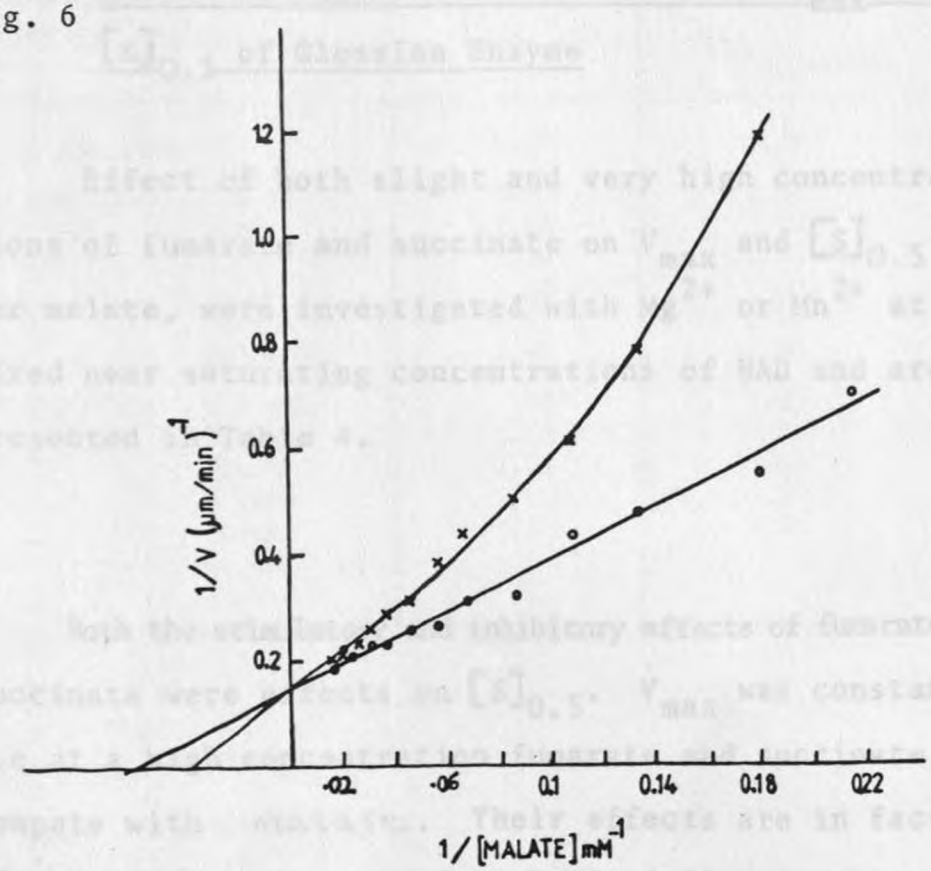
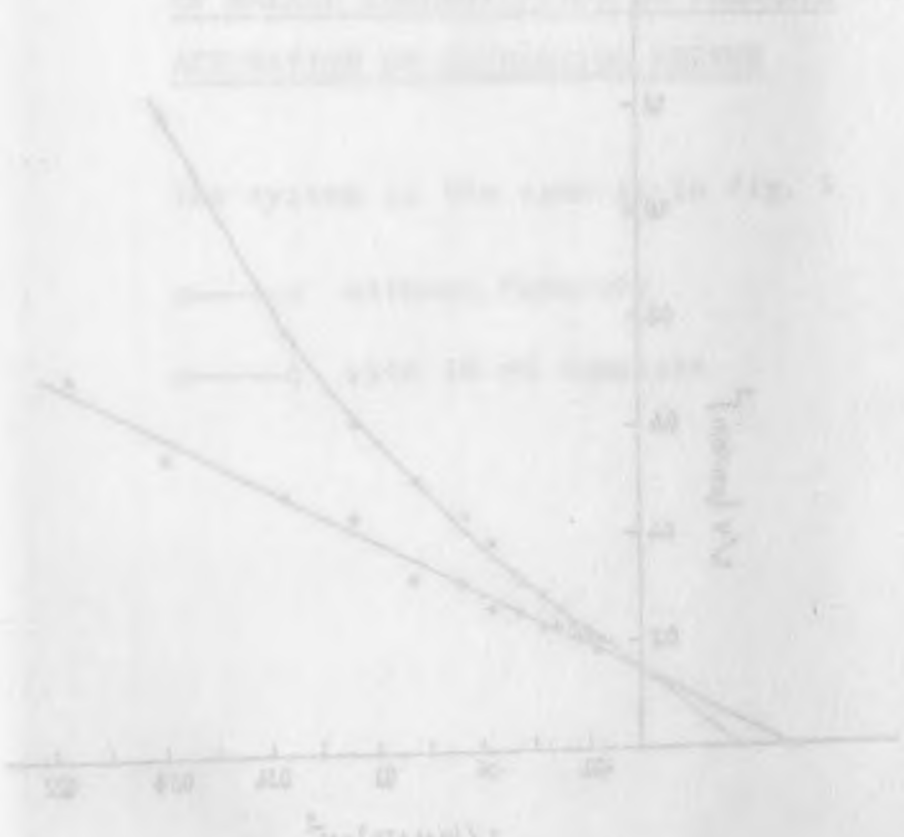

V(µm/min)
0.2
0.4
0.6
0.8
1.0
5
10
15
20
25
30
35
[MALATE] mM

Fig. 6


1/V (µm/min)⁻¹
0.2
0.4
0.6
0.8
1.0
1.2
-02
-06
0.1
0.14
0.18
022
1/[MALATE] mM⁻¹



This was investigated in 50 mM triethanolamine buffer pH 7.8 with 5 mM Mg^{2+} and 2.5 mM malate. The NAD concentration was varied from 0.008 - 1.12 mM in the absence and presence of 2 mM fumarate and results are shown in Fig. 7. Effect of substrate concentration on fumarate stimulation was less marked with NAD than it was with malate (cf Fig. 3). Fumarate appeared to affect both $[S]_{0.5}$ and V_{max} of the NAD plot. A similar result was obtained for the Catharsius enzyme in an experiment in which NAD was varied from 0.098 - 2.8 mM at 4.9 mM malate in Triethanolamine buffer pH 7.2 and 5 mM Mg^{2+} (see Figs. 8, 9.).

3.3.3 Effect of Fumarate and Succinate on V_{max} and $[S]_{0.5}$ of Glossina Enzyme

Effect of both slight and very high concentrations of fumarate and succinate on V_{max} and $[S]_{0.5}$ for malate, were investigated with Mg^{2+} or Mn^{2+} at fixed near saturating concentrations of NAD and are presented in Table 4.

Both the stimulatory and inhibitory effects of fumarate and succinate were effects on $[S]_{0.5}$. V_{max} was constant. i.e at a high concentration fumarate and succinate compete with malate. Their effects are in fact additive as can be seen from Table 4 (i.e compare

TABLE 4: Effects of Fumarate and Succinate Concentration on V_{max} and $[S]_{0.5}$ for malate with Glossina enzyme

With Mg^{2+} as cofactor	$[S]_{0.5}$ mM	$V_{max\ apparent}$ µM/min
no addition	1.56	2.54
+ Fumarate .5 mM	1.09	2.50
" 2 mM	0.65	2.31
" 20 mM	1.18	2.3
" 40 mM	2.03	2.36
+ Succinate 0.5 mM	1.05	2.45
2 mM	0.676	2.34
20 mM	1.07	2.52
40 mM	2.06	2.31
+ Fumarate 1 mM Succinate 1mM	0.64	2.35
with Mn^{2+} as cofactor	$[S]_{0.5}$ mM	$V_{max\ apparent}$
no addition	0.89	2.38
+ Succinate 0.5 mM	0.93	2.31
2 mM	1.23	2.19
20 mM	1.38	2.45
40 mM	2.88	2.64
60 mM	4.24	2.71
80 mM	4.93	2.59
+ Fumarate 0.5 mM	.99	2.43
2 mM	1.19	2.36
20 mM	1.71	2.08
40 mM	2.719	2.23
60 mM	3.248	2.48

$[S]_{0.5}$ with either 2 mM fumarate or 2 mM succinate with that of 1 mM fumarate plus 1 mM succinate with Mg^{2+} as activator).

Inhibition by fumarate was more evident with Mn^{2+} as metal activator. Inhibition by high fumarate concentrations was therefore investigated further in the presence of Mn^{2+}.

The malate concentration was varied from 0.6 - 4 mM at different fixed concentrations of fumarate in triethanolamine buffer pH 7.8, 0.76 mM NAD and 0.5 mM Mn^{2+} using Glossina enzyme. Results are presented in Fig. 10. From this figure it is apparent that fumarate at high concentrations, is a competitive inhibitor with respect to malate.

In summary, fumarate and succinate have K-type effects on both the Glossina or the Catharsius - malic enzymes. Various kinetic parameters obtained in the presence of 2 mM fumarate (the Glossina enzyme) or 10 mM fumarate (the Catharsius enzyme) are included in Tables 2 and 3 respectively.

3.4 PROTEIN PROPERTIES

The Glossina malic enzyme was examined using different gel electrophoresis techniques (see

FIGURE 7: EFFECT OF NAD CONCENTRATION ON FUMARATE ACTIVATION OF GLOSSINA ENZYME

The assay contained

50 mM TEA-buffer pH 7.8

5 mM Mg^{2+}

2.5 mM malate

NAD varied 0.1 - 1 mM

o——o without fumarate

x——x with 2 mM fumarate

FIGURE 8: EFFECT OF FUMARATE ON THE V VERSUS NAD PLOT WITH CATHARSIUS ENZYME

The assay system contained

50 mM TEA - buffer pH 7.2

5 mM Mg^{2+}

5 mM malate

NAD was varied 0.1 - 2.8 mM

o——o without fumarate

x——x with 10 mM fumarate

Fig. 7

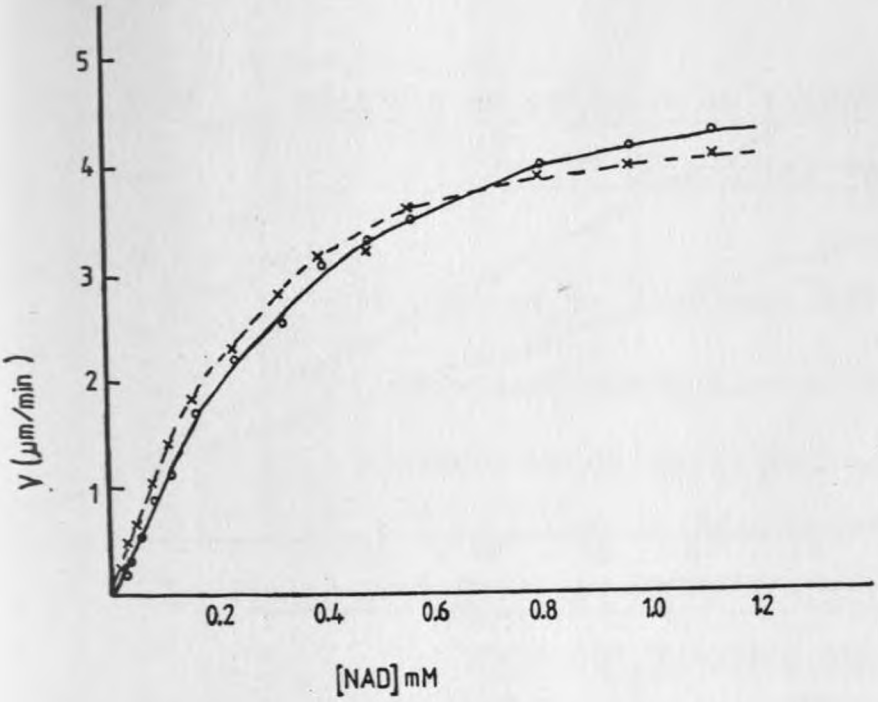

5
4
3
2
1
V (µm/min)
0.2
0.4
0.6
0.8
1.0
1.2
[NAD] mM

Fig. 8

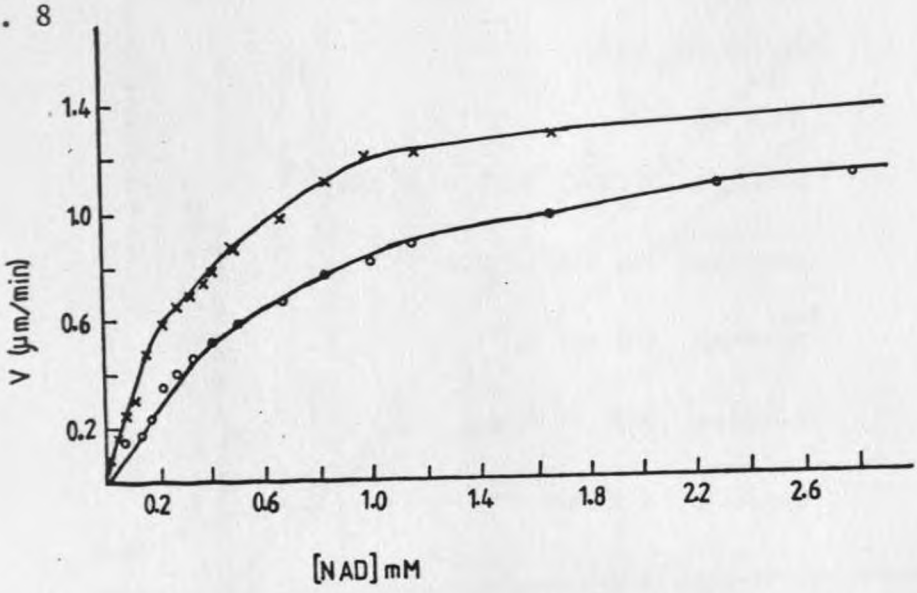

1.4
1.0
0.6
0.2
V (µm/min)
0.2
0.6
1.0
1.4
1.8
2.2
2.6
[NAD] mM

FIGURE 9: EFFECT OF FUMARATE ON $\frac{1}{v}$ VERSUS $\frac{1}{NAD}$ PLOT OF CATHARSIUS ENZYME

The system is as in Fig. 8

o——o without fumarate

x——x with 10 mM fumarate

FIGURE 10: INHIBITION BY FUMARATE WITH MALATE AS THE VARIABLE SUBSTRATE

The system contained

50 mM TEA-buffer pH 7.8

0.76 mM NAD

0.5 mM Mn^{2+}

Malate varied 0.6 - 4 mM

⊙——⊙ no fumarate

x——x 60 mM "

o——o 80 " "

⊗——⊗ 100 mM "

Fig. 9

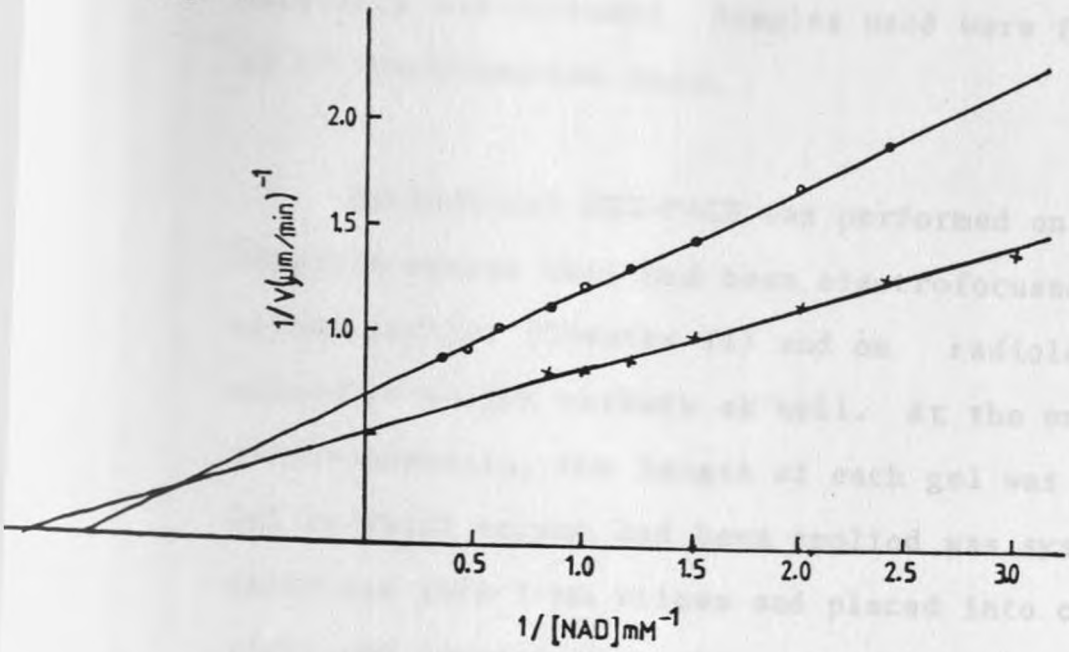
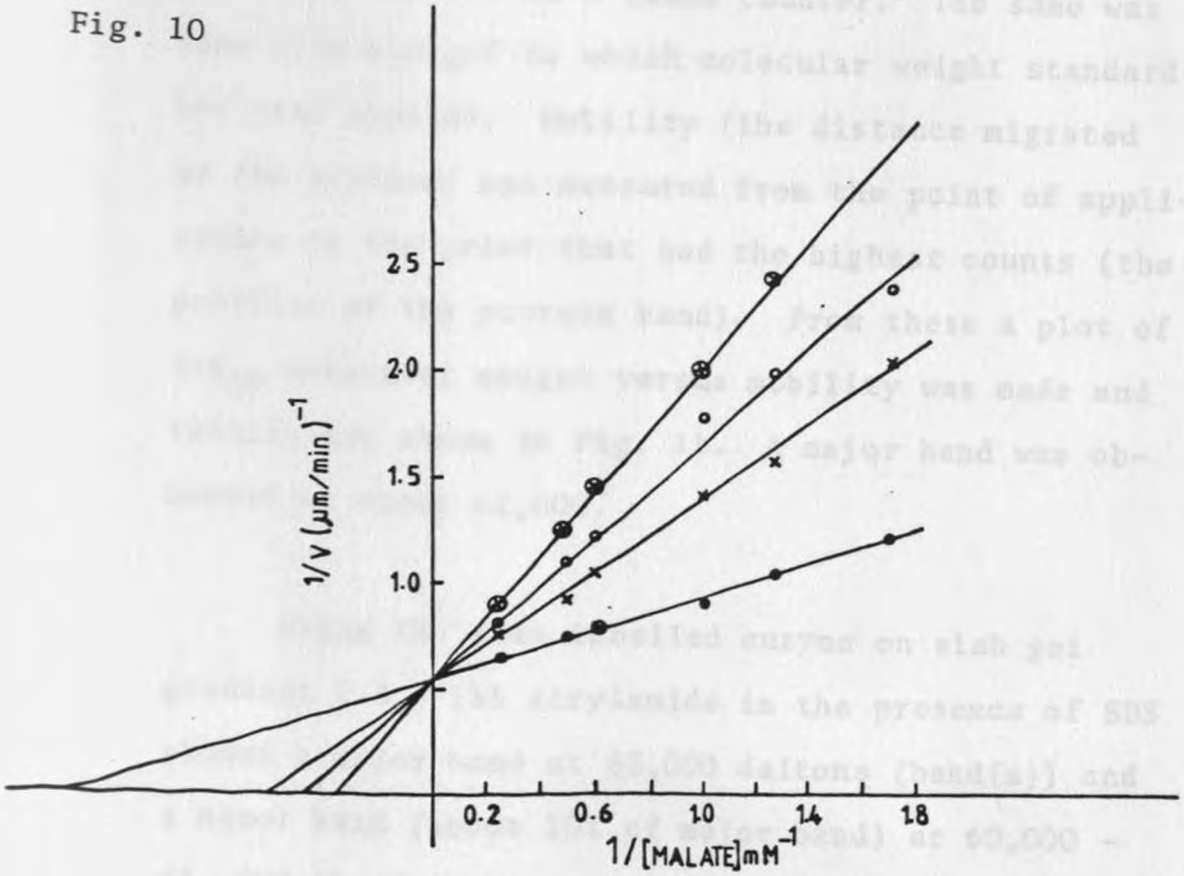

2.0
1.5
1.0
1/V(μm/min)$^{-1}$
0.5
1.0
1.5
2.0
2.5
3.0
1/[NAD]mM^{-1}

Fig. 10


2.5
2.0
1.5
1.0
1/V (μm/min)$^{-1}$
0.2
0.6
1.0
1.4
1.8
1/[MALATE]mM^{-1}

Materials and Methods) Samples used were from the DE 52 fractionation step.

Cylindrical SDS-PAGE was performed on I^{125} labelled enzyme that had been electrofocused as described earlier (Chapter II) and on radiolabelled molecular weight markers as well. At the end of electrophoresis, the length of each gel was measured. Gel to which enzyme had been applied was systematically cut into 1 mm slices and placed into clean vials and counted in a gamma counter. The same was done with each gel to which molecular weight standard had been applied. Mobility (the distance migrated by the protein) was measured from the point of application to the point that had the highest counts (the position of the protein band). From these a plot of $\log_{10}$ molecular weight versus mobility was made and results are shown in Fig. 11. A major band was observed at about 62,000.

Using the same labelled enzyme on slab gel gradient 7.5 - 15% acrylamide in the presence of SDS showed a major band at 65,000 daltons (band(a)) and a minor band (about 10% of major band) at 60,000 - 63, 000 (band (b)). Results are shown in Fig. 12 and on plate I. Lane 1 shows the protein markers

Lane 2 shows the crude enzyme with more than 10 I^{125} protein bands

FIGURE 11: PAGE OF I^{125} GLOSSINA MALIC ENZYME IN CYLINDERS

The molecular weight markers included

Thyroglobulin.	330×10^3
Ferritin,	220×10^3
Albumin	67×10^3
Catalase	60×10^3
Lactate D.H.	36×10^3
$Ferritin_2$	18.2×10^3

Fig. 11

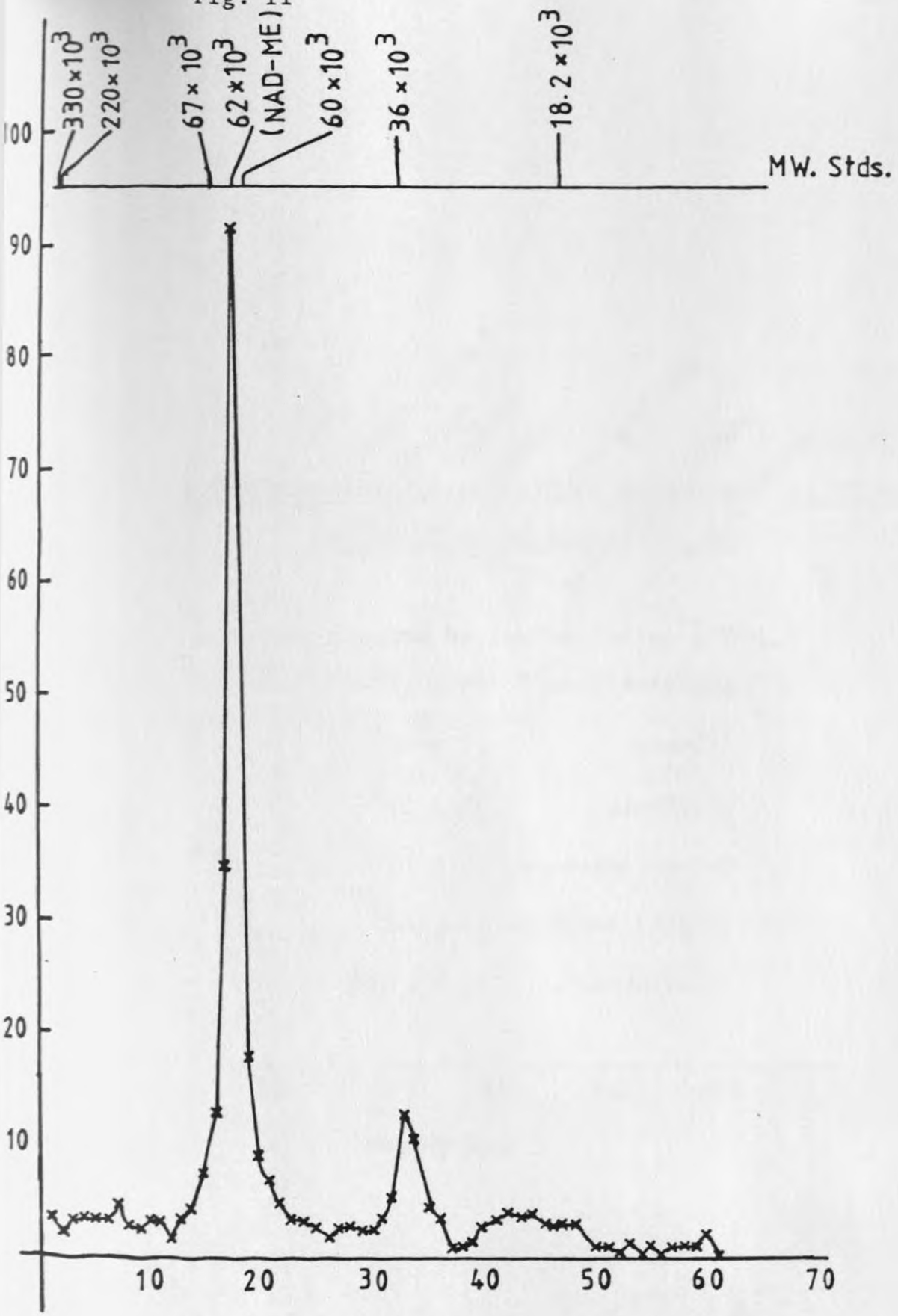

330×10³
220×10³
67×10³
62×10³
(NAD-ME)
60×10³
36×10³
18.2×10³
MW. Stds.
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
Fraction number (mm)

FIGURE 12: SLAB GEL GRADIENT 7.5 - 15% ACRYLAMIDE OF I^{125} GLOSSINA MALIC ENZYME

The molecular weight markers included

phosphorylase 6	94×10^3
Albumin	67×10^3
Ovalbumin	43×10^3
Carbonic anhydrase	30×10^3
Trypsin inhibitor	20×10^3
α-Lactalbumin	14.4×10^3

Fig. 12

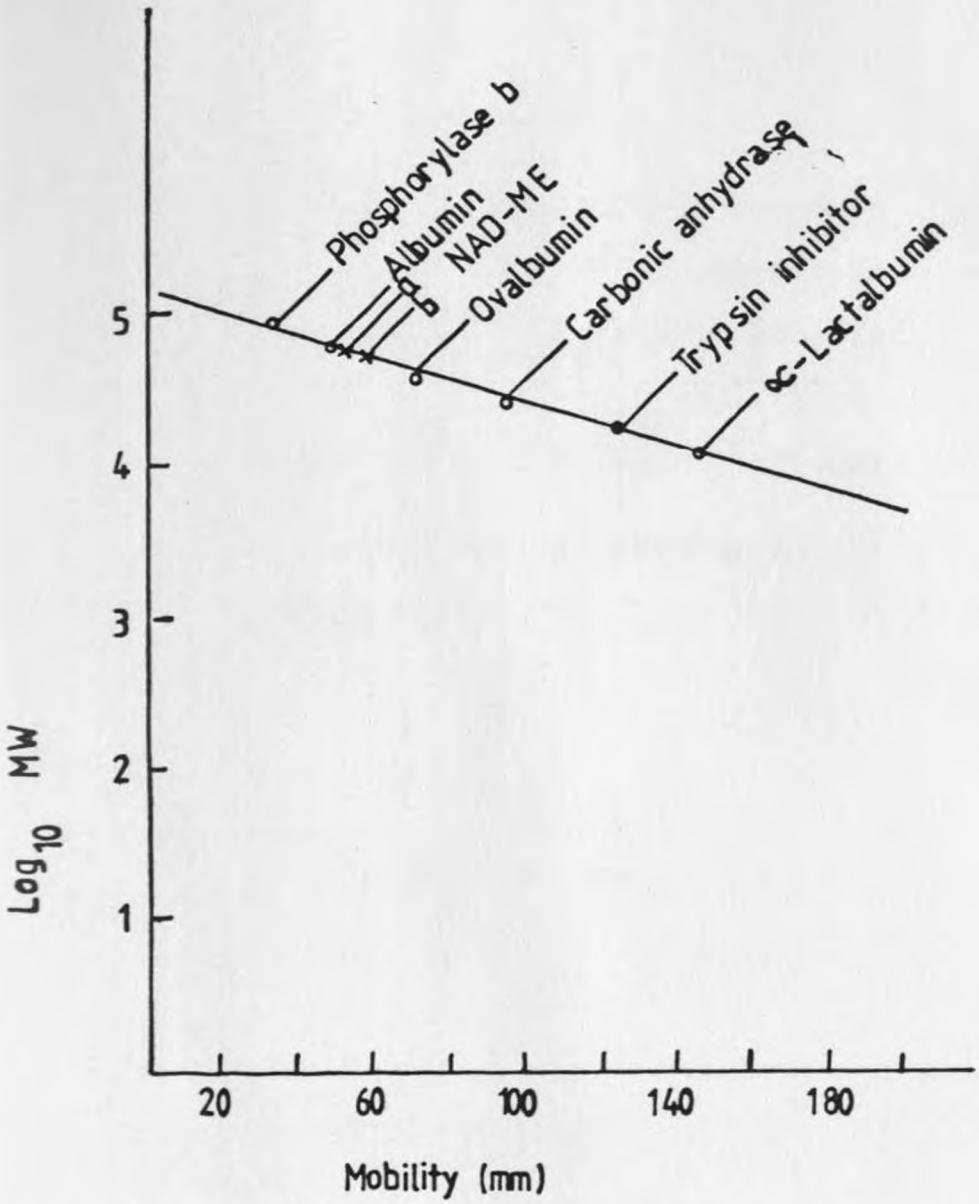

Phosphorylase b
Albumin
a
b
NAD-ME
Ovalbumin
Carbonic anhydrase
Trypsin inhibitor
α-Lactalbumin
5
4
3
2
1
Log10 MW
20
60
100
140
180
Mobility (mm)

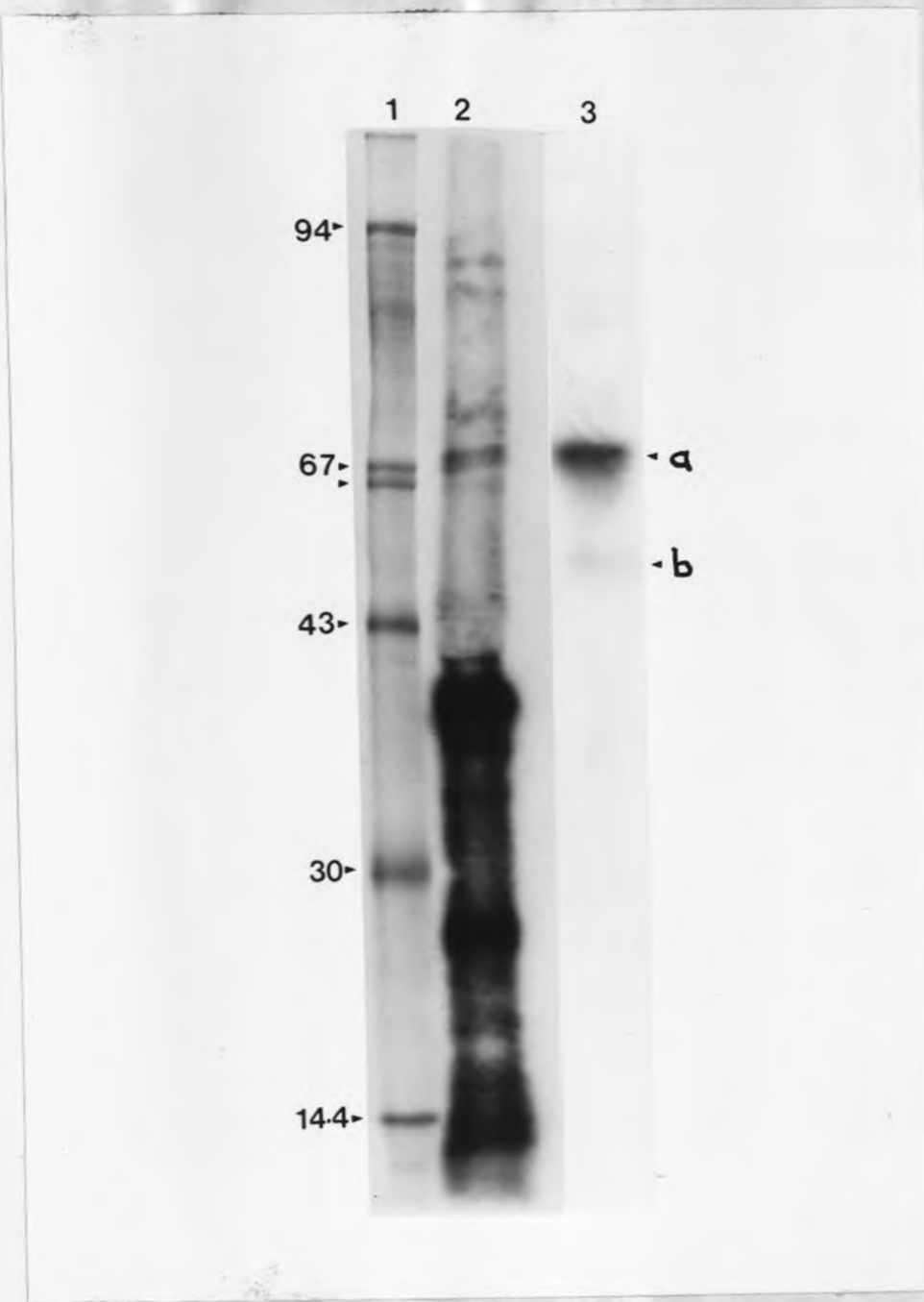
PLATE I: Slab gel gradient 7.5 - 15% acrylamide /SDS stained with coomassie blue using *Glossina* malic enzyme

Lane 1 Shows protein markers

2 Shows crude enzyme

3 Deae 52 enzyme fraction

PLATE I



Lane 3 shows the DEAE 52 enzyme fraction focused for 20 hours as described in Chapter II. Protein band (a) 65,000 daltons is heavily labelled and protein band (b) 60,000 daltons is slightly labelled.

A similar result of unlabelled enzyme is shown as a silver stain of the 7.5% acrylamide/SDS gel. Plate II. Lanes 1 and 2 are as in plate I

Lane 3 is the DE 52 enzyme fraction

Lane 3b is the DE 52 enzyme fraction focused for 20 hrs. Two bands at about 67,000 daltons (band a) and 60,000 daltons band (b) can be observed. The focusing step purified the enzyme slightly but could not be used as a routine purification step since the enzyme lost a lot of activity as stated in Chapter II.

Lane 4 is the sephacryl enzyme fraction and it is evident that the DEAE 52 step effectively cleans up the enzyme.

Electrofocusing of unlabelled malic enzyme was carried out on sucrose gradient and results are shown in Fig. 13. Two peaks of protein were obtained and both had malic enzyme activity. Enzyme Peak (a), however, had a very low specific activity compared to the major Peak (b). Results show that Peak (a)

PLATE II: 7.5% acrylamide/SDS gel shown as a silver stain using *Glossina* malic enzyme

Lane 1 Shows protein markers

2 Shows crude enzyme

3 Shows, the DE 52 enzyme

3b Shows, the DE 52 enzyme fraction focused for 20 hrs

4 The sephacryl enzyme fraction

PLATE II

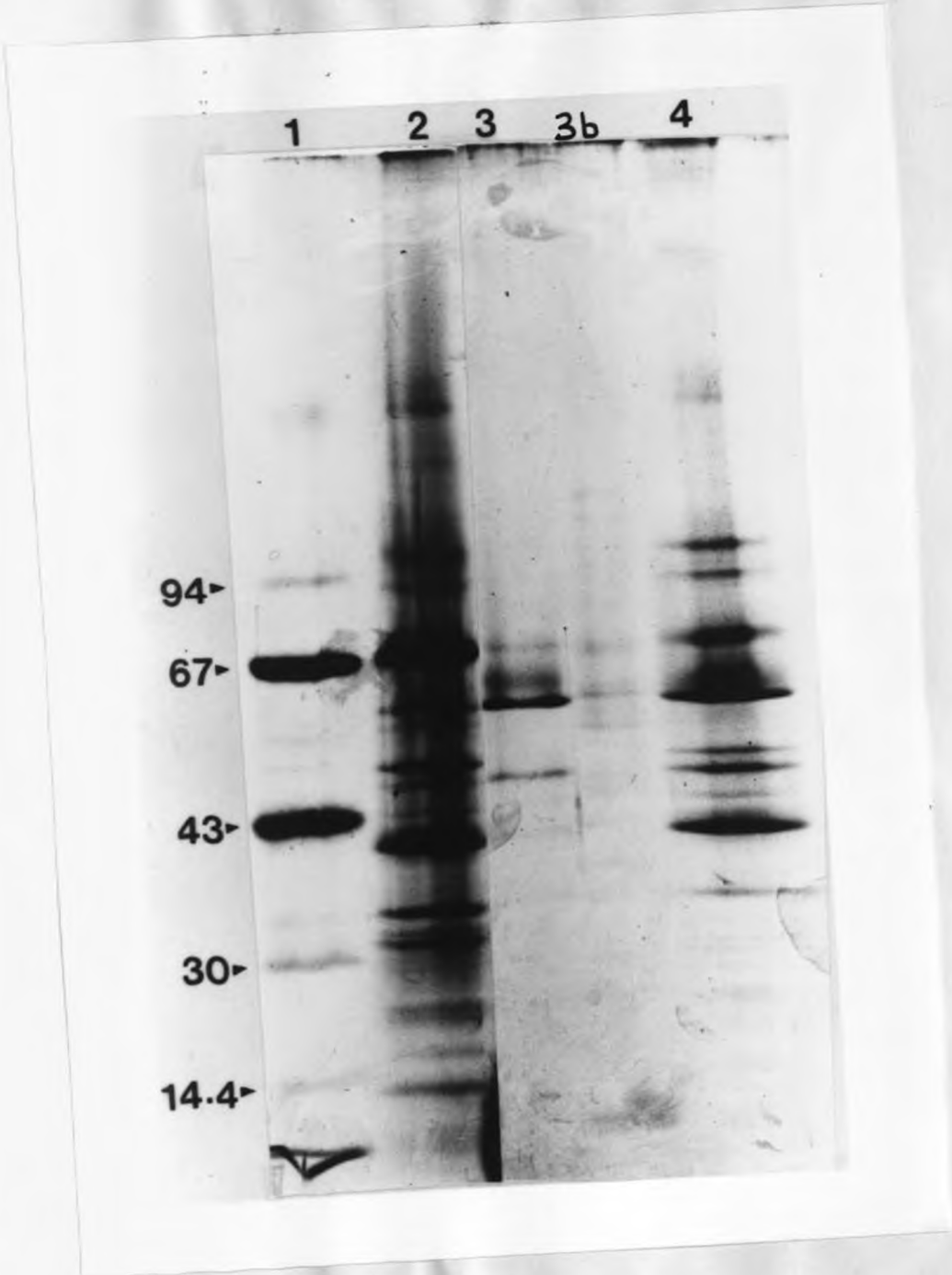
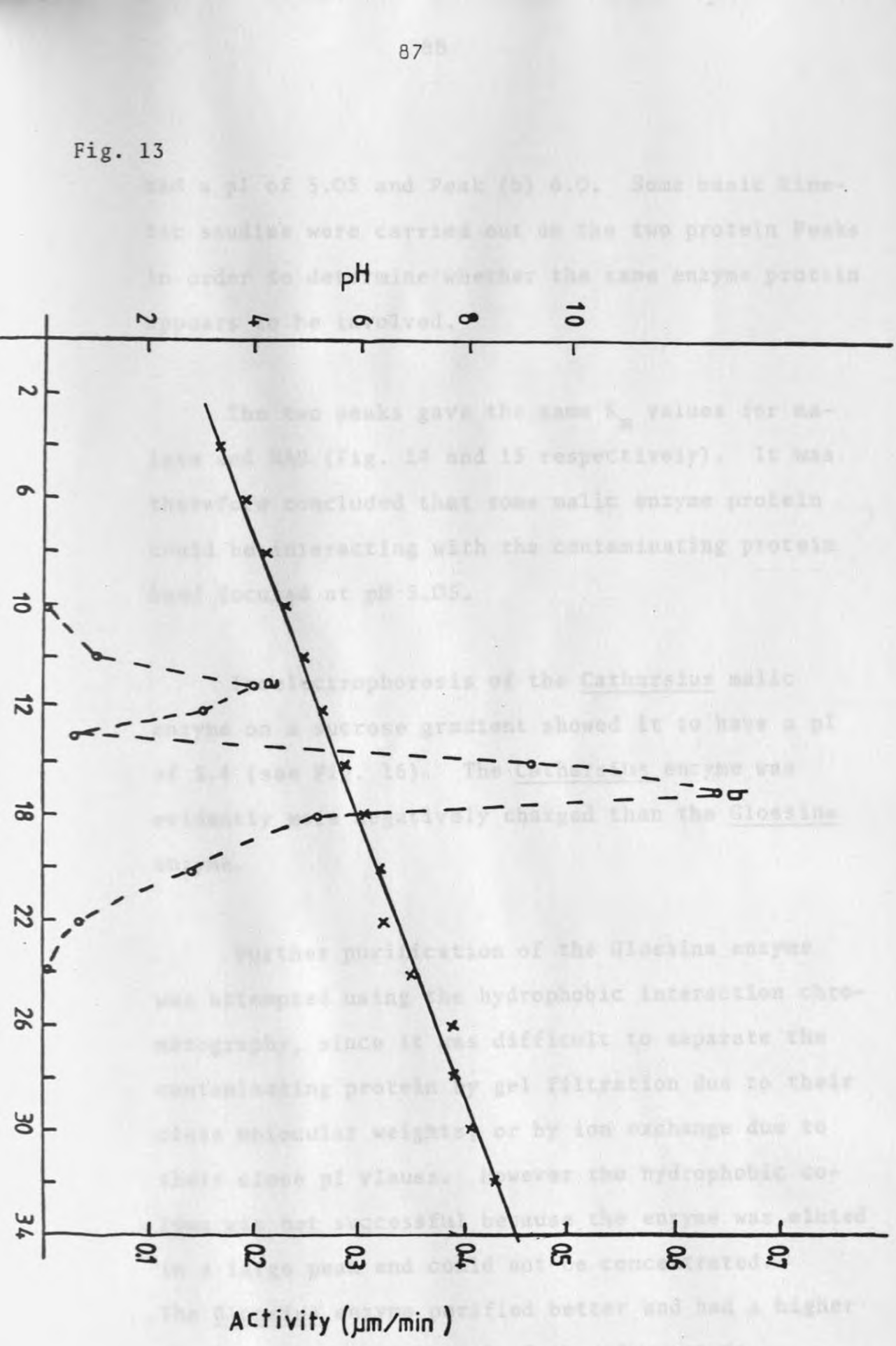


FIGURE 13: ISOELECTROFOCUSING OF *GLOSSINA* MALIC ENZYME ON SUCROSE GRADIENT

x———x pH gradient

O-----o malic enzyme activity

Fig. 13


pH
2
4
6
8
10
2
6
10
12
18
22
26
30
34
a
b
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Activity (µm/min)

had a pI of 5.05 and Peak (b) 6.0. Some basic kinetic studies were carried out on the two protein Peaks in order to determine whether the same enzyme protein appears to be involved.

The two peaks gave the same K_m values for malate and NAD (Fig. 14 and 15 respectively). It was therefore concluded that some malic enzyme protein could be interacting with the contaminating protein band focused at pH 5.05.

Isoelectrophoresis of the Catharsius malic enzyme on a sucrose gradient showed it to have a pI of 5.4 (see Fig. 16). The Catharsius enzyme was evidently more negatively charged than the Glossina enzyme.

Further purification of the Glossina enzyme was attempted using the hydrophobic interaction chromatography, since it was difficult to separate the contaminating protein by gel filtration due to their close molecular weights, or by ion exchange due to their close pI vlaues. However the hydrophobic column was not successful because the enzyme was eluted in a large peak and could not be concentrated.
The Glossina enzyme purified better and had a higher specific activity than the Catharsius enzyme.

FIGURE 14: COMPARISON OF K_m VALUES OF MALATE FOR THE FOCUSED MALIC ENZYME IN PEAK (a) AND (b)
using Glossina malic enzyme

The system contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

0.76 mM NAD

Malate was varied 0.4 - 4 mM

o——o peak (a)

x——x peak (b)

FIGURE 15: COMPARISON OF K_m VALUES FOR NAD FOR THE FOCUSED MALIC ENZYME PEAK (a) AND (b)
using Glossina malic enzyme

The assay system contained

50 mM TEA pH 7.8

5 mM Mg^{2+}

10 mM malate

NAD varied .05 - 1 mM

o——o peak a

x——x peak b

Fig. 14

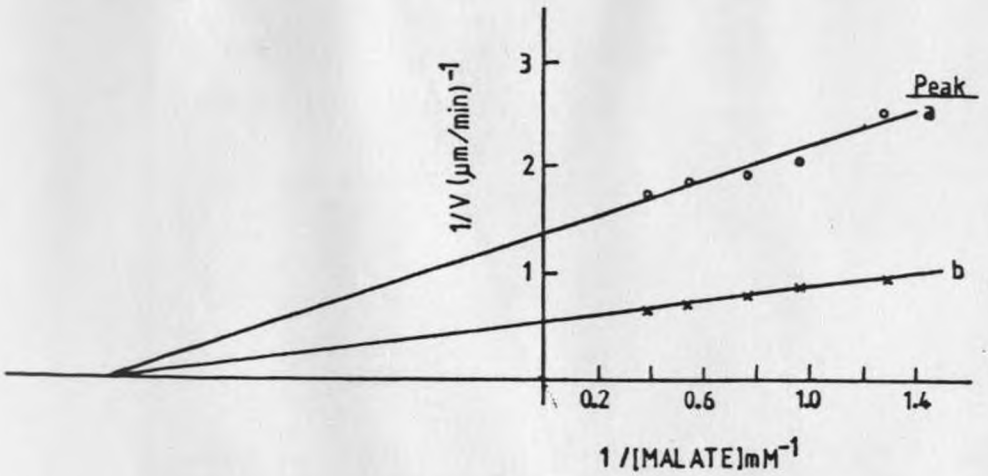

1/V (µm/min)⁻¹
3
2
1
Peak
a
b
0.2
0.6
1.0
1.4
1 /[MALATE]mM⁻¹

Fig. 15

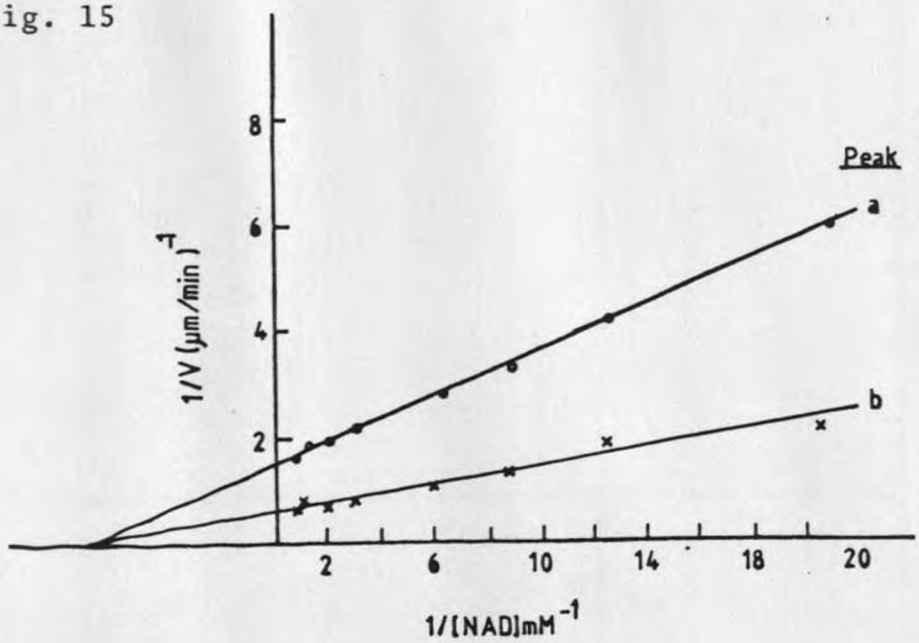

1/V (µm/min)⁻¹
8
6
4
2
Peak
a
b
2
6
10
14
18
20
1/[NAD]mM⁻¹

FIGURE 16: ISOELECTROFOCUSING OF CATHARSIUS ENZYME

x———x pH gradient

---o--- malic enzyme activity

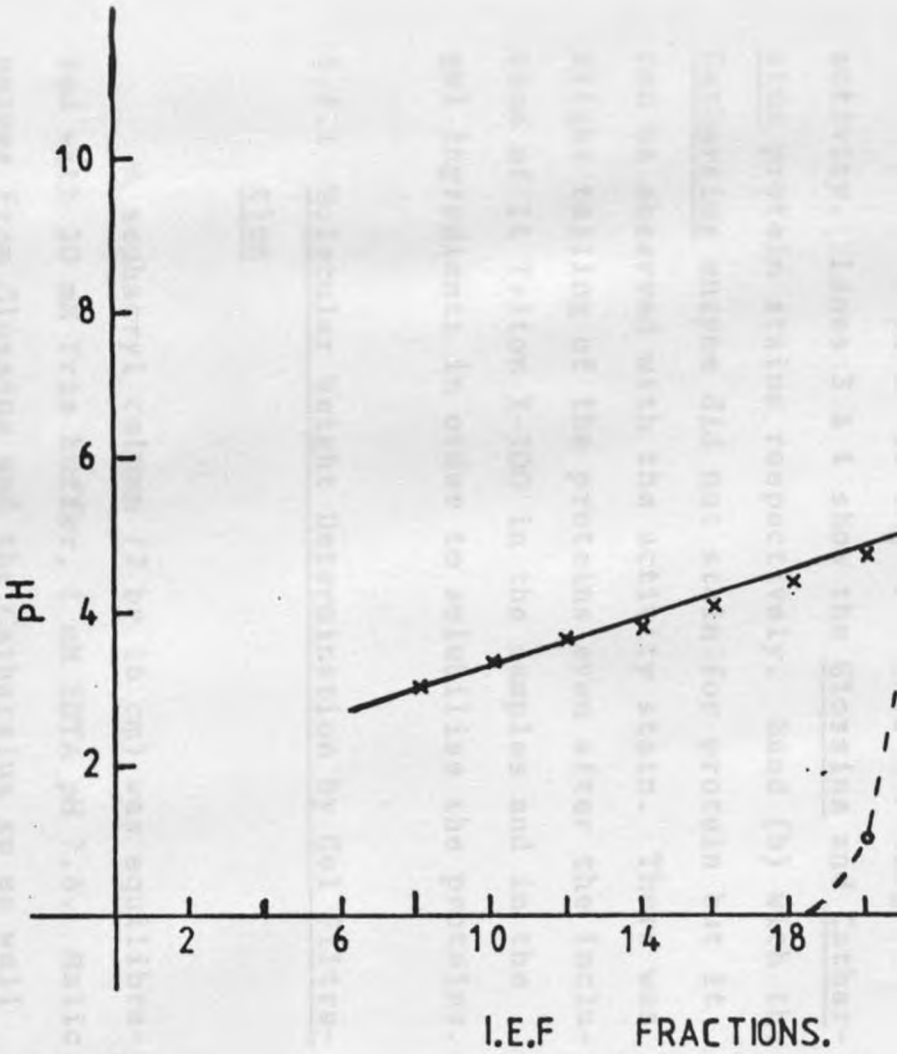

10
8
6
4
2
pH
2
6
10
14
18
I.E.F FRACTIONS.

Fig. 16

Plate III shows coomassie stain of 7.5% SDS acrylamide gel of the DEAE 52 fraction of

Lane 1, Glossina enzyme

Lane 2 Catharsius enzyme

The two main staining protein bands (a) and (b) run slowly i.e are heavier in the Catharsius than the Glossina enzyme.

Plate IV shows an activity stain of a 5% acrylamide (non denaturing) gel of the hydroxyapatite enzyme fraction - Lane 1 shows the Catharsius enzyme activity stain. Lane 2 the Glossina enzyme activity stain both of them showing two bands staining for activity. Lanes 3 & 4 show the Glossina and Catharsius protein stains respectively. Band (b) with the Catharsius enzyme did not stain for protein but it can be observed with the activity stain. There was slight tailing of the proteins even after the inclusion of 1% Triton X-100 in the samples and in the gel ingredients in order to solubilise the proteins.

3.4.1 Molecular Weight Determination by Gel Filtration

A sephacryl column (2 by 16 cm) was equilibrated with 10 mM Tris buffer, 1 mM EDTA pH 7.6. Malic enzyme from Glossina and the Catharsius sp as well as standard proteins, were loaded on the column one

PLATE III: Coomassie blue stain of DE 52 enzyme fraction

Lane 1 *Glossina* enzyme

2 *Catharsius* enzyme

PLATE III

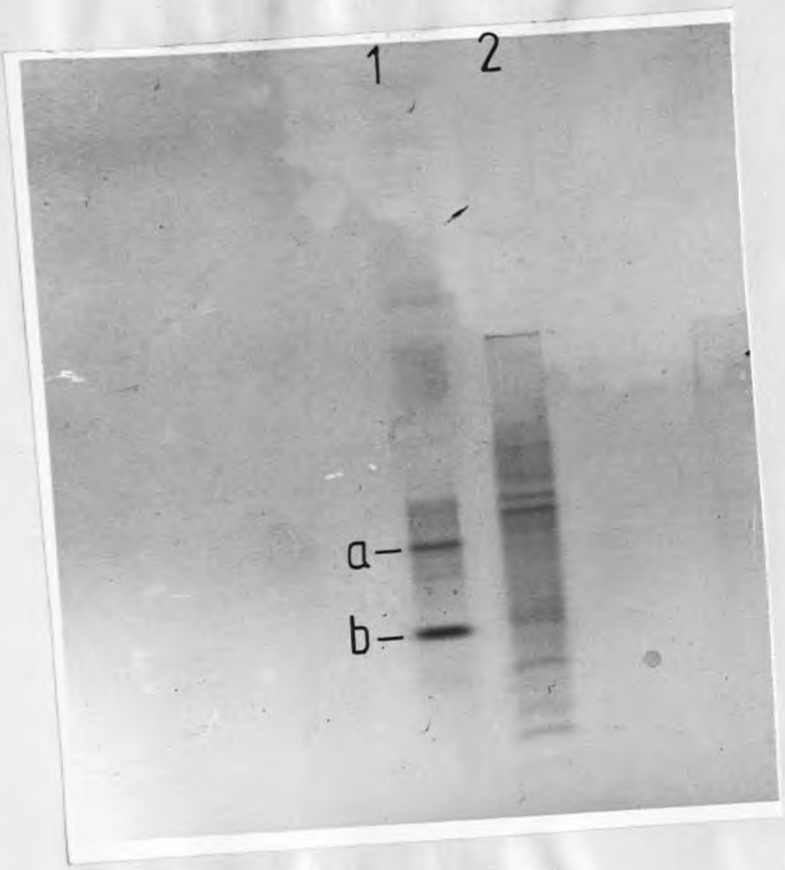


PLATE IV: The 5% acrylamide non denaturing gel; showing:-

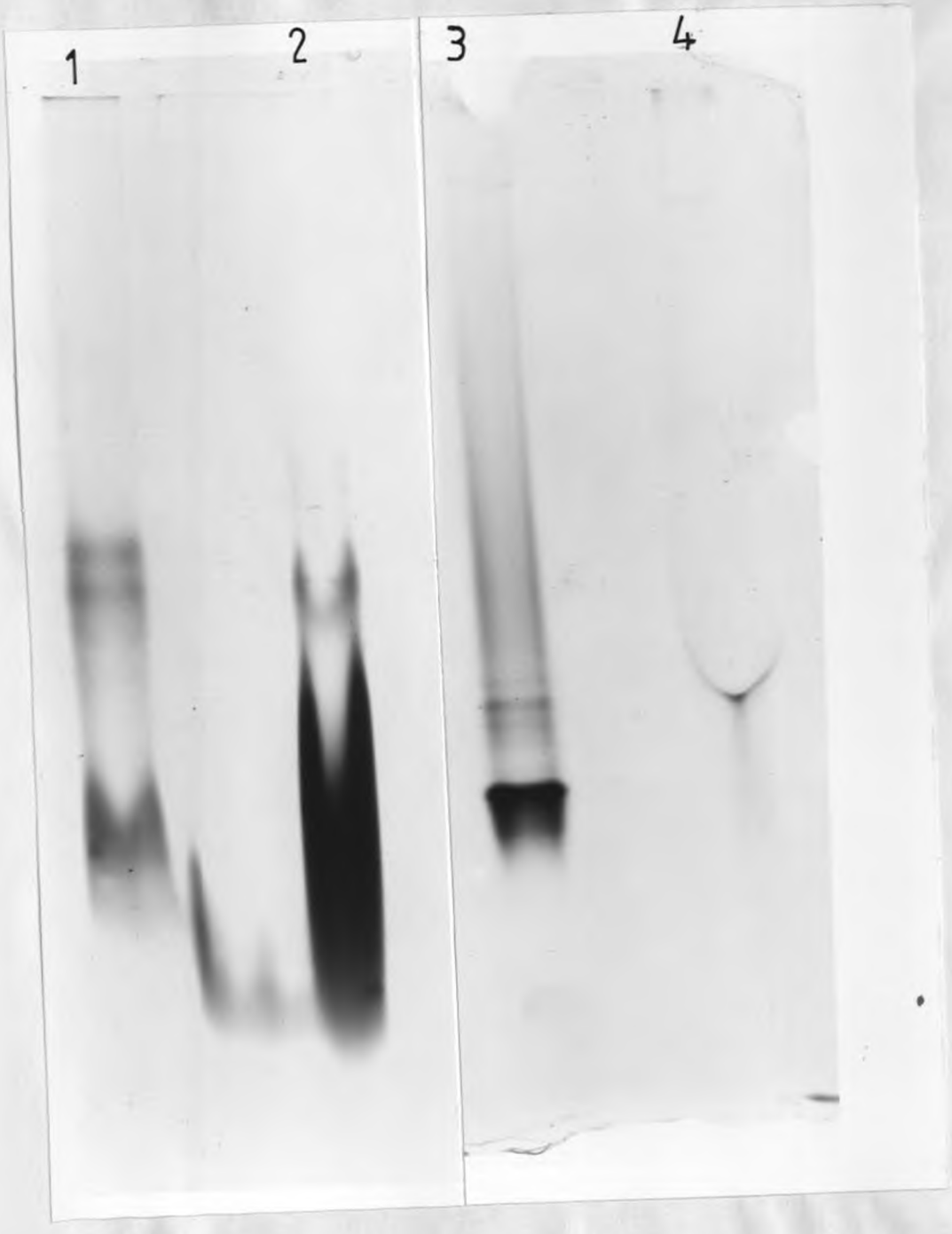
Lane 1 Catharsius enzyme activity stain

2 The Glossina enzyme activity stain

3 The Glossina enzyme coomassie blue protein stain

4 The Catharsius enzyme coomassie blue protein stain

PLATE IV



at a time and eluted with the equilibration buffer. The proteins in the fractions were located by colour, if they were coloured proteins or by assaying for their activity as outlined in materials and methods. The fraction with the highest colour intensity or activity was taken as the peak and elution volume noted. A graph of $\log_{10}$ of molecular weight against the elution volume was constructed and is presented in Fig. 17.

From this figure, malic enzyme from Glossina or Catharsius are very close in their native molecular weights. (Olembo 1980)

3.4.2 Effects of Substrate on the Molecular Weight of the Glossina malic enzyme

Data obtained from Sepharose gel filtration and SDS gel electrophoresis indicated that the NAD-malic enzyme was tetrameric. It was of interest to determine whether the enzyme underwent changes in structure in the presence of various substrates as has been shown to be the case in some plant malic enzymes (Grover et al 1981, 1984). It was therefore incubated for 10 minutes at 30°C with different substrates before being subjected to gel filtration and eluted with 10 mM Tris buffer pH 7.6 containing the respective metabolite. The enzyme was located by its

FIGURE 17: MOLECULAR WEIGHT DETERMINATION OF GLOSSINA AND CATHARSIUS MALIC ENZYMES ON SEPHACRYL S-200 SUPERFINE

Molecular weight standards included

Pyruvate kinase (Rabbit muscle) 230×10^3

G6PD (Yeast) 220×10^3

LDH (Bovine Heart) 150×10^3

Myoglobin 17.8×10^3

Cytochrome C 7.5×10^3

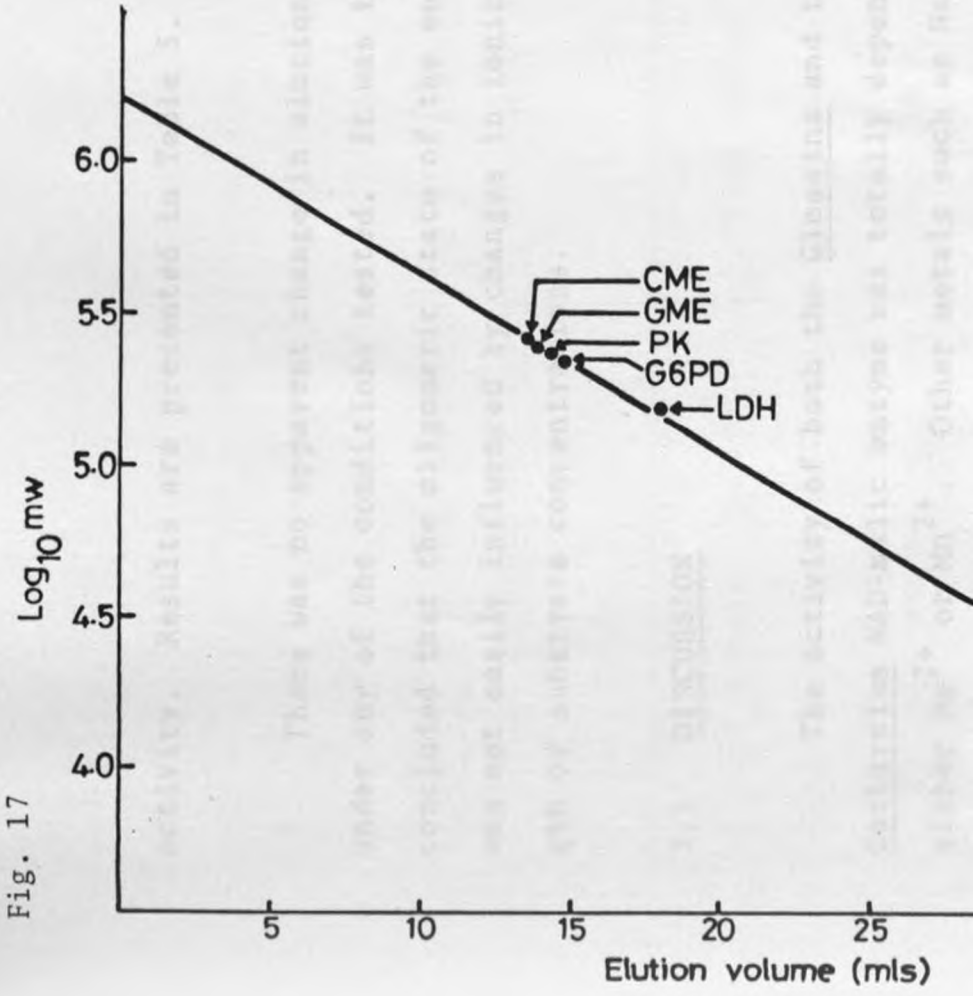

6.0
5.5
5.0
4.5
4.0
Log_{10} mw
CME
GME
PK
G6PD
LDH
5
10
15
20
25
Elution volume (mls)

Fig. 17

activity. Results are presented in Table 5.

There was no apparent change in elution volume under any of the conditions tested. It was therefore concluded that the oligomeric state of the enzyme was not easily influenced by changes in ionic strength or substrate concentrations.

3.5 DISCUSSION

The activity of both the Glossina and the Catharsius NAD-malic enzyme was totally dependent on either Mg^{2+} or Mn^{2+}. Other metals such as Na^{+}, K^{+} could not support the activity, whilst heavy metals as well as Ca^{2+} were inhibitory. For both enzymes, the metal concentration required for full activity was far lower with Mn^{2+} (0.5 mM) than with Mg^{2+} (5 mM).

With the Glossina enzyme, V_{max} and K_m values for both substrates were the same whether Mg^{2+} or Mn^{2+} were used. With the Catharsius enzyme on the other hand the K_m for malate and NAD were two times lower in the presence of Mn^{2+} than with Mg^{2+}, though V_{max} was again unaffected.

The activity of both insect enzymes could be stimulated by fumarate and succinate. However, the

TABLE 5: Effect of Substrates on the Elution Volumes obtained for Glossina NAD-Malic Enzyme

Sample	Elution volume (mls)
NAD-malic enzyme	16.7
NAD-ME plus	
malate (60 mM)	16.7
NAD (8 mM)	16.7
Fumarate (60 mM)	16.7
NaCl (100 mM)	16.7
Tris/HCl pH 7.8 (100 mM)	16.7
Mn^{2+} (20 mM)	16.7

Catharsius enzyme needed a higher concentration (10 mM) for maximum stimulation to be achieved.

The fact that fumarate gave competitive inhibitory effects with malate implies that fumarate might be binding at a malate binding site.

Fumarate and succinate probably bind at the same site, for their actions were additive, and neither had any effects on V_{max}; as well as having a similar magnitude in the lowering of $K_{m_{apparent}}$. This is unlike what was observed for the Crassula enzyme in which fumarate and CoA increased V_{max} as well as lowered K_m, but it could be that they had an effect on the aggregatory state of the enzyme similar to increasing malate concentration (Wedding et al 1881).

Mn^{2+} also appeared to have effects on the enzyme similar to fumarate and succinate i.e removing sigmoidicity and lowering $K_{m_{apparent}}$ for the Catharsius enzyme but not for Glossina enzyme. In fact fumarate gave no stimulation in the presence of Mn^{2+} probably because Mn^{2+} had already abolished the interactions that could be existing between the malate sites.

Fumarate has two effects i.e. removing sigmoi-

dicity and inhibiting competitively at high concentration. Both effects might be explained by fumarate binding at malate site.

Stimulation by fumarate and succinate has been shown with other NAD and NADP malic enzymes (Sauer 1973, Landsperger and Harris 1976, Frenkel 1972, Swierczynski et al 1982 a,b). This could have important physiological significance since like malic enzyme fumarate and succinate exist within the mitochondria.

From gel chromatography studies, it appears that both enzymes are tetrameric with molecular weight at 240,000 - 260,000 although the Catharsius enzyme seems slightly heavier than the Glossina enzyme. (Glossina enzyme 237,000 - 240,000 Catharsius enzyme 250,000 - 260,000 Daltons).

The two enzymes were however different in their pI values, the Glossina enzyme having a pI value of 6.0 and the Catharsius enzyme 5.4. This suggests the Catharsius malic enzyme to be more acidic hence more negatively charged. Interestingly, a correspodding differences exists between the pH optima of the two enzymes, that of the Glossina enzyme being about 0.6 pH units higher than the Catharsius.

CHAPTER IV

4.0 MECHANISM OF SUBSTRATE ADDITION AND EFFECTS OF pH

4.1 EFFECT OF SUBSTRATE CONCENTRATION ON RATE OF REACTION CATALYSED BY THE GLOSSINA ENZYME

The effect of varying both substrates was investigated in 50 mM Triethanolamine buffer pH 7.8. 2 mM fumarate was included to obtain linear kinetics. In each experiment, a two substrate plot was obtained by varying the concentration of one substrate at six different fixed concentrations of the other. Primary data were treated using an Eadie Hofstee plot (v versus v/[S]). Data from the v versus v/[A] plot were replotted against $\frac{1}{[B]}$. In the investigations as reported in this chapter, 1-2 µg of enzyme were used in 2 mls with a 1 cm pathlength cuvette, to obtain ΔOD/min values in the range 0.06 - 0.3.

Initial investigations were carried out using a relatively high range of malate concentration and a relatively low range of NAD concentration. L-malate was varied from about 0.4 - 4 mM and NAD from 0.1 - 1.3 mM. The primary plots of reciprocal of initial velocity against reciprocal of NAD concentration gave a pattern of straight lines intersecting on or just

behind the $\frac{1}{v}$-axis (Fig. 18).

The reciprocal plot of $\frac{1}{v}$ against $\frac{1}{[\text{malate}]}$ values from the same experiment gave a series of lines intersecting further behind the $\frac{1}{v}$-axis as shown in Fig. 19.

These results suggested that the rate equation might be of the relatively simple form

$$v = \frac{V[A][B]}{\bar{K}_A K_{m_B} + K_{m_B}[A] + [A][B]} \qquad (6)$$

where A = malate

B = NAD

$\bar{K}_A$ = limiting K_m for A as [B] tends to zero

K_{m_B} = limiting K_m for B as [A] tends to infinity.

K_{m_A}, the limiting K_m for A as [B] tends to infinity would be zero. Such an equation would apply if malate bound to the enzyme compulsorily before NAD and this binding reaction was near equilibrium.

To ascertain whether K_{m_A} was or was not zero, the experiment was repeated using a slightly higher range of NAD concentrations and a lower range of malate concentrations. (NAD varying from 0.16 - 2 mM and malate from 0.05 - 0.8 mM). The plots shown in

FIGURE 18: DOUBLE RECIPROCAL PLOT WITH NAD AS VARIABLE SUBSTRATE (AT HIGH MALATE CONCENTRATIONS)

using Glossina malic enzyme

The system contained
50 mM TEA buffer pH 7.8
2 mM Fumarate
5 mM Mg^{2+}
NAD varied 0.1 - 1.3 mM
at ▫———▫ 0.47 mM malate
⊙———⊙ 0.93 " "
⊙———⊙ 1.3 " "
x———x 1.86 " "
o———o 2.8 " "
x———x 4.1 " "

FIGURE 19: DOUBLE RECIPROCAL PLOT WITH MALATE AS THE VARIABLE SUBSTRATE (AT LOW FIXED NAD CONCENTRATION)

using Glossina malic enzyme

The assay consisted of
50 mM TEA-buffer pH 7.8
2 mM Fumarate
Malate concentration as in Fig. 18

◇———◇ 0.09 mM NAD
▫———▫ 0.13 " "
⊙———⊙ 0.23 " "
o———o 0.39 " "
⊗———⊗ 0.55 " "
x———x 1.29 " "

Fig. 18

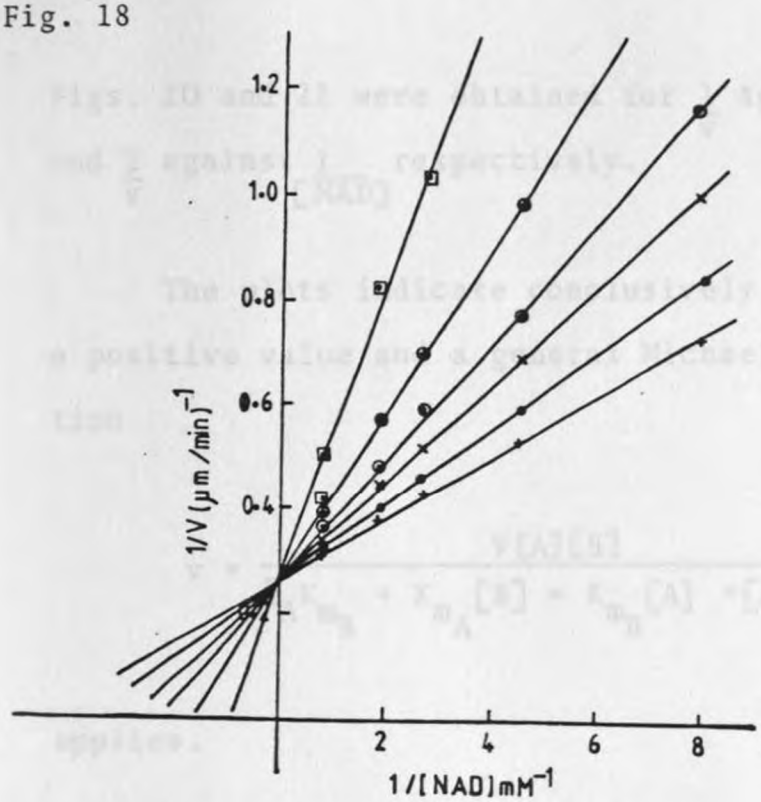
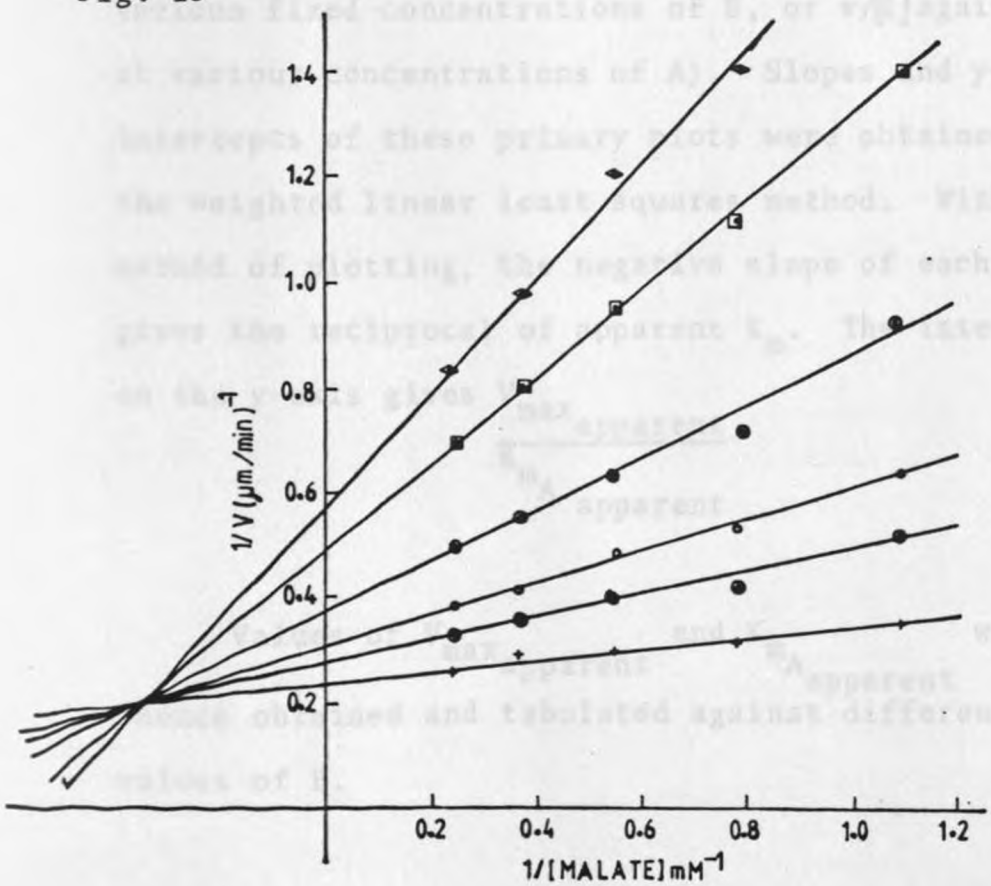

1.2
1.0
0.8
0.6
0.4
1/V (µm/min)$^{-1}$
2
4
6
8
1/[NAD]mM^{-1}

Fig. 19


1.4
1.2
1.0
0.8
0.6
0.4
0.2
1/V (µm/min)$^{-1}$
0.2
0.4
0.6
0.8
1.0
1.2
1/[MALATE]mM^{-1}

Figs. 20 and 21 were obtained for $\frac{1}{v}$ against $\frac{1}{[\text{malate}]}$ and $\frac{1}{v}$ against $\frac{1}{[\text{NAD}]}$ respectively.

The plots indicate conclusively that K_{m_A} has a positive value and a general Michaelis rate equation

$$v = \frac{V[A][B]}{\bar{K}_A K_{m_B} + K_{m_A}[B] + K_{m_B}[A] + [A][B]} \qquad (7)$$

applies.

The primary data from the same experiment were plotted as an Eadie Hofstee plot (v against $\frac{v}{[A]}$ at various fixed concentrations of B, or $v/[B]$ against v at various concentrations of A). Slopes and y-axis intercepts of these primary plots were obtained by the weighted linear least squares method. With this method of plotting, the negative slope of each line gives the reciprocal of apparent K_m. The intercept on the y-axis gives

$$\frac{V_{max_{apparent}}}{K_{m_A}{}_{apparent}}$$

Values of $V_{max_{apparent}}$ and $K_{m_{A_{apparent}}}$ were thence obtained and tabulated against different values of B.

FIGURE 20: DOUBLE RECIPROCAL PLOT WITH MALATE AS VARIABLE SUBSTRATE (AT HIGH FIXED NAD CONCENTRATION)

using Glossina malic enzyme

The assay system contained

50 mM TEA buffer pH 7.8
2 mM Fumarate
5 mM Mg^{2+}

Malate varied 0.05 - 0.8 mM

at x——x 0.16 mM NAD
⊕——⊕ 0.32 " "
◈——◈ 0.48 " "
o——o 0.8 " "
⊖——⊖ 1.28 " "
⊡——⊡ 1.93 " "

FIGURE 21: DOUBLE RECIPROCAL PLOT WITH NAD AS VARIABLE SUBSTRATE (AT LOW FIXED MALATE CONCENTRATION)

using Glossina malic enzyme

The assay system NAD and Malate
The system contained concentrations
as in Fig. 20

⊕——⊕ 0.053 mM malate
⊡——⊡ 0.08 " "
◈——◈ 0.1 " "
o——o 0.26 " "
x——x 0.53 " "
⊖——⊖ 0.8 " "

Fig. 20

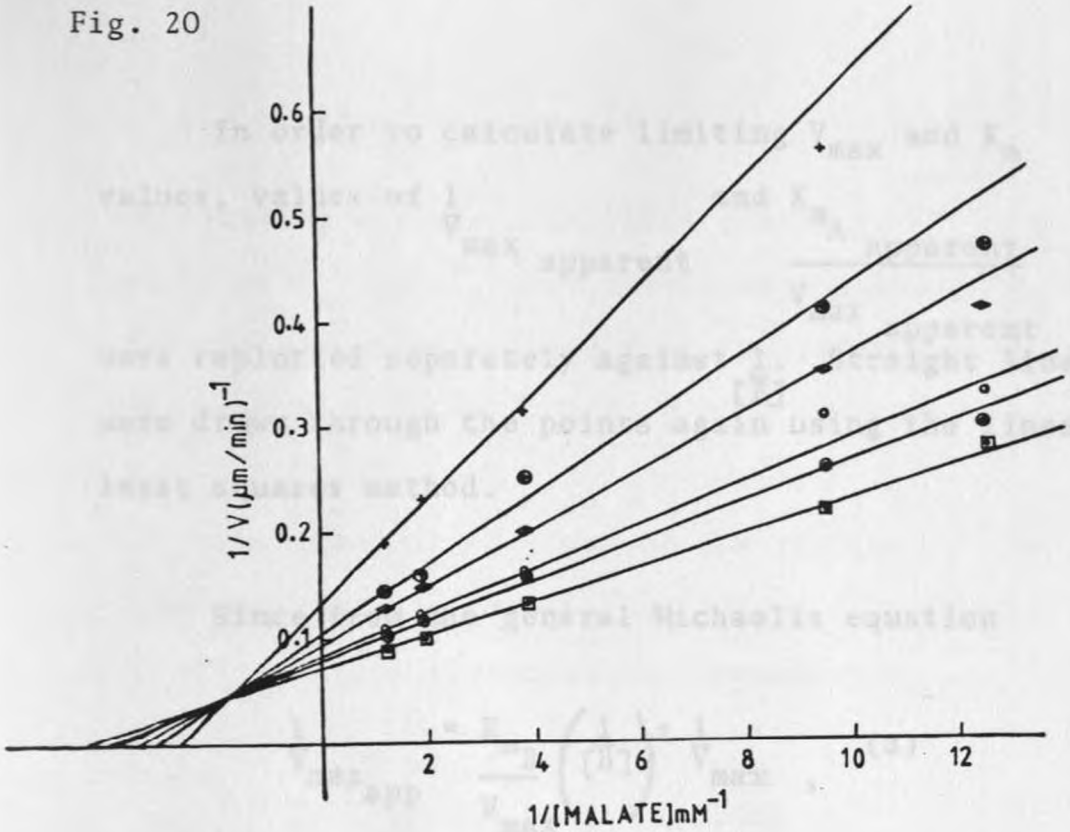
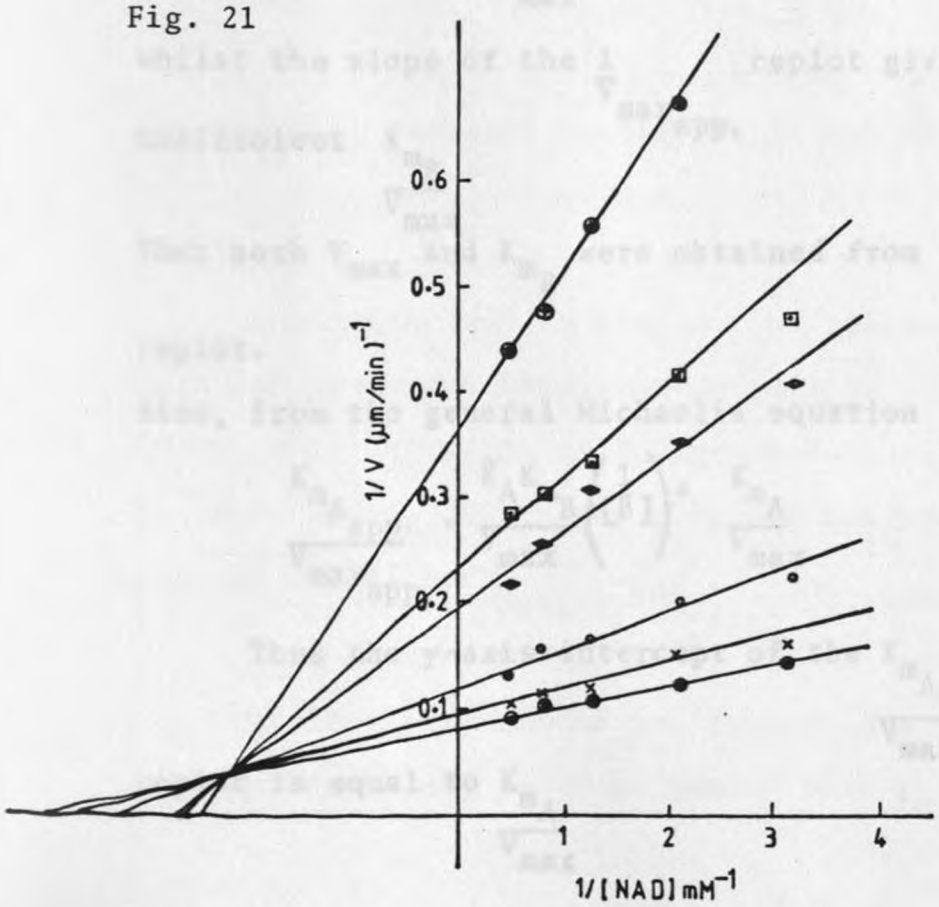

0.6
0.5
0.4
0.3
0.2
0.1
1/V(μm/min)⁻¹
2
4
6
8
10
12
1/[MALATE]mM⁻¹

Fig. 21


0.6
0.5
0.4
0.3
0.2
0.1
1/V (μm/min)⁻¹
1
2
3
4
1/[NAD] mM⁻¹

In order to calculate limiting V_{max} and K_m values, values of $\frac{1}{V_{max}}_{apparent}$ and $\frac{K_{m_A}}{V_{max}}_{apparent}$ were replotted separately against $\frac{1}{[\bar{B}]}$. Straight lines were drawn through the points again using the linear least squares method.

Since from the general Michaelis equation

$$\frac{1}{V_{max_{app}}} = \frac{K_{m_B}}{V_{max}}\left(\frac{1}{[\bar{B}]}\right) + \frac{1}{V_{max}} \quad , \qquad (8)$$

the y-axis intercept of the $\frac{1}{V_{max_{app}}}$ replot gives the limiting value $\frac{1}{V_{max}}$

whilst the slope of the $\frac{1}{V_{max_{app}}}$ replot gives the coefficient $\frac{K_{m_B}}{V_{max}}$

Thus both V_{max} and K_{m_B} were obtained from the $\frac{1}{V_{max_{app}}}$ replot.

Also, from the general Michaelis equation

$$\frac{K_{m_{A_{app}}}}{V_{max_{app}}} = \frac{\bar{K}_A K_{m_B}}{V_{max}}\left(\frac{1}{[\bar{B}]}\right) + \frac{K_{m_A}}{V_{max}} \qquad (9)$$

Thus the y-axis intercept of the $\frac{K_{m_{A_{app}}}}{V_{max_{app}}}$ replot is equal to $\frac{K_{m_A}}{V_{max}}$

and the slope of this replot is equal to $\frac{\bar{K}_A K_{m_B}}{V_{max}}$ substituting already calculated values of V_{max}, K_{m_B}, values of K_{m_A} and $\bar{K}_A$ were therefore obtained. $\bar{K}_B$ when needed was calculated from the relationship

$$\bar{K}_A K_{m_B} = \bar{K}_B K_{m_A}$$

Treatment in this way of the results of the experiment illustrated in Figs 20 and 21 gave the following values for Michaelis parameters:

K_{m_B} = 0.208 mM; K_{m_A} = 0.178 mM;
V_{max} = 3.82 μM/min; $\bar{K}_A$ = 1.12mM;
$\bar{K}_B$ = 1.31 mM.

The value of the ratio $\frac{K_{m_A}}{\bar{K}_A} = \left(\frac{K_{m_B}}{\bar{K}_B}\right)$, subsequently referred to here as α, was obtained as 0.17.

For comparison, a similar two substrate experiment was performed using the Catharsius enzyme, but in 50 mM Triethanolamine buffer pH 7.2, with 10 mM fumarate added. The plots obtained are presented in Fig. 22 and 23 for $\frac{1}{[\text{malate}]}$ and $\frac{1}{[\text{NAD}]}$ respectively.

Earlier experiments had suggested a rapid equilibrium compulsory order mechanism with K_{m_A} close to

zero. When the experiment was repeated as above (Figs. 22 and 23), using higher NAD or lower malate concentration ranges, and primary plots analysed by the least squares method, K_{m_A} was found not to be zero and α was determined as having the finite value of 0.13. Values of 1.89 mM and 0.59 mM were determined for $K_{m_{malate}}$ and $K_{m_{NAD}}$ respectively and 14.1 mM was obtained for $\bar{K}_{malate}$.

4.2 PRODUCT INHIBITION

NAD-malic enzyme has three products, pyruvate, carbon dioxide and NADH. The effect of each of these products on enzyme activity was investigated in the case of the Glossina enzyme by varying one substrate at fixed saturating or non-saturating concentrations of the other, in the absence and presence of various chosen fixed concentrations of product. These experiments were performed in a buffer system containing 50 mM Triethanolamine buffer pH 7.8 and 5 mM Mg^{2+}; 2 mM fumarate was also included in the system to obtain linear double reciprocal plots.

4.2.1 Inhibition by NADH

With near saturating levels of malate (10 mM), the NAD concentration was varied from 0.083 - 2 mM at six different concentrations of NADH. Inhibition

FIGURE 22: DOUBLE RECIPROCAL PLOT WITH MALATE AS THE VARIABLE SUBSTRATE WITH CATHARSIUS ENZYME

The assay system contained

50 mM TEA buffer pH 7.2
5 mM Mg^{2+}
10 mM Fumarate
Malate varied 0.5 - 3.7 mM

◇——◇ 0.51 mM NAD
0——0 0.85 " "
+——+ 1.36 " "
□——□ 2.04 " "
o——o 3.24 " "
x——x 5.29 " "

FIGURE 23: DOUBLE RECIPROCAL PLOT WITH NAD AS THE VARIABLE SUBSTRATE USING CATHARSIUS ENZYME

The assay system, malate and NAD concentration as in Fig. 22

◇——◇ 0.56 mM malate
0——0 0.75 " "
+——+ 0.94 " "
□——□ 1.31 " "
o——o 1.87 " "
x——x 3.7 " "

Fig. 22

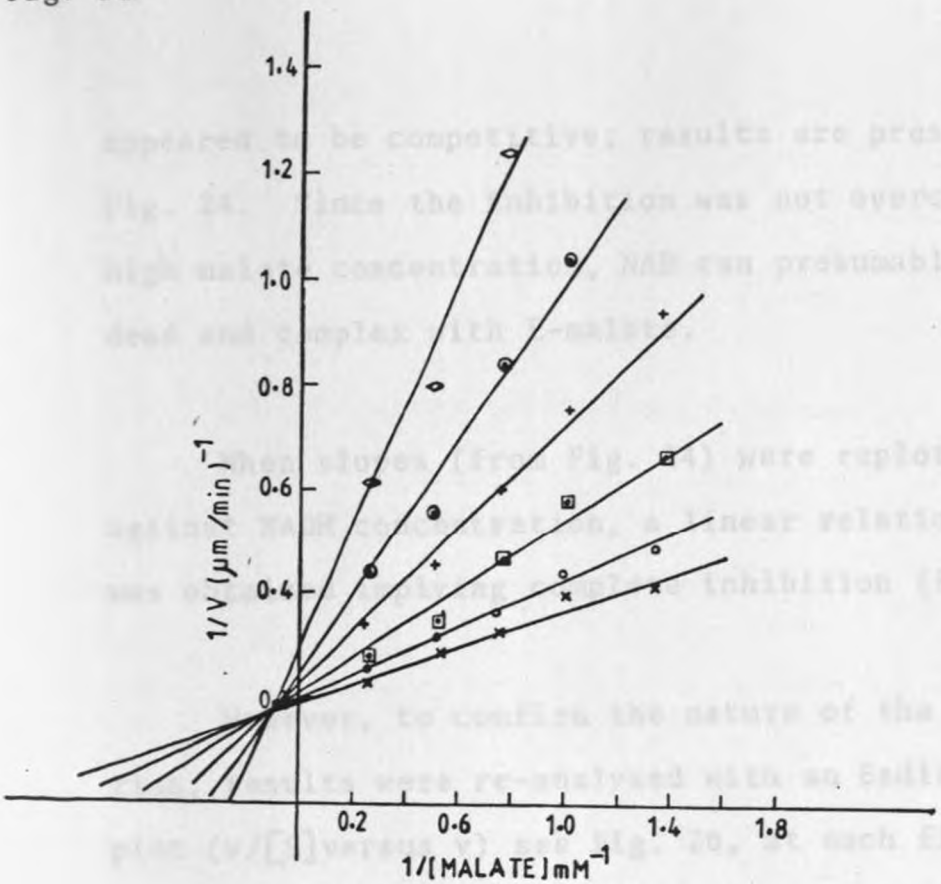
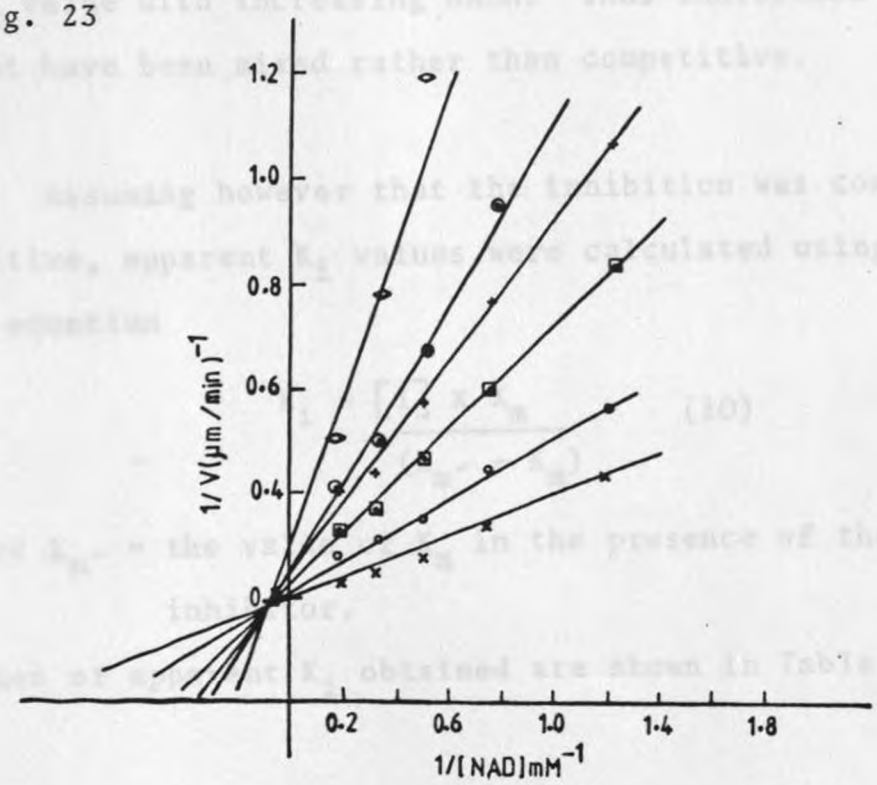

1.4
1.2
1.0
0.8
0.6
0.4
0
1/V (µm/min)$^{-1}$
0.2
0.6
1.0
1.4
1.8
1/[MALATE]mM^{-1}

Fig. 23


1.2
1.0
0.8
0.6
0.4
0
1/V(µm/min)$^{-1}$
0.2
0.6
1.0
1.4
1.8
1/[NAD]mM^{-1}

appeared to be competitive; results are presented in Fig. 24. Since the inhibition was not overcome by high malate concentration, NAD can presumably form a dead end complex with E-malate.

When slopes (from Fig. 24) were replotted against NADH concentration, a linear relationship was obtained implying complete inhibition (Fig. 25).

However, to confirm the nature of the inhibition, results were re-analysed with an Eadie Hofstee plot (v/[S]versus v) see Fig. 26, at each fixed inhibitor concentration. Values of apparent V_{max} and apparent K_m were determined as before by the least squares method and presented in Table 6. There appears to be a slight, but consistent reduction in V_{max} value with increasing NADH. Thus inhibition might have been mixed rather than competitive.

Assuming however that the inhibition was competitive, apparent K_i values were calculated using the equation

$$K_i = \frac{[I] \times K_m}{(K_{m'} - K_m)} \qquad (10)$$

where $K_{m'}$ = the value of K_m in the presence of the inhibitor.

Values of apparent K_i obtained are shown in Table 6.

FIGURE 24: EFFECT OF NADH ON $\frac{1}{v}$ VERSUS $\frac{1}{NAD}$ PLOT AT SATURATING MALATE CONCENTRATION

using *Glossina* malic enzyme

The system contained

50 mM TEA buffer pH 7.8

5 mM Fumarate

10 mM Malate

NAD varied 0.083 - 2 mM

o——o no addition

x---x 0.025 mM NADH

o---o 0.05 " "

●——● 0.075 " "

x——x 0.1 " "

o——o 0.125 " "

FIGURE 25: $\frac{K_{m_{app}}}{V_{max_{app}}}$ at different NADH concentration

The same experiment shown in Fig. 24.

Fig. 24

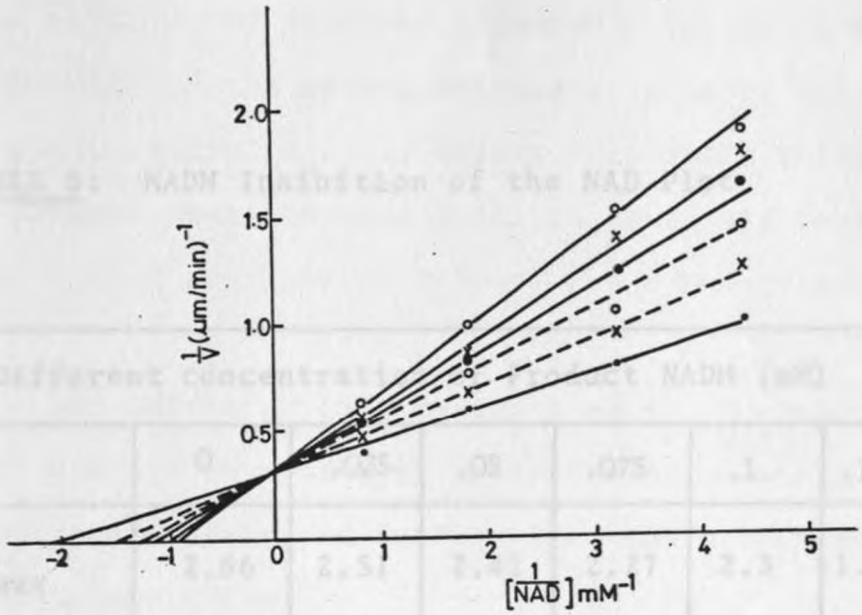

2.0
1.5
1.0
0.5
-2
-1
0
1
2
3
4
5
$\frac{1}{V}$ (µm/min)$^{-1}$
$\frac{1}{[NAD]}$ mM^{-1}

Fig. 25

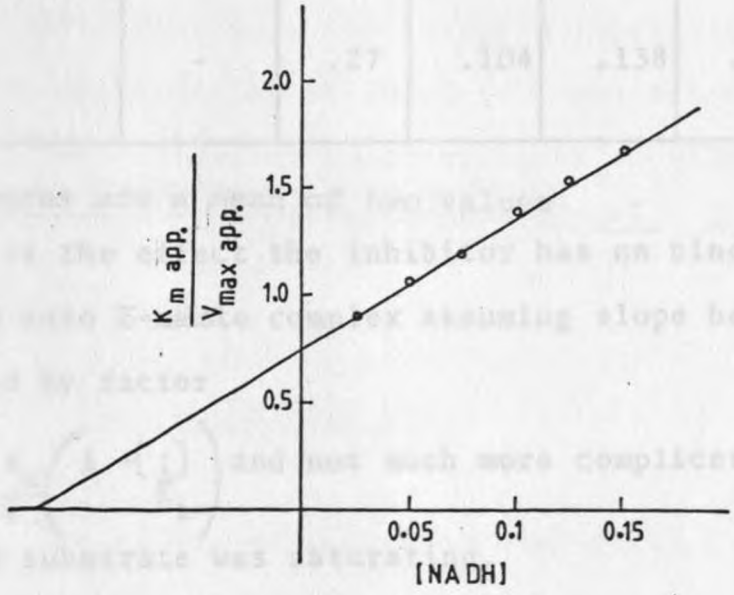

2.0
1.5
1.0
0.5
0.05
0.1
0.15
$\frac{K_m \text{ app.}}{V_{max} \text{ app.}}$
[NADH]

TABLE 6: NADH Inhibition of the NAD Plot

Different concentration of Product NADH (mM)						
	0	.025	.05	.075	.1	.125
V_{max}	2.66	2.51	2.41	2.27	2.3	1.97
K_m	.131	.143	.194	.202	.25	.34
K_i	-	.27	.104	.138	.109	.083

Figures are a mean of two values

K_i is the effect the inhibitor has on binding of NAD onto E-malate complex assuming slope being affected by factor

$$\frac{K_m}{v}\left(1 + \frac{[I]}{K_i}\right)$$

and not much more complicated since 2nd substrate was saturating.

The fact that these values decreased with increase in concentration of NADH was presumably because the concentration of the second substrate (malate) was not strictly saturating. If malate were fully saturating, the values obtained should all be equal and represent the binding constant of NADH to the E-malate complex.

The effect of NADH on the $\frac{1}{\bar{v}}$ versus $\frac{1}{[\bar{N}AD]}$ plot was also studied at unsaturating levels of malate (1 mM). Results are presented in Fig. 27. Inhibition was again competitive.

With malate as the variable substrate at high (0.76 mM) and also at lower (0.1 mM) NAD concentration, several fixed concentrations of NADH were used. Mixed inhibition patterns were obtained as shown in Fig. 28.

4.2.2 Inhibition with HCO_3^-

The effect of bicarbonate on initial velocity was examined at high levels of NAD (0.76 mM) as well as at 0.32 mM NAD. Malate concentration was varied from 0.13 - 5.26 mM at several fixed concentrations of HCO_3^-. The pH was strictly controlled and kept at 7.8 in all the assays performed with this inhibitor. A competitive inhibition pattern as presented in Figs. 29 and 30 was obtained.

FIGURE 26: EADIE HOFSTEE PLOT ON EFFECT OF NADH ON $\frac{1}{v}$ VERSUS $\frac{1}{NAD}$ PLOT

Results plotted from the experiment shown in Fig. 24.

Fig. 26

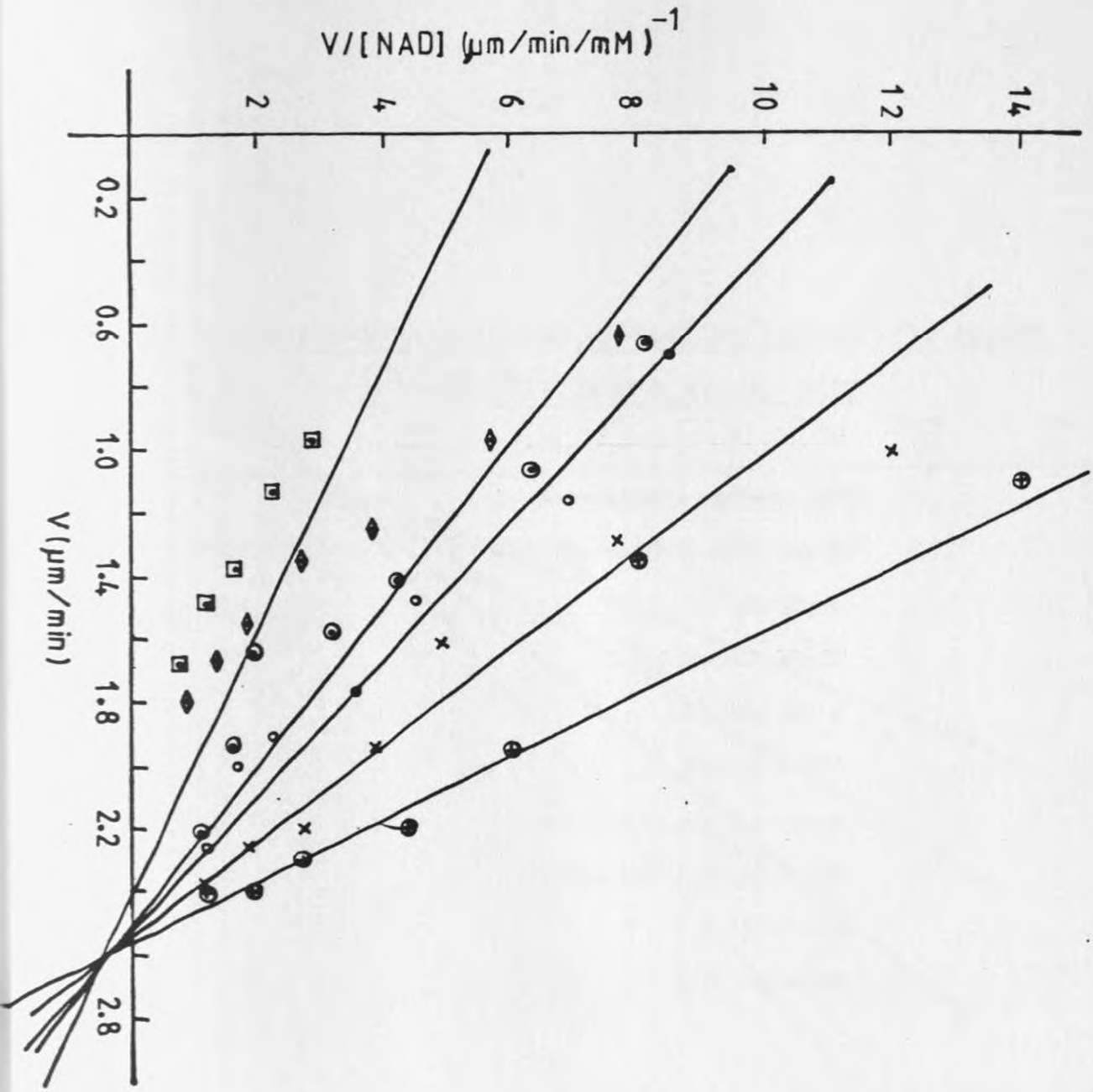


FIGURE 27: EFFECT OF NADH ON NAD PLOT AT UNSATURATING MALATE CONCENTRATIONS

using *Glossina* malic enzyme

The system contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

2 mM Fumarate

1 mM malate

NAD varied 0.1 - 1.25 mM

x——x no addition

o——o 0.1 mM NADH

⊡——⊡ 0.2 " "

◍——◍ 0.3 " "

Fig. 27

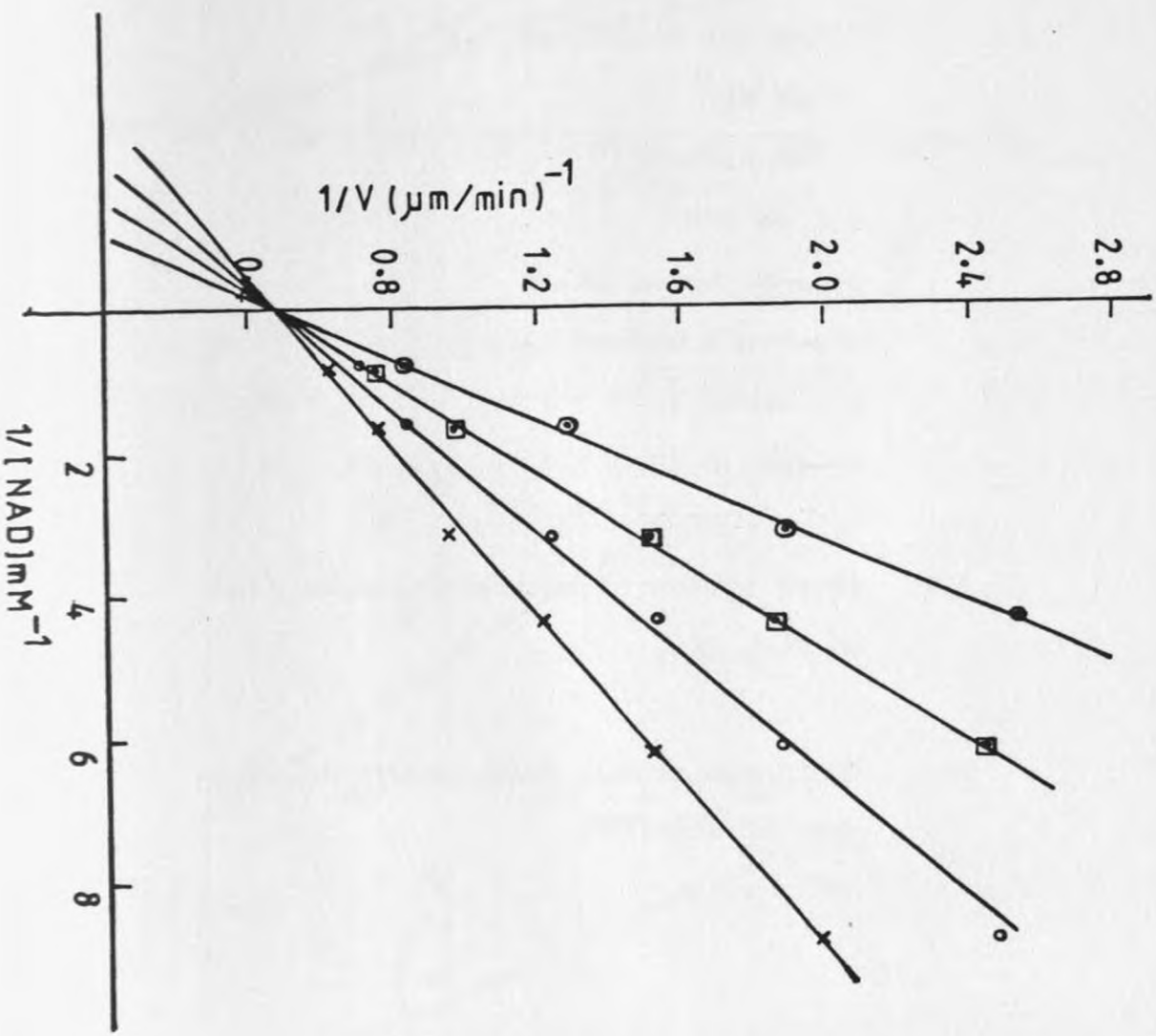


FIGURE 28a: EFFECT OF NADH ON $\frac{1}{v}$ VERSUS $\frac{1}{\text{malate}}$ PLOT

The system contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

2 mM Fumarate

0.1 mm NAD

●——● no addition

·-·-·-· 0.025 mM NADH

—o—o— 0.05 " "

●----●- 0.1 " "

x——x— 0.15 " "

using Glossina malic enzyme

28b. Slope versus NADH concentration plot of Fig. 28a.

28c. Intercept versus NADH concentration plot of Fig. 28a.

Fig. 28a

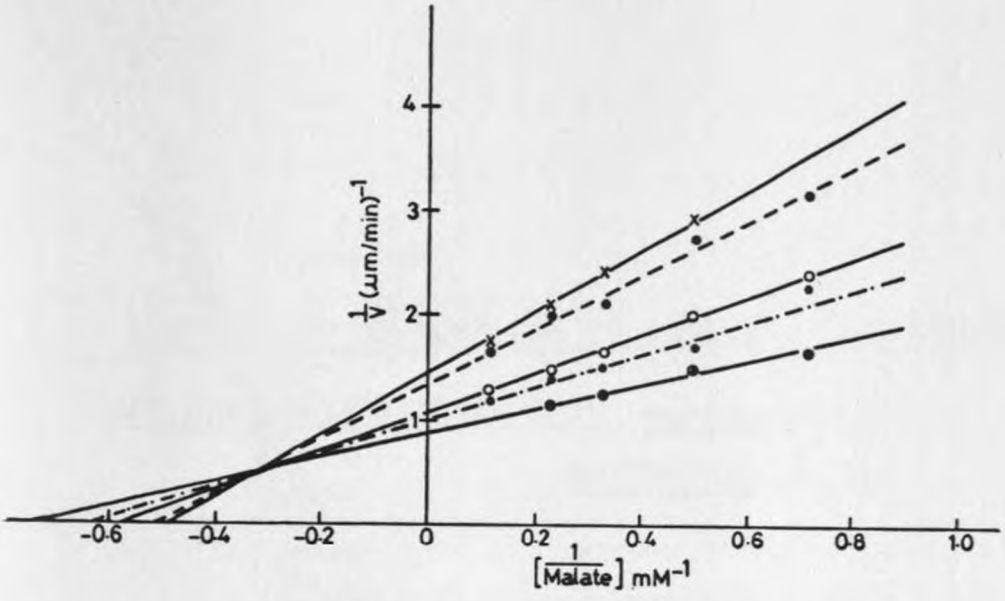


Fig. 28b

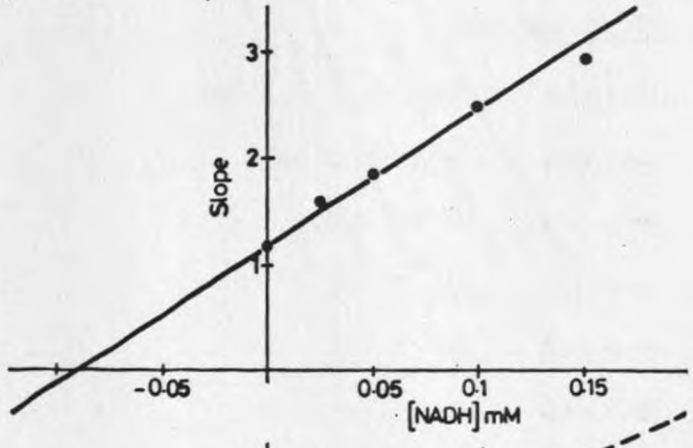


Fig. 28c

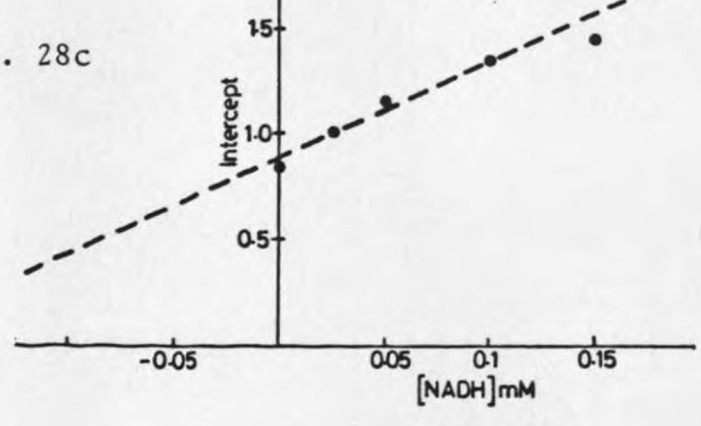


FIGURE 29: EFFECT OF BICARBONATE ON $\frac{1}{v}$ VERSUS $\frac{1}{\text{malate}}$ PLOT AT SATURATING NAD CONCENTRATION

using Glossina malic enzyme

The assay consisted of

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

2 mM NAD

0.76 mM NAD

Malate varied 0.1 - 8 mM

x----x no addition

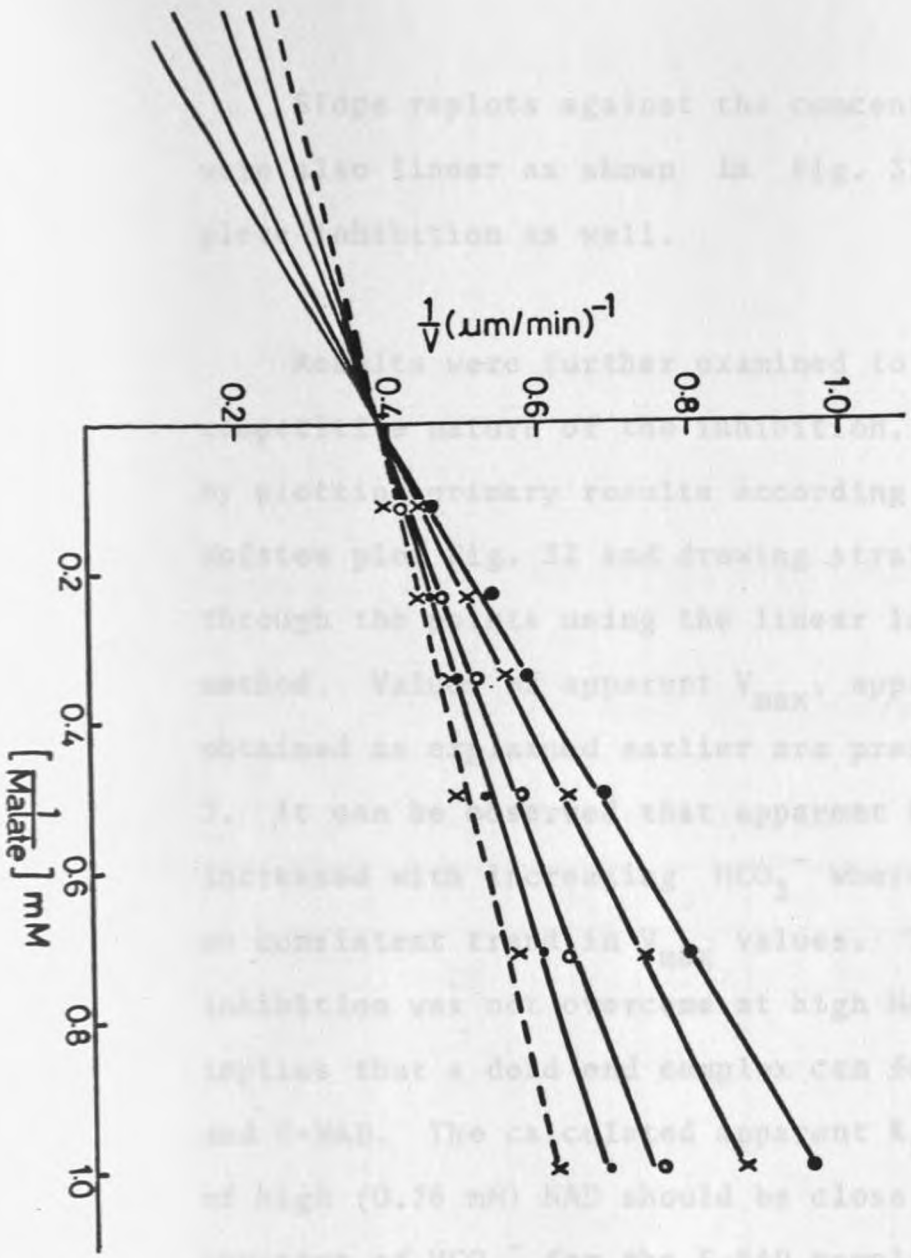
●——● 10 mM HCO_3^-

o——o 20 " "

x——x 30 " "

●——● 40 " "

Fig. 29



Slope replots against the concentration of HCO_3^- were also linear as shown in Fig. 31, implying complete inhibition as well.

Results were further examined to confirm the competitive nature of the inhibition. This was done by plotting primary results according to Eadie-Hofstee plot Fig. 32 and drawing straight lines through the points using the linear least squares method. Values of apparent V_{max}, apparent K_m and K_i obtained as explained earlier are presented in Table 7. It can be observed that apparent K_m was markedly increased with increasing HCO_3^- whereas there was no consistent trend in V_{max} values. The fact that inhibition was not overcome at high NAD concentration implies that a dead end complex can form between CO_2 and E-NAD. The calculated apparent K_i in the presence of high (0.76 mM) NAD should be close to the binding constant of HCO_3^- for the E-NAD complex.

With NAD as the variable substrate HCO_3^- gave mixed inhibition when an unsaturating malate level (1 mM) was used (Fig. 33), this was much reduced by raising the malate concentration to 10 mM (Fig. 34).

4.2.3 Inhibition with Pyruvate

The effects of pyruvate on the initial velocity

TABLE 7: HCO_3^- Inhibition with Malate as Variable Substrate using *Glossina* malic enzyme

	HCO_3^- Concentration (mM)					
	0	10	20	30	40	50
V_{max}	2.82	2.9	2.92	2.84	2.90	2.61
K_m	.66	.82	1.01	1.33	1.52	1.76
K_i	-	41	37	29.6	30.6	30.27

FIGURE 30: EFFECT OF HCO_3^- ON $\frac{1}{v}$ VERSUS $\frac{1}{\text{malate}}$ PLOT OF UNSATURATING NAD CONCENTRATION

using Glossina malic enzyme

The system contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

2 mM Fumarate

0.32 mM NAD

Malate varied 0.13 - 8 mM

x——x— no addition

o——o 10 mM HCO_3^-

⓪——⑩ 20 " "

▣——▣ 30 " "

FIGURE 31: EFFECT OF HCO_3^- CONCENTRATION ON

$\longrightarrow \frac{K_{m_{app}}}{V_{max_{app}}}$

same experiment as Fig. 30

Fig. 30

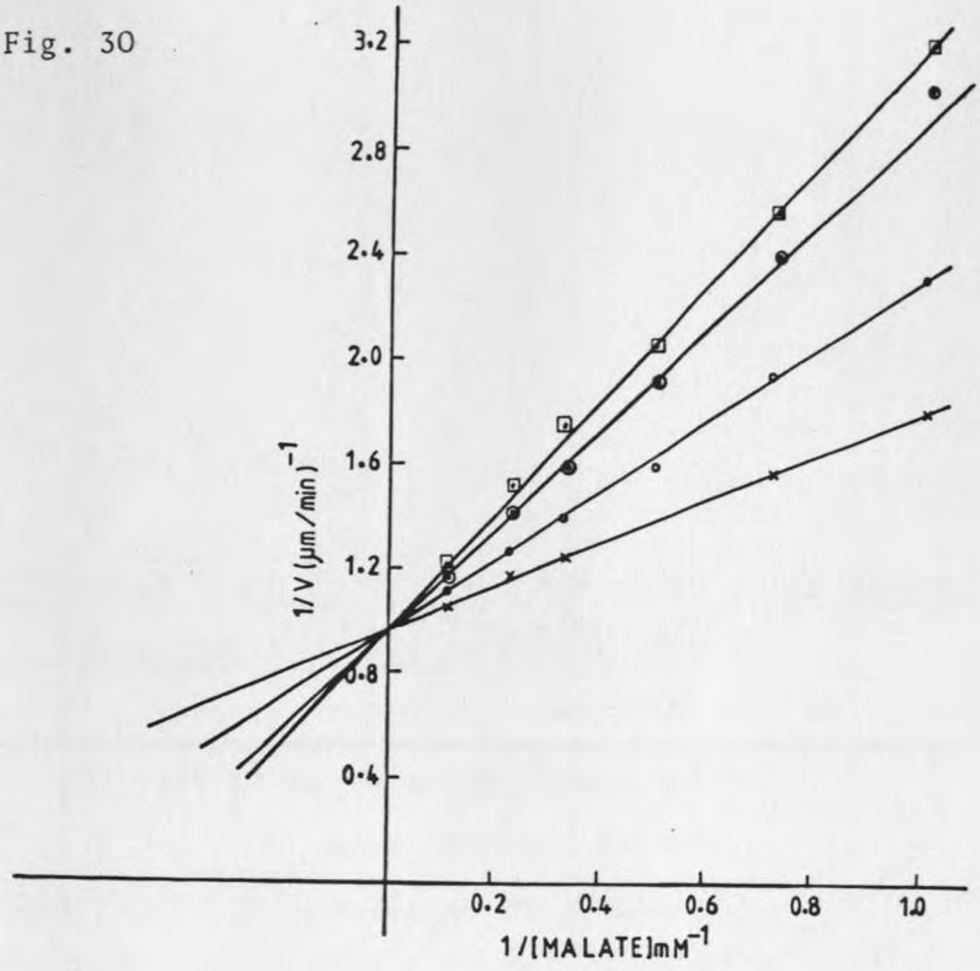

3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
1/V (µm/min)$^{-1}$
0.2
0.4
0.6
0.8
1.0
1/[MALATE]mM^{-1}

Fig. 31

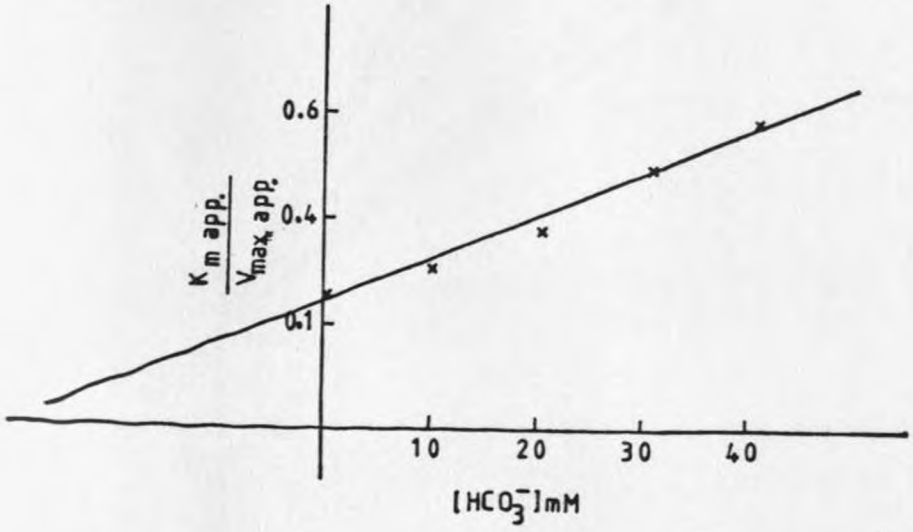

0.6
0.4
0.1
$\frac{K_{m\ app.}}{V_{max.\ app.}}$
10
20
30
40
[HCO_3^-]mM

FIGURE 32: EADIE HOFSTEE PLOT ON HCO_3^- INHIBITION WITH MALATE AS VARIABLE SUBSTRATE

using *Glossina* malic enzyme

The assay system is as in Fig. 29.

⊕——⊕ no addition

x——x— 10 mM malate

o——o 20 " "

◇——◆ 30 " "

⊙——◐ 50 " "

⊟——◪ 60 " "

Fig. 32

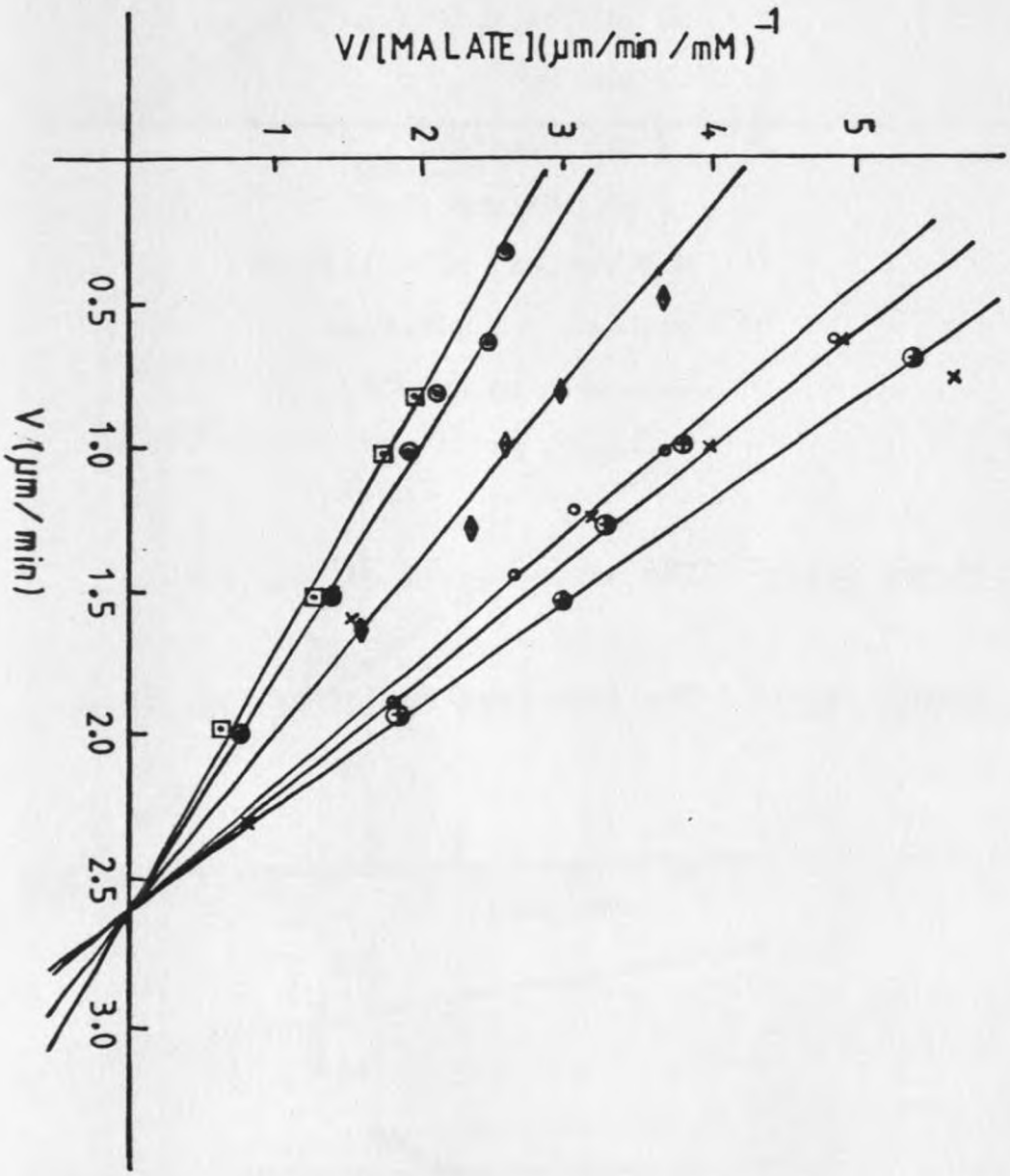


FIGURE 33(a): EFFECT OF HCO_3^- ON THE $\frac{1}{v}$ VERSUS $\frac{1}{NAD}$ PLOT AT LOW MALATE CONCENTRATIONS using *Glossina* malic enzyme

The assay system contained

50 mM TEA buffer pH 7.8

2 mM Mg^{2+}

2 mM Fumarate

1 mM Malate

NAD varied .2 - 1.25 mM

●——● no addition

o----o 10 mM HCO_3

o——o— 20 " "

FIGURE 33(b): The slope replot of Fig. 33(a).

FIGURE 33(c): The intercept replot of Fig. 33(a).

Fig. 33

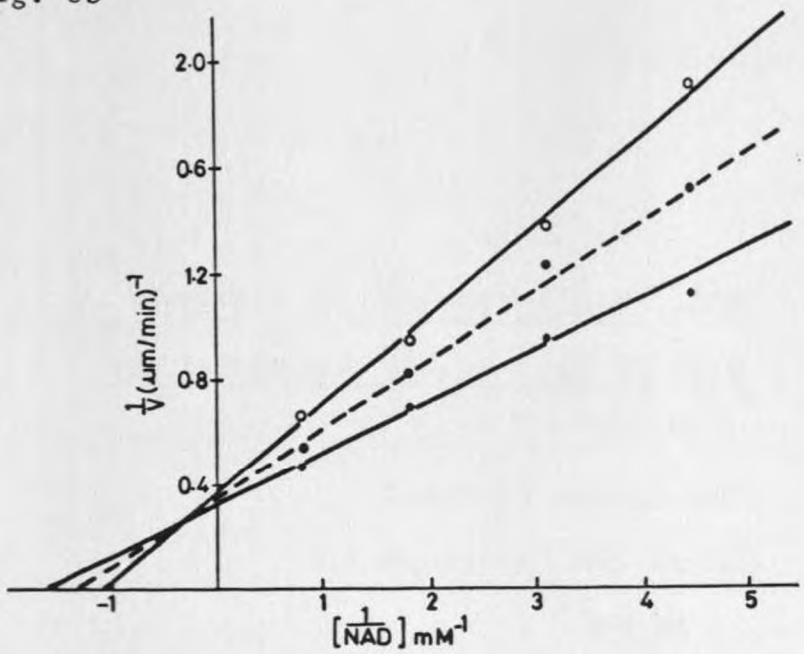


Fig. 33b

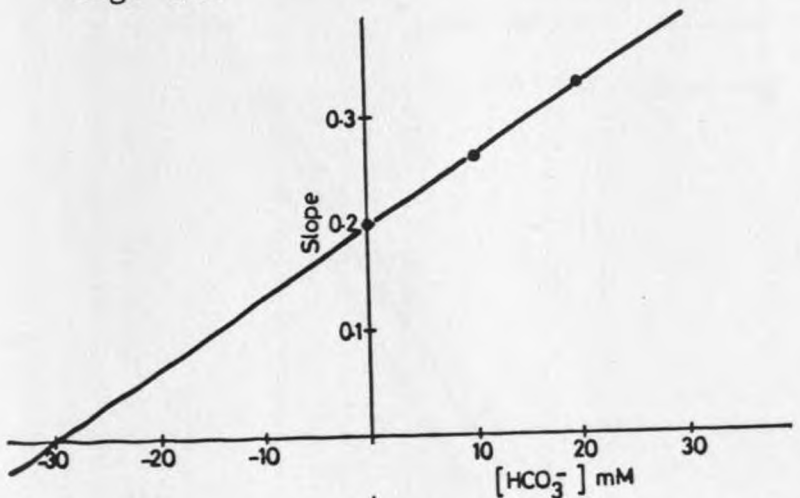


Fig. 33c

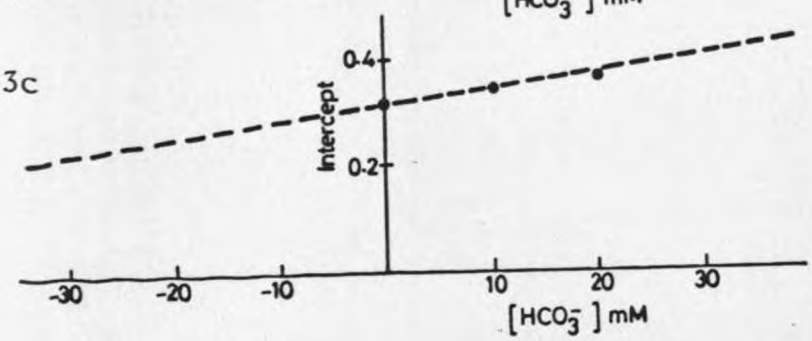


FIGURE 34: HCO_3^- INHIBITION OF THE $\frac{1}{v}$ VERSUS $\frac{1}{NAD}$ PLOT AT HIGH MALATE CONCENTRATION

using *Glossina* malic enzyme

The system included

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

2 mM Fumarate

10 mM Malate

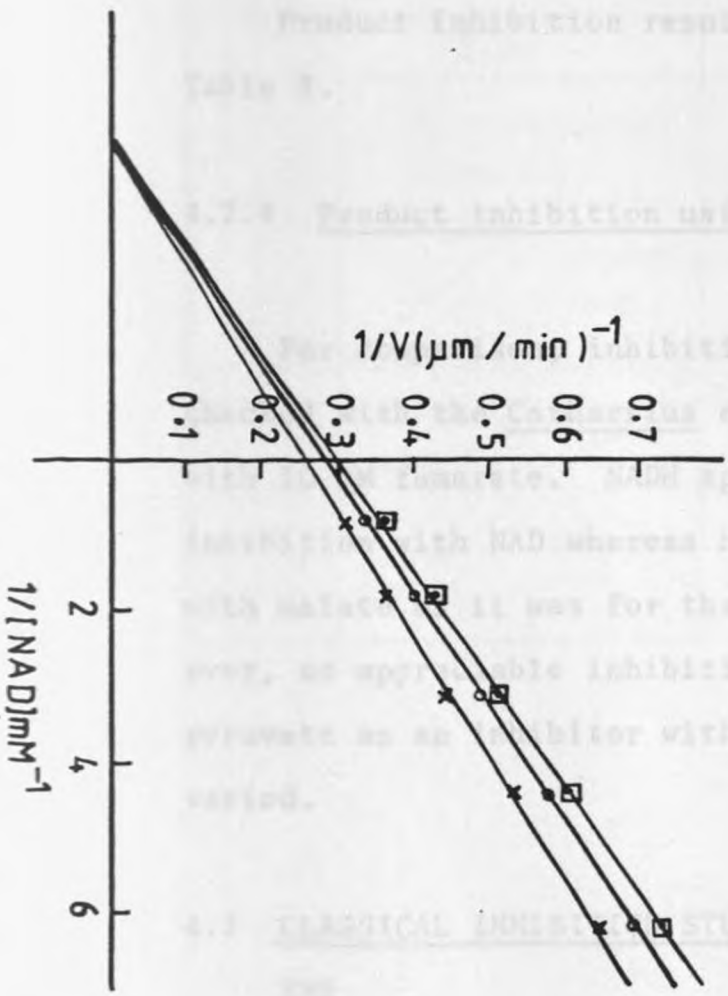
NAD varied 0.12 - 1.25 mM

x——x— no addition

o——o 20 mM HCO_3^-

⊡——⊡ 30 " "

Fig. 34



with either NAD or malate concentrations varied, are shown in Figs. 35 and 36 respectively In both cases, pyruvate gave mixed inhibition. The inhibition patterns did not change whether saturating or unsaturating levels of the second substrate were used.

Product inhibition results are summarised in Table 8.

4.2.4 Product inhibition using the Catharsius enzyme

For comparison, inhibition by products was checked with the Catharsius enzyme, at pH 7.2 and with 10 mM fumarate. NADH appeared to give mixed inhibition with NAD whereas HCO_3^- was competitive with malate as it was for the Glossina enzyme. However, no appreciable inhibition was obtained with pyruvate as an inhibitor with either NAD or malate varied.

4.3 CLASSICAL INHIBITION STUDIES WITH GLOSSINA ENZYME

The inhibition of enzyme activity by various compounds was investigated in the presence of 2 mM fumarate. Malate was varied from 0.4 - 4.4 mM in the presence of 0.76 mM NAD and at various fixed concentrations of the inhibitor. Where NAD was the

TABLE 8: Summary of Product Inhibition on Glossina NAD-malic Enzyme

	NADH	Pyruvate	HCO_3^-
NAD	(2nd substrate saturating) comp / (unsat.) comp.	mixed / mixed	mixed / mixed
Malate	mixed / mixed	mixed / mixed	comp. / comp.

FIGURE 35: PYRUVATE INHIBITION OF $\frac{1}{v}$ VERSUS $\frac{1}{NAD}$ using Glossina malic enzyme

The system contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

2 mM Fumarate

10 mM Malate

NAD varied 0.1 - 1.25 mM

⊗——⊗ no addition

+——+ 10 mM Pyruvate

o——o 20 " "

⊙——⊙ 30 " "

FIGURE 36: PYRUVATE INHIBITION WITH RESPECT TO MALATE using Glossina malic enzyme

The system is same as Fig. 35

at 0.76 mM NAD

Malate varied 1 - 10 mM

⊕——⊕ no addition

o——o 5 mM pyruvate

+——+— 10 " "

⊙——⊙— 20 " "

+——+— 30 " "

Fig. 35

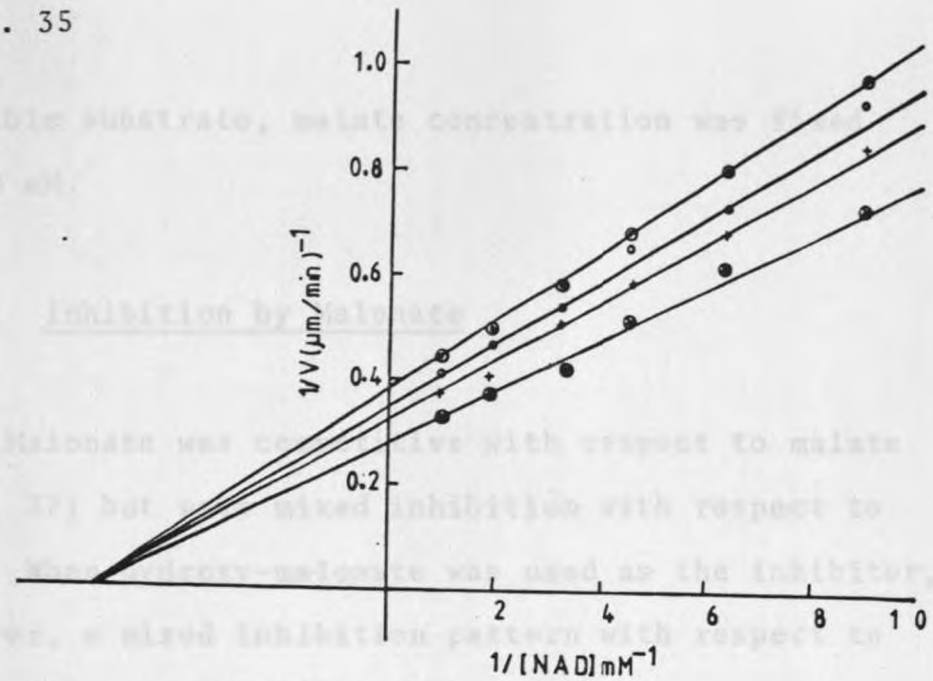
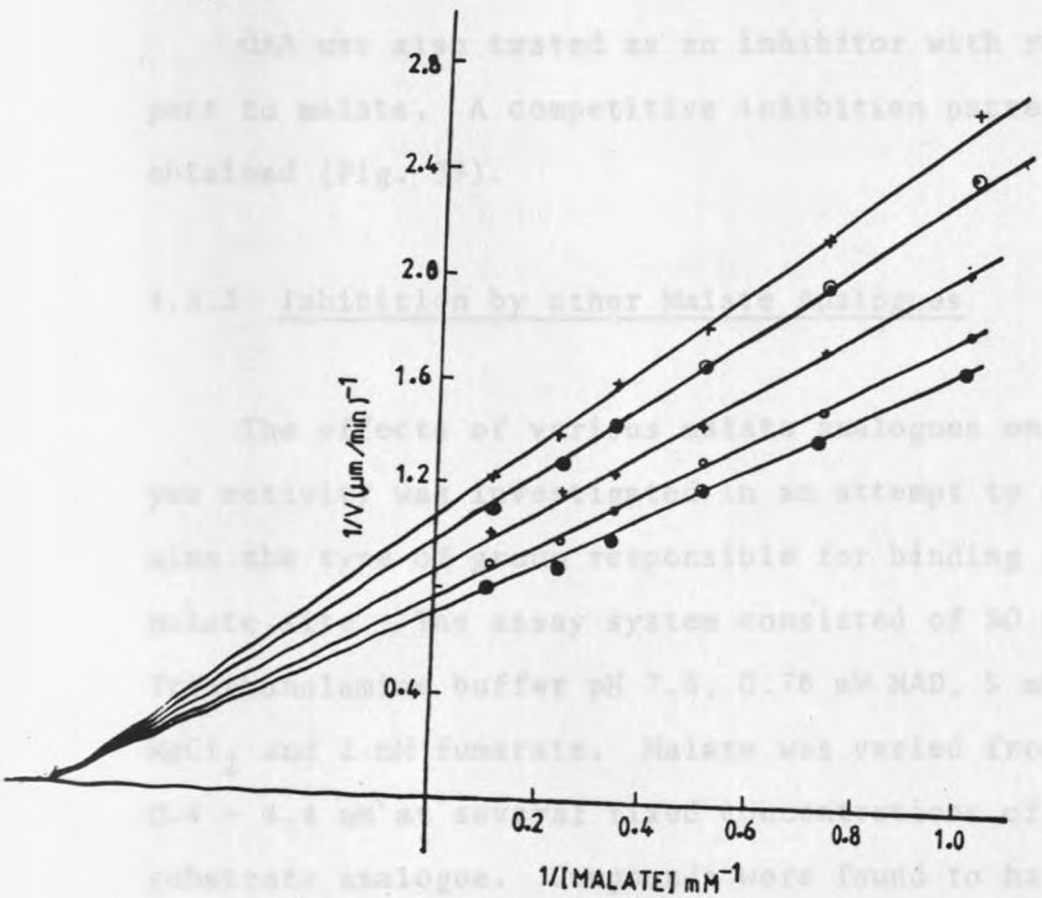

1.0
0.8
0.6
0.4
0.2
1/V(µm/min)⁻¹
2
4
6
8
10
1/[NAD]mM⁻¹

Fig. 36


2.8
2.4
2.0
1.6
1.2
0.4
1/V(µm/min)⁻¹
0.2
0.4
0.6
0.8
1.0
1/[MALATE]mM⁻¹

variable substrate, malate concentration was fixed at 10 mM.

4.3.1 Inhibition by Malonate

Malonate was competitive with respect to malate (Fig. 37) but gave mixed inhibition with respect to NAD. When hydroxy-malonate was used as the inhibitor, however, a mixed inhibition pattern with respect to malate was obtained (Fig. 38).

4.3.2 Inhibition by OAA

OAA was also tested as an inhibitor with respect to malate. A competitive inhibition pattern was obtained (Fig. 39).

4.3.3 Inhibition by other Malate Analogues

The effects of various malate analogues on enzyme activity was investigated in an attempt to determine the type of group responsible for binding at a malate site. The assay system consisted of 50 mM Triethanolamine buffer pH 7.8, 0.76 mM NAD, 5 mM $MgCl_2$ and 2 mM fumarate. Malate was varied from 0.4 - 4.4 mM at several fixed concentrations of the substrate analogue. Compounds were found to have plot patterns complying with either competitive or

FIGURE 37: MALONATE INHIBITION WITH RESPECT TO MALATE

using *Glossina* malic enzyme

The assay contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

0.76 mM NAD

2 mM Fumarate

Malate varied 0.8 - 5 mM

⊕——⊕ no addition

+——+ 10 mM Malonate

o——o— 20 " "

x——x 30 " "

FIGURE 38: HYDROXY-MALONATE INHIBITION WITH RESPECT TO MALATE

using *Glossina* malic enzyme

The system was as for Fig. 37

⊕——⊕ no addition

⊙——⊙ 3 mM OH-malonate

x——x— 5 " "

o——o— 7 " "

+——+— 10 " "

Fig. 38

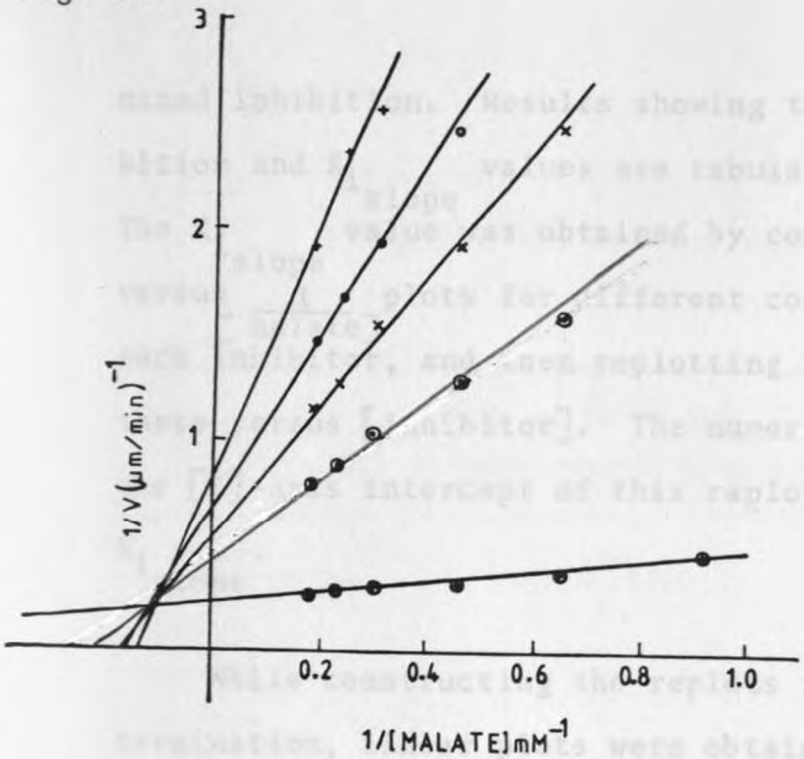
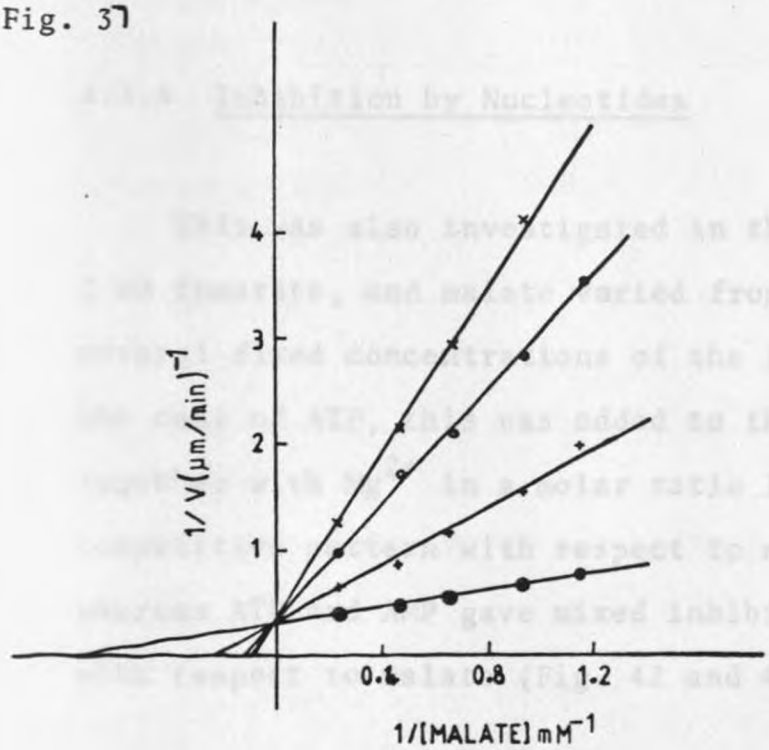

3
2
1
1/V (µm/min)$^{-1}$
0.2
0.4
0.6
0.8
1.0
1/[MALATE] mM^{-1}

Fig. 37


4
3
2
1
1/V (µm/min)$^{-1}$
0.8
1.2
1/[MALATE] mM^{-1}

mixed inhibition. Results showing the type of inhibition and $K_{i_{slope}}$ values are tabulated in Table 9. The $K_{i_{slope}}$ value was obtained by constructing $\frac{1}{v}$ versus $\frac{1}{[\text{malate}]}$ plots for different concentrations of each inhibitor, and then replotting the slopes of these versus [inhibitor]. The numerical value of the [I]-axis intercept of this replot was taken as $K_{i_{slope}}$.

While constructing the replots for $K_{i_{slope}}$ determination, linear plots were obtained (see e.g Fig. 40), implying that complete inhibition was occurring. None of these analogues could support enzyme activity in the absence of L-malate.

4.3.4 Inhibition by Nucleotides

This was also investigated in the presence of 2 mM fumarate, and malate varied from 1 - 8.4 mM at several fixed concentrations of the inhibitor. In the case of ATP, this was added to the assay system together with Mg^{2+} in a molar ratio 1:1, ADP gave a competitive pattern with respect to malate (Fig. 41) whereas ATP and AMP gave mixed inhibition patterns with respect to malate (Fig. 42 and 43 respectively).

Experiments were repeated with NAD as the varied substrate, varying from 0.1 - 1.3 mM. ATP, ADP

TABLE 9: Inhibition of Glossina NAD-malic Enzyme by Malate and NAD analogues

Analogue	Mode of Inhibition (with respect to malate)	K_i slope (mM)
∝-ketobutyrate	competitive	14.9*
Maleate	"	8.0
Malonate	"	3.0
Fumarate	"	14
Succinate	"	21
OAA	"	2.5
ADP	"	1.3
Glutarate	mixed	5.2
L-∝-OH-Glutarate	"	2.3
D-malate	"	30
Ketomalonate	"	0.27
OH-malonate	"	0.7
∝-ketoglutarate	"	23
β-OH-Butyrate	"	63
AMP	"	3.5
ATP	"	1.8

* Estimated (the replot not quite linear).

Fig.39

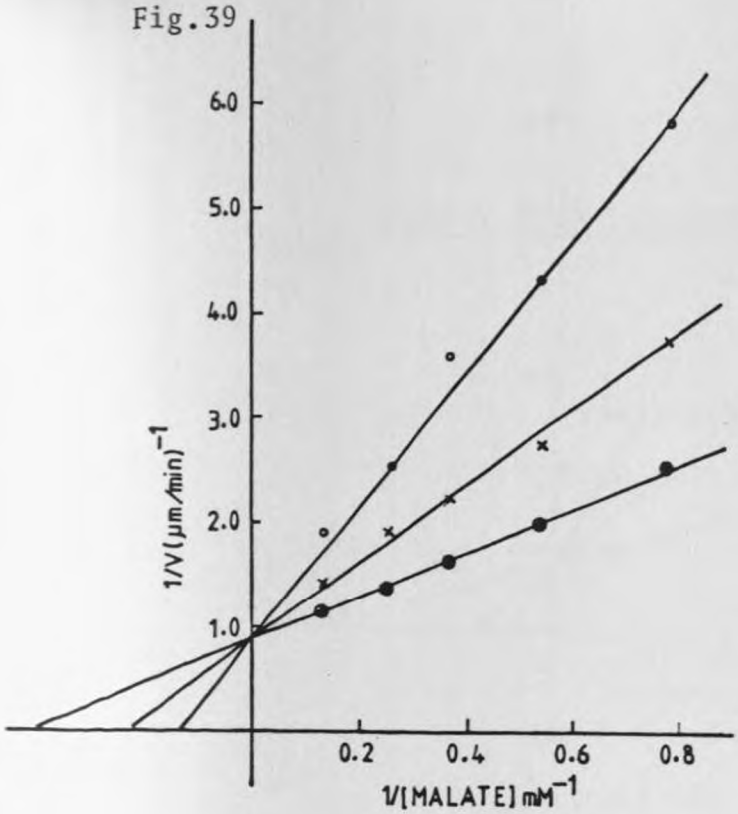

6.0
5.0
4.0
3.0
2.0
1.0
1/V (µm/min)$^{-1}$
0.2
0.4
0.6
0.8
1/[MALATE] mM^{-1}

Fig. 40

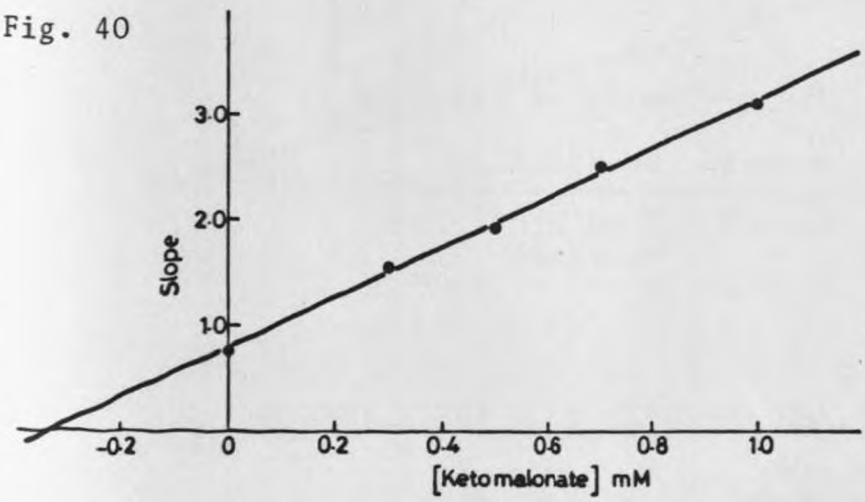

3.0
2.0
1.0
Slope
-0.2
0
0.2
0.4
0.6
0.8
1.0
[Ketomalonate] mM

FIGURE 39: EFFECT OF OAA ON $\frac{1}{v}$ VERSUS $\frac{1}{\text{malate}}$ PLOT

using Glossina malic enzyme

The assay consisted of

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

2 mM Fumarate

0.76 mM NAD

Malate varied 1.2 - 8 mM

⌀——⌀ no addition

x——x 2.5 mM OAA

o——o 5 " "

FIGURE 40: SLOPE REPLOT OF EFFECT OF KETOMALONATE ON $\frac{1}{v}$ VERSUS $\frac{1}{\text{malate}}$ PLOT

The assay system is as for Fig. 39

Fig.39

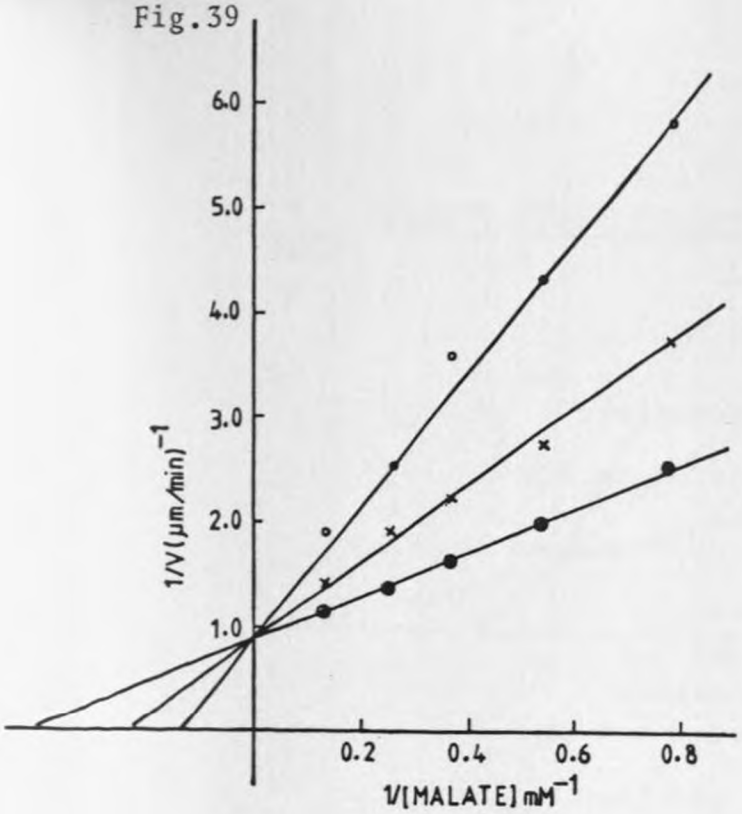

6.0
5.0
4.0
3.0
2.0
1.0
1/V(μm/min)⁻¹
0.2
0.4
0.6
0.8
1/[MALATE] mM⁻¹

Fig. 40

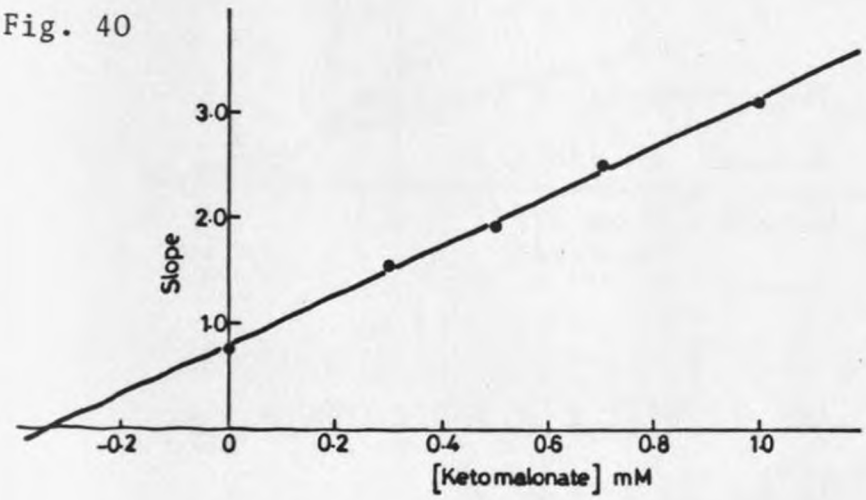

3.0
2.0
1.0
Slope
-0.2
0
0.2
0.4
0.6
0.8
1.0
[Ketomalonate] mM

FIGURE 41: ADP INHIBITION OF THE $\frac{1}{v}$ VERSUS $\frac{1}{\text{malate}}$ PLOT

The system contained

50 mM TEA buffer pH 7.8

0.76 mM NAD

2 mM Fumarate

5 mM Mg^{2+}

Malate varied 1 - 8 mM

●——● no addition

x——x 3 mM ADP

o——o 5 " "

FIGURE 42: ATP INHIBITION OF $\frac{1}{v}$ VERSUS $\frac{1}{\text{malate}}$ PLOT

The system is as for Fig. 41

●——● no addition

o——o 3 mM ATP

x——x 5 " "

FIGURE 43: AMP INHIBITION OF THE $\frac{1}{v}$ VERSUS $\frac{1}{\text{malate}}$ PLOT

The same system as for Fig. 41

x——x no addition

o——o 3 mM AMP

■——■ 5 " "

Fig. 41

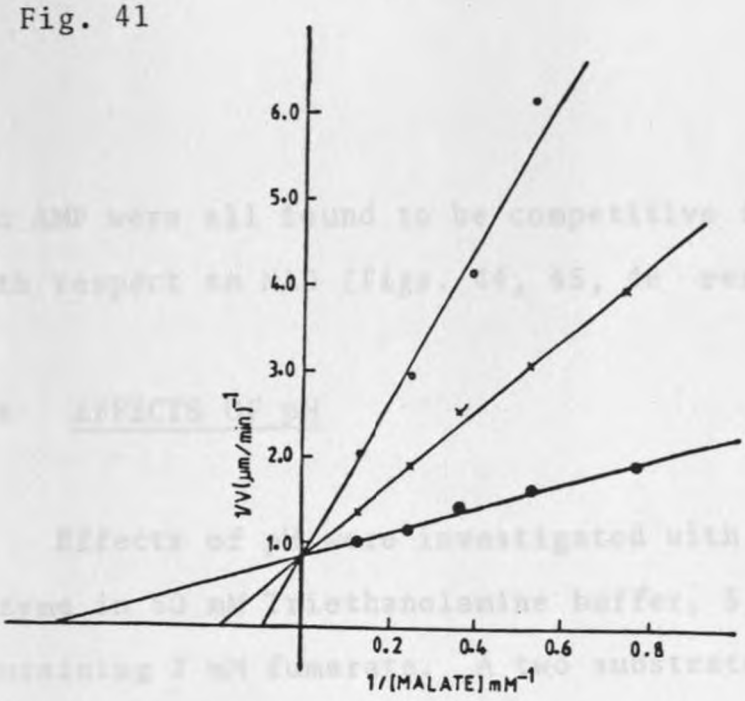


Fig. 42

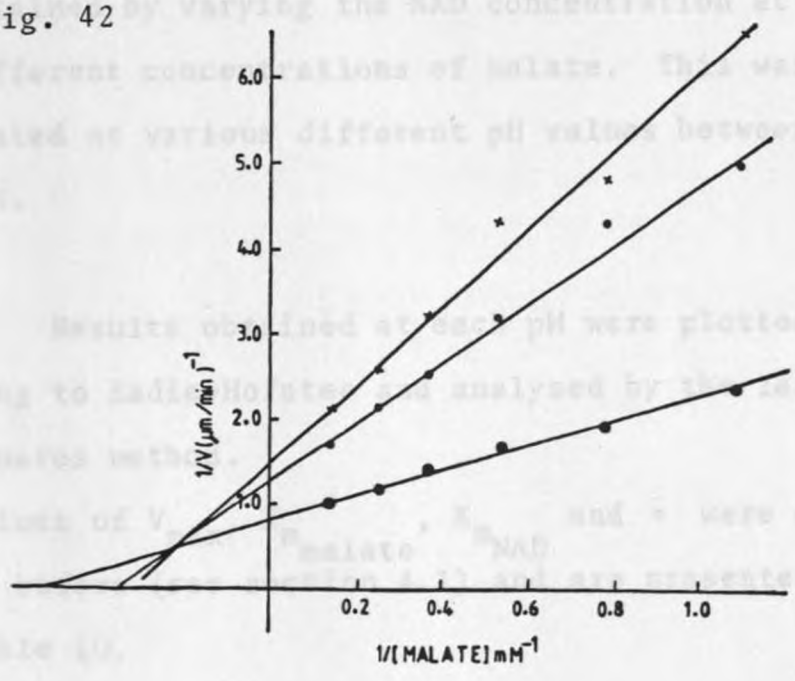
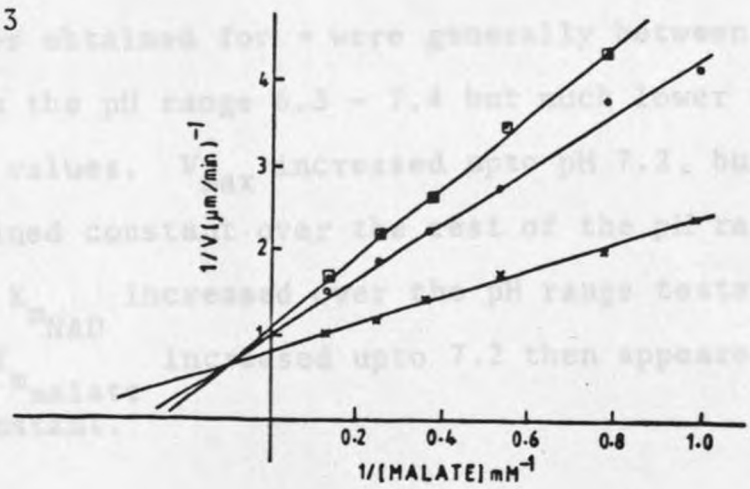


Fig. 43



and AMP were all found to be competitive inhibitors with respect to NAD (Figs. 44, 45, 46 respectively).

4.4 EFFECTS OF pH

Effects of pH were investigated with the Glossina enzyme in 50 mM Triethanolamine buffer, 5 mM Mg^{2+} containing 2 mM fumarate. A two substrate plot was obtained by varying the NAD concentration at six different concentrations of malate. This was repeated at various different pH values between 6.3 - 8.4.

Results obtained at each pH were plotted according to Eadie-Hofstee and analysed by the least squares method.
Values of V_{max}, $K_{m_{malate}}$, $K_{m_{NAD}}$ and α were obtained as before (see section 4.1) and are presented in Table 10.

Values obtained for α were generally between 0.3 - 1 in the pH range 6.3 - 7.4 but much lower at higher pH values. V_{max} increased upto pH 7.2, but then remained constant over the rest of the pH range. Values of $K_{m_{NAD}}$ increased over the pH range tested although $K_{m_{malate}}$ increased upto 7.2 then appeared to remain constant.

FIGURE 44: ATP INHIBITION WITH RESPECT TO NAD

The assay consisted of

50 mM TEA buffer pH 7.8

2.5 mM Malate

5 mM Mg^{2+}

2 mM Fumarate

NAD varied 0.1 - 1.3 mM

x——x no addition

●——● 3 mM ATP

o——o 5 " "

FIGURE 45: ADP INHIBITION WITH RESPECT TO NAD

System as for Fig. 44

x——x no addition

o——o 3 mM ADP

▣——▣ 5 " "

FIGURE 46: AMP INHIBITION WITH RESPECT TO NAD

System as for Fig. 44

x——x no addition

o——o 3 mM AMP

▣——▣ 5 " "

Fig. 44

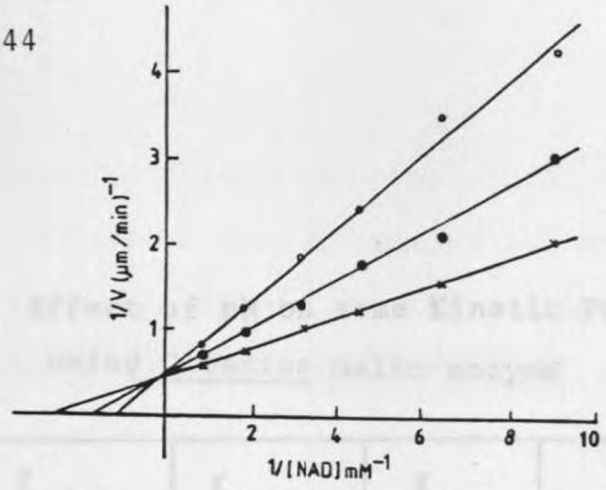


Fig. 45

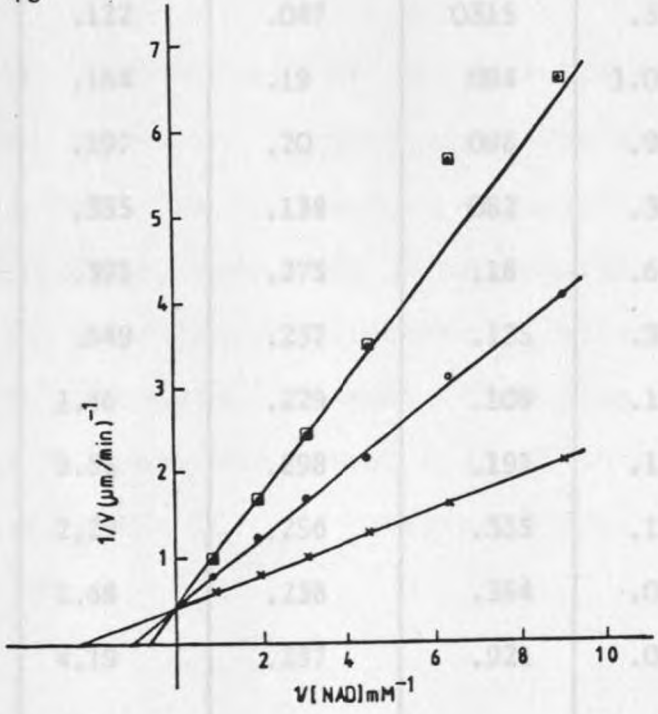


Fig. 46

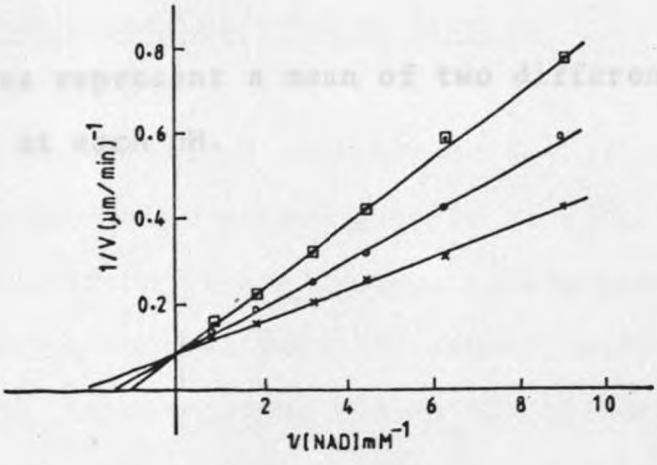


TABLE 10: Effect of pH on some Kinetic Parameters using Glossina malic enzyme

pH	$\bar{K}_{malate}$	$K_{m_{malate}}$	$K_{m_{NAD}}$	α	V_{max}
6.3	.122	.047	0315	.37	.722
6.6	.164	.19	094	1.09	1.29
6.8	.197	.20	098	.96	1.22
7.0	.355	.138	082	.386	2.34
7.2	.391	.275	.18	.699	5.34
7.4	.649	.237	.135	.357	4.1
7.6	1.46	.229	.109	.173	4.36
7.8	2.55	.298	.191	.116	4.38
8.0	2.29	.256	.335	.117	4.84
8.2	2.68	.238	.354	.084	4.64
8.4	4.19	.287	.921	.063	5.76

Figures represent a mean of two different experiments at each pH.

In order to investigate the effect of pH on the ionisation of functional groups on the enzyme or enzyme-substrate complexes, results obtained in Table 10 were used for constructing the Dixon plots presented in Fig. 47. pK values of 6.7 - 6.8, 7.2, 7.6 and 8.1 were deduced.

The effect of pH on the substrate kinetics of the Catharsius enzyme was also reinvestigated. Due to residual sigmoidicity of v versus [S] plots, even with 10 mM fumarate present, it was not possible to determine K_m values accurately at higher pH values. However, the value of α appeared to decrease from about 0.15 at pH 6.5 to 0.03 at pH 8.4, while $K_{m_{malate}}$ increased from 1.9 mM to about 6 mM.

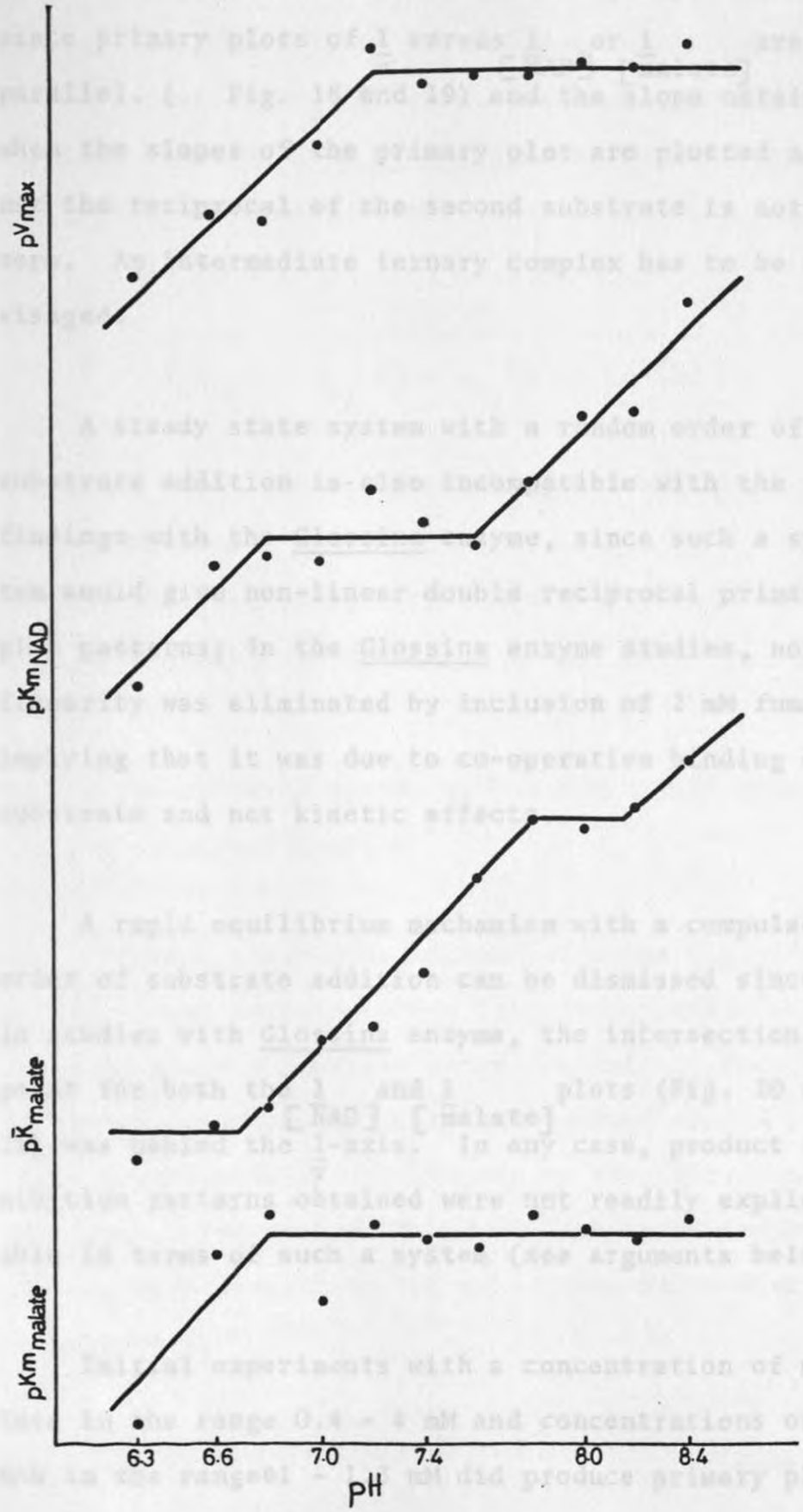
4.5 DISCUSSION

4.5.1 Substrate and Product Effects

The mechanism of decarboxylation of malate by Glossina NAD-malic enzyme appears to involve the random addition of substrates. These conclusions are based on initial velocity studies with varying substrate concentrations and on the influence of products on the enzyme reaction rate.

FIGURE 47: DIXON PLOTS ON EFFECT OF pH ON CERTAIN KINETIC PARAMETERS

Fig. 47



A double displacement mechanism can be ruled out since primary plots of $\frac{1}{v}$ versus $\frac{1}{[NAD]}$ or $\frac{1}{[malate]}$ are not parallel. (Fig. 18 and 19) and the slope obtained when the slopes of the primary plot are plotted against the reciprocal of the second substrate is not zero. An intermediate ternary complex has to be envisaged.

A steady state system with a random order of substrate addition is also incompatible with the findings with the Glossina enzyme, since such a system would give non-linear double reciprocal primary plot patterns; In the Glossina enzyme studies, non-linearity was eliminated by inclusion of 2 mM fumarate implying that it was due to co-operative binding of substrate and not kinetic effects.

A rapid equilibrium mechanism with a compulsory order of substrate addition can be dismissed since in studies with Glossina enzyme, the intersection point for both the $\frac{1}{[NAD]}$ and $\frac{1}{[malate]}$ plots (Fig. 20 and 21) was behind the $\frac{1}{v}$-axis. In any case, product inhibition patterns obtained were not readily explicable in terms of such a system (see arguments below).

Initial experiments with a concentration of malate in the range 0.4 - 4 mM and concentrations of NAD in the range 0.1 - 1.3 mM did produce primary plot

patterns suggesting the compulsory binding of malate before NAD. However, it was subsequently established that the limiting K_m for malate was not zero and that the rate equation with Glossina malic enzyme was of the general Michaelis form.

$$v = \frac{V[A][B]}{\bar{K}_A K_{m_B} + K_{m_A}[B] + K_{m_B}[A] + [A][B]} \qquad (11)$$

There are two different mechanisms which would yield such an equation

a) A rapid equilibrium system with a random order of substrate binding

or

b) A steady state system with a compulsory order of substrate binding.

Product inhibition studies with the Glossina enzyme showed patterns which were incompatible with a steady state compulsory order mechanism. Such a mechanism predicts that only one product should inhibit competitively with respect to the corresponding substrate. Yet both NADH and CO_2 inhibited competitively, with respect to the substrates NAD and malate respectively.

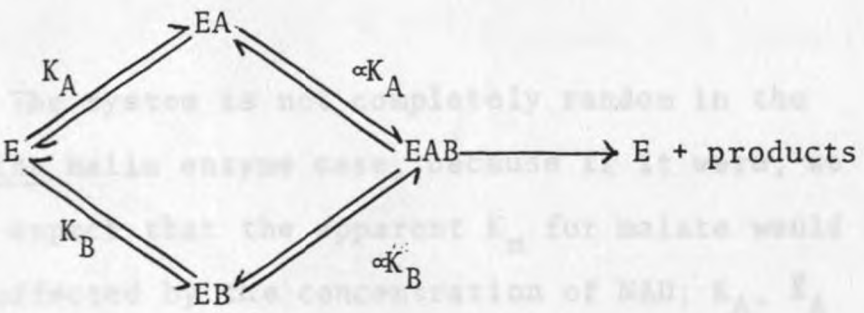
The product inhibition results with Glossina

malic enzyme were in some respects analogous to those obtained for isocitrate dehydrogenase from the mussel, Mytilus edulis L. (Head 1980). However, whereas the decarboxylated ketoacid competes in the isocitrate dehydrogenase case for the "corresponding" hydroxy-acid substrate, in the malic enzyme case, it is CO_2 and not pyruvate which competes with malate.

It is likely that the Glossina malic enzyme obeys a random order of addition mechanism, that is, either substrate could bind first to the enzyme and all steps are rapid except the interconversion of the ternary complex which is slow and rate limiting.

Assuming a rapid equilibrium random mechanism of substrate binding to the Glossina malic enzyme, according to the initial reaction scheme 10.

SCHEME 10:



A = malate

B = NAD

The initial rate equation could be written:-

$$v = \frac{V[A][B]}{\alpha K_A K_B + \alpha K_A[B] + \alpha K_B[A] + [A][B]} \qquad (12)$$

i.e the $K_{m_A} = \alpha K_A$

$K_{m_B} = \alpha K_B$

$\bar{K}_A = K_A$

where K_A is the dissociation constant for the binding of malate to free enzyme

αK_A is the dissociation constant for the binding of malate to the E-NAD complex

K_B is the dissociation constant for the binding of NAD to the free enzyme

αK_B is the dissociation constant for binding NAD to the E-malate complex

$\frac{1}{\alpha}\left(\frac{\bar{K}}{K_m}\right)$ would be the enhancement of the binding of the second substrate by the first substrate to bind on the enzyme.

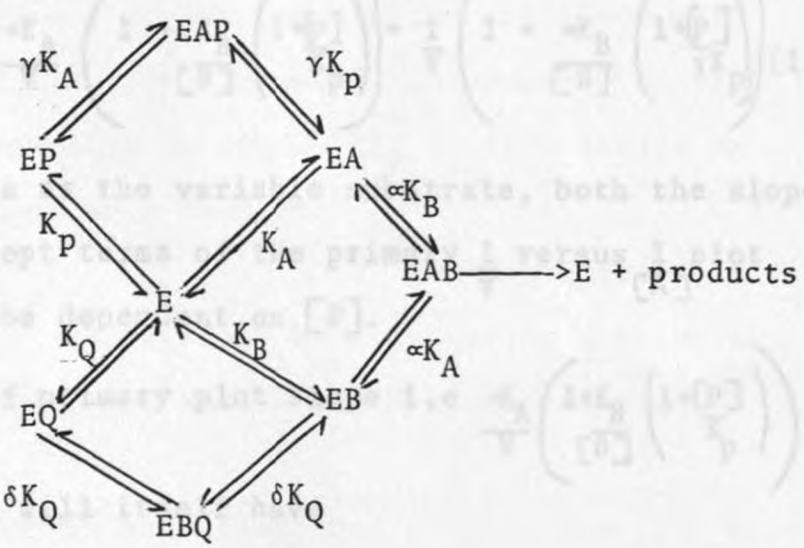
The system is not completely random in the Glossina malic enzyme case, because if it were, we would expect that the apparent K_m for malate would be unaffected by the concentration of NAD; K_A, $\bar{K}_A$ and all apparent K_m values would be the same, and α would be equal to unity. With the Glossina enzyme, the apparent K_m for malate was always depen-

dent on NAD concentration, and vice versa and the value of α was less than unity.

The intersecting primary double reciprocal patterns obtained indicated that the true dissociation constant of E-NAD or E-malate was higher than the apparent Michaelis constant values.

NADH is a competitive inhibitor with respect to NAD and a mixed inhibitor with respect to malate, with inhibition not being overcome by raising the second substrate to high concentrations. Such inhibition is compatible with a scheme in which NADH not only binds to free enzyme E, but forms a dead end complex with E-malate as shown in scheme 11, Thus:

SCHEME 11:



(P = NADH, A = malate, B = NAD, Q = HCO_3^-)

If NADH occupied the NAD binding site only in the absence of malate then it should be competitive with respect to malate as well as with respect to NAD and inhibition would be reduced by raising either substrate to a high concentration.

Scheme 11 will give a rate equation

$$v = \frac{V[A][B]}{\alpha K_A K_B\left(1 + \frac{[P]}{K_p}\right) + \alpha K_B[A]\left(1 + \frac{[P]}{\gamma K_p}\right) + \alpha K_A[B] + [A][B]} \quad (13)$$

in reciprocal form:

$$\frac{1}{v} = \frac{1}{V}\left(\frac{\alpha K_A K_B}{[A][B]}\left(1 + \frac{[P]}{K_p}\right) + \frac{\alpha K_B}{[B]}\left(1 + \frac{[P]}{\gamma K_p}\right) + \frac{\alpha K_A}{[A]} + 1\right) \quad (14)$$

This can be rearranged:

$$\frac{1}{v} = \frac{1}{A} \cdot \frac{\alpha K_A}{V}\left(1 + \frac{K_B}{[B]}\left(1 + \frac{[P]}{K_p}\right)\right) + \frac{1}{V}\left(1 + \frac{\alpha K_B}{[B]}\left(1 + \frac{[P]}{\gamma K_p}\right)\right) \quad (15)$$

with malate as the variable substrate, both the slope and intercept terms of the primary $\frac{1}{v}$ versus $\frac{1}{[A]}$ plot will thus be dependent on $[P]$.

A replot of primary plot slope i.e $\frac{\alpha K_A}{V}\left(1 + \frac{K_B}{[B]}\left(1 + \frac{[P]}{K_p}\right)\right)$ versus $[P]$ will itself have

slope = $\frac{\alpha K_A \cdot K_B}{V K_p [B]}$ and intercept $\frac{\alpha K_A}{V}\left(1 + \frac{K_B}{[B]}\right)$

A replot of primary plot intercept i.e

$$\frac{1}{V}\left(1 + \frac{\alpha K_B}{[B]} + \frac{\alpha K_B [P]}{[B]\gamma K_p}\right) \text{ versus } [P] \text{ will have}$$

$$\text{slope} = \frac{\alpha K_B}{V_o \gamma K_p [B]}$$

slope of slope versus [P] from results in Fig. 28(b)

= 14.28

since other values are known at pH 7.8

(K_A = 2.55 mM, K_B = 1.64 mM, α = 116, [B] = 0.1 mM, V = 2.35 µM/min)

by substitution, therefore K_p for NADH = .144 mM

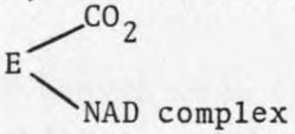
slope of intercept versus [P] (see Fig. 28(c))

= 5.43

other values are K_B = 1.64mM, α = .116, [B] = .1mM, V = 2.35µm/min

Therefore by substituting get γK_p for NADH = .148 mM.

Similarly, HCO_3^- (Q) is a competitive inhibitor with respect to malate and a mixed inhibitor with respect to NAD. It presumably interacts with the enzyme, according to scheme 11, to form either an E-CO_2 complex or a dead end



such a case is described by an equation similar to equation (15), but with A and B terms reversed.

If slopes and intercepts of $\frac{1}{v}$ versus $\frac{1}{[NAD]}$ plots are replotted separately against HCO_3^- concentration, the resulting slopes give K_Q and δK_Q respec-

tively, after substituting approximate values for K_A, K_B, α and [A]. Such an exercise performed on the data plotted in Fig. 34 gives the following results: K_Q = 11.6 mM δK_Q = 16.4 mM (see Fig. 34b and 34c) V for this experiment was 6.25.

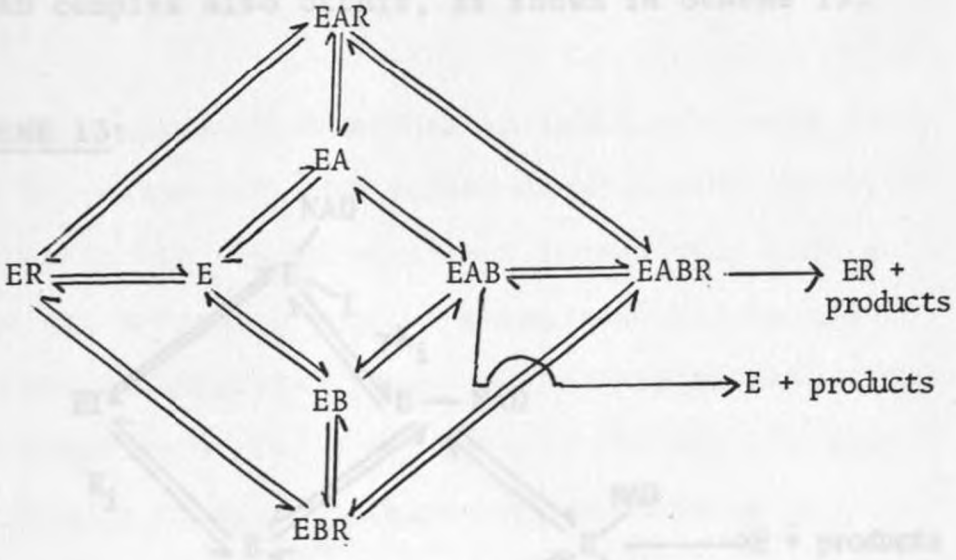
γK_p was obtained as .148 mM and is in agreement with a value of .141 mM (see Table 6) obtained for $K_{i_{app.}}$ NADH. It could be that binding of the first substrate does not affect binding of products related to 2nd substrate.

The value of δK_Q (16.4 mM) obtained is slightly lower than the $K_{i_{app.}}$ for HCO_3^- (30 mM Table 7), probably because during calculations of apparent K_i, it was assumed that NAD was near saturating whereas in calculations of δK_Q actual value of NAD concentration was used. It was difficult to saturate the system with NAD as NAD concentrations of above 1 mM caused substrate inhibition.

Pyruvate showed mixed inhibition with respect to either substrate. Pyruvate could be having its own binding site as an inhibitor which may be the same site it's released from as a product. Pyruvate reduces the binding of both malate and NAD and since mixed inhibition is obtained apparently with high second substrate concentrations, pyruvate must also

be reducing or completely blocking the catalytic reaction i.e pyruvate can bind to all E-S forms probably to E also as shown in Scheme 12.

SCHEME 12:



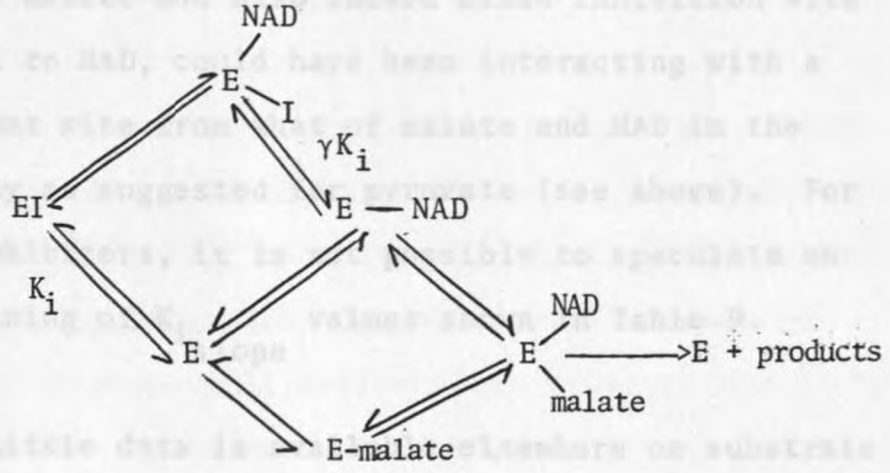
scheme 12 must necessarily be speculative

4.5.2 Inhibition by Substrate Analogues and other Compounds

Malonate and its 3C derivatives were strongly inhibitory whereas most 4 and 5C dicarboxylic acids tested were only moderately inhibitory. The 4C monocarboxylic acid with a hydroxyl group in the β position (β-OH Butyrate), was a weak inhibitor. (see Table 9) whereas OH-malonate and L-α-OH Glutarate, the two homologues of L-malate were both strongly inhibitory.

Competitive inhibitors among the substrate analogues presumably act by binding at the malate site on the enzyme. In the case of malonate, inhibition with respect to NAD was also tested and found to be mixed, indicating that binding to the E-NAD complex also occurs, as shown in Scheme 13.

SCHEME 13:



For these competitive inhibitors, the K_i slope should equal

$$\frac{\gamma K_i \left(1 + \frac{K_B}{[B]}\right)}{\left(1 + \frac{\gamma K_B}{[B]}\right)}$$

At high [NAD] therefore, the numerical values shown in Table 9 should approximate to the value of γK_{is}, the binding of I to the E-NAD complex.

The fact that malonate gave mixed inhibition

with respect to NAD, further supports the random order substrate binding mechanism proposed earlier. If a compulsory ordered mechanism applied, then one would expect malonate to be an uncompetitive inhibitor with respect to NAD, because prior NAD binding should be obligatory for malonate binding.

Compounds which are mixed inhibitors with respect to malate and also showed mixed inhibition with respect to NAD, could have been interacting with a different site from that of malate and NAD in the same way as suggested for pyruvate (see above). For such inhibitors, it is not possible to speculate on the meaning of $K_{i\,slope}$ values shown in Table 9.

Little data is available elsewhere on substrate analogue inhibition of NAD-malic enzyme (Landsperger et al 1978), but Schimerlik and Cleland (1977) have used a wide range of substrate analogues as inhibitors of the pigeon liver NAD-malic enzyme and their results support their own proposed ordered kinetic mechanism for this enzyme. The present inhibition findings with the Glossina enzyme are broadly in agreement with those of Schimerlik and Cleland, in that 3C acids appeared to be more strongly inhibitory than 4C acids. One could also suggest as did these authors, that the active site for malate on the Glossina enzyme is small and the 4C acids are bound with strain which

could enhance decarboxylation.

Analogues of NAD gave competitive inhibition with respect to NAD. This is compatible with the assumption that they were binding to the NAD site either on the free enzyme or on the E-malate complex. ADP was competitive with respect to both substrates; this could be explained if this compound occupied the NAD site only on the free enzyme.

Analogues of NAD and malate yielded only competitive or mixed inhibition patterns. Uncompetitive patterns were never encountered. Although inhibition was not studied for all compounds with respect to both substrates, this nevertheless provides additional support for the assumption of a random order rather than a fixed order substrate binding mechanism.

The Catharsius enzyme had earlier been assumed to follow a rapid equilibrium compulsory mechanism with malate binding before coenzyme (Imbuga and Pearson 1982). However, the fact that the value of $K_m/\bar{K}$ i.e α is a finitive number, makes this mechanism unlikely. It now seems probable that the enzyme follows a partial random rapid equilibrium mechanism like the Glossina enzyme.

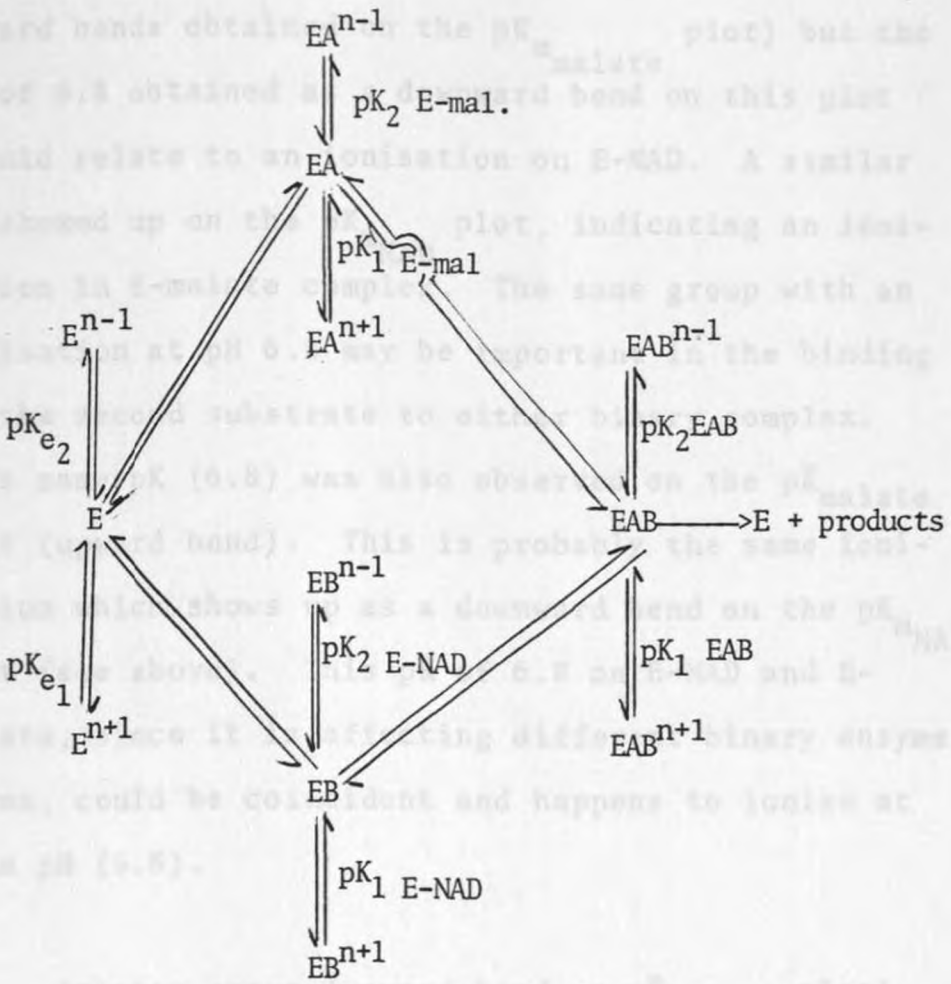
4.5.3 pH Effects

For one substrate system, Dixon (1953) showed that upward bends on the pK_m plot represent ionisation in the E-S-complex and should show up as downward bends in the pV_{max} plot. But downward bends in the pK_m plot represent ionisations in the free enzyme or substrate and do not show up in the pV_{max} plot.

For a two substrate system, downward bends in the pV_{max} plot should indicate ionisation of an important group in the ternary complex that is responsible for catalysis. An upward bend on the pK_{m_A} plot should also relate to the ternary complex, whilst a downward bend on this plot should relate to an ionisation of the binary complex E-B. Similarly the downward bend on the pK_{m_B} plot should indicate an ionisation of the E-A complex important in the binding of the second substrate, whilst the upward bends on this plot should relate to the ternary complex EAB. Downward bends on the pK_A plot relate to ionisation on the free enzyme whereas upward bends should relate to the E-A binding complex.

Scheme 14 may be envisaged for the ionisation of Glossina malic enzyme, where all the enzyme species can ionise but only EAB gives products.

SCHEME 14:



Studies on the Glossina enzyme revealed five pK values at 6.7 - 6.8, 7.2, 7.6, 7.8 and 8.2, none which could correspond to an ionisation on either of the two substrates. Two ionisations appear to relate to the ternary complex, at pH 7.2 (from pV_{max} plot) and at pH 7.6 (from the $pK_{m_{NAD}}$ plot).
This suggests that there are two important groups ionising in the ternary complex, at pH 7.2 and 7.6, the first affecting catalysis and the second affecting dissociation of NAD. There was no evident ioni-

sation relating to dissociation of malate (i.e no upward bends obtained on the $pK_{m_{malate}}$ plot) but the pK of 6.8 obtained as a downward bend on this plot should relate to an ionisation on E-NAD. A similar pK showed up on the $pK_{m_{NAD}}$ plot, indicating an ionisation in E-malate complex. The same group with an ionisation at pH 6.8 may be important in the binding of the second substrate to either binary complex. This same pK (6.8) was also observed on the $p\bar{K}_{malate}$ plot (upward bend). This is probably the same ionisation which shows up as a downward bend on the $pK_{m_{NAD}}$ plot (see above). This pK of 6.8 on E-NAD and E-malate, since it is affecting different binary enzyme forms, could be coincident and happens to ionise at same pH (6.8).

Another group (upward bend on $p\bar{K}_{malate}$ plot) was indicated for the E-malate complex with pK 8.2. This suggests there are two different groups ionising in the E-malate complex at 6.8 and 8.2.

Although pKs 7.6 and 7.8 are close, that of 7.6 involves ternary complex whereas that of 7.8 involves dissociation of free enzyme.

The groups ionising could be attributed to cysteine and histidine which usually have pK values in the range 6.5 - 8.5. Another possibility is the

existence of terminal end α amino group which usually ionises with pK 8.3.

CHAPTER V

5.0 THE CO-OPERATIVE BEHAVIOUR OF INSECT NAD-MALIC ENZYME

The response of initial velocity to substrate concentration was found to be sigmoidal with either the Glossina or the Catharsius enzyme. The latter however, appeared to be more strongly co-operative. It was also observed that both enzymes were more sigmoidal in the presence of Mg^{2+} than Mn^{2+}.

In all co-operativity studies, 50 mM triethanolamine buffer containing 5 mM $MgCl_2$ was used unless otherwise specified. Results that showed a sigmoidal dependence of initial velocity on substrate concentration were treated using the linear transformation of the Hill equation.

$$\log \frac{v_o}{V_{max} - v_o} = n_H \log S - \log K \quad (16)$$

where: v_o is initial velocity

V_{max} is maximum initial velocity (determined by a reciprocal plot of $\frac{1}{v}$ vs $\frac{1}{[S]}$)

n_H is the Hill coefficient

K is a constant related to [S] that gives half maximal velocity ($[S]_{0.5}$)

The best line was fitted to the Hill plot either by eye or by linear regression analysis. The Hill coefficient nH was obtained as the slope of the line.

5.1 EFFECT OF METAL ION

The activity of the Glossina enzyme was determined in the presence of different concentrations of malate, over the range 0.1 - 60 mM. The system contained 50 mM Triethanolamine buffer pH 7.8, 5 mM Mg^{2+} and 0.32 mM NAD. From the results, a Hill plot was constructed which gave a value 1.36 ± 0.14 (8) for the Hill coefficient. Results of one experiment are shown in Fig. 48a.

Substitution of Mn^{2+} for Mg^{2+} in the above experiment removed sigmoidicity (see Fig. 48b) and a Hill coefficient of 0.92 ± .09 (8) was obtained.

Using the Catharsius enzyme, co-operativity was again studied in 50 mM Triethanolamine buffer pH 7.2 in the presence of Mg^{2+} and Mn^{2+}, malate being varied from 0.2 - 4 mM. Hill plots were constructed from the results and nH values of 1.56 ± 0.21 and 1.07 ± 0.1 obtained in the presence of Mg^{2+} and Mn^{2+} respectively (Fig. 49).

FIGURE 48: EFFECT OF METAL ION ON THE CO-OPERATIVITY OF THE GLOSSINA MALIC ENZYME WITH RESPECT TO MALATE

The assay contained

50 mM TEA buffer pH 7.8

0.32 mM NAD

Malate varied 0.1 - 6 mM

a) o——o with 5 mM Mg^{2+}

b) •——• with 5 mM Mn^{2+}

FIGURE 49: EFFECT OF METAL ION ON THE CO-OPERATIVITY OF THE CATHARSIUS MALIC ENZYME WITH RESPECT TO MALATE

The system consisted of

50 mM TEA buffer pH 7.2

0.32 mM NAD

Malate varied 0.2 - 4 mM

a) o——o with 5 mM Mg^{2+}

b) •——• with 5 mM Mn^{2+}

Fig. 48

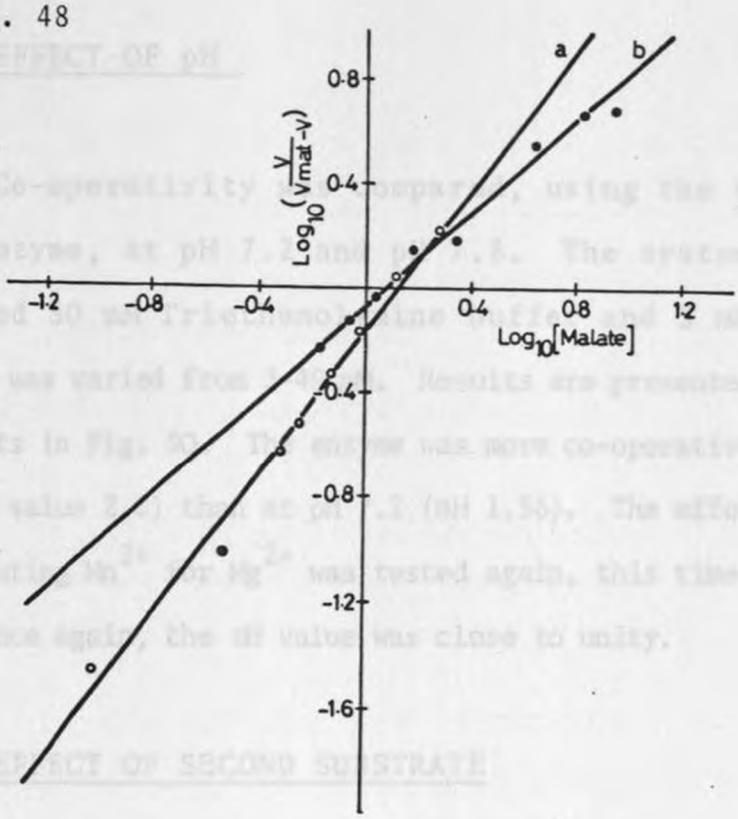
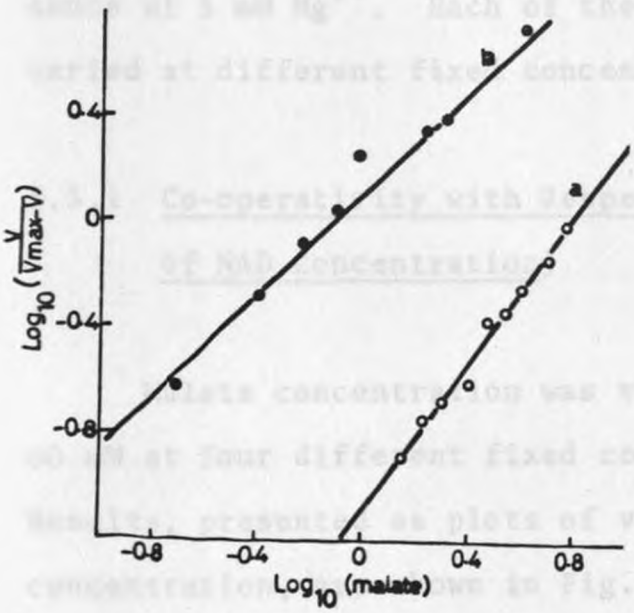

a
b
0·8
0·4
-0·4
-0·8
-1·2
-1·6
-1·2
-0·8
-0·4
0·4
0·8
1·2
$\log_{10}\left(\frac{v}{V_{mal}-v}\right)$
$\log_{10}$[Malate]

Fig. 49


b
a
0·4
0
-0·4
-0·8
-0·8
-0·4
0
0·4
0·8
$\log_{10}\left(\frac{v}{V_{max}-v}\right)$
$\log_{10}$ (malate)

5.2 EFFECT OF pH

Co-operativity was compared, using the Catharsius enzyme, at pH 7.2 and pH 7.8. The system containined 50 mM Triethanolamine buffer and 5 mM Mg^{2+}. Malate was varied from 3-49 mM. Results are presented as Hill plots in Fig. 50. The enzyme was more co-operative at pH 7.8 (nH value 2.2) than at pH 7.2 (nH 1.56). The effect of substituting Mn^{2+} for Mg^{2+} was tested again, this time at pH 7.8. Once again, the nH value was close to unity.

5.3 EFFECT OF SECOND SUBSTRATE

The effect of second substrate concentration on sigmoidicity was investigated with the Catharsius enzyme in Triethanoloamine buffer pH 7.8, in the presence of 5 mM Mg^{2+}. Each of the substrates was varied at different fixed concentrations of the other.

5.3.1 Co-operativity with Respect to Malate (Effect of NAD Concentration)

Malate concentration was varied from 1.0 - 60 mM at four different fixed concentrations of NAD. Results, presented as plots of velocity versus malate concentration, are shown in Fig. 51. Hill plots constructed from these results are also shown in Fig. 52. Values of nH, $S_{0.5}$ and apparent V_{max} are

Fig. 50

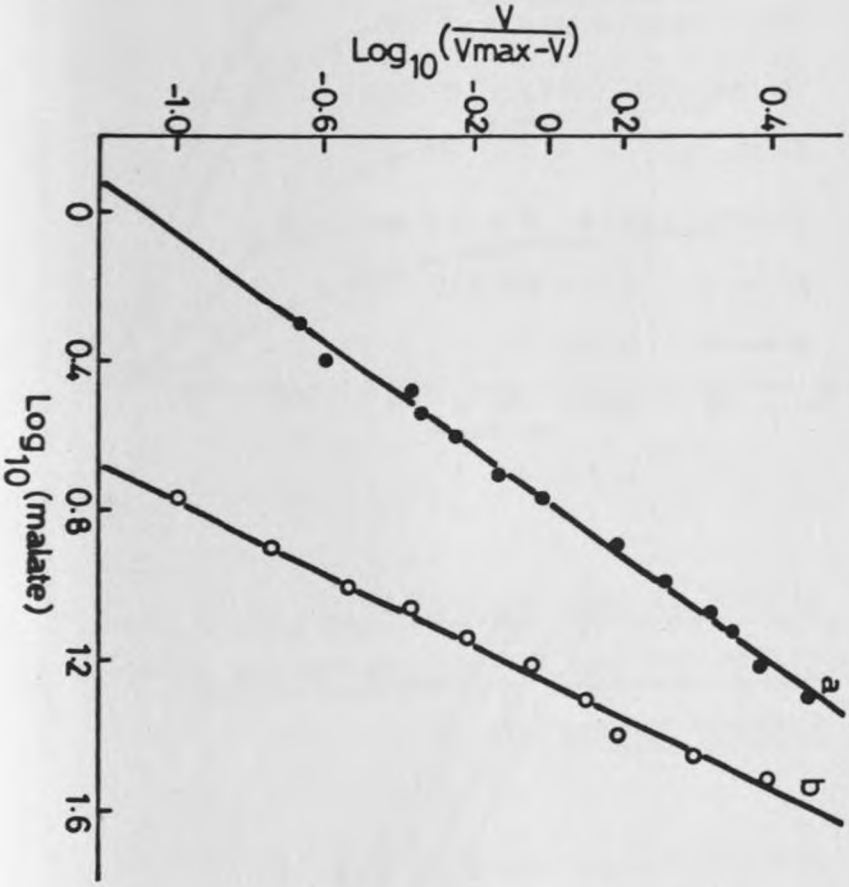


FIGURE 50: EFFECT OF pH ON THE CO-OPERATIVITY OF THE CATHARSIUS ENZYME

The assay system contained

0.32 mM NAD

5 mM Mg^{2+}

Malate varied 3 - 49 mM

50 mM TEA buffer

a) ●——● pH 7.2

b) o——o pH 7.8

Fig. 50

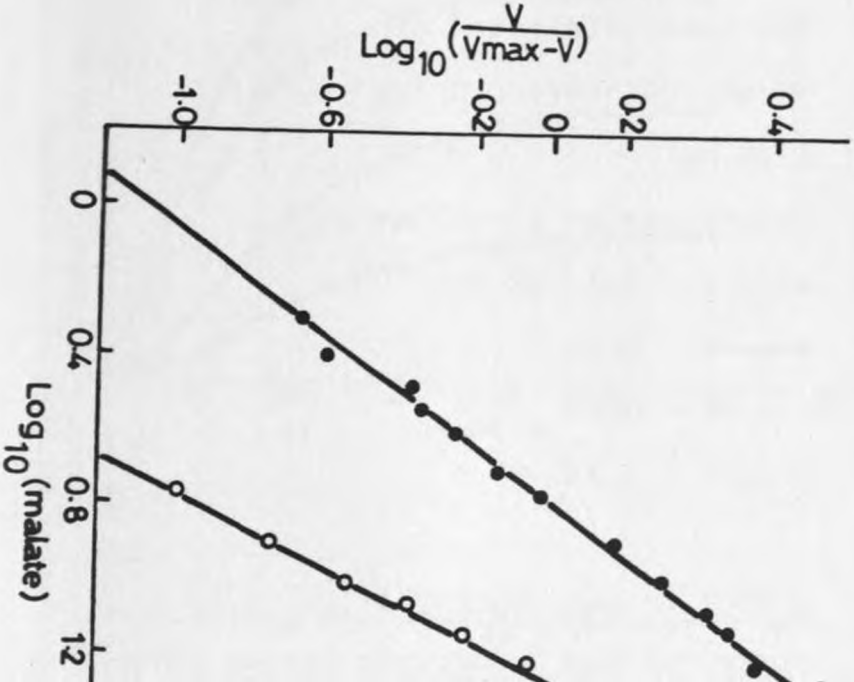


FIGURE 51: EFFECT OF NAD CONCENTRATION ON SIGMOIDICITY OF v VERSUS MALATE CONCENTRATION PLOTS USING CATHARSIUS ENZYME

The assay consisted of

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

Malate varied 1 - 59 mM at

●——● 0.17 mM NAD

●——● 0.42 " "

x——x— 0.83 " "

o——o 1.25 " "

FIGURE 52: EFFECT OF NAD CONCENTRATION ON CO-OPERATIVITY OF THE CATHARSIUS ENZYME WITH RESPECT TO MALATE

The same experiment as Fig. 51

●——● .17 mM NAD

o——o 0.42 " "

x——x 0.83 " "

.——. 1.25 " "

Fig. 51

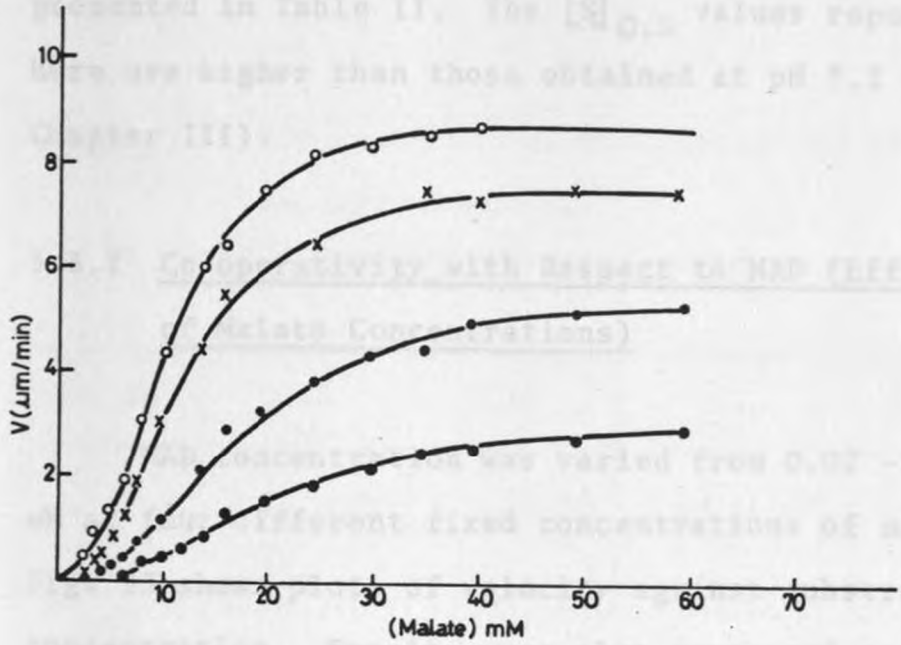
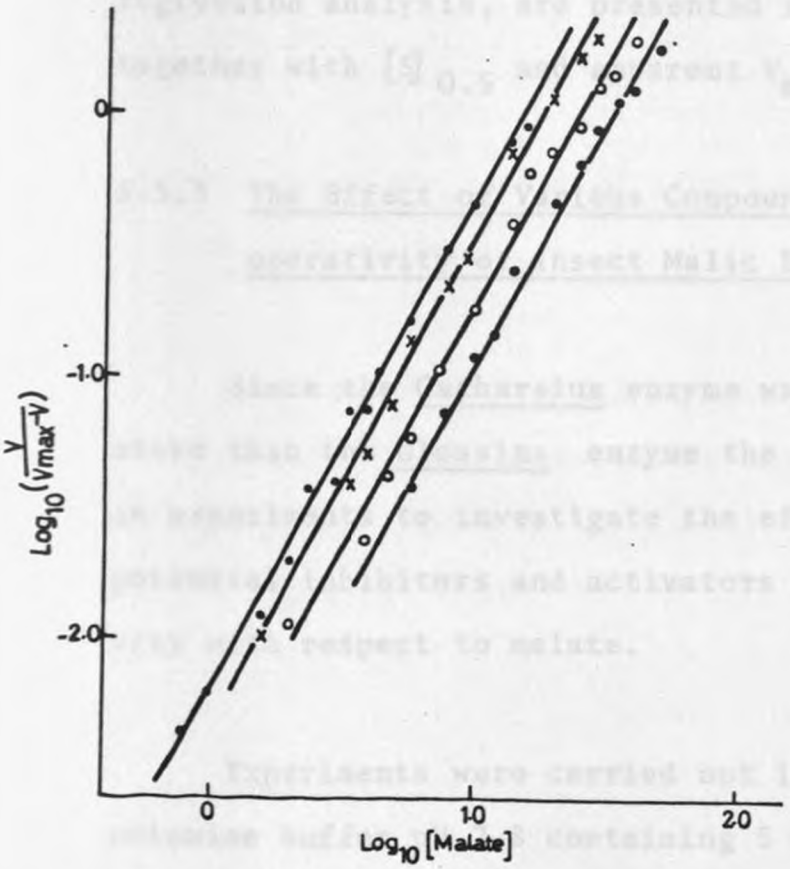

V(μm/min)
10
8
6
4
2
10
20
30
40
50
60
70
(Malate) mM

Fig. 52


0
-1.0
-2.0
Log10 (V/(Vmax-V))
0
1.0
2.0
Log10 [Malate]

presented in Table II. The $[S]_{0.5}$ values reported here are higher than those obtained at pH 7.2 (see Chapter III).

5.3.2 Co-operativity with Respect to NAD (Effect of Malate Concentrations)

NAD concentration was varied from 0.02 - 8.3 mM at four different fixed concentrations of malate. Fig. 53 shows plots of velocity against substrate concentration. Results were also expressed as Hill plots (Fig. 54) and nH values, obtained by linear regression analysis, are presented in Table 12 together with $[S]_{0.5}$ and apparent V_{max} values.

5.3.3 The Effect of Various Compounds on the Co-operativity of Insect Malic Enzyme

Since the Catharsius enzyme was more co-operative than the Glossina enzyme the former was used in experiments to investigate the effects of various potential inhibitors and activators on co-operativity with respect to malate.

Experiments were carried out in 50 mM Triethanolamine buffer pH 7.8 containing 5 mM Mg^{2+} and 0.32 mM NAD. Malate was varied 3 - 50 mM in the presence and absence of a fixed concentration of each modu-

TABLE 11: Effect of NAD on Co-operativity with respect to Malate using Glossina malic enzyme

NAD mM	1.25	0.83	0.42	0.12
nH	1.67±.41(3)	1.78±.36(8)	1.66±.38(3)	1.64±.3(3)
$[S]_{0.5}$ (mM)	16.4±1.7(3)	18.5±1.08(3)	24.4±1.3(3)	33±1.38(3)
$V_{max\ app}$ (µM/min)	4.41±.17(3)	3.67±.63(3)	3.49±.34(3)	1.44±.36(3)

(Means given for number of experiments shown in parenthesis ± S.D.)

NAD concentration had no apparent effect on co-operativity with respect to malate, though increasing NAD concentrations, raised the apparent V_{max} and decreased the $[S]_{0.5}$ value.

TABLE 12: Effect of Malate on the Sigmoidicity of the NAD plot.

using Glossina malic enzyme

Malate mM	19.7	9.87	4.83	1.97
n^H	1.06±.1(4)	1.38±.18(4)	1.55±.27(3)	1.95±.34(4)
$[S]_{0.5}$ (mM)	1.21±.5(4)	1.05±.17(4)	2.88±.16(3)	4.23±.2(4)
$V_{max\ app}$ (μM/min)	6.06±2.3(4)	3.57±1.4(4)	2.43±1.19(3)	0.67±.02(4)

(Means given for number of experiments shown in parenthesis ± S.D.)

The sigmoidicity of the velocity versus NAD concentration plots was found to increase with decreasing malate concentration. Increasing malate concentration also lowered $[S]_{0.5}$ and raised apparent V_{max} values.

FIGURE 53: EFFECT OF MALATE CONCENTRATION ON V VERSUS NAD PLOT USING CATHARSIUS ENZYME

The experiment contained

50 mM Triethanolamine buffer pH 7.8

5 mM Mg^{2+}

NAD varied 0.02 - 8 mM at

●——● 1.97 mM Malate

●——● 4.83 " "

x——x 9.8 " "

o——o 19.7 " "

FIGURE 54: EFFECT OF MALATE CONCENTRATION ON CO-OPERATIVITY WITH RESPECT TO NAD

The same experiment as Fig. 53

●——● 1.97 mM Malate

o——o 4.93 " "

x——x 9.8 " "

●——● 19.7 " "

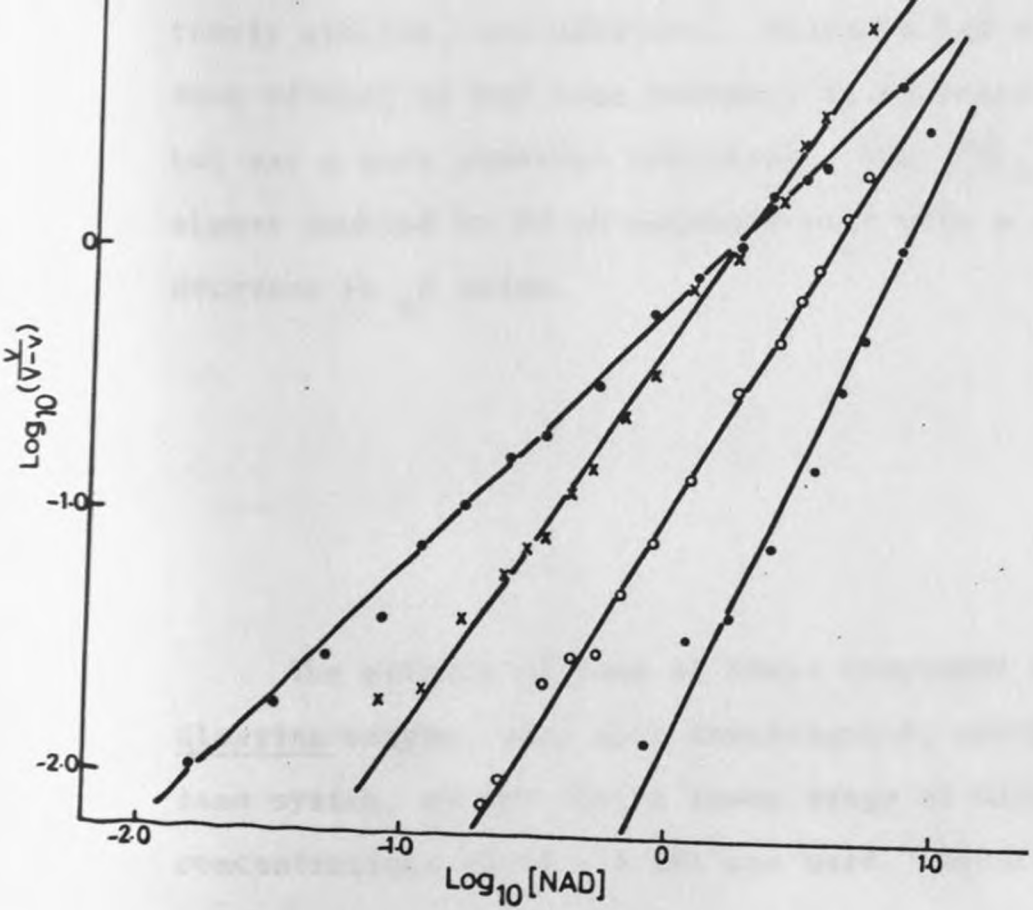

0
-1·0
-2·0
$\log_{10}(\frac{v}{V-v})$
-2·0
-1·0
0
1·0
$\log_{10}$[NAD]

Fig. 53

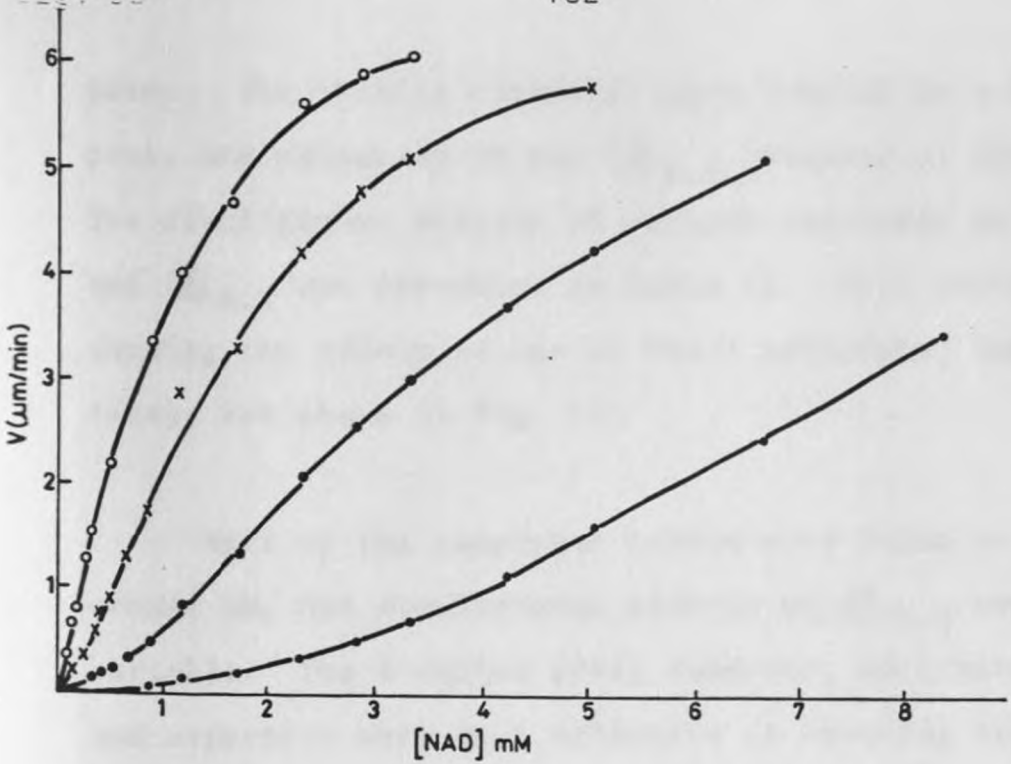


Fig. 54

lator. The results obtained, were treated by a Hill plot, and values of nH and $[S]_{0.5}$ obtained as before. The simultaneous effects of various compounds on nH and $[S]_{0.5}$ are presented in Table 13. Hill plots showing the effect of one of these modifiers, (aspartate), are shown in Fig. 55.

Most of the compounds tested were found to decrease nH, but simultaneous effects on $[S]_{0.5}$ were variable. The 4-carbon acids fumarate, succinate and aspartate were most effective in removing sigmoidicity, but at the same time, had little effect on $[S]_{0.5}$. Their effects were proportional to their concentration and were quantitatively and qualitatively similar, and additive. Malonate had a different effect; it had some tendency to decrease $_nH$, but was a more powerful inhibitor. Thus $[S]_{0.5}$ was almost doubled by 10 mM malonate with only a small decrease in $_nH$ value.

The effects of some of these compounds on the Glossina enzyme, were also investigated, using the same system, except that a lower range of malate concentrations (0.05 - 6 mM) was used. Results are

TABLE 13: Effects of Various Compounds on Values of n_H and $[S]_{0.5}$ for Malate, obtained with the Catharsius enzyme (NAD = 0.32 mM)

Modulator	n_H	$[S]_{0.5}$ (mM)
Enzyme only	1.97±.13(6)	29.3±5.1(6)
Enzyme plus		
Fumarate 2 mM	1.68	25.9
5 mM	1.52	23.7
10 mM	1.34	25.2
20 mM	1.19	26.5
Succinate 20 mM	1.04	25.1
Aspartate 5 "	1.74	24.0
10 "	1.54	29.2
20 "	1.38	31
Aspartate 10 mM + Fumarate 20 "	0.91	27.7
Aspartate 5 " + Fumarate 10 "	1.43	19.4
Malonate 10 "	1.74	51.9
20 "	1.33	65
Malonate 10 " + Fumarate 20 "	.94	54.6
ATP 3 mM	1.93	34.9
5 mM	1.59	36.3
ATP 5 mM + Fumarate 10 mM	1.3	15.1

Values are means of two separate determinations. Only experiments that gave a correlation coefficient for Hill plot y and and x values greater that 0.9 are included.

summarised in Table 14. The effects of fumarate on co-operativity of the Glossina enzyme are shown in Fig. 56.

Various compounds generally had the same effect on the Glossina enzyme as on the Catharsius enzyme, except that only 2 mM fumarate was needed to completely remove sigmoidicity where 10 mM fumarate was required with the Catharsius enzyme. Negative co-operativity was in some cases found with the Glossina enzyme when 4-carbon acids were added.

With the Catharsius enzyme, the effect of various compounds on the sigmoidicity of the velocity versus NAD concentration plot was also investigated with the malate concentration fixed at 10 mM. The NAD concentration was varied from 0.08 - 2.8 mM. The effect of ATP and fumarate on co-operativity with respect to NAD is shown in Fig. 57 and 58 respectively. 5 mM ATP or 10 mM fumarate reduced the nH value of the NAD plot from about 1.67 to a value close to unity.

5.4 ACTIVATION EFFECTS

Since they lowered nH without greatly affecting $[S]_{0.5}$ various potentially inhibitory compounds were in fact stimulatory at low substrate concentrations,

TABLE 14: Effect of Various Modulators on Co-operativity of the Glossina Enzyme

Modulator		nH	$[S]_{0.5}$ (mM)
Enzyme only		1.51±.13(4)	1.35±.23(4)
Enzyme plus Fumarate	2 mM	1.04	1.16
	5 "	.91	1.25
	10 "	.87	1.16
Succinate	2 mM	.94	1.49
	5 "	.82	1.66
Aspartate	2 mM	1.3	1.27
	5 "	1.10	1.4
	10 "	.84	1.44
ATP	1 mM	1.35	1.47
	3 "	1.24	1.55
	5 "	1.18	1.62
ATP 3 mM Fumarate	2 "	.88	1.04
ATP 5 mM Fumarate	2 "	.83	1.32

Values are mean of two separate determinations only plots with a correlation coefficient above 0.9 are increased.

FIGURE 55: EFFECT OF ASPARTATE ON THE CO-OPERATIVITY OF THE CATHARSIUS ENZYME WITH RESPECT TO MALATE

The assay contained
50 mM TEA buffer pH 7.8
5 mM Mg^{2+}
0.32 mM NAD
Malate varied 3 - 50 mM
●——● no addition
o——o with 20 mM aspartate

FIGURE 56: EFFECT OF FUMARATE ON CO-OPERATIVITY OF GLOSSINA ENZYME WITH RESPECT TO MALATE

The assay as in Fig. 55
Malate varied 0.05 - 6 mM
●——● without fumarate
o——o with 2 mM fumarate

Fig. 55

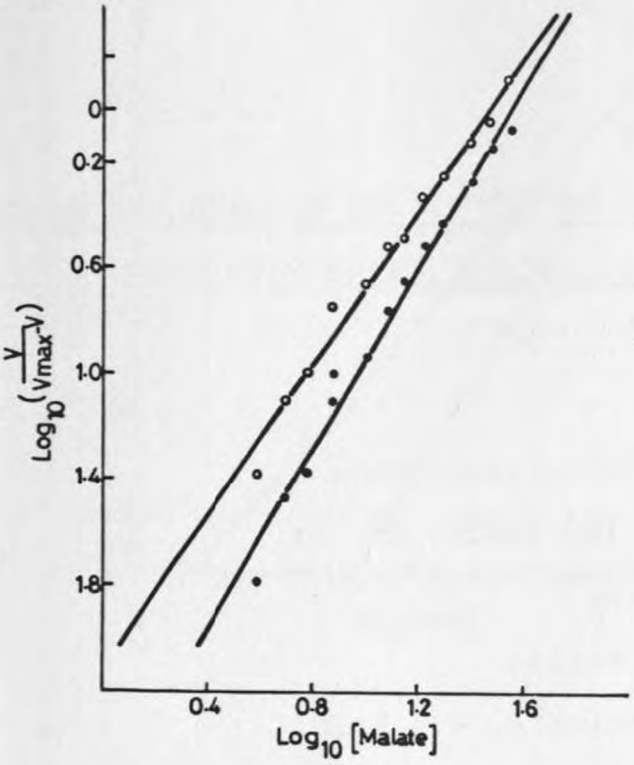

0
0·2
0·6
1·0
1·4
1·8
$\log_{10}\left(\frac{V}{V_{max}-V}\right)$
0·4
0·8
1·2
1·6
$\log_{10}$ [Malate]

Fig. 56

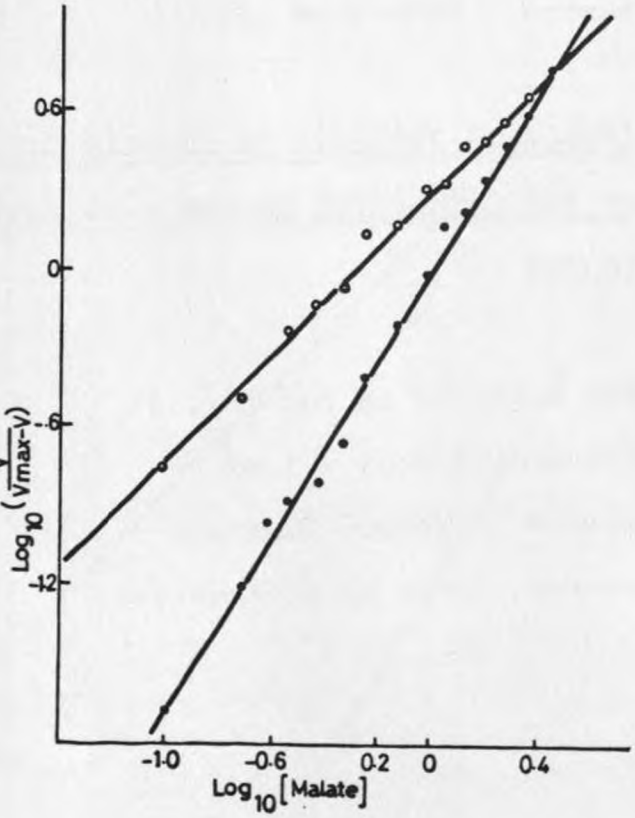

0·6
0
-·6
-1·2
$\log_{10}\left(\frac{V}{V_{max}-V}\right)$
-1·0
-0·6
0·2
0
0·4
$\log_{10}$ [Malate]

FIGURE 57: EFFECT OF ATP ON THE CO-OPERATIVITY OF THE CATHARSIUS ENZYME WITH RESPECT TO NAD

The system contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

10 mM Malate

NAD varied 0.08 - 3 mM

●——● without ATP

o——o with 5 mM ATP

FIGURE 58: EFFECT OF FUMARATE ON CO-OPERATIVITY OF THE CATHARSIUS ENZYME WITH RESPECT TO NAD

The assay is as for Fig. 57

NAD varied 0.08 - 3 mM

●——● without fumarate

o——o with 10 mM Fumarate

Fig. 57

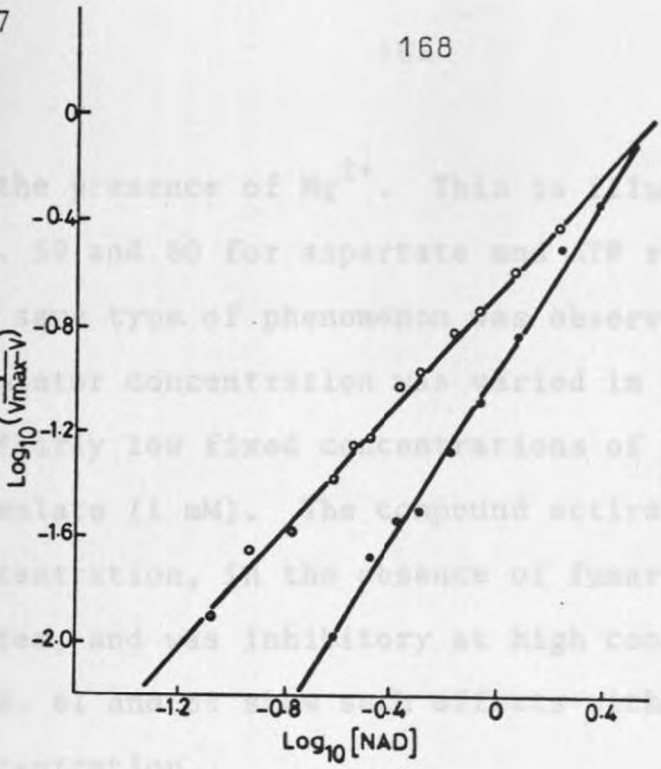
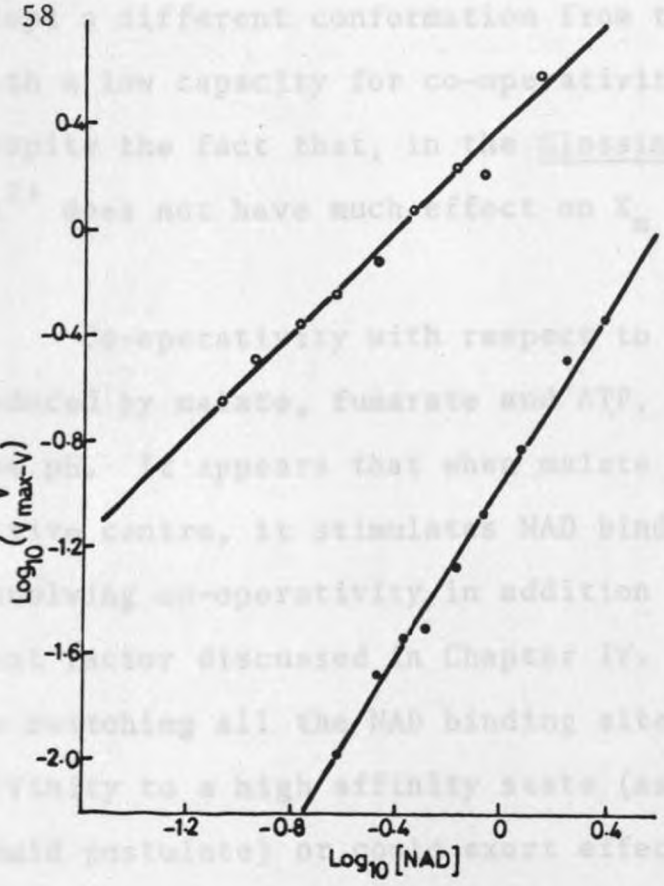

0
-0.4
-0.8
-1.2
-1.6
-2.0
$\text{Log}_{10}\left(\frac{V}{V_{max}-V}\right)$
-1.2
-0.8
-0.4
0
0.4
Log_{10} [NAD]

Fig. 58


0.4
0
-0.4
-0.8
-1.2
-1.6
-2.0
$\text{Log}_{10}\left(\frac{V}{V_{max}-V}\right)$
-1.2
-0.8
-0.4
0
0.4
Log_{10} [NAD]

in the presence of Mg^{2+}. This is illustrated in Fig. 59 and 60 for aspartate and ATP respectively. The same type of phenomenon was observed when the modulator concentration was varied in the presence of fairly low fixed concentrations of NAD (0.32 mM) or malate (1 mM). The compound activated at low concentration, in the absence of fumarate in the system, and was inhibitory at high concentrations. Figs. 61 and 62 show such effects with varied ATP concentration.

5.5 DISCUSSION

In the presence of Mn^{2+}, the active centre must adopt a different conformation from that with Mg^{2+} with a low capacity for co-operativity. This is despite the fact that, in the Glossina enzyme case, Mn^{2+} does not have much effect on K_m values.

Co-operativity with respect to NAD could be reduced by malate, fumarate and ATP, and by lowering the pH. It appears that when malate binds at the active centre, it stimulates NAD binding by a factor involving co-operativity in addition to the enhancement factor discussed in Chapter IV. Malate could be switching all the NAD binding sites from a low affinity to a high affinity state (as the WC model would postulate) or could exert effects on NAD co-

FIGURE 59: STIMULATORY AND INHIBITORY EFFECTS OF ASPARTATE ON GLOSSINA ENZYME

The system contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

0.32 mM NAD

Malate varied 0.4 - 6 mM

.——. without aspartate

o----o with 20 mM aspartate

FIGURE 60: STIMULATORY AND INHIBITORY EFFECTS OF ATP ON CATHARSIUS ENZYME

The assay consisted of

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

0.32 mM NAD

Malate varied 0.4 - 40 mM

.——. without ATP

o----o with 5 mM ATP

Fig. 59

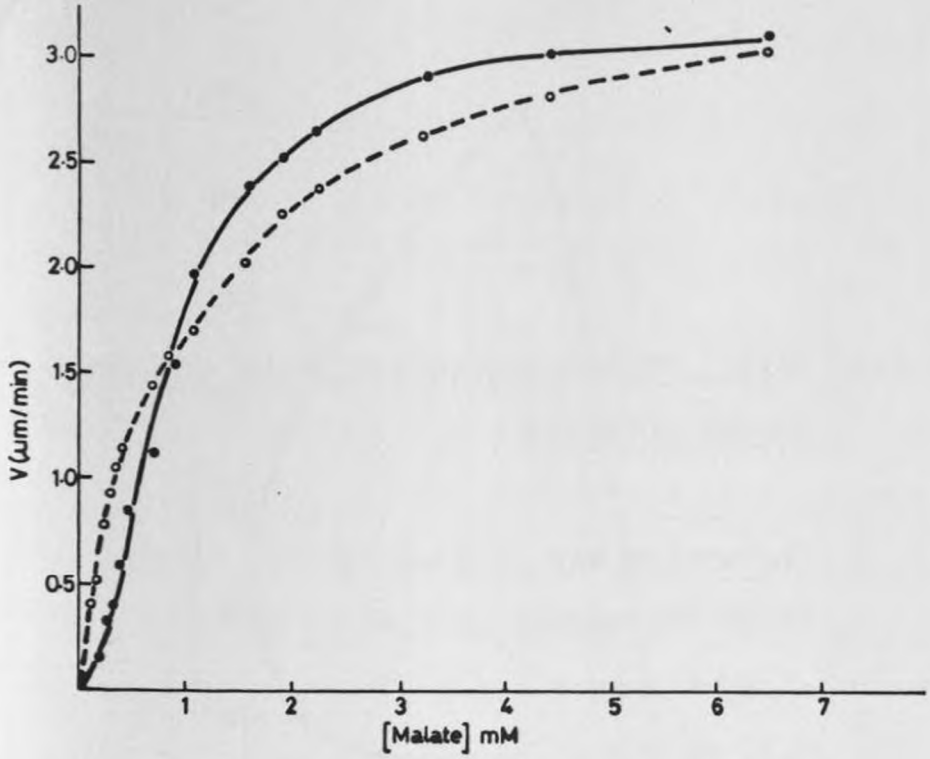

3·0
2·5
2·0
1·5
1·0
0·5
V (µm/min)
1
2
3
4
5
6
7
[Malate] mM

Fig. 60

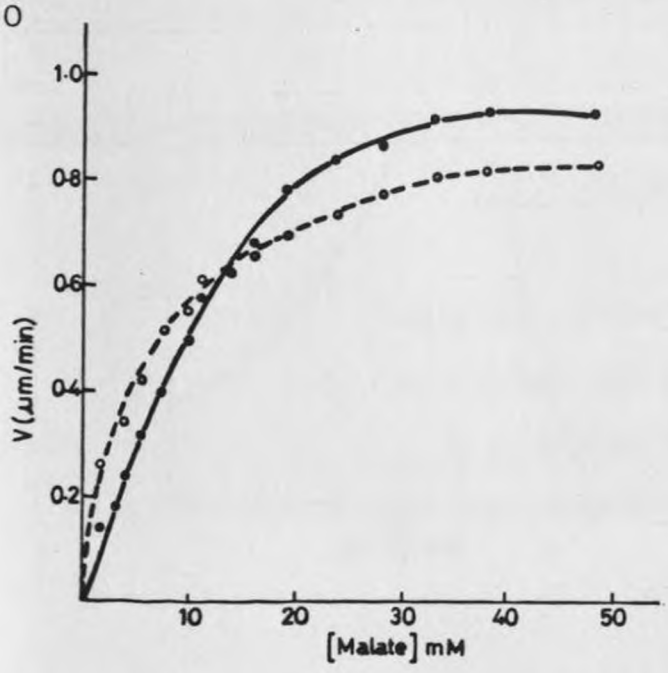

1·0
0·8
0·6
0·4
0·2
V (µm/min)
10
20
30
40
50
[Malate] mM

FIGURE 61: EFFECT OF ATP CONCENTRATION ON GLOSSINA ENZYME ACTIVITY

The system was made up of

50 mM TEA buffer pH 7.8

1 mM Malate

0.32 mM NAD

o——o with 5 mM Mg^{2+}

●——● with 5 mM Mn^{2+}

FIGURE 62: EFFECT OF ATP CONCENTRATION ON CATHARSIUS ENZYME ACTIVITY

The system contained

50 mM TEA buffer pH 7.2

1 mM malate

0.32 mM NAD

Fig, 61

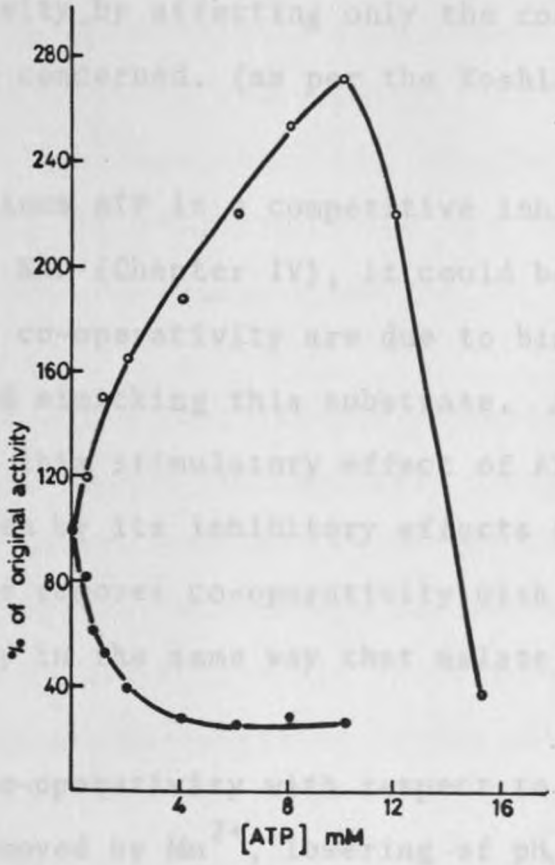
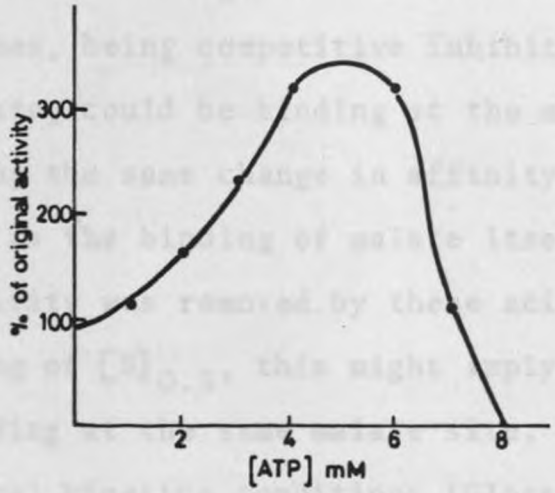

280
240
200
160
120
80
40
% of original activity
4
8
12
16
[ATP] mM

Fig. 62


300
200
100
% of original activity
2
4
6
8
[ATP] mM

operativity by affecting only the conformation of the subunit concerned. (as per the Koshland model idea).

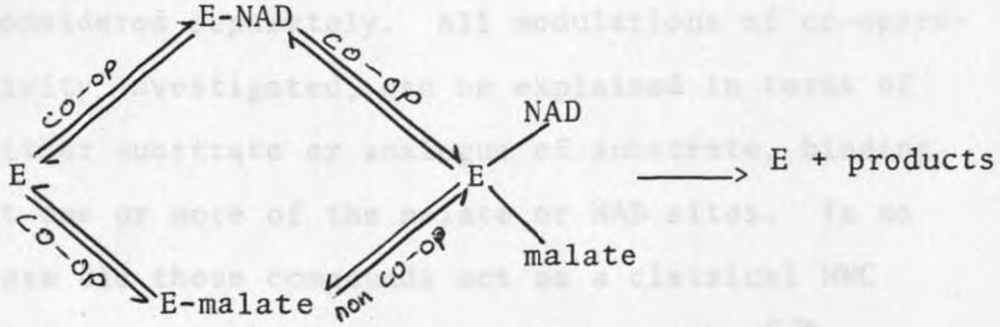
Since ATP is a competitive inhibitor with respect to NAD (Chapter IV), it could be that its effects on co-operativity are due to binding at the NAD site and mimicking this substrate. At high concentration, this stimulatory effect of ATP is presumably overidden by its inhibitory effects (see Chapter IV). Fumarate removes co-operativity with respect to NAD, probably in the same way that malate does.

Co-operativity with respect to malate, was also removed by Mn^{2+}, lowering of pH, fumarate and other malate analogues. Fumarate and other malate analogues, being competitive inhibitors with respect to malate, could be binding at the malate site and inducing the same change in affinity, in unoccupied sites, as the binding of malate itself. When co-operativity was removed by these acids without a lowering of $[S]_{0.5}$, this might imply two effects due to binding at the same malate site. Operating under classical kinetics conditions (Glossina enzyme in presence of 2 mM initial addition of fumarate-see Chapter IV), such acids have been shown to be weak competitive inhibitors with respect to malate. In the co-operativity studies with Catharsius enzyme, they could be binding to the substrate site and

mimicking the substrate with regard to co-operativity, but also blocking its binding. At low malate concentration therefore, they are stimulatory, but at high substrate concentration, they are inhibitory, as is illustrated in Figs. 59 and 60.

Co-operativity should probably be considered in malate or NAD binding sites independently, because, whereas malate reduced co-operativity with respect to NAD, NAD did not reduce co-operativity with respect to malate. Scheme 15 illustrates the steps that would appear co-operative during substrate binding:-

SCHEME 15:



Malate binding would seem to induce a conformation in the NAD site that is non-co-operative, whereas NAD binding seems to leave the malate site still co-operative. Malate and its 4-carbon analogues, i.e fumarate, seem to exert strong effects on co-operativity with respect to both malate and NAD.

Although it is also presumed to bind at the malate site (see Chapter IV), malonate had qualitatively different effects than, for example, fumarate. At a highly inhibitory concentration, it had little effect on nH. Removal of sigmoidicity by malate-like substrates would thus seem to be more than just a function of malate site occupancy, and to depend on the identity of the compound occupying the site. The NAD analogue, ATP, like NAD itself, had little effect on co-operativity with respect to malate.

From the results obtained in this Chapter, it is difficult to explain co-operativity as simply a change from low to high affinity enzyme forms involving the whole protein, but each site has to be considered separately. All modulations of co-operativity investigated, can be explained in terms of either substrate or analogue of substrate, binding at one or more of the malate or NAD sites. In no case did these compounds act as a classical MWC activator i.e lowering nH and decreasing $[S]_{0.5}$. Such an MWC activator acts by stabilising the enzyme in a high affinity form and leaving all substrate sites available. The fact that $[S]_{0.5}$ does not decrease might still admit the MWC model but inhibitor is binding at active center, mimicking the substrate and blocking some substrate binding sites. Whereas in simple MWC model, the inhibitor has its own bind-

ing site and even if nH decreases, it cannot be lower than unity. However, there is no real evidence to suggest binding of any of these co-operative modulators other than at the NAD or malate site. MWC model cannot explain two things

a) The fact that the Glossina enzyme showed negative co-operativity.

b) As argued above co-operativity has to be considered at NAD and malate sites separately, therefore we should be dealing with more than just two forms of enzyme.

CHAPTER VI

6.0 METAL INDEPENDENT REDUCTION OF NAD BY MALATE

At pH 10, when NAD-malic enzyme is normally inactive, slow reduction of NAD by malate is observed in the absence of metal ions. The Glossina malic enzyme appears to function like MDH. It is not however possible to check for products at this pH since OAA decarboxylates non-enzymically to pyruvate. Because no metal ion was involved (indeed malic enzyme is inactive at this pH with Me^{2+} present), it is assumed that this activity could be an MDH activity. The metal independent activity of malic enzyme was only observed with the Glossina enzyme and not with the Catharsius enzyme. In all studies described in this section the Glossina NAD-malic enzyme was used.

6.1 KINETIC STUDIES OF METAL INDEPENDENT ACTIVITY OF MALIC ENZYME

To investigate the dependency of this activity on malate concentration, the enzyme was dialysed thoroughly against 10 mM Tris-HCl pH 7.6 containing 1 mM EDTA. 0.2 mM EDTA was included in the experimental buffer system to completely eliminate any metal ion that might support the full decarboxylating malic

enzyme activity.

In absence of metal ion, malate was varied from 6-50 mM in 0.1 M Glycine-KOH buffer pH 10, containing 1 mM NAD. Results are shown in Fig. 63. The apparent K_m value was very high (21.9 mM) and the apparent V_{max} value was 1.6% of the malate decarboxylation activity at pH 7.8 (cf Olembo 1980).

The effect of NAD concentration on the metal ion independent activity was also investigated in the same coupled system using 100 mM malate and NAD varied from .08 - 1.29 mM. Results are shown in Fig. 64. $K_{m_{app}}$ for NAD was 0.3 mM.

At pH 7.4 without metal ion, NAD reduction by malate in the presence of the Glossina enzyme is slow and quickly ceases. This suggests that equilibrium in an MDH type of reaction is being obtained, rather than malic enzyme activity which should be continuous. This assumption is supported by the fact that addition of citrate synthase and acetyl CoA allows slow rate of NAD reduction to proceeed linearly. The effect of malate concentration on the metal ion independent activity was investigated at pH 7.4 in 50 mM Triethanolamine buffer containing 0.1 M acetyl CoA, 5 µg citrate synthase and 1 mM NAD. The apparent K_m was 156 mM (Table 15), and the V_{max} was 1.7% the rate of

FIGURE 63: EFFECT OF MALATE CONCENTRATION ON METAL INDEPENDENT ACTIVITY OF MALIC ENZYME using *Glossina* malic enzyme

The assay system consisted of

0.1 M Glycine-KOH pH 10

1 mM NAD

Malate varied 6 - 50

FIGURE 64: EFFECT OF NAD CONCENTRATION ON METAL INDEPENDENT ACTIVITY OF MALIC ENZYME using *Glossina* malic enzyme

The system contained

0.1 M Glycine-KOH pH 10

100 mM Malate

NAD varied 0.08 - 1.3 mM

Fig. 64

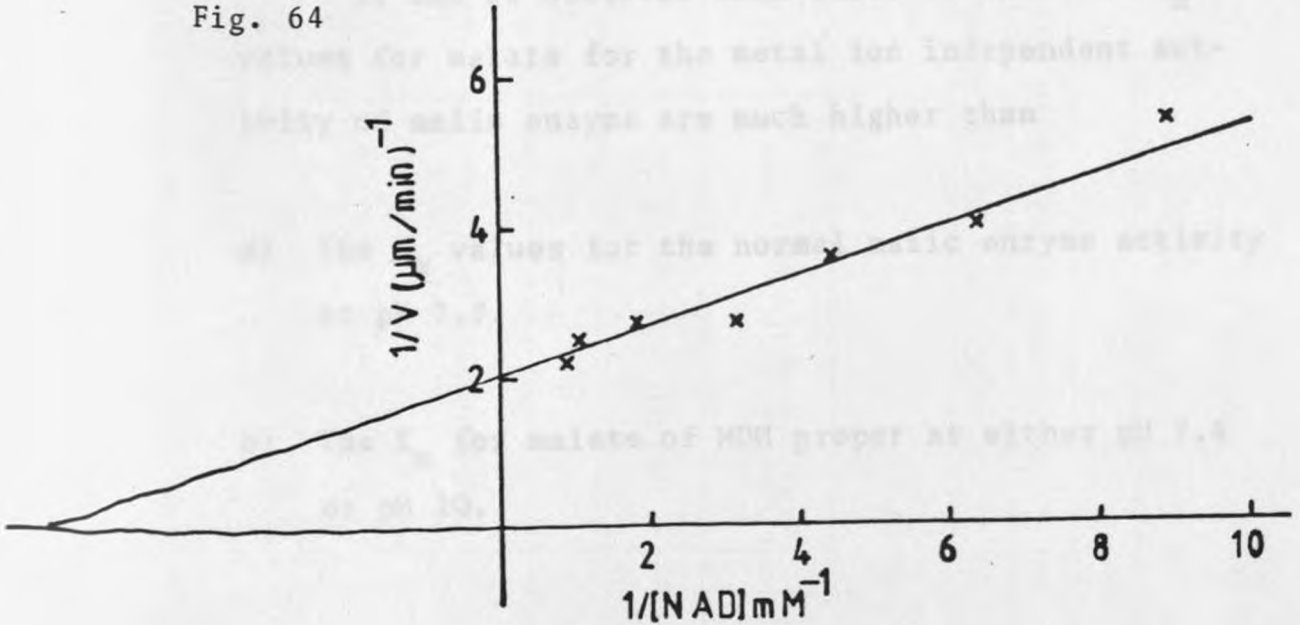
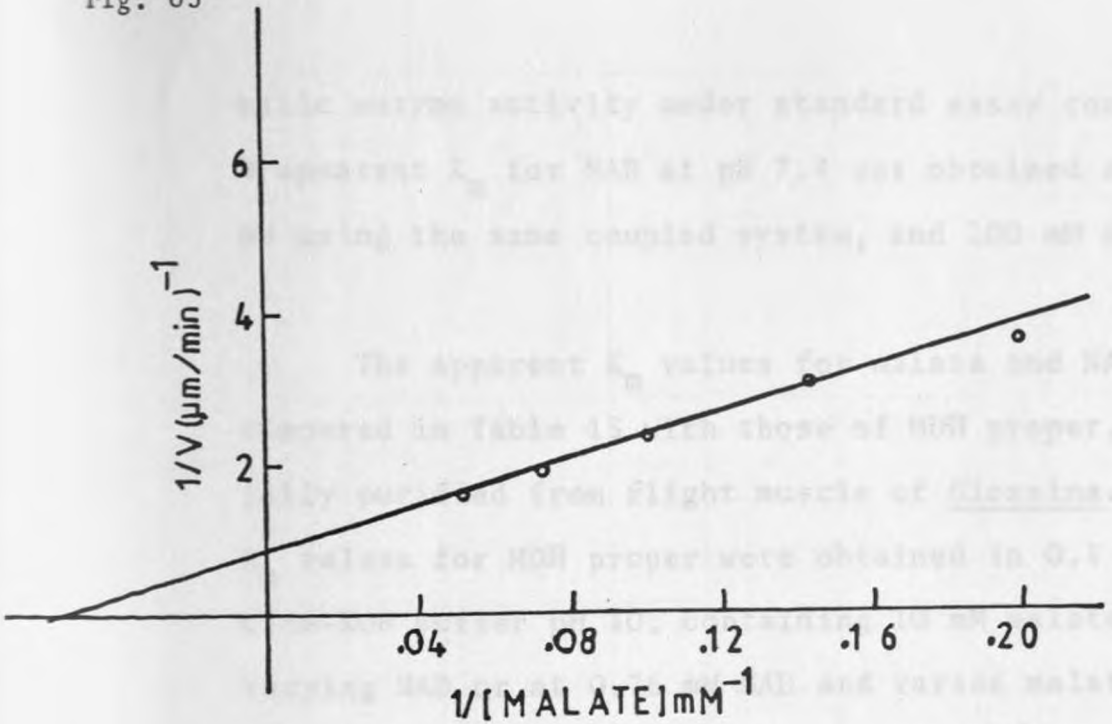


Fig. 63



malic enzyme activity under standard assay conditions. An apparent K_m for NAD at pH 7.4 was obtained as 0.82 mM using the same coupled system, and 100 mM malate.

The apparent K_m values for malate and NAD are compared in Table 15 with those of MDH proper, partially purified from flight muscle of Glossina. These K_m values for MDH proper were obtained in 0.1 M Glycine-KOH buffer pH 10; containing 10 mM malate and varying NAD or at 0.76 mM NAD and varied malate. K_m values were also obtained for the MDH proper at pH 7.4 by coupling the system to citrate synthase. The system contained 50 mM Triethanolamine buffer pH 7.4, 0.1 M acetyl CoA, 5 µg citrate synthase. NAD was fixed at 0.76 mM for the determination of apparent K_m for NAD. Results are included in Table 15.

It can be observed from Table 15 that the K_m values for malate for the metal ion independent activity of malic enzyme are much higher than

a) The K_m values for the normal malic enzyme activity at pH 7.8.

b) The K_m for malate of MDH proper at either pH 7.4 or pH 10.

TABLE 15: Kinetic Parameters for the Metal Independent Activity of Malic Enzyme from Glossina compared with those for the True MDH Enzyme from the same Insect Flight Muscle

Substrate	$K_{m_{malate}}$ (mM)		$K_{m_{NAD}}$ (mM)		V_{max} (μM/min/mg protein	
pH	7.4	10	7.4	10	7.4	10
Me^{2+}-independent activity of Glossina MDH	156±54(3)	21.98±1.23(3)	0.82±.066(3)	0.30±.05(3)	0.16±.004(4)	0.16±.05(4)
Glossina MDH	3.64±.16(3)	1.23±.18(6)	0.185±.01(3)	0.204±.003(3)	0.91±.3(4)	3.37±.66(4)

Values shown are a mean of the number of determinations shown in parenthesis.

For NAD on the other hand, the apparent K_m values of the metal independent activity at either pH 10 or 7.4 was similar to both that of MDH proper and also to that of the standard malic enzyme activity.

6.2 ANALYSIS OF PRODUCTS OF THE METAL INDEPENDENT ACTIVITY OF MALIC ENZYME

It was necessary to investigate whether the metal independent activity of malic enzyme was a malate oxidation producing OAA or a residual oxidative decarboxylation that could occur in the absence of metal activator.

The experiment was carried out at pH 7.4 to prevent non-enzymic decarboxylation of OAA, using the citrate synthase/Acetyl CoA trapping system. The system included 50 mM Triethanolamine pH 7.4 100 mM malate, 1 mM NAD, 0.1 mM acetyl CoA, 5 µg citrate synthase and about 20 µl of appropriately diluted malic enzyme in a total volume of 2 mls. This was incubated for 30 minutes at 30°C. Aliquots of 0.2 ml were withdrawn and mixed with 50 µl of 35% v/v perchloric acid then neutralised with Tris-KOH. Pyruvate and citrate present in the neutralised extract were assayed after centrifugation, as described in section 2.4.4 and 2.4.5.respectively. Results

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were presented in Table 16. Extracts were also assayed for OAA but none was found.

6.3 OAA REDUCTASE ACTIVITY

Without metal ion, malic enzyme appears to reduce OAA to malate with consequent oxidation of NADH. This reaction was studied at pH 7.4 in 50 mM triethanolamine buffer. For the determination of the K_m value for OAA, NADH was fixed at 0.15 mM. (see Fig. 65); for determination of the K_m value of NADH, OAA was fixed at 2.5 mM (see Fig. 66). Results for apparent K_m and V_{max} values are presented in Table 17 together with values obtained with the proper OAA reductase activity of the MDH proper from Glossina flight muscle, obtained under similar conditions.

6.4 PRODUCT INHIBITION STUDIES OF MDH PROPER

Since malic enzyme and MDH complete for the same substrate, malate, it was of interest to note whether the two enzymes had the same mechanism of substrate binding. In the case of MDH, this was investigated by varying one substrate at a fixed concentration of the other with or without the product in question. The results are shown in Fig. 67, 68 and 69. For the NADH inhibition of the $\frac{1}{v}$ versus $\frac{1}{NAD}$ plot, $\frac{1}{v}$ versus $\frac{1}{malate}$ plot and on the OAA inhibition of the

TABLE 16: Product Formation during the Metal Independent Malic Enzyme Activity using *Glossina* malic enzyme

Enzyme	Rates of product formation per ml incubation	
	Citrate (µm/min)	Pyruvate (µm/min)
Metal independent ME activity	0.35	1.2
Proper ME	-	57.8

It appears that about three quarters of the metal independent activity is actually malic enzyme (i.e malate decarboxylation producing pyruvate) and only about a third of the OAA leaves the enzyme without being decarboxylated.

TABLE 17: Kinetic Parameters of OAA Reductase Activity of MDH proper and Me^{2+}- independent activity of ME from Glossina

Enzyme	apparent K_m (mM)	
	OAA	NADH
OAA reductase of ME	0.12 ± .016 (3)	0.032 ± .001 (3)
Proper OAA reductase	0.26 ± .007 (3)	0.34 ± .01 (3)

Values given are the mean of the numbers shown in parenthesis.

The $K_{m_{app}}$ values for OAA and NADH were similar for the two activities but $V_{max_{apparent}}$ for the OAA reductase activity of metal independent activity of malic enzyme was 2.1% of the normal malic enzyme activity at pH 7.8.

FIGURE 65: EFFECT OF OAA CONCENTRATION ON OAA REDUCTASE ACTIVITY OF MALIC ENZYME

The assay consisted of
50 mM TEA buffer pH 7.4
0.15 mM NADH
OAA varied 0.1 - 0.3 mM

FIGURE 66: EFFECT OF NADH CONCENTRATION ON OAA REDUCTASE ACTIVITY OF MALIC ENZYME

The system contained
50 mM TEA buffer pH 7.4
2.5 mM OAA
NADH varied 0.001 - 0.1 mM

Fig. 65

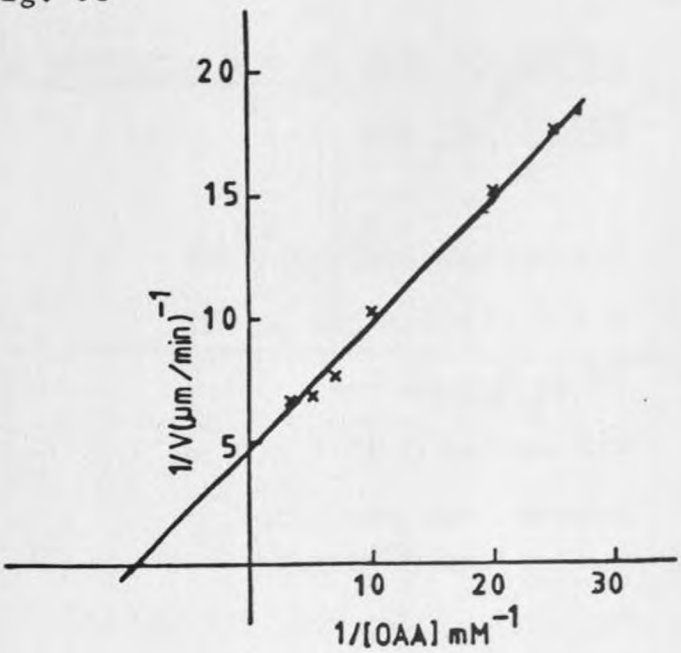

20
15
10
5
1/V(µm/min)$^{-1}$
10
20
30
1/[OAA] mM^{-1}

Fig. 66

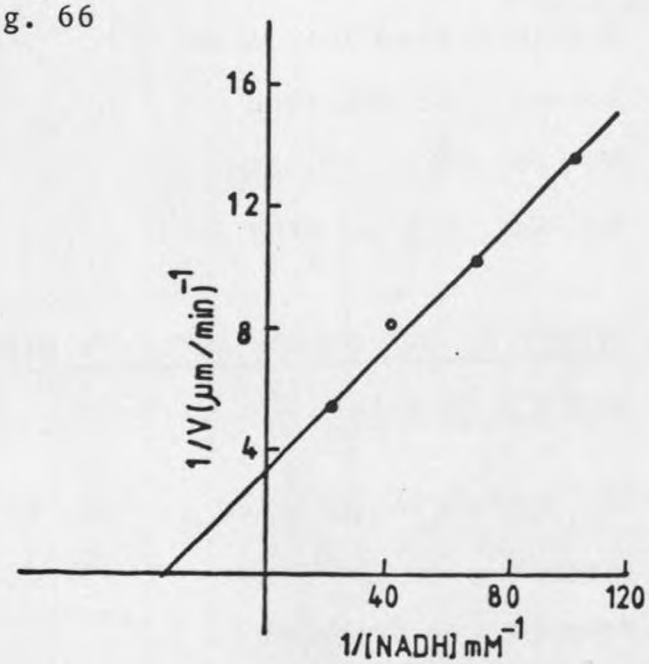

16
12
8
4
1/V(µm/min)$^{-1}$
40
80
120
1/[NADH] mM^{-1}

FIGURE 67: EFFECT OF NADH ON MDH ACTIVITY WITH RESPECT TO NAD

The system consisted of

0.1 M Glycine-KOH pH 10

10 mM Malate

NAD varied 0.05 - 1.3 mM

x——x no addition

o——o 0.1 mM NADH

FIGURE 68: EFFECT OF NADH ON MDH ACTIVITY WITH RESPECT TO MALATE

The assay consisted of

0.1 M Glycine-KOH pH 10

0.76 mM NAD

Malate varied 1 - 15 mM

x——x no addition

●——● 0.05 mM NADH

●——●— 0.1 mM NADH

FIGURE 69: EFFECT OF OAA ON MDH ACTIVITY WITH RESPECT TO MALATE

The system as outlined in Fig. 68

x——x no addition

●——● 0.3 mM OAA

■——■ 0.5 mM OAA

Fig. 67

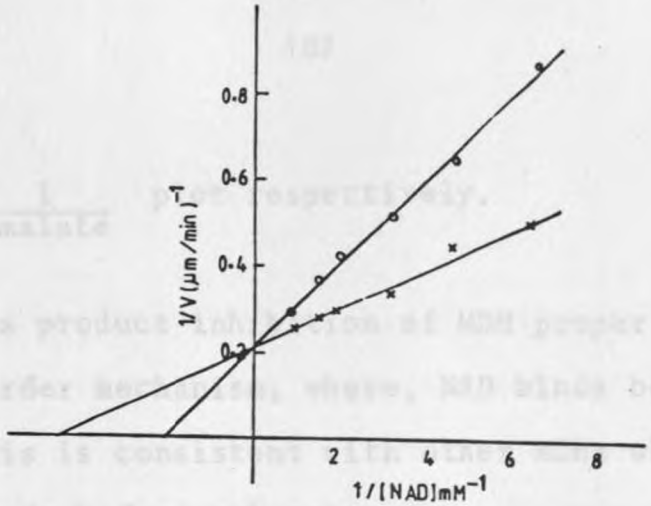


Fig. 68

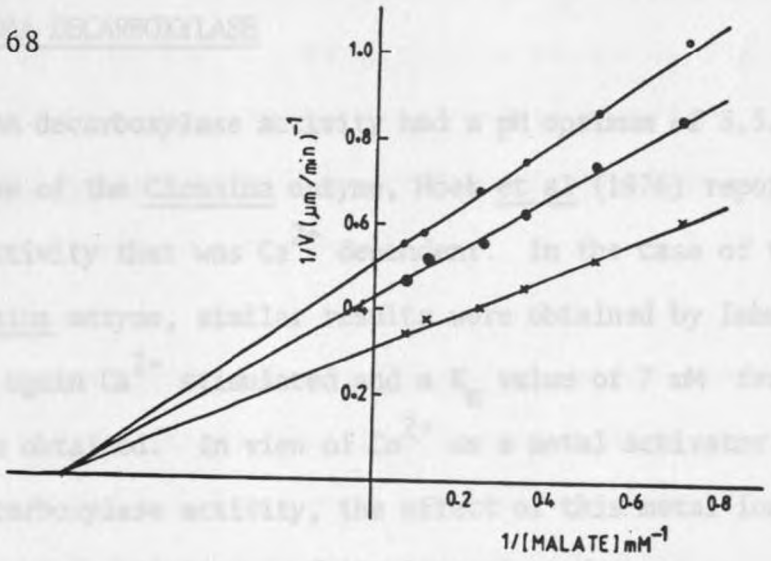
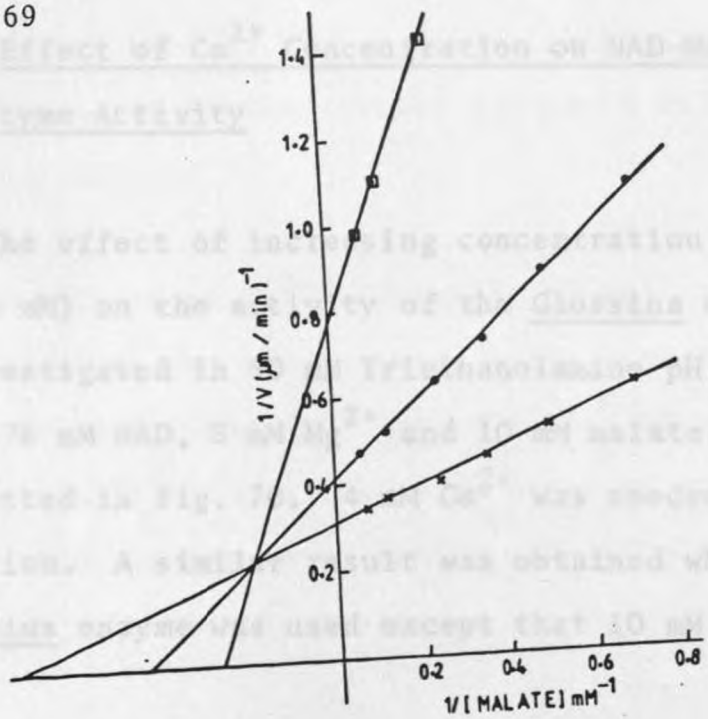


Fig. 69



$\frac{1}{v}$ versus $\frac{1}{[malate]}$ plot respectively.

Thus product inhibition of MDH proper fits with a fixed order mechanism, where, NAD binds before malate. This is consistent with other MDHs where coenzyme binds before substrate.

6.5 OAA DECARBOXYLASE

OAA decarboxylase activity had a pH optimum of 5.5. In the case of the Glossina enzyme, Hoek et al (1976) reported high activity that was Ca^{2+} dependent. In the case of the Catharsius enzyme, similar results were obtained by Imbuga (1979) again Ca^{2+} stimulated and a K_m value of 7 mM for OAA was obtained. In view of Ca^{2+} as a metal activator of OAA decarboxylase activity, the effect of this metal ion on malic enzyme activity was fully investigated.

6.5.1 Effect of Ca^{2+} Concentration on NAD-Malic Enzyme Activity

The effect of increasing concentration of Ca^{2+} (0 - 15 mM) on the activity of the Glossina enzyme was investigated in 50 mM Triethanolamine pH 7.8 and with 0.76 mM NAD, 5 mM Mg^{2+} and 10 mM malate. Results are plotted in Fig. 70. 4 mM Ca^{2+} was needed for 50% inhibition. A similar result was obtained when the Catharsius enzyme was used except that 10 mM Ca^{2+}

caused 50% inhibition.

Calcium inhibition of the Glossina enzyme was also studied in relation to malate concentration. The system contained 50 mM Triethanolamine buffer pH 7.8, 0.76 mM NAD and 5 mM Mg^{2+}. Malate was varied from 0.5 to 2.7 mM with and without a fixed concentration of 5 mM Ca^{2+}. Results are shown in Fig. 71. A competitive inhibition pattern was obtained. With the Catharsius enzyme, a similar competitive inhibition pattern was obtained.

The activity of the Glossina enzyme was also determined in 50 mM Triethanolamine buffer pH 7.8 with 5 mM Mg^{2+} and a fixed concentration of 13 mM malate, NAD being varied from 0.1 - 1.5 mM with and without a fixed concentration of 5 mM Ca^{2+}. Results are presented in Fig. 72. A mixed inhibition pattern was obtained. A similar result was given using the Catharsius enzyme.

The effect of Ca^{2+} on binding of Mg^{2+} to the Glossina enzyme was investigated in an experiment that involved varying Mg^{2+} from 0.4 to 5.99 with and without 5 mM Ca^{2+} in an otherwise standard assay system. Results are shown in Fig. 73 and a mixed inhibition pattern was obtained.

FIGURE 70: EFFECT OF Ca^{2+} CONCENTRATION ON MALIC ENZYME ACTIVITY

The system contained

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

0.76 mM NAD

10 mM Malate

FIGURE 71: EFFECT OF Ca^{2+} ON MALIC ENZYME ACTIVITY WITH RESPECT TO MALATE

The assay consisted of

50 mM TEA buffer pH 7.8

0.76 mM NAD

5 mM Mg^{2+}

Malate varied 0.5 - 2.7 mM

x——x no addition

o——o with 5 mM Ca^{2+}

Fig. 71

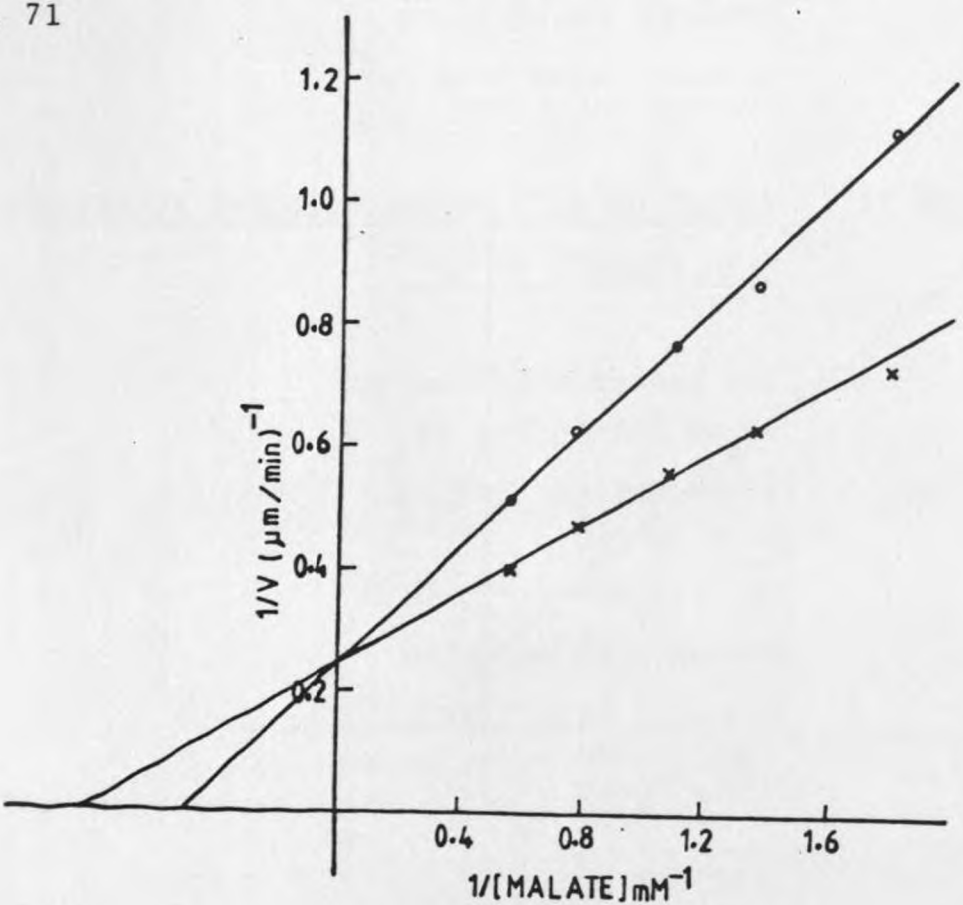


Fig. 70

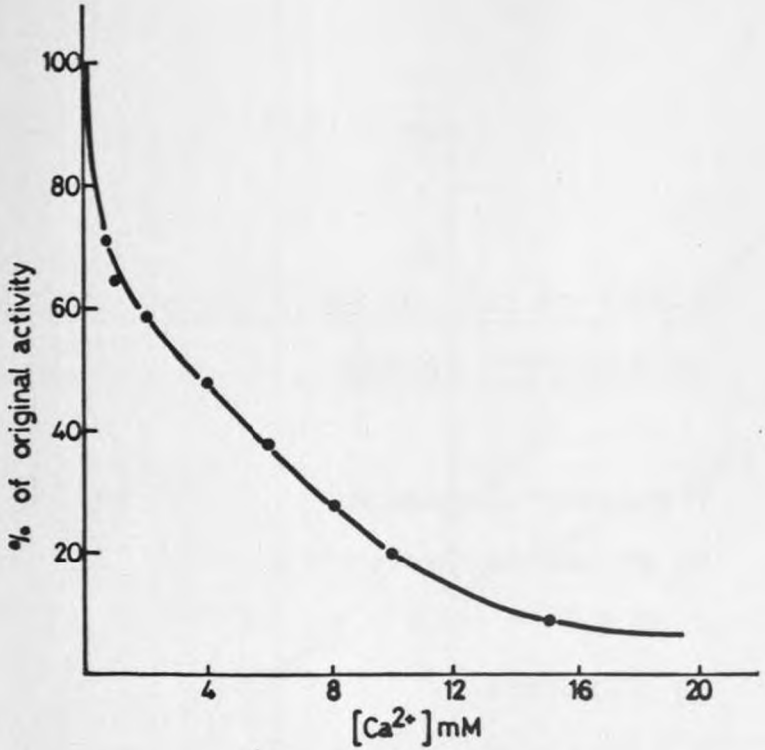


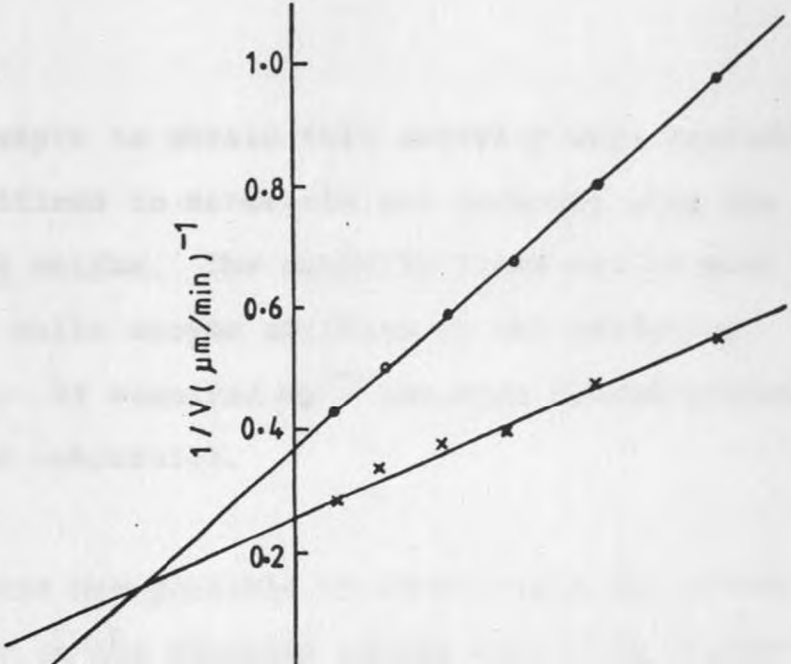
FIGURE 72: EFFECT OF Ca^{2+} ON MALIC ENZYME ACTIVITY WITH RESPECT TO NAD

The assay contained
50 mM TEA buffer pH 7.8
5 mM Mg^{2+}
13 mM Malate
NAD varied 0.1 - 1.5 mM
x——x no addition
o——o with 5 mM Ca^{2+}

FIGURE 73: EFFECT OF Ca^{2+} ON MALIC ENZYME ACTIVITY WITH RESPECT TO Mg^{2+}

The system consisted of
50 mM TEA-buffer pH 7.8
13 mM Malate
0.76 mM NAD
Mg^{2+} varied 0.4 - 6 mM
x——x no addition
o——o with 5 mM Ca^{2+}

Fig. 72



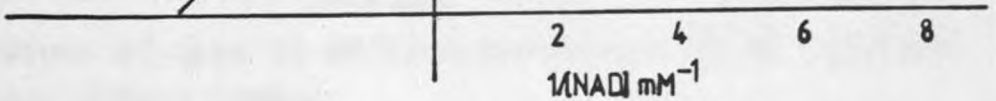
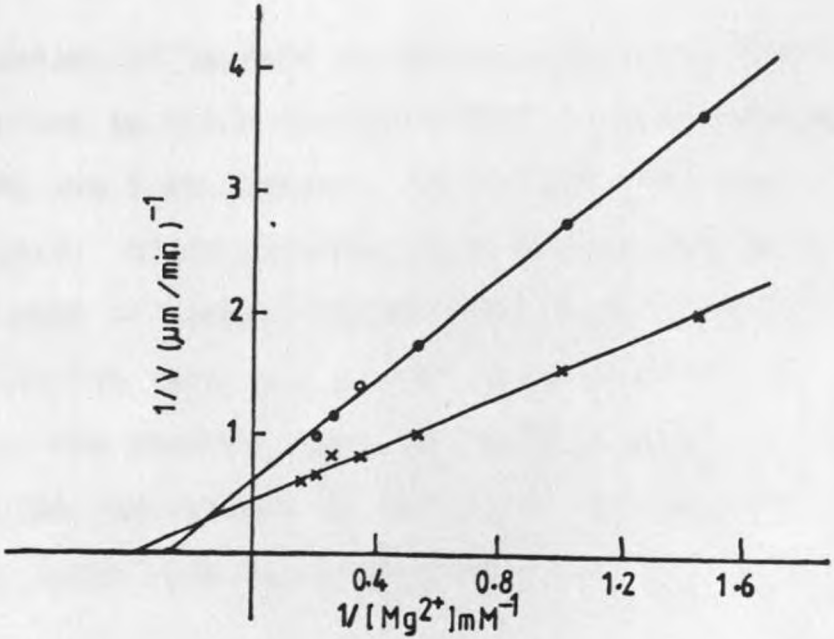


Fig. 73



6.6 REDUCTIVE PYRUVATE CARBOXYLATION BY MALIC ENZYME

Attempts to obtain this activity were carried out as outlined in materials and methods, with the Catharsius enzyme. The activity found was at most 4% of the malic enzyme activity in the oxidative direction. It required Mg^{2+} and high concentrations of all the substrates.

It was not possible to demonstrate any activity whatsoever in the Glossina enzyme even with concentrations of upto 50 mM bicarbonate and 30 mM pyruvate. (See Olembo 1980).

6.7. OXIDATION OF LACTATE BY MALIC ENZYME

Oxidation of lactate to pyruvate by malic enzyme was checked in 0.1 M Glycine buffer pH 9 containing 0.76 mM NAD and 5 mM lactate. No activity was however detected. Since pyruvate clearly does not react with NADH with or without bicarbonate present, there is no indication that this enzyme could catalyse the reaction in the reverse reaction. This therefore rules out the possibility of malate being converted to lactate first then oxidised to pyruvate.

6.8 SENSITIVITY TO SULFHYDRIL REAGENTS

Studies of pH effects on Glossina NAD-malic enzyme revealed important pKs at pH values between 7.5 and 8.2 which could perhaps be due to -SH groups associated with the active centre. The sensitivity of the enzyme to sulfhydril reagents was therefore investigated.

6.8.1 Effect of Certain Sulfhydril Reagents on Malic Enzyme Activity

Using a standard assay system of 50 mM Triethanolamine buffer pH 7.8, 5 mM Mg^{2+}, 10 mM malate and 0.76 mM NAD, the effects of various concentrations of p-hydroxy-mercuribenzoate (HMB) were tested. The enzyme was exposed to HMB for about 1-2 minutes before the assay. Results, presented as percentage of original activity against HMB concentration, are shown in Fig. 74. About 0.2 µM HMB was needed for 50% inhibition.

The same procedure was used to test effects of various heavy metals Hg^{2+}, Cu^{2+} and Zn^{2+}. These were all also found to inhibit the Glossina enzyme, the strength of inhibition decreasing in that order. The fall off in Fig. 74 is not linear i.e doesn't appear to be titration of 1:1 and could be a reversible

effect since it is a curve.

6.8.2 Effect of Incubating Malic Enzyme with Sulfhydril Reagents

The effect of incubating Glossina malic enzyme at 30°C with sulfhydril inhibitors for periods of upto 30 minutes was also investigated.

The 2 ml incubation mixture included 50 mM Triethanolamine pH 7.8 and 5 µg of enzyme together with 50 µM iodobenzoate (IBA) 30 µM Ethylmalemide (EMD) or 1.3 µM HMB; a control out. Aliquots were withdrawn after different time intervals and tested for activity using the standard assay system. Results, plotted as log $\frac{vt}{vo}$ against time, are presented in Fig. 75 (vt = enzyme activity left at time t, vo = initial velocity at time 0 i.e original activity). HMB was the most potent. Its effect seemed to involve two phases, an initial fast and a subsequent less potent and more gradual. IBA inhibition also appeared to be biphasic.

6.8.3 Effect of Substrates on the Inhibition of Malic Enzyme Activity by Sulfhydril Reagents

Since sulfhydril reagents were found to inhibit Glossina malic enzyme, it was of interest to find

FIGURE 74: EFFECT OF p-HYDROXY-MERCURIBENZOATE ON MALIC ENZYME ACTIVITY

The assay contained

50 mM TEA pH 7.8

5 mM Mg^{2+}

10 mM Malate

0.76 mM NAD

p-hydroxy-mercuribenzoate varied 0 - 1 mM

FIGURE 75: INACTIVATION OF MALIC ENZYME ACTIVITY BY VARIOUS SULFHYDRIL REAGENTS

After treatment as outlined in text.

Enzyme assayed in

50 mM TEA buffer pH 7.8

5 mM Mg^{2+}

0.76 mM NAD

10 mM Malate

—●——●— Enzyme only

x——x with 30 µM IBA

o----o- with 30 µM EMD

●——●— with 1.3 µM HMB

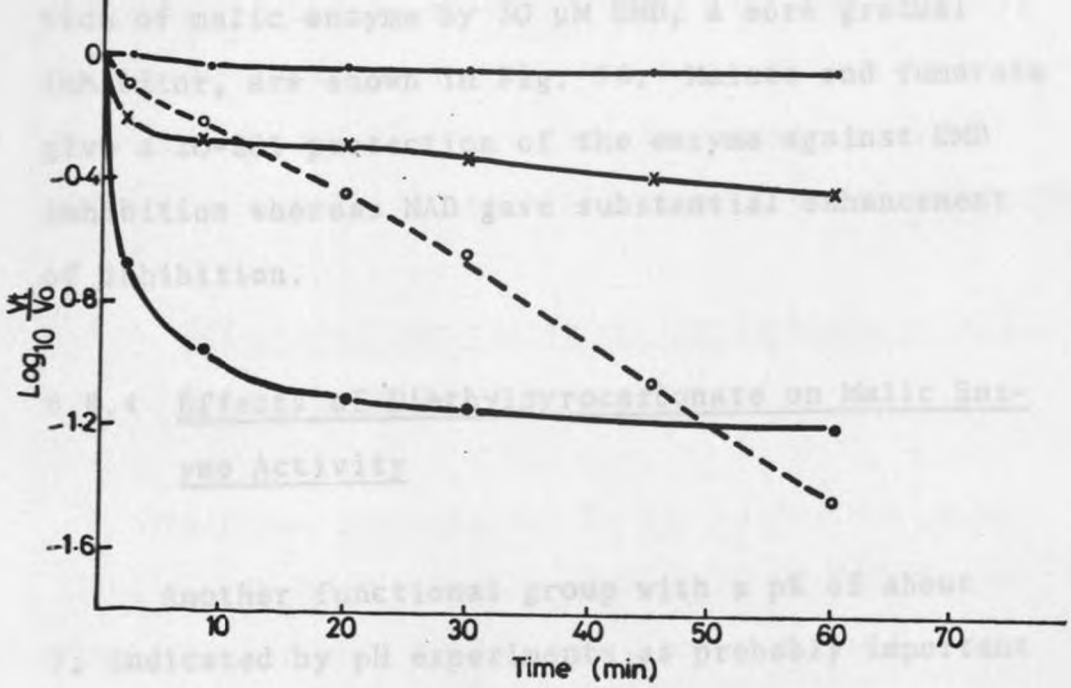

0
-0.4
-0.8
-1.2
-1.6
$\log_{10} \frac{V_t}{V_0}$
10
20
30
40
50
60
70
Time (min)

Fig. 74

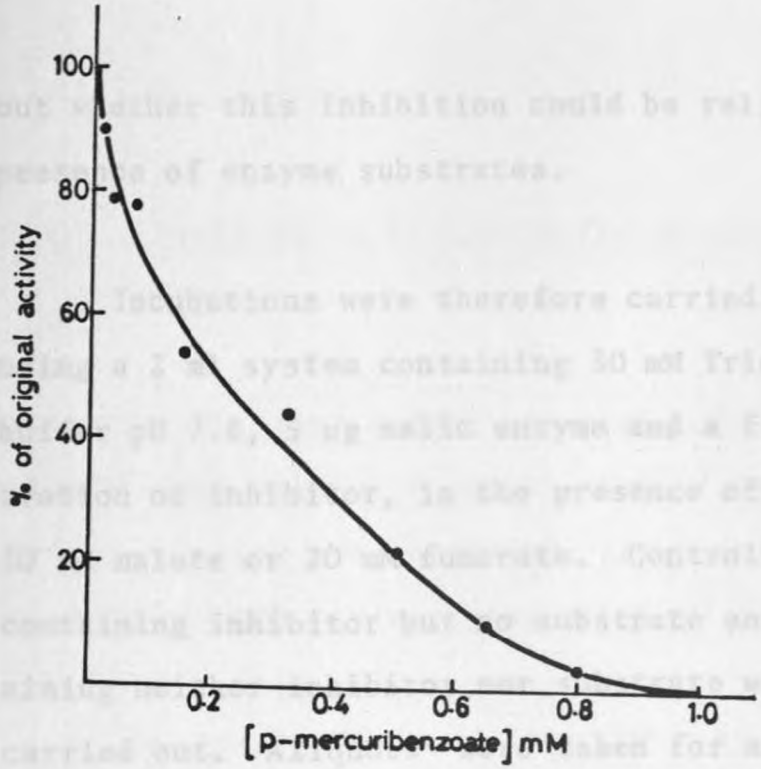

100
80
60
40
20
% of original activity
0·2
0·4
0·6
0·8
1·0
[p-mercuribenzoate] mM

Fig. 75

out whether this inhibition could be relieved by the presence of enzyme substrates.

Incubations were therefore carried out at 30°C using a 2 ml system containing 50 mM Triethanolamine buffer pH 7.8, 5 µg malic enzyme and a fixed concentration of inhibitor, in the presence of 1 mM NAD, 20 mM malate or 20 mM fumarate. Control incubations containing inhibitor but no substrate and also containing neither inhibitor nor substrate were also carried out. Aliquots were taken for assay after various times of incubation. Substrates did not have any effect on the HMB inhibition of malic enzyme. Results showing the effects of substrates on inhibition of malic enzyme by 30 µM EMD, a more gradual inhibitor, are shown in Fig. 76. Malate and fumarate give a 10-20% protection of the enzyme against EMD inhibition whereas NAD gave substantial enhancement of inhibition.

6.8.4 Effects of Diethylpyrocarbonate on Malic Enzyme Activity

Another functional group with a pK of about 7, indicated by pH experiments as probably important in active centre functioning, is the imidazole group of histidine. It was therefore of interest to investigate further whether such a group was essential

for enzyme activity.

6.8.5 Inactivation of the Enzyme by Diethylpyrocarbonate

The Glossina enzyme was preincubated for three minutes with various concentrations of pyrocarbonate before reaction was initiated, under standard assay conditions with the addition of 10 mM malate.

Results are shown in Fig. 77. Pyrocarbonate at about 2 mM concentration was required for 50% inactivation of the enzyme. Pyrocarbonate does not appear to give a stoichiometric binding and could be a reversible inhibition effect. Since the concentration of pyrocarbonate needed for 50% inactivation is high, it could be that the histidine functional group at the active centre is not highly reactive with pyrocarbonate.

6.8.6 Effects of Substrates on Inactivation of Malic Enzyme by Diethyl Pyrocarbonate

This was investigated by incubating the enzyme at 30°C with 4 mM pyrocarbonate in 50 mM Triethanolamine buffer pH 7.8 with and without various substrates, for a period of 30 minutes. Aliquots of the incubation mixture were withdrawn after suitable time intervals and assayed for enzyme activity under stan-

FIGURE 76: EFFECT OF SUBSTRATES ON INACTIVATION OF MALIC ENZYME BY 30 μM EMD

After treatment as outlined in text

Enzyme assay in standard assay system as Fig. 75

●----● E-only

x----x- E + EMD + 10mM Fumarate +0.32mM NAD

●-----●-- E + EMD + 10 mM Malate

o——o E + EMD only

●——●— E + EMD + 0.32 mM NAD

FIGURE 77: EFFECT OF PYROCARBONATE CONCENTRATION ON GLOSSINA MALIC ENZYME ACTIVITY

The system contained

50 mM TEA buffer pH 7.8

10 mM Malate

0.76 mM NAD

5 mM Mg^{2+}

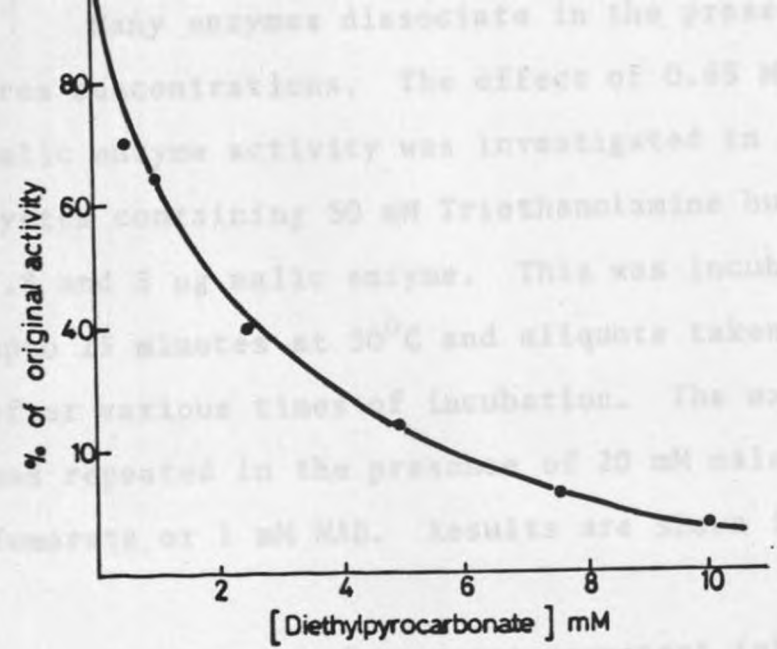

80
60
40
10
% of original activity
2
4
6
8
10
[Diethylpyrocarbonate] mM

Fig. 76

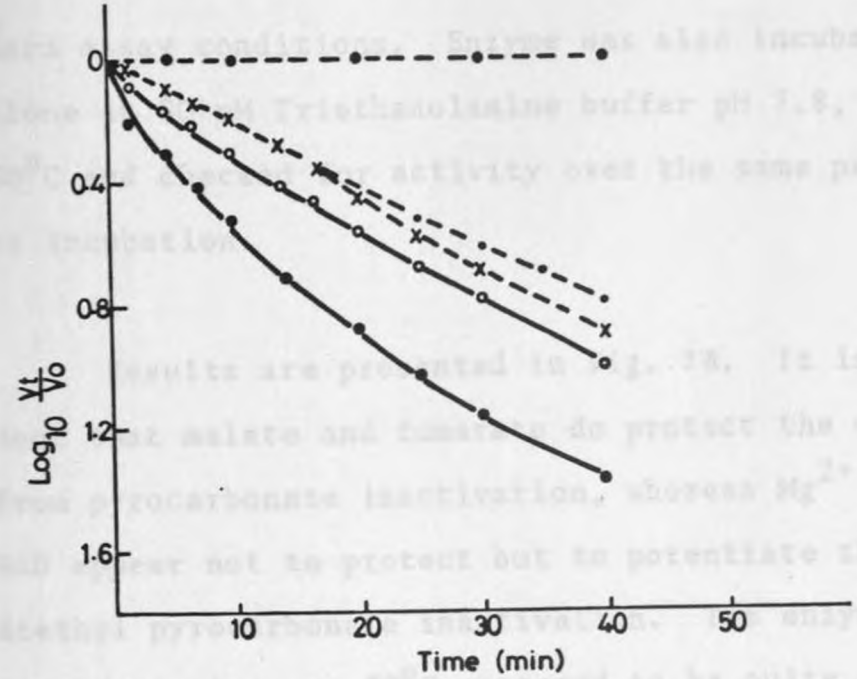


Fig. 77

dard assay conditions. Enzyme was also incubated alone in 50 mM Triethanolamine buffer pH 7.8, at 30°C and checked for activity over the same period of incubation.

Results are presented in Fig. 78. It is evident that malate and fumarate do protect the enzyme from pyrocarbonate inactivation, whereas Mg^{2+} and NAD appear not to protect but to potentiate the diethyl pyrocarbonate inactivation. The enzyme when incubated alone at 30°C appeared to be quite stable.

6.8.7 Effect of Urea on Malic Enzyme Activity

Many enzymes dissociate in the presence of high urea concentrations. The effect of 0.65 M urea on malic enzyme activity was investigated in a 2 ml system containing 50 mM Triethanolamine buffer pH 7.8 and 5 µg malic enzyme. This was incubated for upto 15 minutes at 30°C and aliquots taken for assay after various times of incubation. The experiment was repeated in the presence of 20 mM malate, 20 mM fumarate or 1 mM NAD. Results are shown in Fig. 79.

Urea appeared to cause permanent inhibition which was time dependent. It is assumed since urea is inert chemically it could be due to osmolarity i.e due to some sort of enzyme dissociation which is of

FIGURE 78: EFFECT OF SUBSTRATES ON DIETHYL PYROCARBONATE (PC) INACTIVATION OF MALIC ENZYME ACTIVITY

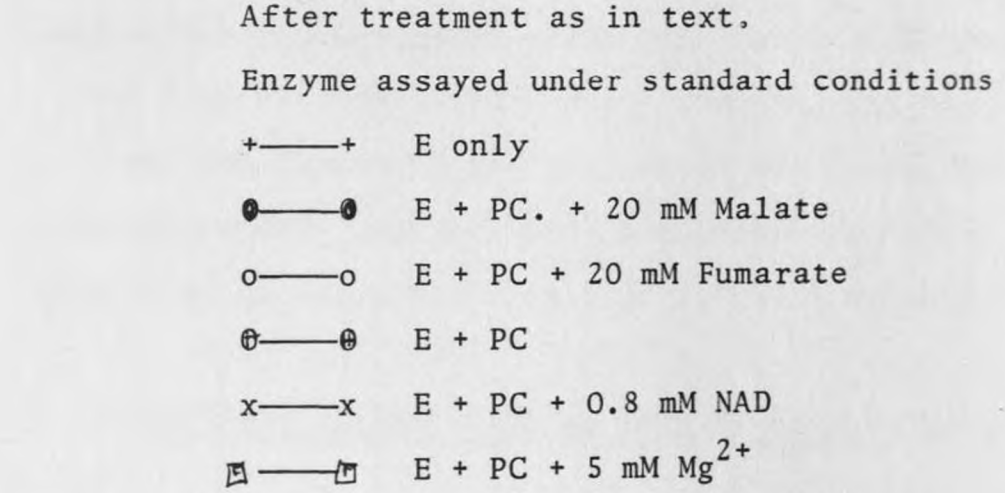


FIGURE 79: EFFECTS OF SUBSTRATES ON INACTIVATION OF MALIC ENZYME BY 0.65 M UREA

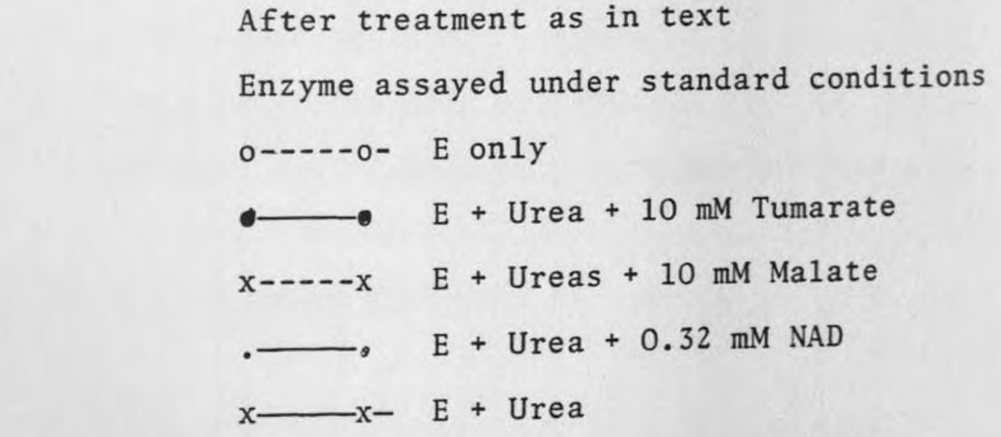


Fig. 78

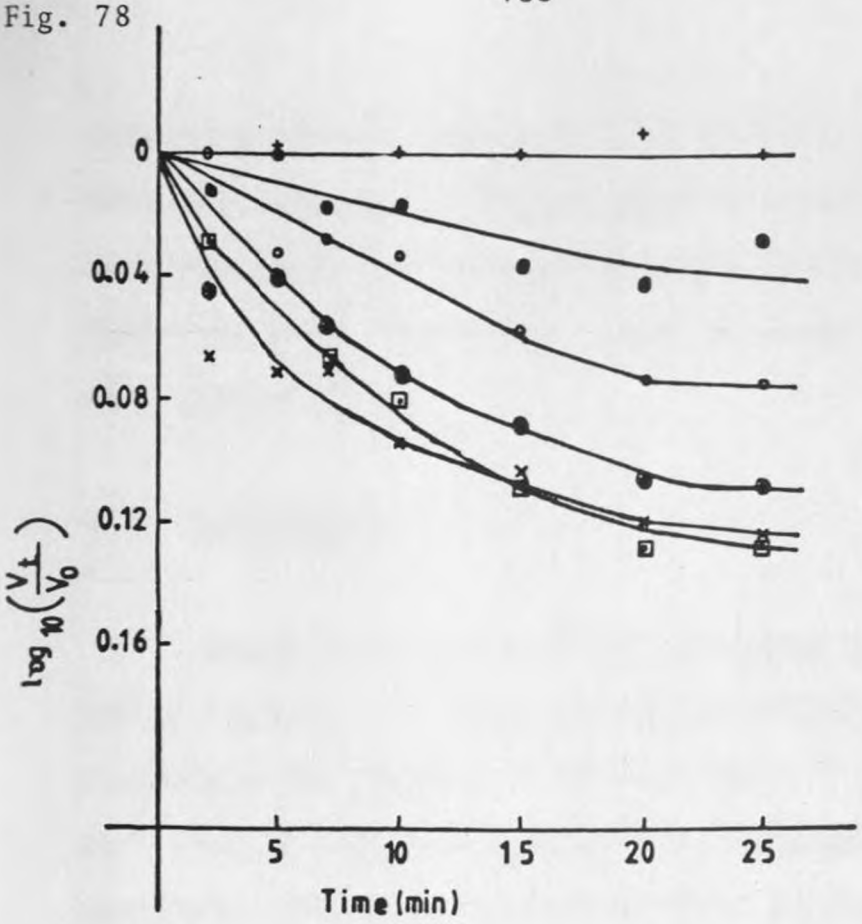
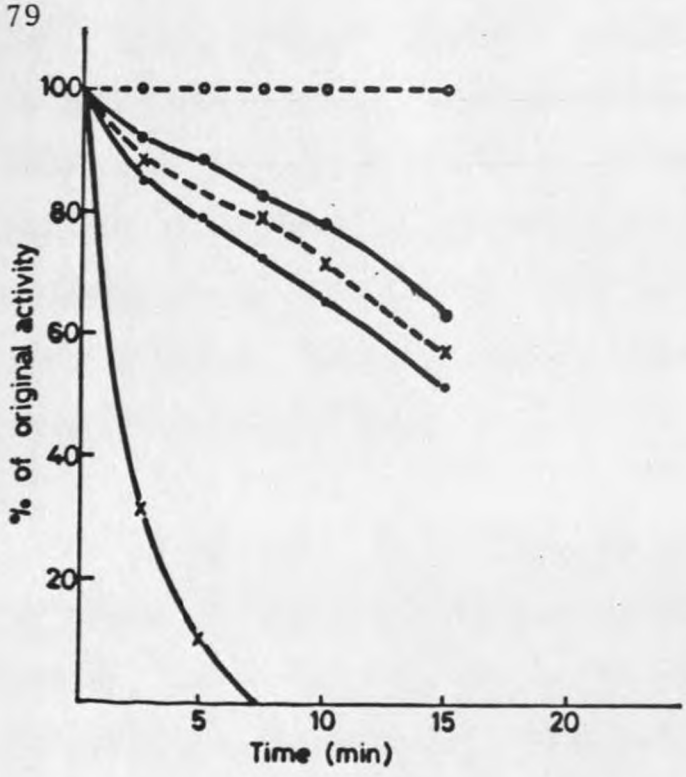

0
0.04
0.08
0.12
0.16
$\log_{10}\left(\frac{V_t}{V_0}\right)$
5
10
15
20
25
Time (min)

Fig. 79


100
80
60
40
20
% of original activity
5
10
15
20
Time (min)

permanent nature since, after withdrawing aliquots were not assayed in the presence of urea but still obtained high inhibition. Against this type of inhibition both substrates and fumarate were protective.

6.9 DISCUSSION

Without metal ion, the OAA formed from malate has a tendency to leave the enzyme without being decarboxylated, whereas in the presence of Mg^{2+} or Mn^{2+} the so formed bound OAA is efficiently decarboxylated. Probably, metal provides the proper orientation and interactions for this decarboxylation. Ca^{2+}, being heavier than Mg^{2+} and Mn^{2+}, presumably causes conformational changes in the enzyme molecule that lead to lowered affinity for malate, but increases the efficiency of decarboxylation of OAA in the absence of bound NADH. It seems probable that reduction of malate does not require metal ion but decarboxylation of OAA does.

In the case of the Glossina malic enzyme, the K_m value for OAA (with respect to OAA reductase activity), was lower than the K_m for malate with respect to malic enzyme activity. This indicates tighter binding of the former than the latter. Also, the K_m value for OAA (with respect to OAA reductase)(0.12mM)

was far lower than its K_i (with respect to malic enzyme activity) (2.5 mM). OAA was competitive with respect to malate, but since it could presumably bind to free enzyme or to the E-NAD complex, the value of the K_i obtained could have been near the binding constant of OAA to E-NAD, for NAD was near saturating in the experiments concerned. The K_m for OAA of the OAA reductase could have been close to the binding constant for OAA of the free enzyme, or of an E-NADH binary complex, but again this may well have been affected (lowered) by the absence of metal.

The metal independent activity of malic enzyme was apparently a combination of malic enzyme and MDH-type activity. It is unlikely to be of physiological importance in view of its low V_{max} and the extremely high K_m values for substrates. The Catharsius enzyme clearly differed from the Glossina enzyme in being devoid of metal independent activity.

With the true Glossina MDH activity studied at pH 7.4, a low K_m for OAA and a high K_m for malate were obtained, a result which could be related to the equilibrium constant. Since V_{max} was about the same for the forward and back reactions, it was expected that the K_m for OAA would be relatively low. The Haldane equation would predict that

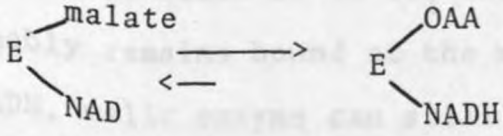
$$K_{eq} = \frac{V_{max_f} \cdot K_{m_p} \cdot \bar{K}_Q}{V_{max_b} \cdot K_{m_B} \cdot \bar{K}_A}$$

If $\bar{K}_Q$ and $\bar{K}_A$, the binding constants for NAD and NADH, were similar, then the ratio $\frac{K_{m_{OAA}}}{K_{m_{malate}}} \times \frac{V_{max_f}}{V_{max_b}}$ should approximate to K_{eq}

K_{eq} is in fact 10^{-4}. At pH 7.4, substituting values, the obtained K_{eq} was 1.3×10^{-2} (K_{m_p} = .26 mM $\bar{K}_{m_B}$ = 3.64mM V_{max_f} = 0.91 µm/min V_{max_b} = 4.7 µm/min).

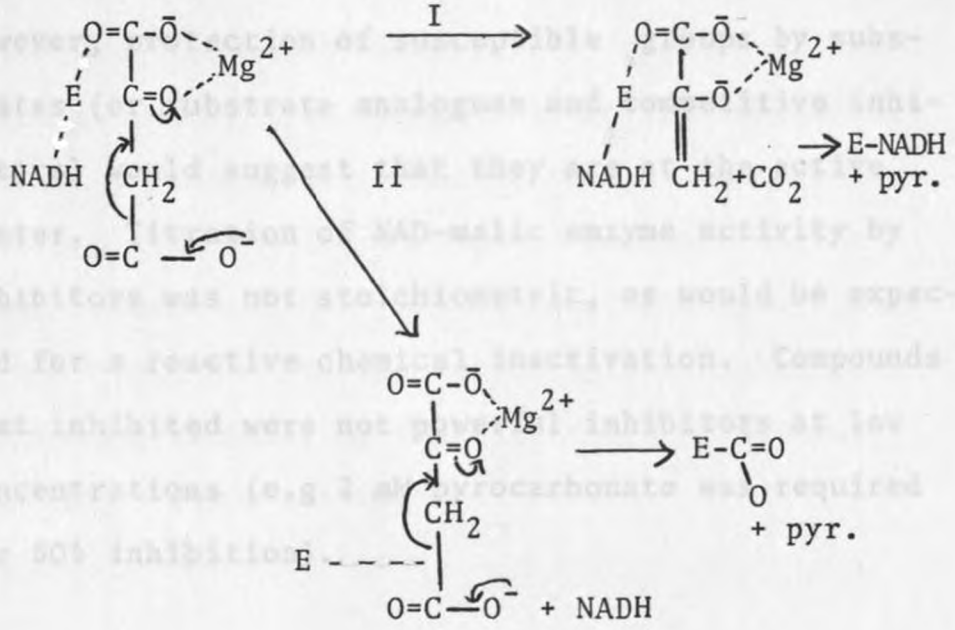
The enzymatic decarboxylation of OAA was dependent on Ca^{2+} activation. Ca^{2+} was however an inhibitor of malic enzyme activity. Presumably Ca^{2+} causes conformational changes on the enzyme molecule that are conducive to OAA binding and decarboxylation but unfavourable for malate oxidation.

The findings of the present study are consistent with a molecular mechanism in which malic enzyme reaction occurs in two steps, oxidation followed by decarboxylation. Probably the first step (oxidation), as in a non decarboxylating MDH, does not require metal ion.



However, OAA is not normally released as a product, but remains enzyme bound for the second step of the reaction. The second step (decarboxylation) requires metal ion which probably causes interactions as proposed by Dixon and Webb (1979) (although Schimerlik and Cleland 1977 have proposed a different mode of metal interaction). The metal ion could interact with OAA, weakening the bonds for decarboxylation as shown in Scheme 16.

SCHEME 16:



In scheme 16, route II fits product inhibition results (see Chapter IV), since it is CO_2 that is competitive and presumably remains bound at the malate site. Without NADH, malic enzyme can still bind OAA but in this case its decarboxylation appears to need heavier

divalent metal ions, i.e Ca^{2+} (or apparently Mn^{2+}), to confer proper orientation in the binary E-OAA complex. Probably Ca^{2+} changes the molecule so that malate binding is reduced since Ca^{2+} inhibited competitively with respect to malate.

Irreversible inhibitors have been much used to identify residues that are essential for enzyme activity. However, it has been pointed out by Dixon and Webb (1979) that inactivation could be due to chemical modification at specific groups on the enzyme surface and not necessarily at the active site. However, protection of susceptible groups by substrates (or substrate analogues and competitive inhibitors) would suggest that they are at the active center. Titration of NAD-malic enzyme activity by inhibitors was not stoichiometric, as would be expected for a reactive chemical inactivation. Compounds that inhibited were not powerful inhibitors at low concentrations (e.g 2 mM pyrocarbonate was required for 50% inhibition).

Both the Glossina and Catharsius malic enzymes were sensitive to sulfhydril reagents, suggesting that they have SH-groups which are important for activity. However, since substrates could only slightly relieve this inactivation, it seems unlikely that these SH-groups have a direct role in catalysis,

though they are presumably at positions greatly affecting the active site or active conformation of the enzyme.

Inactivation of malic enzyme by diethylpyrocarbonate (imidazole reagent) was greatly reduced by the presence of malate or fumarate. Some histidine residues could be at the active site, although not highly reactive towards the inhibitor.

Since Glossina malic enzyme activity was greatly affected by urea, it appears that the enzyme is inactive when quaternary structure is modified. The dissociation by urea appeared to be time dependent but was permanent in the sense that it was not reversed within a few minutes by exposure to the assay system. Either substrates or fumarate, gave marked protection to the enzyme against inhibition by urea, presumably by reducing the tendency for quaternary dissociation.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSION

7.0 The NAD-malic enzyme from Glossina was purified further than that from Catharsius. The former enzyme was deduced to be about 90% pure with a specific activity of 50 units/mg protein at 30°C. This compares favourably with the highest specific activities reported for the plant NAD-malic enzyme (Canellas et al 1983) and enzyme from Ascaris (Allen and Harris 1982) and mammalian tissue (e.g Nagel and Sauer 1982).

Enzymes from both insect sources were found to be tetrameric although the Catharsius enzyme appears to be slightly heavier with a native molecular weight of 250,000 daltons as compared to 237,000 daltons for the Glossina enzyme. These values agree with figures reported for enzyme from Ascaris (Fodge et al 1972) and rabbit muscle (Lin and Davis 1974). It may be that there are two forms of the enzyme, as two activity bands persist on native gels despite further purification, and two protein staining bands close in molecular weight persist on SDS-gels: Neither of the insect enzymes was observed to associate to higher levels, even in the presence of substrates, in the manner reported for certain plant enzymes (Grover and Wedding 1984, Davis and Patil

1975). The substrates were however able to protect the Glossina enzyme against urea inactivation, presumably by preventing quarternary dissociation. Malate has been reported to protect the plant enzyme from dissociating to a tetrameric or dimeric state from a more active, more highly polymerised form (Grover and Wedding 1984). As suggested in Chapter VI, these substrates, on binding probably create a conformation that is resistant to dissociation.

Some differences in protein characteristics between the two insect enzymes included the pI values (5.4 for Catharsius, 6.0 for Glossina enzyme), with the Catharsius enzyme thus being the more negatively charged. pI values in the same range have been obtained for other malic enzymes (Nagel and Sauer 1982, Fodge et al 1972).

Both enzymes had a requirement for metal ion, either Mg^{2+} or Mn^{2+}, as has been reported for all other malic enzymes investigated (e.g. Canellas et al 1983, Nagel and Sauer 1982, Landsperger et al 1978, Schimerlik and Cleland 1977). The two insect enzymes also had high K_m values for either substrate. Other NAD-malic enzymes have been reported to have K_m values in the range 1 - 3 mM for malate and 50 - 700 µM for NAD (Lin and Davis 1974, Landsperger et al 1978 It seems likely that this activity would commonly be

substrate limited in vivo. By contrast pigeon liver cytoplasmic NADP-malic enzyme had low K_m values for its two substrates (Hsu and Lardy 1967). The mitochondrial NADP malic enzymes however, tended to have high K_m values for malate as was found with the NAD-malic enzymes (Sauer et al 1980). The K_m values for NADP of the mitochondrial NADP malic enzymes was also slightly higher than with the cytoplasmic NADP malic enzyme (Davis and Patil 1974, Sauer et al 1980). With Mn^{2+} as activator, the K_m values of the Catharsius enzyme were greatly reduced but those of Glossina enzyme were not much changed. Mn^{2+} also tended to reduce K_m values for malate in some other malic enzymes (Lin and Davis 1974), as well as reducing V_{max} value (Grover et al 1981) although with Glossina and Catharsius enzymes replacing Mg^{2+} for Mn^{2+} had no effect on V_{max}. Mn^{2+} apparently induces a different conformation in both insect enzymes, however, for it tended to abolish co-operativity (see Chapter V). In some malic enzymes however, co-operativity still persisted in the presence of Mn^{2+} (Swierczynski et al 1980).

In the presence of Mg^{2+}, both insect enzymes were found to have regulatory properties. Both displayed positive co-operativity with respect to either substrate, this tendency being being more marked in the Catharsius enzyme case.

As with a number of other malic enzymes described, both insect enzymes were subject to activation by dicarboxylic acids such as fumarate (see also Davis and Patil 1975, Swierczynski et al 1980) succinate (see Sauer and Dauchy 1978, Mandella and Sauer 1975, Frenkel and Cobo-Frenkel 1973) and aspartate (see also Takeo et al 1967, Frenkel and Cobo-Frenkel 1973). This stimulation involved the removal of positive co-operativity and was only evident at low substrate concentration. None of the authors cited above reported a dual effect of these dicarboxylic acids, inhibitory at high concentration but stimulatory at low concentrations as was observed in the present study.

Other malic enzymes for which activation by dicarboxylic acids has been reported, showed regulatory properties, and were positively co-operative, and the nature of the activation would seem to be similar to that found in this study. For certain malic enzymes, inhibition has been described but without activation. Thus some authors found these dicarboxylic acids to be inhibitory but working at high malate concentrations or working with enzymes that were not co-operative, like in the case of the Colorado and Japanese malic enzymes, expected to be similar to Catharsius malic enzyme, were found to be inhibited, but this was using high malate concentra-

tion (Weeda et al 1980, Hansford and Johnson 1975).

In certain malic enzymes described by Lin and Davis (1974) and Mandella and Sauer (1975) no co-operativity or stimulation by fumarate and succinate was observed. Probably inhibition by fumarate or succinate at high malate concentration might have been observed but was not checked.

The Catharsius enzyme was more co-operative than the Glossina enzyme and needed a higher fumarate concentration to produce Michaelis kinetics. Co-operativity in these insect enzymes could also be abolished by a decrease in pH or (in the case of NAD-co-operativity) by an increase in the concentration of the second substrate.

As discussed in Chapter V, the co-operativity observed with these two insect enzymes, cannot be explained in terms of a simple MWC model. In particular, the Glossina enzyme displayed negative co-operativity, in the presence of high concentrations of dicarboxylic acids(Olembo (1980).

Malic enzymes studied so far have been reported to have various mechanisms of substrate addition, basing deductions mainly on primary velocity versus substrate concentration plots, and product inhibition

patterns. The much studied pigeon liver NADP-malic enzyme was found to follow an ordered mechanism with coenzyme binding before malate (Hsu et al 1967, Schimerlik and Cleland 1977), and this mechanism has also been deduced some other malic enzymes (Coleman and Palmer 1972, Sanwal and Smando 1969).

A random order of substrate addition has been proposed for the highly purified NAD-malic enzyme from Ascaris suum (Landsperger et al 1978), and for the cauliflower enzyme (Valenti and Pupillo 1981, Canellas et al 1983).

The Glossina enzyme, and probably also the Catharsius enzyme, would seem to follow a rapid equilibrium random order mechanism, in which binding of the first substrate facilitates binding of the second. The extent of this facilitation, seemed to depend greatly on pH. Al low pH, the mechanism appears to be completely random, so that apparent K_m values for malate were unaffected by NAD concentrations and vice versa, and K_A $\bar{K}_A$ and all K_m values were the same. (see Table 10).

There could be slight differences between the two enzymes as regards the order of product release, for pyruvate slightly inhibited the Glossina enzyme but had no detectable effect on the Catharsius

enzyme. Also, NADH, a competitive inhibitor with respect to NAD with the Glossina enzyme, was a mixed inhibitor in the case of the Catharsius enzyme. The fact that double reciprocal plots for both substrates intersected behind the Y-axis; and no parallel patterns were observed with any of the products as inhibitors, points to a random rather than to an ordered mechanism for both insect enzymes.

From pH studies with the Glossina enzyme, several pK values were indicated. These were in the range 6.7 - 8.2 and could therefore correspond with the ionisation of vital sulfhydril and histidine groups. The importance of these groups in the maintainance and functioning of the active centre was also indicated using specific inhibitors. It could be therefore that both these functional groups are important in substrate binding and/or catalysis.

The existence of OAA decarboxylase and OAA reductase activities associated with the malic enzyme protein, as well as the inhibitory effect of OAA on malic enzyme activity, would support a molecular mechanism in which OAA is an enzyme bound reaction intermediate. Such a mechanism has been proposed for the pigeon liver NADP-malic enzyme (Hsu 1970, Garrison and Cleland 1985). A similar molecular mechanism was also proposed for the analogous NADP-

isocitrate dehydrogenase in which oxalosuccinate was identified as an enzyme bound intermediate (Siebert et al 1957) on the basis of decarboxylation and reduction activities corresponding to those exhibited by malic enzyme.

With insect malic enzyme therefore, it is likely that oxidation occurs before decarboxylation. Metal ion appears to be necessary for the second stage of the reaction but not for the first. OAA reductase works without metal ion and low activities of metal independent OAA producing MDH was exhibited by the enzyme.

If the molecular mechanism involved decarboxylation before oxidation, with a bound lactate intermediate, it seems unlikely that CO_2 would remain bound to the enzyme during the second stage of reaction, and highly unlikely in this case that it would be CO_2 occupying the malate site (Lactate in this case occupying a different site for oxidation). Yet product inhibition by CO_2 was competitive with respect to malate, suggesting that it could be released last from the malate site.

This alternative mechanism with lactate as an enzyme bound intermediate, is unlikely, in any case as no LDH activity could be demonstrated with lactate

and NAD as substrates. Also lactate itself had no inhibitory effects on malic enzyme activity. A very weak LDH activity was demonstrated with the cytoplasmic NADP-malic enzyme (Hsu et al 1967, Hsu 1970) but after detailed investigation, Hermes et al (1982) and Grissom and Cleland (1985) proved that this enzyme has a molecular mechanism involving OAA as an enzyme bound intermediate.

The maximum OAA reductase and OAA decarboxylase activities obtained with the Glossina malic enzyme, were less than 10% of the malic enzyme activity, and moreover K_m values of these activities for OAA were high (above 0.1 mM and 1 mM respectively) higher than typical estimates for concentration of OAA in the cell. The contribution of these side activities in vivo to the synthesis of malate and pyruvate is probably therefore negligible. In the case of the decarboxylase, the optimal pH was far below physiological.

Various physiological roles have been proposed for NAD-malic enzymes (e.g Coleman and Palmer 1972, Chapman and Hatch 1977, Saz and Hubbard 1957, Sauer et al 1980, Swierczynski et al 1980, Hoek et al 1976). It is assumed that the insect malic enzymes studied here are important in the metabolism of proline as proposed by Hoek et al (1976) and Weeda et al 1980

(see Scheme 7). Proline was found to be the most effective substrate supporting respiration in mitochondria from the flight muscle in these two insects (Pearson et al 1979, Bursell and Slack 1976). Thus malic enzyme would compete with MDH for the same mitochondrial substrate, malate. Under optimal conditions, the ratio of MDH activity to malic enzyme activity was about 1:1, both enzymes being very active. K_m values for malate as indicated in Glossina in the present study (with 0.76 mM NAD) were about 3.6 mM for MDH and about 1.2 mM for malic enzyme.

Whereas in the mitochondria in vivo it is likely that MDH might be near equilibrium and opposed by a back reaction, malic enzyme would unlikely be opposed by a substantial back reaction(Olembo 1980). Also , malic enzyme would presumably be more saturated with malate than MDH. Most malate would probably therefore be channelled through the malic enzyme pathway rather than through the MDH route. This might be accentuated by the tendency of pyruvate, a product of malic enzyme activity, to inhibit MDH more strongly than malic enzyme itself.

In view of its complex kinetic properties and the susceptibility to various metabolites, malic enzyme may well be an important regulatory enzyme in the proline oxidation pathway in which pyruvate has

to be generated continously. The activity of the enzyme assayed in optimal conditions is comparable to that of other enzymes in Scheme 7 but since it may be substrate limited, it might still be rate limiting in the proline oxidation pathway and possibly inhibited by cellular concentrations of ATP (Norden and Matanganyidze 1977, Pearson et al 1979, Olembo 1980).

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