GLUTAMATE DEHYDROGENASE FROM THE EXTREMELY THERMOPHILIC ARCHAEBACTERIAL ISOLATE AN1

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PREFACE

This thesis consists of three papers prepared for publication, a series of appendices and a final discussion. Chapter 1, the literature review, has been submitted as a review to Comparative Biochemistry and Physiology, Chapter 2 (a full-length research paper) has been accepted by Biochimica et Biophysica Acta, and Chapter 3 (a full-length research paper) will be submitted to The Journal of Biological Chemistry. These chapters are presented in the same form as that in which they were submitted to the journals, hence there are some differences in format between Chapters 1, 2 and 3. There is also some minor overlap between these chapters, as each is intended to be complete in itself. Some peripheral data which would normally be found in the experimental sections of a thesis, but which is not suitable for inclusion in publications due to length restrictions, has instead been included in appendices. Appendix 1 gives data which pertains to Chapter 2. Appendix 2 describes the crystallography work performed by Dr Gill Norris of Massey University, plus some preliminary crystallisation work performed by R.C.H.

The aim of this project was to study a glutamate dehydrogenase from a thermophilic archaebacterium. The glutamate dehydrogenase from the extremely thermophilic archaebacterial isolate AN1 was chosen when it was found to be present in large amounts in this organism. AN1 is considered a somewhat "primitive" organism (in the sense that it phylogenetic position is near the root of the archaebacterial tree). Although glutamate dehydrogenases have been characterised from many species of eukaryotes and eubacteria, at the time that this study began, the only archaebacterial glutamate dehydrogenases which had been studied were from halophiles. Thus this study extends the field of comparative enzymology (reviewed by Danson, 1988 and 1989) to glutamate dehydrogenases, key enzymes because they form a link between carbon and nitrogen metabolism. Another aim of the project was to carry out a steady state kinetic study with the glutamate dehydrogenase from AN1, something previously only performed with glutamate dehydrogenases from eukaryotes and eubacteria.

ABSTRACT

An NADP-specific glutamate dehydrogenase (E.C. 1.4.1.4) constituted approximately 10% of the soluble cell protein of the extremely thermophilic archaebacterial isolate AN1. This enzyme was purified to homogeneity in high yield, using column chromatography on Reactive Red-Sepharose CL-6B and hydroxyapatite.

The glutamate dehydrogenase was a tetramer of Mr 204 000. It was highly thermostable with half-life values of 12.5 h and 47 min at 90°C and 103°C, respectively. The pI was 4.0 and the pH optimum was 8.25 for both oxidative deamination and reductive amination reactions. Activity in the oxidative deamination direction was markedly enhanced by calcium, magnesium and manganese ions. Glutamate synthase activity was about 50% of the glutamate dehydrogenase activity. The thermostability and pH optimum of the glutamate synthase activity was the same as for the glutamate dehydrogenase activity. Apparent Km determinations at 40, 60, and 80°C revealed increases in apparent Km's with increasing temperature.

The kinetic mechanism of the forward and reverse reactions of the glutamate dehydrogenase was investigated using initial velocity studies and inhibition studies with products and substrate analogues. Many of the product inhibitions were of a nonlinear (parabolic) nature. The results of the experiments were consistent with a steady state random mechanism but not with an ordered mechanism or a rapid-equilibrium random mechanism. The results also indicated that there was a preference for the cofactors to bind the enzyme first (both reaction directions), and for 2-ketoglutarate to bind the enzyme second (reductive amination reaction). The true Km values (mM) for $NADP^+$, glutamate, NADPH, 2-ketoglutarate and ammonia were 0.086, 2.0, 0.015, 0.71 and 10.7, respectively. The true K_i (dissociation constant) values (mM) were 0.054, 1.27, 0.018, 1.79 and 8.03 for $NADP^+$, glutamate, NADPH, 2-ketoglutarate and ammonia, respectively.

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LIST OF ABBREVIATIONS

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ADP	Adenosine 5'-diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$[Ca]_{0.5}$	Concentration of calcium giving half maximal activation
CHES	2-(Cyclohexylamino)-1-ethane sulphonic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	[(Ethylenedioxy)diethylenedinitrilo]tetra-acetic acid
EPPS	*N*-(2-Hydroxyethyl)piperazine-*N'*-3-propanesulphonic acid
GDH	Glutamate dehydrogenase
GTP	Guanosine triphosphate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HPLC	High performance liquid chromatography
MPD	Methylpentenediol
MOPS	3-(*N*-Morpholino)propanesulphonic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
PEG	Polyethyleneglycol
PEP	Phosphoenolpyruvate
PAGE	Polyacrylamide-gel electrophoresis
P_{FR}	Phytochrome (far red), the active form of plant phytochrome
SDS	sodium dodecyl sulphate
$t_{1/2}$	Half life
TCA cycle	Tricarboxylic acid cycle

T_m	"Melting temperature" in the literal sense. Refers to a temperature at which a protein undergoes a major conformational change, such as irreversible denaturation.
Tris	Tris[hydroxymethyl]-aminomethane

CHAPTER 1

L-GLUTAMATE DEHYDROGENASES:

Distribution, Properties and Mechanism

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1. Assimilation of Nitrogen

The bulk of the nitrogen in the biosphere is in the form of nitrogen gas (N_2), which is the most stable form of the element, but a form which few organisms can assimilate into organic compounds. Ammonia and nitrate are the two most common sources of inorganic nitrogen. Nitrite, hydroxylamine, cyanide, cyanate, thiocyanate and cyanamide are other sources which are used by some microorganisms. Many number of organisms, including all vertebrates and some microorganisms, require an organic nitrogen source, i.e. proteins or amino acids synthesized by other organisms.

For a general summary of the pathways of nitrogen assimilation see Brock *et al.* (1984).

2. Pathways of Ammonia Assimilation

There are two major routes for the assimilation of ammonia into amino acids: the glutamate dehydrogenase reaction (reaction [1]) and the sequential reactions catalysed by glutamine synthetase and glutamate synthase (reactions [2] and [3], respectively).

[1] 2-ketoglutarate + NH_3 + NAD(P)H ---------> glutamate + $NAD(P)^+$

[2] glutamate + NH_3 + ATP ---------> glutamine + ADP +P_i

[3] glutamine + 2-ketoglutarate +NAD(P)H ---------> 2 glutamate + $NAD(P)^+$

The net result of reactions [2] and [3] is essentially the same as that of reaction [1]; both routes produce one molecule of glutamate and use the reducing potential of one molecule of reduced nicotinamide dinucleotide. The difference is that phosphate bond energy in the form of ATP is required for the glutamine synthetase/glutamate synthase route. In organisms which have both routes, ammonia will generally be assimilated via the glutamate dehydrogenase (GDH) reaction when its external concentration is high, and via the glutamine synthetase/glutamate synthase route when it is scarce. The latter route is known as the "high affinity" pathway, since the enzyme glutamine synthetase has an exceptionally low Km for ammonia, considerably lower than that of GDH. The disadvantage of this route is its extra energy requirement.

Glutamate, the amino acid product of the two pathways above, is an important central metabolite and several other amino acids may be synthesized directly from it via the action of transaminases (these enzymes catalyse the transfer of an α -amino group from an α -amino acid to the α -carbon atom of an α -keto acid). Most transaminases are substrate specific for a glutamate/2-ketoglutarate substrate pair.

Ammonia may also be assimilated via the action of amino acid dehydrogenases which catalyse reactions analogous to the GDH reaction, but are specific for other keto acid/amino acid substrate pairs (e.g.. alanine dehydrogenase catalyses the reductive amination of pyruvate to alanine). However, in most species, glutamate is the only amino acid with a corresponding highly active dehydrogenase.

3. Dissimilation of Ammonia

Glutamate dehydrogenase is an important enzyme in the context of the formation of nitrogenous excretory products (dissimilation of ammonia) as well as in the recycling of nitrogen within the organism. The GDH reaction [1], unlike the reactions catalysed by glutamine synthetase [2] and glutamate synthase [3], is reversible, although the equilibrium of the reaction lies strongly in the direction of glutamate formation.

The majority of terrestrial vertebrates excrete nitrogen in the form of urea. GDH has an important function in feeding amino groups into the urea cycle (which operates in

the livers of these animals). One amino group enters the cycle as free ammonia, following the GDH-catalysed oxidative deamination of glutamate in the mitochondria, while the other enters in the form of aspartate (formed via transamination from glutamate). Free ammonia is highly toxic to these ureotelic vertebrates, chiefly because its presence would result in depletion of 2-ketoglutarate from the TCA cycle (due to GDH-catalysed reductive amination), leading to inhibition of brain respiration and excess ketone body formation from acetyl-CoA in the liver. For this reason GDH and some of the other enzymes which feed amino groups into the urea cycle are localised in the mitochondria.

Ammonotelic organisms, which include aquatic vertebrates, excrete the ammonia produced by the action of GDH directly into the environment.

In order for amino acids to be catabolised as energy sources, their amino groups must be removed. The amino acids arginine, glutamine, histidine and proline are converted by a series of reactions into glutamate, and their carbon skeletons enter the TCA cycle as 2-ketoglutarate, formed by the action of GDH. GDH therefore may serve an anaplerotic role in replenishing the TCA cycle with intermediates.

Amino acid oxidases play a minor role in oxidative deamination (see Lehninger, 1975 for a description). In skeletal muscle, which contains negligible GDH, deamination occurs by way of the purine nucleotide cycle. The net result of this cycle (catalysed by the enzymes adenylate deaminase, adenylosuccinate synthetase and adenylosuccinate lyase) is shown in reaction [4] (Lehninger (1975) details the individual enzymic reactions of the cycle).

[4] Aspartate + GTP + H_2O ---------> Fumarate + NH_3 + GDP +P_i

Note that the TCA cycle intermediate fumarate is produced, thus the purine nucleotide cycle fulfils an anaplerotic function in a tissue which does not have the capacity for other reactions of this type.

Some organisms are known to catabolise glutamate via a route which does not involve GDH. The amino group of glutamate may be transferred to oxaloacetate by transamination, thus forming aspartate and 2-ketoglutarate. The 2-ketoglutarate enters the

TCA cycle, while the aspartate is deaminated by aspartase to form fumarate, which also enters the TCA cycle. *Escherichia coli* and *Salmonella typhimurium* use such a mechanism (Miyamoto and Katsuki, 1992).

4. Glutamate Dehydrogenases

Glutamate dehydrogenases (E.C. 1.4.1.2-4) catalyse the reversible oxidative deamination of L-glutamate to 2-ketoglutarate and ammonia:

[5] glutamate + $NADP^+$ ---------> 2-ketoglutarate + NH_3+NADPH

This is the same as reaction [1], above, but written in reverse.

GDH's are widely distributed throughout the eukaryotic, eubacterial and archaebacterial kingdoms, with few organisms being known to lack them. These enzymes are of major significance because they occupy a pivotal position between carbon and nitrogen metabolism, and extensive research on GDH's from a wide range of species has been carried out. There are three basic types of GDH: those that are cofactor specific for NAD (E.C. 1.4.1.2), those that are specific for NADP (E.C. 1.4.1.4) and those which can use either cofactor (E.C. 1.4.1.3) (dual coenzyme specific GDH's).

Most GDH's are homopolymers consisting of two to six subunits of molecular weight 40 000 to 60 000 (fungal NAD-specific GDH's are an exception). The most common number of subunits is six. In general GDH's catalyse the oxidative deamination of glutamate optimally at alkaline pH (8-10), while the pH optimum for reductive amination will generally be 0.5 to 2 pH units lower.

In microorganisms, the biosynthetic (anabolic) or ammonia assimilating role of GDH (reductive amination) is usually carried out by NADP-GDH's, while oxidative deamination (the catabolic reaction direction) is usually catalysed by NAD-specific GDH's. There is much evidence to support that this is the case, especially in the form of induction/repression data. A number of examples of this type are given by Goldin and Frieden (1971). It is possible that the dual specific enzymes of animal cells usually carry

out oxidative deamination using NAD and glutamate synthesis using the phosphorylated cofactor, but due to the complex biochemical situation in these cells and to the complexity of their GDH's, no generalisation as to their metabolic role can be made. Even for microorganisms, there are some exceptions to the general rule that NAD^+ is used to oxidise glutamate and NADPH to reduce 2-ketoglutarate.

The NAD-specific GDH's of some fungi belonging to the Phycomycetes are one exception. A number of Phycomycetes from the taxonomic subclass Oomycetes (which produce only NAD-specific GDH) were studied by Le John and Stevenson (1970), and complex regulatory effects were discovered. GDH was present only in low levels in cells grown on glucose, but was induced by glutamate; the NADP-specific isocitrate dehydrogenase of these organisms responded in the opposite manner. The reductive amination reaction catalysed by GDH is subject to activation (by compounds which include $NADP^+$, NADPH, ATP and PEP) and inhibition (by citrate and isocitrate), as well as allosteric inhibition by the substrates. Le John *et al* (1970) have proposed that the NAD-specific GDH enzymes catalyse reductive amination when there is an abundance of high energy compounds such as ATP and PEP in the cells, and that GDH couples with isocitrate dehydrogenase to form a transhydrogenase system (see below).

[6] Isocitrate $+NADP^+$ ---------> 2-ketoglutarate + NADPH + CO_2

[7] 2-ketoglutarate + NH_3 + NADH ---------> Glutamate + NAD^+

NET: Isocitrate $+NH_3$ $+NADP^+$ +NADH ---------> Glutamate +NADPH + NAD^+ + CO_2

Hence it seems that these fungi have evolved a GDH with complex regulatory properties, such that the one enzyme may perform both a catabolic and a biosynthetic role without having dual coenzyme specificity.

The agent of avian malaria, the intracellular parasite *Plasmodium lophurae*, has an NADP-specific GDH which appears to have an *in vivo* role in glutamate oxidation, in addition to ammonium assimilation. This organism is capable of net CO_2-fixation, and produces glutamate as one of the products of this. The initial products of ^{14}C-glutamate

metabolism were found to be 2-ketoglutarate (mostly) and other TCA cycle intermediates, while the final product is principally CO_2 (Sherman *et al.*, 1971). These authors suggest that the catabolism of glutamate via GDH followed by the TCA cycle may provide a source of energy for this organism which supplements that produced by glycolysis. Turner and Lushbaugh (1988) proposed that in *Trichomonas vaginalis*, another protozoan parasite, the NADP-GDH catalyses predominantly the deamination reaction *in vivo*.

4.1 Eukaryotic Glutamate Dehydrogenases

4.1.1 Glutamate Dehydrogenases from Vertebrates

The GDH's of vertebrates are dual coenzyme-specific. They have complex regulatory effects mediated by purine nucleoside phosphates and a variety of other metabolic intermediates, including hormones. Their *in vivo* reaction direction may be dependent on the biochemical status of the cell, or on the tissue where they are found. Intracellular compartmentation of nicotinamide cofactors, and of the GDH enzymes themselves, is a feature which further controls their reaction direction, and the destination of their end products. In vertebrate cells, GDH appears to be localised primarily in the mitochondrial matrix (Smith *et al.*, 1975; Goldin and Frieden, 1971), but Colon *et al.* (1986) have isolated both a soluble (mitochondrial matrix) and a particulate (membrane bound) GDH from rat brain; the properties of these enzymes were significantly different (with respect to their thermal stability and susceptibility to GTP inhibition), which suggests that they play different roles in this tissue. In vertebrates, GDH has a significant role in controlling levels of ammonia and glutamate, the latter being important as a neurotransmitter (Shoemaker and Haley, 1993).

The GDH from bovine liver is undoubtedly the most extensively studied GDH. Its molecular weight is 332,000, and it consists of six subunits (Smith *et al.*, 1975). The enzyme molecules are known to undergo a polymerisation process, in which the enzyme hexamers aggregate to form a high molecular weight species. The polymerisation is promoted by a high concentration of enzyme in solution and by high ionic strength

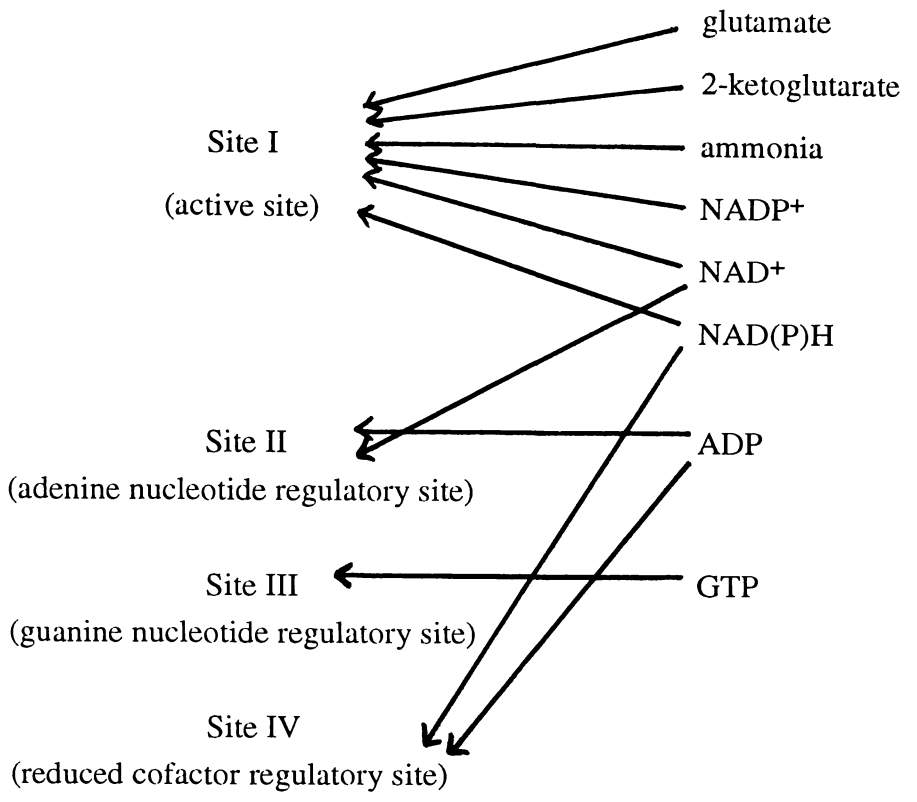
(Bitensky *et al.*, 1965). Allosteric modifiers, such as purine nucleoside phosphates and L-leucine, have also been said to influence the polymerisation equilibrium (Bitensky *et al.*, 1965), although Goldin and Frieden (1971) dispute this. The polymerisation process may perhaps be an *in vivo* control mechanism, since the concentration of GDH in some tissues is high enough (>2 mg/ml in some cases) to promote polymerisation, while in other tissues (such as skeletal muscle) it is so low that it is unlikely that any polymerisation occurs.

The effects of nucleosides on bovine liver GDH are complex. The review by Goldin and Frieden (1971) gives a detailed account of their effect on catalytic activity, enzyme polymerisation and the competition between nucleosides for enzyme binding sites. In summary, guanosine and inosine nucleotides inhibit the enzyme, while ADP and AMP activate it. The effect of ATP is complex: it has little effect on the rate of NADPH oxidation in the absence of other nucleoside phosphates (although it does bind to the enzyme), but will antagonise both the inhibition by GTP and the activation by ADP (therefore it is an activator in the former case and an inhibitor in the latter). NADH, when present in high levels, causes the substrate inhibition of its own oxidation; under these conditions, ATP itself inhibits the reaction.

A variety of techniques (fluorescence spectroscopy, circular dichroism, transfer nuclear overhauser effects and direct binding studies) have been used to show that the bovine liver GDH has at least four binding sites per subunit: the active site (site I), the adenine nucleotide regulatory site (site II), the guanine nucleotide regulatory site (site III) and the reduced coenzyme regulatory site (which will be called site IV here) (Bayley and O'Neill, 1980; Banerjee *et al.*, 1987; Lark and Colman, 1990). NAD^+ binds to site II as well as to site I, while $NADP^+$ binds effectively only to site I (Banerjee *et al.*, 1987). The presence of a dicarboxylate coligand (such as 2-ketoglutarate or the glutamate analogue glutarate) increases the affinity of cofactor binding to site I but not site II. The transfer nuclear overhauser effect study by Banerjee *et al.* (1987) indicated that NAD^+ binds to sites I and II with equal affinity in the absence of dicarboxylate coligands, but Bayley and O'Neill (1980), using circular dichroism difference spectroscopy, found that NAD^+ preferentially to binds site II in the absence of dicarboxylates. The latter finding correlates with the observation by Chen and Engel (1974) that NAD^+ does not protect the enzyme

against inactivation by pyridoxal 5'-phosphate unless a dicarboxylate is also present. When a dicarboxylate and high levels of NAD^+ are present together, NAD^+ binds site II as well as site I, with the site II binding causing a decrease in the coenzyme affinity at the active site (similar to the effect of ADP) which is seen as substrate activation (Bayley and O'Neill, 1980). The transfer nuclear overhauser effect study of Banerjee *et al.* (1987) provided evidence, which was supported by the fluorescence stopped-flow studies of the binding of a fluorescent NAD^+ analogue by Favilla *et al.* (1988), that sites I and II of each subunit are close together. ADP, as well as NAD(P)H, can bind the reduced coenzyme regulatory site (site IV), which has also been found to be close to the active site (Lark and Colman, 1990). Recently, Shoemaker and Haley (1993) identified the guanine nucleotide binding site within bovine liver GDH using the GTP photoaffinity analogues [α-^{32}P]8N_3GTP and [γ-^{32}P]8N_3GTP. This study detected only one GTP binding site per subunit (NADH-independent), despite an earlier report (Pal and Colman, 1979) that there are two (one NADH-dependent and one NADH-independent). Figure 1 is a diagrammatic representation of the substrate and regulatory binding sites of the bovine liver GDH; the distances between the sites are those given by Lark and Colman (1990) and Jacobson and Colman (1983 and 1984). Lark and Colman point out that there is no evidence to rule out the possibility that regulatory sites on one subunit may modify the catalytic site on an adjacent subunit.

A



B

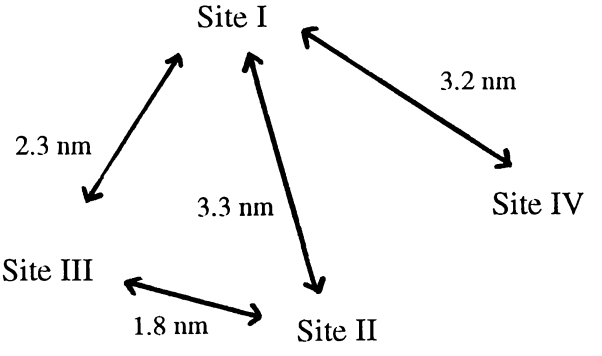


Fig. 1. Schematic representation of the catalytic and regulatory sites of the bovine liver glutamate dehydrogenase. Fig 1**A** shows which substrates and effectors bind particular sites. Fig 1**B** shows the distances between those sites for which they are known. The distances are those given by Lark and Colman (1990) and Jacobson and Colman (1983 and 1984). This diagram is not intended to imply any particular orientation of sites.

The hormones diethylstilbestrol, estradiol, progesterone, Δ^4-androstene-3,17-dione, testosterone and thyroxine inhibit bovine GDH activity, while some L-amino acids (including leucine, isoleucine and methionine) stimulate it (Goldin and Frieden, 1971).

Bovine liver GDH can catalyse the oxidative deamination of a number of L-amino acids other than glutamate, and also the reductive amination of a number of 2-keto acids other than 2-ketoglutarate. However, at physiological pH values (~7.4) the activity with these alternative substrates is relatively low, since the pH optima for activity with these substrates is higher than that for glutamate (pH 8.5-9.0) or 2-ketoglutarate (pH 7.8) (Smith *et al.*, 1975). L-Norvaline and L-alanine are among the amino acids that GDH has activity against. It is interesting that some effectors, for example GTP or steroid hormones, increase the ratio of alanine dehydrogenase to glutamate dehydrogenase activity (Bitensky *et al.*, 1965). Glutamine may replace ammonia as the nitrogen donor for bovine GDH, i.e. it has glutamate synthase activity (~40% of its GDH activity) (Smith *et al.*, 1975).

The GDH's from other vertebrates are similar in some respects to the bovine liver enzyme. Although they are all dual coenzyme-specific, some GDH's show a preference for a particular cofactor; for example, the frog and dogfish liver enzymes are more active with the non-phosphorylated cofactor (Fahien *et al.*, 1965; Electricwala and Dickinson, 1979), whereas many mammalian GDH's have similar activities with either cofactor (Smith *et al.*, 1975). In all known cases, vertebrate GDH's are hexameric. The rat, chicken, tuna and dogfish liver enzymes have molecular weights (see Table 1) which are not dissimilar to that of the bovine enzyme, while the frog liver enzyme is smaller, having a molecular weight of only 250,000. The reported molecular weight for a given enzyme is often variable, and this is probably a reflection of the difficulty encountered in determining the true molecular weights of enzymes which aggregate. The human, pig, chicken and frog liver enzymes are all known to associate, although the latter two do this to a lesser extent than the bovine liver enzyme (Smith *et al.*, 1975). The enzymes from rat kidney, and from rat, tuna and dogfish liver do not aggregate (Smith *et al.*, 1975). Most vertebrate GDH's are, like the bovine liver enzyme, inhibited by GTP and activated by ADP, but there are differences in the way these enzymes behave towards the purine nucleoside phosphates.

Table 1 gives the molecular weights of GDH's from some vertebrates and other eukaryotes.

4.1.2. Glutamate Dehydrogenases from Non-Vertebrate Animals

NADP-GDH's have been isolated from several species of coelenterates, including the staghorn coral *Acropora formosa* (Catmull *et al.*, 1987) and the sea anemone *Anthopleura xanthogrammica* (Male and Storey, 1983). These two enzymes, like the GDH's from other coelenterates, differ from the GDH's of other animals (i.e. vertebrates) in that they are mono-coenzyme specific and are not regulated by nucleotides (Hoffman *et al.*, 1978). The *An. xanthogrammica* enzyme uses glutamine as an alternative amino group donor (i.e. it has glutamate synthase activity), this activity being 50% of glutamate dehydrogenase activity at pH 8 (Male and Storey, 1983). The *Ac. formosa* enzyme does not have glutamate synthase activity (Catmull *et al.*, 1987). Male and Storey (1983) found that the *An. xanthogrammica* GDH was activated in the reductive amination direction by sodium and potassium salts, suggesting that the enzyme may have an important role in amino acid biosynthesis during hyperosmotic stress. Both the above enzymes are hexameric (Male and Storey, 1983; Catmull *et al.*, 1987). The molecular weights of these enzymes are included in Table 1.

4.1.3. Glutamate Dehydrogenases from Higher Plants

GDH is ubiquitous in higher plant tissues, where it is often found in high concentrations. A number of isozymes are usually present in a single species. Some isozymes are inducible, hence the isozyme pattern may vary with nutritional and environmental conditions (Srivastava and Singh, 1987). Plant GDH's generally have molecular weights in the range 208 000 - 270 000, and are composed of four or six subunits. The enzyme from pea (*Pisum sativum*) seeds formed six electrophoretic bands after crosslinking with diimidates (indicating a hexameric structure) (Kindt *et al.*, 1980),

but SDS electrophoresis and EM studies showed it to be tetrameric (Srivastava and Singh, 1987).

It is thought that the assimilation of nitrogen into amino acids in higher plant cells is achieved mainly via the concerted action of glutamine synthetase and glutamate synthase (reactions 2 and 3) since the concentration of ammonia in plant cells is normally quite low under physiological conditions (Bray, 1983). Glutamine synthetase has a much higher affinity for ammonia than GDH (the apparent Km values for ammonia are in the millimolar range for GDH's from most sources, whereas they are in the micromolar range for glutamine synthetases). However, Yamaya *et al.* (1984) proposed that NAD-specific GDH has a significant role in ammonium assimilation in corn shoot mitochondria, and that furthermore, the enzyme may provide an additional source of glutamate to sustain the glutamine synthetase reaction - consistent with the enhanced rate of protein synthesis in light treated leaves. They simulated the *in vivo* conditions of mitochondria with respect to GDH substrates and Ca^{2+} and found that the equilibrium of the reaction lay in the direction of glutamate formation.

GDH is thought to be an important enzyme in the assimilation of ammonia when there is a high concentration of ammonia in the plant's environment, or under stress conditions such as dark starvation, high temperature, salinity, water stress, environmental pollution and senescence since GDH activity increases under such conditions (Givan, 1979; Srivastava and Singh, 1987). An excess of ammonia is toxic to plants. When there is a large exogenous source of ammonia, GDH may supplement the glutamine synthetase/glutamate synthase pathway in assimilating it into organic compounds (thus detoxifying it) (Givan, 1979). GDH probably also has an important role in detoxifying the ammonia produced from amino acid breakdown during senescence, when there is a rapid decrease in glutamine synthetase and glutamate synthase (Srivastava and Singh, 1987). Glutamate synthase becomes uncoupled from glutamine synthetase during higher water stress; under these conditions GDH is activated and may provide glutamate for the glutamine synthetase reaction. GDH is a more stable enzyme than the glutamine synthetase/glutamate synthase pathway enzymes under stress conditions (e.g. it is more

thermostable than glutamine synthetase), and may play a significant role in maintaining plant metabolism under such conditions (Srivastava and Singh, 1987).

Multiple forms of GDH from higher plants can use both NAD(H) and NADP(H). However, in most cases NAD(H) is preferred (Srivastava and Singh, 1987). Rice plants have been reported to contain both a mitochondrial and a cytosolic NAD-specific GDH, as well as an inducible dual specific enzyme (Kanamori *et al.*, 1972). Plant NAD-specific GDH has generally been considered to be a mitochondrial matrix (soluble) enzyme, but Yamaya *et al.* (1984) found that the majority of the NAD-specific GDH of corn shoot mitochondria was associated with the mitochondrial membrane fraction when the organelles were disrupted by a gentle method. They concluded that the NAD-specific GDH was loosely associated with the mitochondrial membrane. A chloroplast GDH (NADP-linked), distinct from mitochondrial GDH, has been isolated from many species (Srivastava and Singh, 1987; Stewart *et al.*, 1980).

GDH's from plants are thought to be metalloenzymes, and the influence of divalent metal cations on plant GDH's is well documented (Srivastava and Singh, 1987; Itagaki *et al.*, 1990; Kindt *et al.*, 1980; Yamaya *et al.*, 1984; Das *et al.*, 1989). For example, Kindt *et al.* (1980) found that NAD-linked GDH (isozyme number 7) from pea seeds (*Pisum sativum*) is almost inactive in the absence of divalent metal ions, calcium ions being the most effective activator ($[Ca^{2+}]_{1/2}$ = 5 μM). This is in contrast to the earlier finding of Joy (1973) that zinc ions activate the enzyme from *Pisum sativum* roots at lower concentrations than calcium ions, although higher concentrations of zinc (1.5 mM or above) cause inhibition. Garland and Dennis (1977) also found that low concentrations of zinc ions activate the GDH from pea stem mitochondria (NAD-linked), but like Kindt *et al.*, found calcium to be the most effective activator. Calcium ions not only activate the NAD-specific GDH from turnip (*Brassica rapa* L.) roots, they also reverse substrate inhibition by ammonium (Itagaki *et al.*, 1990).

There has been some controversy as to whether plant GDH's require metal ions for activity only in the reductive amination of 2-ketoglutarate by NADH, or whether they require them in order to display any significant activity. For the GDH's from pea root and stem mitochondria, it has been reported that only the NADH-dependent amination of 2-

ketoglutarate is activated by divalent metal ions, with NAD^+-dependent deamination (and also NADPH-dependent amination in the former case) reported to be not effected or slightly inhibited by metal ions (Joy, 1973; Garland and Dennis, 1977). However, Kindt *et al.* (1980), who used a chelating resin to ensure that assay solutions for metal ion experiments were virtually free of divalent metal ions, found that NAD-linked GDH (isozyme 7) from pea seeds was essentially inactive unless divalent metals ions, or alternatively high concentrations of sodium ions, were present. The NAD(H)-dependent (but not NADPH-dependent) activity of GDH from *Lemna minor* (duckweed) is abolished by the addition of EDTA; calcium or magnesium ions restore the NAD^+-dependent activity, but NADH-dependent activity is restored only by calcium ions (Ehmke and Hartmann, 1976). The turnip root NAD-linked GDH is known to require greater levels of calcium for full activation in the reductive amination reaction (60 μM) than in the oxidative deamination reaction (7-8 μM), which suggests that calcium may have a role in determining the *in vivo* direction of the reaction (Itagaki *et al.*,1988).

The NAD-specific GDH from maize (*Zea mays*) was activated by calcium ions, and this effect was found to be mediated indirectly through calmodulin (Das *et al.*,1989). These workers also found a marked increase in the GDH activity of 5 day old plants which had been exposed to red light; this effect was photoreversible, showing that P_{FR} (the active form of plant phytochrome) exerts a regulatory effect on GDH *in vivo*.

Table 1 shows the molecular weights of some plant GDH's. More extensive information is contained in the review by Srivastava and Singh (1987).

4.1.4. Glutamate Dehydrogenases from Eukaryotic Microorganisms

4.1.4.1. Fungal Glutamate Dehydrogenases

In fungi, primary ammonium assimilation is mainly achieved by the successive activities of NADP-specific GDH and glutamine synthetase (Brun *et al.*, 1992). Fungal GDH's are usually soluble cytoplasmic enzymes (Smith *et al.*, 1975). The higher fungi of the classes Deuteromyces, Ascomycetes and Basidiomycetes produce both an NAD- and

an NADP-specific GDH, while the lower fungi of the Phycomycetes and Myxomycetes have only an NAD-specific GDH (Le John, 1971). *Dictyostelium discoidium* has a cytoplasmic NAD-specific GDH plus a dual coenzyme specific mitochondrial enzyme (Smith *et al.*, 1975). Smith *et al.* suggest that mitochondrial GDH's evolved as dual specific enzymes sometime later than their monocoenzyme-specific cytoplasmic counterparts. However, the presence of dual specific GDH's in two species of archaebacteria (Consalvi *et al.*, 1991a and b) sheds doubt on this theory, since the archaebacterial kingdom is considered to be the least evolved of the three kingdoms. NAD- and NADP-specific GDH's are also found in archaebacteria (Bonete *et al.*, 1986,1987; Leicht *et al.*, 1978).

The GDH's of *Neurospora crassa* are inversely regulated enzymes. The NADP-specific GDH is induced by ammonium and glucose but repressed by glutamate and carbon limitation (Smith *et al.*, 1975; Goldin and Frieden, 1971; Vierula and Kapoor, 1989). The opposite is true for the NAD-specific GDH: it is derepresssed during growth on glutamate or by carbon starvation, and repressed by ammonium (Goldin and Frieden, 1971; Vierula and Kapoor, 1989). This indicates that the NADP-specific GDH is chiefly a biosynthetic enzyme while the NAD-specific GDH serves a mainly catabolic function. A number of mutant strains of *N. crassa* have been isolated which require amino acids for normal growth (Goldin and Frieden, 1971). These mutants have a defective NADP-specific GDH; the NAD-specific GDH is unaffected. The NAD-specific GDH is produced upon germination, and these mutants will grow normally on unsupplemented media after a prolonged lag phase, probably due to the NAD-specific GDH functioning in a biosynthetic role in this case (Smith *et al.*, 1975; Goldin and Frieden, 1971). The NADP- and NAD-linked GDH's of *N. crassa* are encoded by unlinked genes and have quite different properties. The NADP-specific GDH is a hexamer with a molecular weight of 288 400 (Blumenthal and Smith, 1973); in this respect it is similar to the vertebrate enzymes. Like the bovine liver GDH, the *N. crassa* NADP-specific GDH has a relatively broad substrate specificity: it deaminates 4 amino acids other than L-glutamate and aminates eight 2-keto acids other than 2-ketoglutarate (Barratt and Strickland, 1963). The NAD-specific GDH, on the other hand, is a tetramer with a subunit molecular weight of 116 000 (Veronese *et*

al., 1974). It is unusual both in its tetrameric structure and its large subunit size, approximately twice the size of the subunits of most other GDH's (Smith *et al.*, 1975).

The yeast *Saccharomyces cerevisiae* is another species which has a hexameric NADP-specific GDH (subunit molecular weight 48 000) (Smith *et al.*, 1975), and an NAD-specific GDH which is thought to be tetrameric and have a subunit molecular weight of 100 000 (Uno *et al.*, 1984). *Pichia jadinii* (formerly *Candida utilis*) also has a tetrameric NAD-specific GDH with a large subunit size (116 000) (Hemmings, 1980). The NAD-specific GDH's of the yeasts *S. cerevisiae* and *P. jadinii* are both regulated by phosphorylation-dephosphorylation system, the dephosphorylated form being more active (Uno *et al.*, 1984; Hemmings, 1980). The NAD-specific GDH of *S. cerevisiae* was shown to be inactivated by cAMP-dependent and cAMP-independent protein kinases *in vitro*; activity could be restored by treatment with bovine alkaline phosphatase (Uno *et al.*, 1984).

The spores of *Phycomyces blakesleeanus* contain a dimeric NAD-specific GDH with a molecular weight of 98 000 (Van Laere, 1988), making it one of the smallest GDH's ever reported. Van Laere found no evidence for the regulation of this enzyme by phosphorylation, but found it to be virtually inactive in the absence of AMP (9.5 and 43 μ M gave half-maximal activation in the reductive amination and the oxidative deamination reactions, respectively). ATP, at a concentration of 1mM, inhibited the enzyme by 40% in the direction of oxidative deamination (Van Laere, 1988). Le John (1971) studied an NAD-specific GDH from the mycelia of *Phycomyces* which did not possess the above regulatory properties; Van Laere (1988) proposed that the NAD-specific GDH from the spores of *P. blakesleeanus* is an enzyme unique to spores and has an important role in the breakdown of glutamine reserves which occurs as the spores germinate.

Le John (1971) classified the NAD-specific GDH's of lower fungi into three types on the basis of their regulatory properties: type I enzymes are unregulated; type II enzymes are effected by adenylates (when the energy charge is high, glutamate biosynthesis is stimulated while its catabolism is inhibited) and influenced unidirectionally (in nonequilibrium conditions) by divalent metal ions; type III enzymes are confined to the Oomycetes and Hypochytridiomycetes - their complex regulatory properties are described

above (section 4). He found correlations between enzyme regulation, lysine pathways and cell wall structure, and proposed a pathway for the evolution of fungal NAD-specific GDH's on the basis of this. He presented a theory that the regulators of the NAD-specific GDH's were once substrates of a primitive form of "glutamate dehydrogenase", and that these substrates became effectors as the enzymes evolved.

Finally, in the dimorphic fungus *Benjaminiella poitrasii* which possesses two forms of GDH (NAD- and NADP-specific), there may be a correlation between their relative proportions and dimorphic behaviour. The yeast (Y) to mycelial (M) transitions in *Benjaminiella poitrasii* can be regulated by temperature and by carbon/nitrogen balance (Khale *et al.*, 1992). Khale *et al.* found that NAD-specific GDH levels were 10-fold lower in the Y form than in the M form, NADP-specific GDH levels were 7-fold higher in the Y form than in the M form and that glucose repressed NAD-specific GDH but induced NADP-specific GDH (which is consistent with the fact that the M form grows on a variety of carbon sources while the Y form requires hexose sugars for optimal growth). Furthermore, they found that an increased ratio of NADP-GDH/NAD-GDH preceded the yeast to mycelial transition and that monomorphic mutants which maintained the yeast morphology had a consistently high NADP-GDH/NAD-GDH ratio. In *Mucor* sp., the yeast morphotype similarly has more specific growth requirements than those of the hyphae and also has decreased levels of NAD-GDH (Orlowski. 1991). However, the levels of NAD-specific GDH were found to be regulated by cyclic AMP concentration, and in mutants which maintained the mycelial morphology in air in the presence of exogenous cAMP, NAD-specific GDH was still repressed by cAMP (Paznokas *et al.,* 1982). Orlowski (1991) concluded that there was no direct evidence for a correlation between nitrogen metabolism or GDH levels and morphology in *Mucor* sp. In fact, both NAD- and NADP-GDH's are thought to have a catabolic function in *Mucor* sp., since the levels of both forms are increased during growth on complex organic nitrogen sources (Peters and Sypherd, 1979), and ammonia assimilation was found to be a function of the glutamine synthetase/glutamate synthase pathway (Sypherd *et al.*, 1978).

The molecular weights of some fungal GDH's are listed in Table 1.

4.1.4.2. Algal Glutamate Dehydrogenases

All three types of GDH (NAD-, NADP- and NAD(P)-linked GDH's) have been reported in algae, the NADP-specific GDH often being localised in the chloroplasts (Stewart *et al.*, 1980). *Chlamydomonas reinhardtii* has three dual coenzyme specific hexameric GDH isozymes which are induced under different stress conditions and are thought to be synthesised as precursor proteins in the cytosol before being transported into the mitochondria (Munoz-Blanco *et al.*, 1989; Moyano *et al.*, 1992b). *Chlorella sorokiniana* has two chloroplast-localised isozymes of NADP-specific GDH, one induced during growth on low levels of ammonia, the other during growth on high levels of ammonia (Bascomb and Schmidt, 1987). Both isozymes are homopolymers of six subunits which have different molecular weights (53 000 for the alpha-subunit and 55 500 for the beta-subunit), but have a high degree of sequence homology, similar antigenic properties and may be synthesized *in vitro* from a precursor protein or proteins with a molecular weight of 58 500; hence it is hypothesized that both types of subunits are derived from an identical precursor protein (Bascomb and Schmidt, 1987). *Chlorella sorokiniana* also has a tetrameric mitochondrial NAD-linked GDH (Meredith *et al.*, 1978). Javed and Merrett (1986) characterised a tetrameric cytoplasmic NADP-specific GDH from *Euglena gracilis* which failed to show immunological cross-reactivity with NADP-specific GDH's from several other algae and it was suggested that this may be due to its less common tetrameric subunit structure, since most GDH's, particularly NADP-specific GDH's, are hexameric.

In some species of algae GDH is thought to play an important role in the assimilation of ammonia; this contrasts with the situation in higher plants in which the glutamine synthetase/glutamate synthase route is thought to be more important (see above, section 4.1.3.). The NADP-specific GDH of the soil alga *Stichococcus bacillaris* naeg. has a relatively low Km for ammonium (1.6 mM) and its level increases 10 to 15 fold when the concentration of ammonium in the medium is decreased from 2 to 0.2 mM (Ahmad and Hellebust, 1986). *S. bacillaris* naeg. will grow and assimilate ammonia normally in the presence of methionine sulphoximine (MSX, a potent glutamine synthetase inhibitor), as will *Chlorella autotrophica* and *Chlamydomonas reinhardtii* (Ahmad and Hellebust, 1986

and 1984; Munoz-Blanco *et al.*, 1989), which suggests that GDH may effectively carry out the role of ammonia assimilation in these organisms. *Euglena gracilis*, on the other hand, was unable to assimilate ammonia in the presence of MSX, even though its NADP-specific GDH has a Km for ammonia of only 0.4 mM; it is thought that this may be because the NADP-specific GDH is located in the cytoplasm, whilst the 2-ketoglutarate necessary for the assimilation of ammonia by the GDH reaction is mainly produced in the mitochondria as a result of TCA cycle activity (Javed and Merrett, 1986).

Calcium ions (1mM) stimulated the NAD-specific GDH activity of the three dual coenzyme specific GDH isozymes from *Chlamydomonas reinhardtii*, while several other divalent ions (Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Ba^{2+}) either inhibited the enzyme or had no effect (Moyano *et al.*, 1992a). EDTA and several other chelating agents strongly inhibited the isozymes (Moyano *et al.*, 1992a). Plant NAD-specific GDH's are also markedly activated by calcium ions and inhibited by chelating agents (see above section 4.1.3.), but only micromolar amounts of the metal ion are needed to effect this activation; the effect of micromolar amounts of Ca^{2+} on the *Chlamydomonas reinhardtii* GDH isozymes is not reported. A GDH from a thermophilic archaebacterium (isolate AN1) has also been found to be activated by millimolar concentrations of calcium ions (see below section 4.3.2). Both the NAD- and the NADP-GDH from *Stichococcus bacillaris* naeg. are unaffected by calcium ions and EDTA (Ahmad and Hellebust, 1986).

See Table 1 for some examples of algal GDH's.

4.1.4.3. Glutamate Dehydrogenases from Protozoa

GDH's have been characterised from a number of protozoans, including the parasites which cause American trypanosomiasis (Chagas' disease) and malaria. Due to the status of GDH as a well-studied enzyme and to the marked differences between protozoan and vertebrate GDH's, this enzyme has been a target for studies which attempt to find chemotherapeutic agents to treat these diseases.

Both the NAD- and NADP-specific GDH's from *Trypanosoma cruzi* are hexameric; the former is inhibited by adenine and guanosine nucleosides (high energy

charge is inhibitory), while the latter is unaffected by nucleotides (Cazzulo *et al.*, 1979; Carneiro and Caldas, 1983). The level of NADP-specific GDH in *Try. cruzi* epimastigotes is variable and depends on the culture conditions and the parasite stock; conversely, the levels of NAD-specific GDH are less variable and it is thought to be a constitutive enzyme with a mainly catabolic role (Cazzulo, 1984). The role of the *Try. cruzi* NADP-specific GDH is uncertain; the fact that its levels are increased by the presence of glutamate in the medium (although this is not due to *de novo* protein synthesis), suggest that an anabolic role for this enzyme is unlikely (Carneiro and Caldas, 1983; Cazzulo, 1984). The NADP-specific GDH from the flagellated parasite *Trichomonas vaginalis* (mentioned above, section 4) is, in contrast to the NADP-specific GDH from *Try. cruzi*, effected by some nucleotides: GDP and IDP both increased the Km for 2-ketoglutarate fivefold; GDP also reduced the Km for glutamate by 40%, hence the effect is unidirectional in favour of oxidative deamination (Turner and Lushbaugh, 1988). The NADP-specific GDH from *Plasmodium lophurae* (avian malaria) is mentioned above in Section 4. Further information about some protozoan GDH's is contained in Table 1.

TABLE 1

Some Properties of Eukaryotic Glutamate Dehydrogenases

Source	Coenzyme Specificity	M_r	Subunit M_r	Number Subunits	Reference
Vertebrates					
Bovine liver	Dual	332K	55K	6	Smith *et al.*, 1975; Moon & Smith, 1973
Chicken liver	Dual	334K	56K	6	Moon *et al.*, 1973; Smith & Piszkiewicz 1973
Dogfish liver	Dual	330K	-	-	Corman *et al.*, 1967
Eel liver	Dual	324K	54K	6	Tang *et al.*, 1992
Frog liver	Dual	250K	-	-	Fahien *et al.*, 1965
Human brain	Dual	360K	60K	6	Hussain *et al.*, 1989
Rat liver	Dual	350K	48K	6-8	King & Frieden, 1970
Invertebrates					
Acropora formosa	NADP	360K	56K	6	Catmull *et al.*, 1987
Anthopleura xanthogrammica	NADP	325K	49K	6	Male & Storey, 1983
Plants					
Lemna seeds	NAD	230K	58.5K	4	Srivastava & Singh, 1987
Pea (*Pisum*) seeds	NAD	230K	58.5K	4	"
Turnip (*Brassica rapa*)	NAD	300-310K	43K	6-8	Itagaki *et al.*, 1988

Fungi					
Candida utilis	NAD	460K	116K	4	Hemmings, 1980
Cenococcum graniforme	NADP	320K	48K	6	Martin *et al.*, 1983
Laccaria laccata	NADP	298K	47K	6	Brun *et al.*, 1992
Neurospora crassa	NADP	288K	49K	6	Blumenthal & Smith, 1973
N. crassa	NAD	480K	116K	4	Veronese *et al.*, 1974
Phycomyces spores	NAD	98K	54K	2	Van Laere, 1988
Saccharomyces cerevisiae	NADP	270-290K	48K	6	Smith *et al.*, 1975
S. cerevisiae	NAD	450K	100K	4	Uno *et al.*, 1984
Sphaerostilbe repens	NADP	280K	48K	6	Botton & Msatef, 1983
Algae					
Chlamydomonas reinhardtii[a]	Dual	266-269K	44-46K[a]	6	Moyano *et al.*, 1992a
Chlorella sorokiniana[b]	NADP	-	53&55.5K[b]	6	Bascomb & Schmidt, 1987
C. sorokiniana	NAD	~180K	45K	4	Meredith *et al.*, 1978
Euglena gracilis	NADP	180K	45K	4	Javed & Merrett, 1986
Protozoa					
Trichomonas vaginalis	NADP	230K	-	-	Turner & Lushbaugh, 1988
Trypanosoma cruzi	NADP	265-315K	43-52K	6	Cazzulo, 1984
T. cruzi	NAD	360K	58K	6	Walter & Ebert, 1979

a: *C. reinhardtii* has three GDH isomers, all dual cofactor specific and of similar molecular weights. GDH_1 is a homopolymer of 44KDa subunits, while GDH2 and GDH3 each consist of six similar sized subunits (4 of 44KDa and 2 of 46KDa) (Moyano *et al.*, 1992a).

b: *C. sorokiniana* has two hexameric NADP-GDH isomers which are homopolymers of 53 or 55.5KDa subunits. Both types of subunit are thought to be synthesized from an identical 58.5KDa precursor protein (Bascomb & Schimdt, 1987).

4.2. Eubacterial Glutamate Dehydrogenases

Glutamate dehydrogenases have been isolated and characterised from a large number of eubacteria. They are found in a wide range of physiological groups: nutritional types include heterotrophs, lithotrophs (both nitrifying and sulphur oxidising), phototrophs and methylotrophs. GDH has been isolated from both nitrogen-fixing eubacteria (which only fix nitrogen when the cellular concentration of ammonia is very low) and denitrifying eubacteria. In most cases, GDH's from eubacteria are specific for either NAD or NADP, although dual specific GDH's have been reported in *Mycoplasma laidlawii* (Yarrison *et al.*, 1972), *Azospirillum brazilense* (Maulik and Ghosh, 1986), *Rhodopseudomonas sphaeroides* (Engelhardt and Klemme, 1978), *Bacteroides thetaiotaomicron* (Glass and Hylemon, 1980) and other *Bacteroides* species (Yamamoto *et al.*, 1987). Some eubacteria produce only NAD-specific GDH (e.g.. some clostridia (Goldin and Frieden, 1971)), while many others (including *Escherichia coli* and *Salmonella typhimurium*) produce only NADP-specific GDH. *Thiobacillus novellus, Pseudomonas aeruginosa* and *Streptomyces fraidiae* are among those organisms which produce both NAD- and NADP-specific GDH's (Le John *et al.*, 1968; Janssen *et al.*, 1980; Vancurova *et al.*, 1989). As is the case for eukaryotic GDH's, the most common number of subunits is six (see Table 2).

The NADP-GDH from the extensively studied enteric bacterium *E. coli* has been investigated by a number of groups. The GDH is induced by ammonia and glucose (Veronese *et al.*, 1975), as expected for a biosynthetic enzyme. In this organism, GDH occurs in a complex with glutamate synthase (Savageau *et al.*, 1972), but the ammonia-dependent and glutamine-dependent activities were found not to be additive; this is probably due to the inhibition of GDH by glutamine (competitive with respect to ammonia) (Sakamoto *et al.*, 1975). Recently Lin and Reeves (1991a) found that the enzyme is phosphorylated *in vitro* in an ATP-dependent reaction catalysed by an endogenous protein kinase. The *in vitro* phosphorylated enzyme and the native enzyme (isolated from *E. coli*) have identical molecular weights and isoelectric points, which suggests that the catalytically active form may be the phosphorylated one (Lin and Reeves, 1991b).

TABLE 2

Some Properties of Eubacterial Glutamate Dehydrogenases

Species	Coenzyme Specificity	Molecular Weight	Subunit M_r	Number Subunits	Reference
Gram negative					
Escherichia coli $D_5H_3G_7$	NADP	275K	44.5K	6	Lin & Reeves, 1991b
E. coli B/r	NADP	300K	50K	6	Sakamoto *et al.*, 1975
Salmonella typhimurium	NADP	280K	-	-	Coulton & Kapoor, 1973a
Streptomyces fraidiae	NADP	200K	49K	6	Vancurova *et al.*, 1989
Mycobacterium smegmatis	NADP	245.5K	40K	6	Sarada *et al.*, 1980
Hyphomicrobium X	NADP	380K	63.4K	6	Duchars & Attwood, 1987
Methylobacillus flagellatum	NADP	300K	47K	6	Kiriukhin *et al.*, 1992
Paracoccus denitrificans	NADP	210K[a]	47K	4-6	Kremeckova *et al.*, 1992
Pseudomonas aeruginosa	NADP	325K	54K	6	Smits *et al.*, 1984
Pseudomonas sp. Strain AM1	NADP	190K	50K	4	Bellion & Tan, 1984
Synechocystis PCC6803	NADP	208K	46K	4	Florencio *et al.*, 1987
Thiobacillus novellus	NADP	130K	-	-	Le John *et al.*, 1968
T. novellus	NAD	120K	-	-	"
Azospirillum brasilense	Dual	285K	48K	6	Maulik & Ghosh, 1986
Mycoplasma laidlawii[b]	Dual	250K	48K	?	Yarrison *et al.*, 1972

Gram positive					
Bacillus fastidiosus	NADP	>200K	48K	?	Op Den Camp *et al.*, 1989
B. megaterium	NADP	270K	47K	6	Hemmila & Mantsala, 1978
B. stearothermophilus	NAD	240K	40K	6	Mantsala, 1985
Clostridium botulinum 113B	NAD	251K	42.5K	6	Hammer & Johnson, 1988
C. SB_4	NAD	275K	-	-	Winnacker & Barker, 1970
C. symbiosum	NAD	282K	49K	6	Rice *et al.*, 1985
Peptostreptococcus assacharolyticus	NAD	266K	49K	6	Hornby & Engel, 1984
Lactobacillus fermentum	NADP	300K	50K	6	Misono *et al.*, 1985
Ruminococcus flavefaciens	NADP	280K	48K	6	Duncan *et al.*, 1992

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a :The GDH activity eluted from a gel permeation column in a volume corresponding to a molecular weight of 210K, but two inactive peaks also eluted from the column (molecular weights 140 and 55K) which could have resulted from enzyme cleavage. Presuming the molecular weight of the native enzyme is 210K, the structure is probably tetrameric.

b : *M. laidlawii* is a wall-less organism which stains gram-negative.

Compared to NADP-specific GDH's, fewer NAD-specific GDH's have been characterised from eubacteria. NAD-specific GDH is the first enzyme in the hydroxyglutarate pathway of glutamate fermentation, one of the two main pathways by which anaerobic bacteria ferment glutamate (the other route is the citramalate pathway) (Johnson and Westlake, 1972; Hornby and Engel, 1984). *Peptostreptococcus asaccharolyticus* (formerly *Peptococcus aerogenes* and *Micrococcus aerogenes*), is one organism which uses this pathway (Hornby and Engel, 1984) and when grown on glutamate produces very high levels of NAD-specific GDH (up to 10% of the total protein) (Johnson and Westlake, 1972; Kew and Woolfolk, 1970).

Hammer and Johnson (1988) found high specific activities of NAD-specific GDH in proteolytic strains of *Clostridium botulinum*, whereas this activity was present in low levels or absent altogether in several strains of non-proteolytic *C.botulinum*. It is thought that the oxidative deamination of glutamate by NAD-specific GDH is important in the metabolism of the proteolytic strains (which obtain much of their energy from the fermentation of amino acids) because the reaction generates 2-ketoglutarate as a nitrogen acceptor for transaminase reactions and disposes of excess ammonia. The ammonia released may facilitate the ability of proteolytic *C.botulinum* to grow and produce neurotoxin in highly acidic environments (including foods with pH < 4.6) (Hammer and Johnson, 1988).

NAD-specific GDH's have also been isolated from *Clostridium* SB_4 (Winnacker and Barker, 1970) and *C. symbiosum* (Rice *et al.*, 1985). The latter enzyme, a hexamer of 49K, is the only GDH which has had its structure solved by X-ray-diffraction studies (Rice *et al.*, 1987). Each subunit of the *C. symbiosum* GDH consists of two domains separated by a deep cleft which contains the NAD binding site (Stillman *et al.*, 1992). A comparison of a model of the *C. symbiosum* GDH with an electron micrograph of the bovine liver enzyme (for which attempts to grow crystals suitable for X-ray studies have proved unsuccessful) revealed considerable structural homology (Rice *et al.*, 1987).

The facultative chemoautotroph *Thiobacillus novellus* produces NAD- and NADP-specific GDH's with molecular weights of 120K and 130K (determined by zone

centrifugation in sucrose gradients)[1], respectively (Le John *et al.*, 1968). Assuming that the subunit size is 40-60K, as found in most other GDH's, these enzymes may have as few as two subunits. The only other GDH known to be dimeric is the NAD-specific GDH from the spores of the fungus *Phycomyces blakesleeanus* (Van Laere, 1988) (see above, section 4.1.4.1.). In common with the *P. blakesleeanus* NAD-specific GDH, the *T. novellus* NAD-specific GDH is activated by AMP. The enzyme displays Michaelis-Menten kinetics only in the presence of AMP; this will be discussed further in section 6. The *T. novellus* NADP-specific GDH is not effected by AMP and displays linear kinetics in its absence (Le John *et al.*, 1968).

The molecular weights of some eubacterial GDH's are given in Table 2.

4.3. Archaebacteria and their Glutamate Dehydrogenases.

For many years, organisms were divided into two kingdoms: the eukaryotes and the prokaryotes. Recently, with the advent of phylogenetic analysis (e.g. 16S and 18S ribosomal RNA sequencing), the prokaryotes have been divided into two new kingdoms: the eubacteria ("true" bacteria) and the archaebacteria (Woese and Fox, 1977; Woese, 1987). The archaebacteria are no more related to the eubacteria than they are to the eukaryotes, although phenotypically they resemble eubacteria, being microscopic unicellular organisms. More recently, a new scheme has been proposed which divides all organisms into one of three domains (a new taxon of the highest rank): the Eucarya (= the eukaryotes), the Bacteria (= the eubacteria) and the Archaea (= the archaebacteria) (Wheelis *et al.*, 1992). The Archaea comprises two kingdoms: the Crenarchaeota and the Euryarchaeota (Wheelis *et al.*, 1992).

The archaebacteria (or Archaea) encompasses three basic phenotypes: methanogens, halophiles and thermophiles (or combinations thereof). Many of the thermophiles are sulphur-dependent or acidophilic or both. The methanogens are obligate anaerobes, the halophiles are obligate aerobes, while the thermophiles may be anaerobic

[1]Tris-HCl buffer was used for the zone centrifugation. The NADP-GDH from *Pseudomonas aeruginosa* also appears to have a low molecular weight (110K) when the enzyme is in Tris buffer, but is a hexamer of 325K in phosphate buffer (Janssen *et al.*, 1980; Smits *et al.*, 1984).

(such as the hyperthermophile *Pyrococcus woesei*) or aerobic (such as the thermoacidophile *Sulfolobus solfataricus*). Thus the group covers a broad range of phenotypes, and, probably more than any other group of organisms, has a tendency to occupy niches in extreme environments.

The central metabolism of the archaebacteria has been reviewed by Danson (1988,1989), who outlines hexose and pyruvate catabolism in different groups of archaebacteria and compares it to the situation in eubacteria and eukaryotes. Danson (1988) also describes particular archaebacterial enzymes from the central pathways. One feature of archaebacterial nicotinamide nucleotide-dependent dehydrogenases (particularly those from thermoacidophilic organisms) is that they often have dual cofactor specificity, whereas this is less common in the dehydrogenases from eubacteria and eukaryotes. So far, a relatively small number of archaebacterial glutamate dehydrogenases have been isolated: three from halophiles and five from thermophiles. As yet, there have been no reports of the isolation and properties of glutamate dehydrogenases from methanogens.

4.3.1. Glutamate Dehydrogenases from Halophiles.

Archaebacterial halophiles not only tolerate high salt conditions, but require them. The cell cytoplasm is maintained at a similar ionic strength to the external environment, with the predominant cation inside the cell being potassium which the cells concentrate from their environment (Brock *et al.*, 1984). Although *Halobacterium* sp. are heterotrophic, they are capable of light-generated ATP synthesis (a proton gradient may be established across their cell membranes as a result of light absorption by the cell membrane carotenoid bacteriorhodopsin) (Brock *et al.*, 1984). *Halobacterium halobium* accumulates most amino acids by cotransport with sodium ions or protons (Na^+ and H^+ symporters), but only a sodium ion gradient is necessary for the accumulation of glutamate (Kamo *et al.*, 1988).

Both NAD- and NADP-dependent GDH's have been isolated from the halophile *Halobacterium halobium*, each being completely specific for the cofactors NAD and

NADP, respectively (Bonete *et al.*, 1986, 1987). The molecular weights were estimated by gel filtration to be 148K and 215K for the NAD- and NADP-specific GDH, respectively (Bonete *et al.*, 1987); the subunit structure has not been reported. The NAD-specific GDH is activated by a number of TCA cycle intermediates and amino acids, while the NADP-specific GDH is not. Neither enzyme is effected by purine nucleotides. Both GDH's, as expected for enzymes from halophiles, are activated by sodium or potassium chloride, although the optimal concentrations are different for each enzyme. The NAD-specific GDH has a pH optimum for oxidative deamination (8.0) which is more acidic than that for the reductive amination (9.0) (Bonete *et al.*, 1986). This is unusual, as for most GDH's, the pH optimum for the oxidative deamination is either more alkaline or the same as that for reductive amination (Smith *et al.*, 1975).

Leicht *et al.*, (1978) purified to homogeneity a GDH from another *Halobacterium* sp., isolated from the Dead Sea. The enzyme is NADP-specific although it displays a small amount of activity with NADH (<1% of the activity shown with NADPH). The GDH has a molecular weight of 212K (average from two methods: sedimentation equilibrium and sedimentation velocity + diffusion coefficient) and consists of four 53.5K subunits (determined by SDS-PAGE). The enzyme was found by circular dichroism to have a high content of α -helical structure (almost 60%), which is a typical property of halophilic proteins (Leicht *et al.*, 1978). Salts (sodium chloride or sodium sulphate) activate the GDH and are important for its stability. In the presence of 4.3M sodium chloride, the enzyme has a half life of approximately 11 hours at 65°C, which is remarkable for a mesophilic enzyme (Leicht *et al.*, 1978). The purine nucleotides ADP and ATP did not effect enzyme activity, although 2',5'-ADP caused inhibition (50% at 0.75mM).

Some properties of the three characterised halophilic GDH's are summarised in Table 3.

TABLE 3

Some Properties of Archaebacterial Glutamate Dehydrogenases

	Halobacterium halobium[a,b] (NAD-GDH)	*Halobacterium halobium*[b] (NADP-GDH)	*Halobacterium* sp. of the Dead Sea[c] (NADP-GDH)	*Sulfolobus solfataricus*[d,e,f] (NAD(P)-GDH)	*Pyrococcus furiosus*[g,h] (NAD(P)-GDH)	Isolate AN1[i] (NADP-GDH)
M_r	148K	215K	212K	270K[d,e]	270-290K[g,h]	204K
Subunit M_r	-	-	53.5K	44-46K[d,e,f]	46[g]/48K[h]	47K
No. subunits	-	-	4	6[d,e]	6[g,h]	4
pH optima:						
amination	9	8-8.5	-	9[d]/7.6[e]	9[g]	8.25
deamination	8	10	-	10[d]	9[g]	8.25
pI	-	-	-	5.7[d]/6.2[e]	4.5[g]	4.0
Apparent Km (mM):						
NAD	0.3	-	-	-	0.53[h]	28.6
NADP	-	0.11	0.061	0.05, 0.25[d]*	0.2[g]/0.18[h]	0.038
glutamate	4	4600	14.2	2.5[d]	0.6[g]/1.6[h]	9.12
NADH	0.07	-	-	0.14[d]/0.007[e]	0.36[g]/0.98[h]	0.32
NADPH	-	0.077	0.024	0.01[d,e]	0.012[g]/0.56[h]	0.066
2-ketoglutarate	20.2	13.3	3.0	1.4[d]	0.33[g]/4.0[h]	1.7
ammonia	450	3.1	16.6	4.2, 34[d]*	6, 27[g]*	15.5

Refs. **a**: Bonete *et al.*, 1986; **b**: Bonete *et al.*, 1987; **c**: Leicht *et al.*, 1978; **d**: Consalvi *et al.*, 1991a; **e**: Schinkinger *et al.*, 1991; **f**: Maras *et al.*, 1992; **g**: Consalvi *et al.*, 1991b; **h**: Robb *et al.*, 1992; **i**: Hudson *et al.*, 1993. The apparent Km values given are those determined at 80°C.

*: Apparent Km depended on range of substrate concentration.

4.3.2. Glutamate Dehydrogenases from Thermophilic Archaebacteria.

GDH has been purified and characterised from five extremely thermophilic sulphur-dependent archaebacteria: the acidophilic aerobe *Sulfolobus solfataricus* (Consalvi *et al.*, 1991a; Schinkinger *et al.*, 1991), and the obligate anaerobes *Pyrococcus furiosus* (Consalvi *et al.*, 1991b; Robb *et al.*, 1992), ES4 (pers. comm. Di Ruggiero, Klump and Robb), *Thermococcus* sp. (pers. comm. Kobayashi) and isolate AN1 (Hudson *et al.*, 1993). *S. solfataricus* is facultatively chemolithotrophic (it oxidises hydrogen sulphide to elemental sulphur and elemental sulphur to sulphuric acid) (Brock *et al.*, 1984), while *P. furiosus* is heterotrophic, fermenting carbohydrates to carbon dioxide and hydrogen gas (it reduces sulphur to hydrogen sulphide with the hydrogen, which is a growth inhibitor) (Consalvi *et al.*, 1991b). *P. furiosus* is not actually a sulphur-dependent archaebacterium as it will grow in the absence of sulphur (Klump *et al.*, 1992), and although it ferments carbohydrates, they cannot serve as the organism's sole carbon source - peptides are also required (Robb *et al.*, 1992). Isolate AN1, which like *Pyrococcus* sp. is a member of the order Thermococcales (Zillig, 1987; Adams, 1990), also reduces sulphur to hydrogen sulphide (cystine may serve as an alternative reduced sulphur source). Isolate AN1 grows on either peptones or amino acid mixtures supplemented with a small amount of peptone (Klages, 1991) and may also use carbohydrates (it has extracellular carbohydrate-degrading enzymes (Bragger *et al.*, 1989)), but not as a sole carbon source.

The GDH from *S. solfataricus* is a hexamer with a molecular weight of 270K (Consalvi *et al.*, 1991a; Schinkinger *et al.*, 1991). The enzyme is dual cofactor specific, but shows a preference for NADP: its activity with NADH is 30% of that with NADPH and its Km for NADH is more than ten-fold greater than that for NADPH (Consalvi *et al.*, 1991a). *S. solfataricus* was found to contain high levels of GDH (0.5 mg/g wet cells) when grown on glucose and yeast extract - the enzyme was repressed during growth on glutamate (Consalvi *et al.*, 1991a). Consalvi *et al.* noted that the repression of the enzyme by glutamate and the preference for NADP over NAD indicate a biosynthetic role for the enzyme.

As expected for an enzyme from an extremely thermophilic bacterium (*S. solfataricus* grows optimally at 85°C), the GDH is highly thermostable, although the thermostability was found to be dependent on the protein concentration (proposed by Consalvi *et al.* (1991a) to be due to enzyme aggregation at higher protein concentrations). Its half-lives (at a protein concentration of 0.2 mg/ml) are 15 h, 2 h and 20 min at 80, 85 and 90°C, respectively (Consalvi *et al.* 1991a). Schinkinger *et al.* (1991) report the enzyme to be more stable than this (no significant loss of activity after 1h at 90°C), but do not report the conditions under which they carried out their thermostability experiments. Schinkinger *et al.* (1991) also found the GDH to be highly resistant to the denaturing agents urea and SDS, although it was inactivated by exposure to some organic solvents (ethanol, methanol or isopropanol at 50% v/v).

The sequence of the *S. solfataricus* GDH has been obtained and compared with the sequences of eight other GDH's from eukaryotic and eubacterial sources (Maras *et al.*, 1992). The *S. solfataricus* sequence has an overall identity of 9.2% with the other sequences and shows a symmetrical evolutionary distance from the vertebrate GDH's (from man, ox, rat and chicken) on one side, and fungal and eubacterial GDH's (from *Saccharomyces cerevisiae, Neurospora crassa, Aspergillus nidulans* and *Escherichia coli*) on the other side.

The GDH from *Pyrococcus furiosus* shares many features with the *S. solfataricus* GDH. Its molecular weight has been reported as 270+/-15K (Robb *et al.*, 1992) and 290K (Consalvi *et al.*, 1991b), and, like the *S. solfataricus* GDH, it is hexameric (Consalvi *et al.*, 1991b; Robb *et al.*, 1992). The enzyme has dual cofactor specificity. Its specific activity with NADH as cofactor is equal to that with NADPH as cofactor, although the Km for NADH is more than ten-fold higher than that with NADPH, indicating a preference for the latter coenzyme (as is the case for the *S. solfataricus* GDH) (Consalvi *et al.*, 1991b).

P. furiosus produces extremely high levels of GDH; Consalvi *et al.* (1991b) report that GDH represents 16mg/g wet cells and 20% of the total soluble protein, about twenty times the level found in *S. solfataricus*. However, the purification table of Robb *et al.* (1992) indicated that GDH represented only 1.2% of the soluble protein in their *P.furiosus*

cells, even though this group used a higher g force in the preparation of the cell free extract (50 000 xg) than did Consalvi's group (30 000 xg). The discrepancy could possibly be due to the conditions used to grow the organism: Consalvi *et al.* added elemental sulphur (0.2 g/l) to the medium, while Robb *et al.*, who grew the organism on a large scale, did not.

The GDH from *P. furiosus*, which grows optimally at 100°C, is even more thermostable than the *S. solfataricus* enzyme: Consalvi *et al.* (1991b) report half-lives of 12 h and 25 min at 100 and 110°C, respectively, while Robb *et al.* (1992) report a half-life of 2.3-10 h at 100°C, depending on the protein concentration. Contrary to the findings of Robb *et al.* (1992), Consalvi *et al.* (1991b) found no evidence for the thermostability of the GDH being dependent on the protein concentration. This discrepancy may be due to differences in the conditions under which the thermostability experiments were carried out by the two groups (Consalvi *et al.* used a phosphate buffer containing glycerol, which probably would have prevented any adsorption of the enzyme to the walls of the vessel; the buffer system used by Robb *et al.* is not reported). Klump *et al.* (1992) investigated the conformational changes undergone by the *P. furiosus* GDH during heating by means of differential scanning calorimetry and monitoring of absorbance at 280 nm (A_{280}). Large changes in heat capacity and A_{280} accompany the thermal denaturation of the GDH at 113°C, which was noted by Klump *et al.* as being the highest thermal denaturation temperature observed for any enzyme. The enzyme is inactive below 40°C; relatively small changes in heat capacity and A_{280} occur during the (reversible) thermal reactivation of the enzyme (T_m = 57°C), suggesting the exposure of some hydrophobic residues to the solvent during the process (Klump *et al.*, 1992).

The amino acid composition of the *P. furiosus* GDH is similar to that of the *S. solfataricus* GDH (Consalvi *et al.*, 1991b) and its amino terminal sequence has considerable homology with that of GDH from yeast (Robb *et al.*, 1992).

An NADP-specific GDH has been purified from the hyperthermophilic sulphur-reducer, ES4, which like the enzymes from *S. solfataricus* and *P. furiosus*, is a hexamer of 270 KDa (pers. comm. Di Ruggiero, Klump and Robb). ES4 grows at temperatures up to 110°C, and the thermostabilty of its GDH reflects this ($t_{1/2}$ at 105°C = 3.5 h). The enzyme

has T_m's for denaturation (113°C) and activation (60°C) which are similar to those of the *P. furiosus* GDH.

Hudson *et al.* (1993) have purified and characterised a GDH from the archaebacterial isolate AN1. This enzyme has a molecular weight of 204 K and, like the GDH from *Halobacterium* sp. of the Dead Sea (Leicht *et al.*, 1978), is tetrameric. It is specific for NADP and displays only very low activity with the non-phosphorylated cofactors. The GDH represents about 10% of the soluble cell protein in isolate AN1.

The GDH from isolate AN1 is highly thermostable, having half lives of 12.5 h and 47 min at 90 and 103°C, respectively, but somewhat less so than the *P. furiosus* enzyme, which is expected since isolate AN1 has a lower optimum growth temperature (75-80°C). The thermostability of the enzyme appears not to be effected by protein concentration, but is higher in a zwitterionic buffer (EPPS) than in sodium phosphate buffer.

The GDH's from *S. solfataricus, P. furiosus* and isolate AN1 are unaffected by purine nucleotides and by chelating agents (Consalvi *et al.*, 1991a and b; Hudson *et al.*, 1993). The oxidative deamination activity of the AN1 enzyme is, however, markedly enhanced by millimolar concentrations of calcium, magnesium or manganese ions (Hudson *et al.*, 1993), and although EDTA alone has no effect on the enzyme, marked inhibition is seen when EDTA and magnesium or calcium ions are present together (Hudson and Daniel, unpublished results). Thus we propose that the metal ion activation effect involves the chelation of the metal ion by glutamate, and that metal ion-EDTA inhibits due to its structural similarity to the metal ion-glutamate complex (i.e., the metal ion-EDTA complex may compete with the metal ion-glutamate complex for the binding in the active site). All three enzymes have a narrow substrate specificity, although they do show activity with norvaline and the AN1 enzyme displays glutamate synthase activity (Consalvi *et al.*, 1991a and b; Hudson *et al.*, 1993). Consalvi *et al.* (1991a and b) found that the *S. solfataricus* GDH does not follow Michaelis-Menten kinetics for any of its substrates, while the *P. furiosus* enzyme shows linear kinetics only for the cofactor substrates (NADP, NADPH and NADH). However, the double reciprocal plots were linear

over restricted substrate ranges, allowing apparent Km values to be calculated. Hudson *et al.* (1993) saw no evidence of deviations from Michaelis-Menten kinetics with the AN1 enzyme. Apparent Km values are reported in Table 3 along with other general properties of the enzymes. The kinetic properties of these enzymes will be discussed further in Section 6.

5 Purification of Glutamate Dehydrogenases

A variety of methods have been used in the purification of GDH's, but the following have been frequently used in purification protocols for these enzymes: affinity chromatography on 2',5'-ADP-Sepharose or -Agarose (Catmull *et al.*, 1987; Misono *et al.*, 1985; Sarada *et al.*, 1980; Martinez-Bilbao *et al.*, 1988; Kumar and Nicholas, 1984; Brun *et al.*, 1992) and 5'-AMP-Sepharose (Kindt *et al.*, 1980; Mantsala, 1985; Schinkinger *et al.*, 1991), and dye-ligand chromatography (Consalvi *et al.*, 1991a and b; Male and Storey, 1983; Smits *et al.*, 1984; Lin and Reeves, 1991b; Van Laere, 1988; Hemmings, 1980; Maulik and Ghosh, 1986; Duncan *et al.*, 1992; Kiriukhin *et al.*, 1992; Syed *et al.*, 1991; Hornby and Engel, 1984; Hudson *et al.*, 1993). The two most commonly used dyes are Cibacron Blue F3G-A (the dye used in the commercially prepared matrix Affigel Blue and the dye moiety of Blue Dextran) and Procion Red HE-3B (marketed as Reactive Red 120 by Sigma). Watson *et al.* (1978) tested the effectiveness of these two ligands in the retardation of a range of dehydrogenases, and found that Cibacron Blue F3G-A columns retarded NAD-linked dehydrogenases more effectively than NADP-linked dehydrogenases, while the opposite was true for Procion Red HE-3B columns. However, Cibacron Blue columns have been used in the purification of NADP-linked GDH's, and there are cases where both types of column have been used within a purification procedure (Male and Storey (1983) used both in the purification of NADP-specific GDH from the sea anemone *Anthopleura xanthogrammica*). Hornby and Engel (1984) tested a range of Procion dyes in the purification of NAD-linked GDH from *Peptostreptococcus asaccharolyticus* (high levels of GDH are present in this organism) and developed a high yield, one-step purification procedure. Similarly, Syed *et al.* (1991) were also able to

purify the NAD-linked GDH from *Clostridium symbiosum* (which also contains high levels of the enzyme (10-15%)) in one step using Remazol Brilliant Red GG-Sepharose 6B.

It has been claimed that Cibacron Blue F3G-A binds to the "dinucleotide fold" present in most dehydrogenases (Stellwagen, 1977), but since it will bind proteins which are not dehydrogenases (Watson *et al.* 1978; Orellano *et al.*, 1985), it is probably not strictly specific for such a structure. Orellano *et al.* (1985) found that this dye inhibited the NADP-specific GDH from *Trypanosoma cruzi* competitively with respect to NADPH and noncompetitively with respect to 2-ketoglutarate, which may indicate the presence of a dinucleotide fold. Hudson and Daniel, however, investigated the type of inhibition given by Reactive Red 120 (= Procion Red HE-3B) towards the NADP-linked GDH from the archaebacterial isolate AN1, and found that the inhibition was parabolically competitive with respect to glutamate and noncompetitive towards $NADP^+$ (unpublished results). This surprising result raises questions as to the nature of the specificity of Procion Red HE-3B for GDH's and dehydrogenases in general.

6 The Kinetic Mechanism of the Glutamate Dehydrogenase Reaction

Although glutamate dehydrogenases from a vast number of sources have been studied, relatively few have been the subject of kinetic investigations. Hence for many GDH's, only apparent Km's (which may differ from the true kinetic constants) are known. The Km's (true or apparent) of the GDH's from a representative selection of organisms are listed in Table 4. As noted by Smith *et al.* (1975), there is a tendency for the Km's for 2-ketoglutarate, $NADP^+$, and NADPH to be quite similar for enzymes from a variety of sources, whereas Km's for glutamate, ammonia and NADH may differ widely. The Km's for glutamate and ammonia of the GDH's from a wide selection of organisms are presented in Table 5.

In this section an attempt is made to give a only broad overview of the mechanistic information available to date. The kinetic mechanism of the bovine liver GDH is well known as a result of extensive studies by many groups of researchers, and for detailed

information in this area readers are referred to Adams (1987), Fisher (1985), and Singh *et al.* (1993). GDH is a stereospecific enzyme (*Pro-S*, or B-specific); it binds NAD in *syn* conformation (and NADP in predominantly *syn* conformation) (Adams, 1987) and utilises and produces L-glutamate. Details of the stereospecificity are covered by Smith *et al.* (1975).

The mechanism proposed for the bovine liver GDH has been changed and modified as new information has come to light (see below, 6.1), hence it is difficult to know whether the mechanisms of GDH's from other sources are the same as that of the bovine liver enzyme, as they have not been so thoroughly investigated. However, the results of Le John *et al.* (1968) indicated that the NAD- and NADP-specific GDH's from *Thiobacillus novellus* use different mechanisms (see 6.4), which suggests that the mechanisms all GDH's are not necessarily the same. The presence of allosteric modifiers may influence the mechanism used by a GDH (this occurs with the bovine liver enzyme, see 6.1), as may pH (see 6.1), and possibly temperature (see 6.5).

6.1 Kinetic Studies of Vertebrate Glutamate Dehydrogenases.

The bovine liver GDH has been the subject of numerous kinetic studies which have been complicated by the deviations from Michaelis-Menten kinetics displayed by the enzyme. Barton and Fisher (1971) found that NAD^+, $NADP^+$ and glutamate all exhibit substrate inhibition when present in high concentrations and cause substrate activation at lower concentrations. The substrate activation by NAD^+ and $NADP^+$ is apparent from Lineweaver-Burk plots which show deviations towards higher activity with increasing cofactor concentration (Engel and Dalziel, 1969). When phosphate buffer, pH 7.0, was used, these plots had three or four linear regions (3 for $NADP^+$, 4 for NAD^+) separated by relatively sharp discontinuities (Engel and Dalziel, 1969). Engel and Dalziel explain this behaviour in terms of negative homotropic interactions between the GDH subunits, although the circular dichroism study of Bayley and O'Neill (1980) indicates that this behaviour may be in part due to a second molecule of $NADP^+$ binding at the ADP

TABLE 4

Km Values for some Glutamate Dehydrogenases

Source	Km (mM):							Reference
	NAD+	NADP+	Glutamate	NADH	NADPH	2-ketoglutarate	ammonia	
Bovine liver	0.7	0.047	1.8	0.024	0.025	0.7	3.2	Smith *et al.*, 1975
Frog liver	0.02	0.5	1.8	0.03	0.2	5.0	0.5	Fahien *et al.*, 1965
Turnip*	0.25	-	28.6	0.09	-	2.0	22.2	Itagaki *et al.*, 1988
*Neurospora crassa**	0.33	-	5.5	0.55	-	4.6	17.0	Veronese *et al.*, 1974
*Neurospora crassa**	-	0.05	45.0	-	0.125	5.3	10.0	Blumenthal & Smith, 1973
*Laccaria laccata**	-	0.03	26.0	-	0.01	1.0	5.0	Brun *et al.*, 1992
*Plasmodium lophurae**	-	0.0285	0.467	-	0.0625	0.143	0.0510	Sherman *et al.*, 1971
Clostridium SB4	0.01	-	1.8	0.01	-	0.65	0.32	Winnacker & Barker, 1970
Thiobacillus novellus	0.19	-	13.3	0.004	-	0.66	0.5	Le John *et al.*, 1968
Thiobacillus novellus	-	0.061	36.0	-	0.077	7.4	7.5	"
Escherichia coli (B/r)*	-	0.042	1.3	-	0.04	0.64	1.1	Sakamoto *et al.*, 1975
Mycobacterium smegmatis	-	0.029	62.5	-	0.045	5.0	33.0	Sarada *et al.*, 1980
*Streptomyces fraidiae**	-	0.12	28.6	-	0.07	1.54	30.8	Vancurova *et al.*, 1989

* Apparent Km's

regulatory site. The cofactor activation phenomena are not observed with the poorer substrate norvaline (Engel and Dalziel, 1969) or in the presence of saturating concentrations of ADP (Hornby *et al.*, 1984). In the reductive amination of 2-ketoglutarate, the reduced cofactors NADH and NADPH do not "activate" the enzyme in an analogous manner to the oxidised cofactors, but do inhibit at high concentrations (as a result of their binding to the reduced coenzyme regulatory site (see 4.1.1)) (Lark and Colman, 1990), as does 2-ketoglutarate (Engel and Dalziel, 1970).

Initial rate studies by Frieden (1959) suggested that the mechanism was sequential in both directions (i.e. all reactants bind to the enzyme prior to any release of product) and that the reductive amination followed a compulsory order mechanism in which NADPH was bound first, followed by ammonia and then 2-ketoglutarate. However, later studies using more sensitive methods indicated a random order rapid equilibrium mechanism[2] (Smith *et al.*, 1975). Initial rate and product inhibition studies of both reaction directions, all of which used a sensitive fluorometric method of reaction rate measurement, lent support to, but did not unequivocally prove, the random order rapid equilibrium model (Engel and Dalziel, 1969 and 1970; Engel and Chen, 1975). These studies ruled out all six possible compulsory order mechanisms for the reductive amination reaction, but the possibility of a compulsory order mechanism for oxidative deamination with the cofactor as the leading substrate could not be completely excluded (Engel and Chen, 1975). The results of the product inhibition experiments of Engel and Chen (1975) are not compatible with either a compulsory order or random order rapid equilibrium mechanism without the inclusion of several abortive complexes.

Stopped flow studies of the oxidative deamination reaction (using $NADP^+$ as the cofactor) by Colen *et al.* (1972) provided evidence for a random order rapid equilibrium mechanism[2] for this reaction. The equilibrium kinetic study (using isotopic exchange) of Silverstein and Sulebele (1973) also predicted such a mechanism, as did the steady state kinetic study of Barton and Fisher (1971). The studies of Colen *et al.* (1972) and Silverstein and Sulebele (1973) both indicated cooperativity in the formation of the

[2]A random order rapid equilibrium mechanism is used here to mean a mechanism in which substrates may bind to the enzyme in any order and in which the different binary complexes are in rapid equilibrium with each other. Silverstein and Sulebele (1973) refer to this as an alternative order mechanism.

enzyme-NAD(P)-glutamate ternary complex (as explained in Section 4.1.1). The formation of the abortive complexes enzyme-NAD(P)$^+$-2-ketoglutarate and enzyme-NAD(P)H-glutamate are indicated by a stopped flow study of the oxidative deamination reaction in the presence of 2-ketoglutarate (Colen, 1978) and by the equilibrium kinetic study of Silverstein and Sulebele (1973). Figure 2 shows the kinetic scheme proposed by Silverstein and Sulebele (1973), supported by their experiments and those of others (Barton and Fisher, 1971; Colen *et al.*, 1972; di Franco, 1974; Colen *et al.*, 1975; Colen, 1978). However, the studies of Rife and Cleland (1980b) using NADP(H) as the cofactor (initial velocity studies, dead-end inhibition studies with oxalylglycine, and deuterium isotope effects) indicate that the order of substrate addition for the reductive amination reaction is not entirely random: 2-ketoglutarate obligatorily binds the enzyme-NADPH complex before ammonia (this is in keeping with the proposed chemical mechanism, see below); there is also a preference for NADPH to bind the enzyme first, although 2-ketoglutarate may form a weak but kinetically competent binary complex with the enzyme. Rife and Cleland (1980b) also found that the dissociation of NADP$^+$ from the ternary product complex enzyme-NADP$^+$-glutamate is faster than that of glutamate.

Silverstein (1974) investigated the oxidative deamination of glutamate and of alanine (using isotopic exchange kinetics at equilibrium) at pH 8.8 and 9.5, and found that the mechanism is ordered with cofactor leading at pH 8.8, but is random at pH 9.5. Initial rate studies of the oxidative deamination of glutamate in the presence of saturating concentrations of ADP (which linearises the double reciprocal plots, as discussed above) (Hornby *et al.*, 1984) also indicted an ordered mechanism, but with glutamate leading. These results emphasise the sensitivity of kinetic properties to the environment of the enzyme.

Various schemes for the chemical mechanism of the glutamate dehydrogenase reaction are summarised by Adams (1987). Rife and Cleland (1980a) propose that the chemical mechanism of the reductive amination reaction involves the nucleophilic attack of neutral ammonia on the 2-ketoglutarate moiety of the enzyme-NADPH-2-ketoglutarate complex to form a carbinolamine which further reacts to give an enzyme-bound alpha-iminoglutarate intermediate. Srinivasan *et al.* (1988) are in agreement with this, and

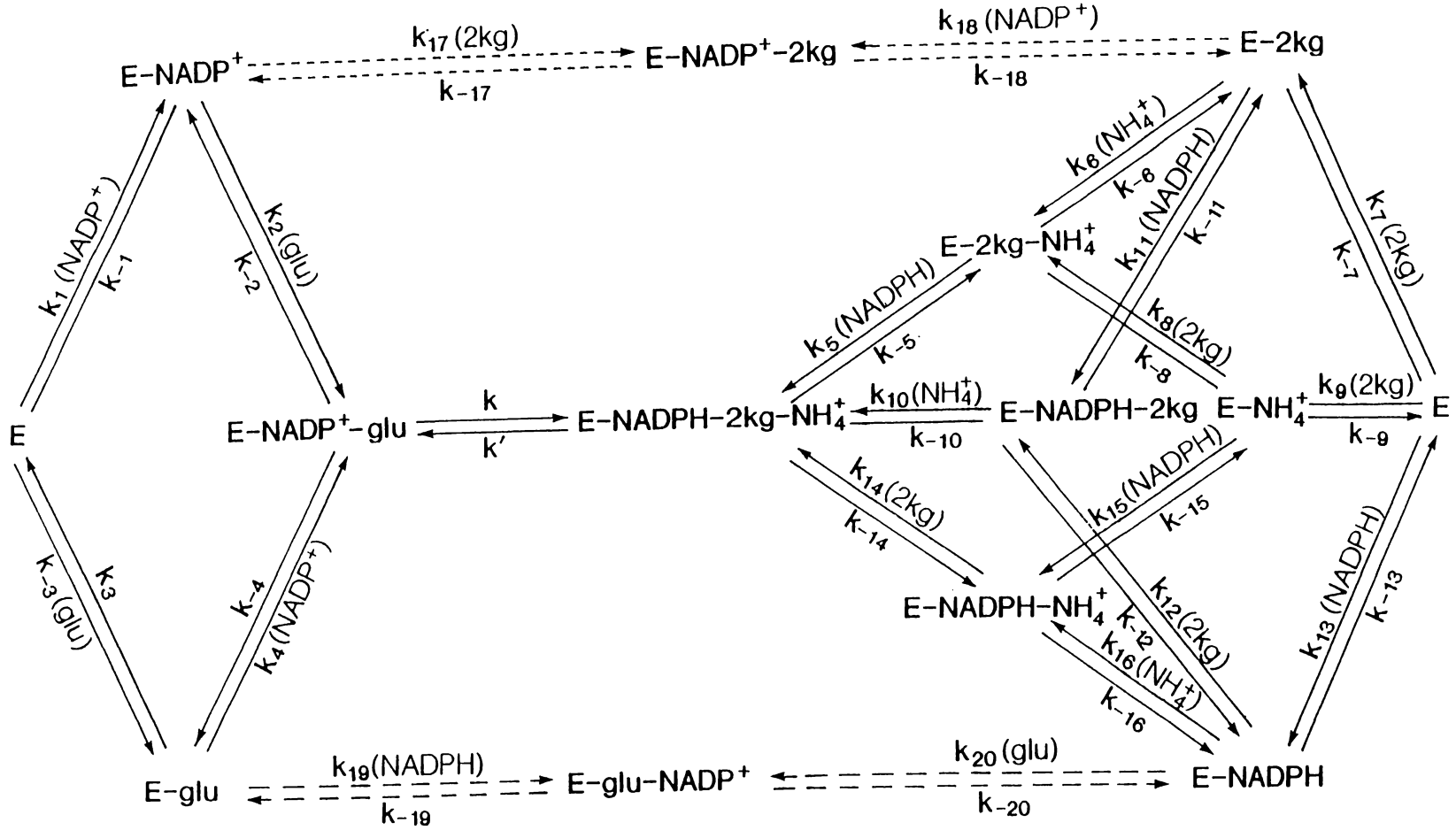
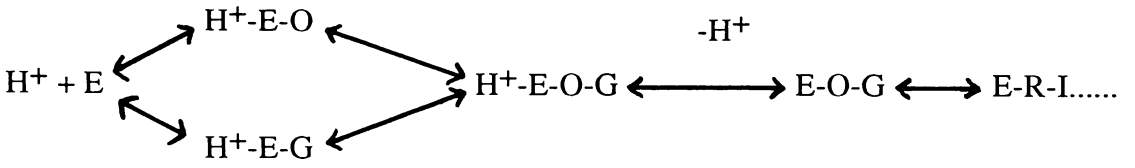


Fig. 2. Kinetic scheme for the bovine liver glutamate dehydrogenase reaction. Catalytically effective steps are indicated by solid lines and catalytically ineffective steps by broken lines. Although NADP(H) has been used in the figure, the scheme holds true for NAD(H) also. Abbreviations: glu, glutamate; 2kg, 2-ketoglutarate. Adapted from Silverstein and Sulebele (1973).

further propose a chemical basis by which the enzyme recognises ammonia. The binding of reduced coenzyme to the GDH is a prerequisite for the formation of α-iminoglutarate from 2-ketoglutarate (in the reductive amination direction), although hydride transfer is not involved (Fisher *et al.*, 1992). Fisher *et al.* (1992) used a stopped-flow indicator dye method to study the oxidative deamination of glutamate and were able to identify the slow release of a proton which preceded hydride transfer. Thus they have refined the reaction sequence to:



where E is the GDH, O is oxidised cofactor, G is glutamate and I is alpha-iminoglutarate. Hydride transfer occurs in the transition from E-O-G to E-R-I. The released proton does not come from the amino group of glutamate since a number of competitive inhibitors of L-glutamate, including isopthalate which does not contain nitrogen, also induced its release (Fisher *et al.*, 1992). Singh *et al.* (1993) used a novel transient state kinetic approach to achieve further insights into the oxidative deamination reaction.

There have been various reports as to which reaction step is rate limiting for the glutamate dehydrogenase (oxidative deamination) reaction. The equilibrium kinetic study of Silverstein and Sulebele (1973) suggested that the release of the cofactor from product complexes is rate limiting for the overall reaction when NAD^+ is the cofactor, but that the release of 2-ketoglutarate is rate limiting when $NADP^+$ is cofactor (unless the glutamate concentration is above 50 mM when NADPH release becomes rate limiting). The results of Colen *et al.* (1975) - who performed a stopped-flow study of D_2O solvent isotope effects - are in agreement with those of Silverstein and Sulebele, suggesting that release of 2-ketoglutarate from the ternary complex enzyme-NADPH-2-ketoglutarate is rate limiting in the steady state. However, di Franco (1974) concluded that the release of NADPH from the abortive complex enzyme-NADPH-glutamate[3] was rate limiting, and the results of d'Albis and Pantaloni (1972) also indicated that the dissociation of this complex was rate

[3] Glutamate displaces 2-ketoglutarate from the enzyme-NADPH-2-ketoglutarate ternary complex in phase III of the reaction. Fisher (1985) details the three spectrophotometrically distinct phases of the GDH reaction.

limiting when the concentration of glutamate was high. ADP activates the oxidative deamination of glutamate by $NADP^+$ because when it binds to the ADP regulatory site, the binding of NADPH at the active site is weakened hence its release from ternary complexes (rate-limiting when the concentration of glutamate is high) is increased (Fisher, 1985). Likewise, GTP inhibits glutamate oxidation because it decreases the rate of NADPH release. The situation with NAD(H) is more complex because both oxidised and reduced NAD may bind to the ADP regulatory site as well as to the active site (Fisher, 1985).

Early studies with frog (Fahien *et al.*, 1965) and dogfish liver GDH's (Corman *et al.*, 1967) predicted that the kinetic mechanism was the same as that found by Frieden (1959) for the bovine liver GDH (i.e. compulsory order). However, more detailed studies with the dogfish liver enzyme by Electricwala and Dickinson (1979) suggested either a rapid equilibrium random order mechanism or a compulsory order mechanism in which the binding order was ammonia, reduced cofactor and then 2-ketoglutarate.

6.2. Kinetic Studies on Plant Glutamate Dehydrogenases.

Stone *et al.* (1980a and b) carried out extensive steady state kinetic studies on the forward and reverse reactions catalysed by the NAD-specific GDH from lupin nodules. The Lineweaver-Burk plots drawn from initial velocity experiments (NAD^+ as variable substrate and glutamate held at varying fixed levels) showed two discontinuities separating linear regions when the concentration of the cofactor was varied over a wide range; this was attributed to substrate activation by NAD^+. Initial velocity studies were backed up by product inhibition and substrate analogue inhibition studies of both directions of reaction. Their results are qualitatively and quantitatively consistent with a fully ordered sequential mechanism in which NAD^+ is the leading substrate in the oxidative deamination reaction, and NADH is the leading substrate in the reductive amination reaction with 2-ketoglutarate and ammonia being the second and third substrates, respectively, to bind the enzyme. The

product inhibition studies indicated that 2-ketoglutarate forms an abortive complex with enzyme-NAD^+.

A compulsory order sequential mechanism the same as that found for the lupin nodule GDH (above) has been proposed for the GDH's from safflower seedlings (Errel *et al.*, 1973) and from soybean cotyledons (King and Wu, 1971). However, for both these kinetic studies, statistical analyses were not performed and the proposed mechanism was not the only one consistent with the results. The results of initial velocity and product inhibition studies on the NAD-specific GDH from pea (*Pisum sativum*) stem mitochondria although inconclusive, are inconsistent with a simple random order mechanism. A partially random mechanism (in which the order of addition of ammonia and 2-ketoglutarate is interchangeable) has been proposed (Garland and Dennis, 1977). The product inhibition pattern found for the pea stem enzyme (Garland and Dennis, 1977) is similar to that of the bovine liver GDH (Engel and Chen, 1975) which is now known to follow a random order rapid equilibrium mechanism (see above). It is of interest that Garland and Dennis (1977) added 1 mM calcium chloride to reaction mixtures for reductive amination, whereas Stone *et al.* (1980a and b) make no mention of adding divalent metal ions to the reaction mixtures in their steady state kinetic studies with the lupin nodule GDH (above).

In an interesting piece of applied kinetic work, Osuji *et al.*, (1991) compared the effects of 2-ketoglutarate on enzymes of ammonia salvage (glutamate synthase, GDH and aspartate transaminase) from protein deficient crops (sweet potato and yam tuber) and a protein rich crop (cream pea). Initial velocity experiments were carried out (using partially purified GDH) in which NADH was held at a constant concentration, ammonia was the variable substrate and 2-ketoglutarate was held at varied fixed levels. 2-Ketoglutarate did not cause substrate inhibition of GDH from cream pea, but competitively inhibited with respect to ammonia in the case of yam tuber GDH and inhibited the sweet potato GDH when present in high levels. Osuji *et al.* proposed that the effects of 2-ketoglutarate on yam tuber GDH (and also sweet potato glutamate synthase) may contribute to the tendency of these crops to lose ammonia during storage, something which does not occur with protein rich crops such as cream pea.

6.3. Some Kinetic Properties of *Neurospora crassa* Glutamate Dehydrogenase

Wootton (1983) found that for the NADP-specific GDH from *Neurospora crassa*, Lineweaver-Burk plots with ammonia as the variable substrate show downwardly convex deviations from linearity which are more pronounced at lower concentrations of 2-ketoglutarate. Rubidium ions (which have a similar ionic radius to ammonium ions) were also found to produce similar activating effects. Wootton explained these results by postulating that ammonium ions, and also rubidium ions, may bind to a cation binding site separate from the active site thus causing an increase in the Vmax and the Km for ammonia. He also pointed out that this phenomenon, which may be a mechanism that protects against the toxic effects of high intracellular ammonium concentrations, may be responsible for the high Km values reported for NADP-linked GDH's from *N. crassa* and other organisms. At very high concentrations of ammonium chloride (>0.3 M) the *N. crassa* NADP-specific GDH is inhibited (Barratt and Strickland, 1963).

6.4.Kinetic Studies on Glutamate Dehydrogenases from Eubacteria

Syed *et al.* (1991) carried out a steady state kinetic analysis of the oxidative deamination and reductive amination catalysed by the NAD-dependent GDH from *Clostridium symbiosum*, an enzyme for which the three dimensional structure has been deduced by X-ray studies (Rice *et al.*, 1987). Syed *et al.* found that this GDH is activated by high concentrations of NAD^+, as is the case for the bovine enzyme, with the Eadie plot showing two linear regions corresponding to concentrations of NAD^+ above and below 60 μM. For the kinetic determinations, NAD^+ concentrations in this higher range were used. The results of initial velocity studies of the oxidative deamination reaction established that the mechanism is sequential, but did not distinguish between random and compulsory order mechanisms. For the reductive amination reaction on the other hand, a random order rapid equilibrium mechanism is the only mechanism compatible with the results of the

initial velocity studies (finite values could be calculated for all eight initial rate parameters of Dalziel's (1975) general rate equation for a three substrate reaction), compulsory order and enzyme substitution mechanisms being ruled out.

For the *Clostridium* SB_4 GDH, initial velocity studies of the oxidative deamination reaction also established that the mechanism was sequential (Winnacker and Barker, 1970). Initial velocity studies of the reductive amination reaction yielded Lineweaver-Burk plots which were intersecting when NADH was held at saturating concentration but parallel when either 2-ketoglutarate or ammonia were held at high and constant concentrations (Winnacker and Barker, 1970). These results qualitatively indicate that the binding order of 2-ketoglutarate and ammonia is interchangeable with NADH being the first (or less plausibly last) substrate to bind the enzyme.

A steady state kinetic study was carried out by Le John *et al.* (1968) on both the GDH's from *Thiobacillus novellus*. The influence of AMP on the NAD-specific GDH is interesting: the enzyme displayed linear kinetics for the oxidative deamination reaction only at high substrate concentrations or in the presence of AMP, while the kinetics of the reductive amination reaction were linear in the presence or absence of AMP although AMP increased the Michaelis constants for the reductive amination substrates by an order of magnitude. On the basis of qualitative examination of Lineweaver-Burk plots obtained from initial velocity studies (in the absence of statistical analysis), the authors proposed that the NADP-specific GDH follows an ordered sequential mechanism with the cofactors as leading substrates and ammonia and 2-ketoglutarate being the second and third substrates, respectively, to bind the enzyme in the reductive amination reaction. The results for the NAD-specific GDH, in the presence of AMP, were analogous, except that the order of addition of ammonia and 2-ketoglutarate appeared to be reversed. The product inhibition data did not suggest the presence of abortive complexes. Although each enzyme was found not to possess transhydrogenase activity, the authors proposed that the NAD- and NADP-specific GDH's may act in unison to produce NADH in favour of NADPH. They suggested that AMP - generated from ATP produced ultimately as a result of glutamate catabolism - would regulate the NAD-GDH in favour of continuous glutamate catabolism.

Initial velocity studies have been performed on both directions of the reaction catalysed by the NADP-specific GDH from *Mycobacterium smegmatis* CDC 46 (Sarada *et al.*, 1980). The authors concluded that the mechanism was sequential and ordered, with the order of substrate addition in the reductive amination reaction being NADPH, followed by ammonia and then 2-ketoglutarate. NADPH caused a quenching of the protein fluorescence spectrum of the GDH at 340 nm (ammonia and 2-ketoglutarate had no effect), suggesting that this substrate may bind the free enzyme. However, the conclusion of an ordered mechanism on the basis of initial velocity and fluorescence studies alone is probably premature, although the initial velocity studies did establish that the mechanism is sequential.

6.5. Some Kinetic Properties of Glutamate Dehydrogenases from Archaebacteria

To date, there has been no published report of a complete kinetic study performed on an archaebacterial glutamate dehydrogenase. However, a steady state kinetic study has been performed on the oxidative deamination and reductive amination reactions catalysed by the NADP-specific GDH from the archaebacterial *Thermococcaceae*, isolate AN1 (Hudson *et al.*, unpublished results). Apparent Km's for six characterised archaebacterial GDH's are known and have been included in Table 3.

The three characterised GDH's from *Halobacterium* sp. display Michaelis-Menten kinetics (Bonete *et al.*, 1986 and 1987; Leicht *et al.*, 1978). Schinkinger *et al.* (1991) and Robb *et al.* (1992) also reported linear kinetics for the GDH's from *Sulfolobus solfataricus* and *Pyrococcus furiosus*, respectively, but Consalvi *et al.* (1991a and b) - who used a wide range of substrate concentrations for their Km determinations - reported departures from Michaelis-Menten kinetics for these two enzymes. These authors found that NADPH caused substrate inhibition of the *S. solfataricus* GDH (above 0.25 mM), as did glutamate with the *P. furiosus* GDH (above 15 mM), while 2-ketoglutarate caused the substrate inhibition of both enzymes (above 20 and 2.0 mM, respectively). Apparent negative cooperativity for $NADP^+$ (in the case of the *S. solfataricus* GDH) and for ammonia (in the case of both the *S. solfataricus* and *P. furiosus* GDH's) was suggested by Hill coefficients

less than unity (Consalvi *et al.*, 1991a and b). At higher concentrations of ammonia the Vmax for these enzymes was increased, but by a lesser magnitude than the increase in the apparent Km for ammonia, indicating that the enzymes are not activated by ammonia but that higher ammonium concentrations are required to saturate the enzyme when the ammonia concentration is high (Consalvi *et al.*, 1991a and b). Consalvi *et al.* (1991a and b) calculated the apparent Km's of these enzymes for ammonia from the two linear regions (corresponding to high and low ammonia concentrations) seen in the Lineweaver-Burk plots; these values are reported in Table 3.

On the basis of initial velocity studies and inhibition studies with products and substrate analogues, the GDH from Isolate AN1 appears to follow a steady state random order mechanism. The effect of substrate saturation on the initial velocities of the reductive amination reaction, and the inhibition studies, indicated a preferred order of substrate addition of $NADP^+$, glutamate for the oxidative deamination, and NADPH, 2-ketoglutarate, ammonia for the reductive amination reaction (Hudson *et al.*, unpublished results). This study was performed at 60°C, because the instability of the oxidised cofactor precludes the acquisition of accurate kinetic data at the growth temperature of the organism (75-80°C). Therefore it is quite possible that at a higher temperature the enzyme-substrate complexes would interchange rapidly, thereby making the mechanism conform to a rapid-equilibrium random type.

7 Conclusions

With few exceptions, GDH's fall into one of three oligomeric classes: hexamers with a subunit molecular weight of around 50K, tetramers with a subunit molecular weight around 50K, and tetramers with a subunit molecular weight of 100K or greater. This last group is comprised entirely of NAD-linked GDH's from fungi which have been suggested to be the products of linked genes. Sequence comparisons of the hexameric enzymes have revealed a high degree of structural similarity, regardless of their coenzyme specificity (Lilley *et al.*, 1991). Britton *et al.*, (1992) compared the sequence of the tetrameric NAD-linked GDH from *Neurospora crassa* with the sequences of a variety of hexameric GDH's,

including that from *Clostridium symbiosum* GDH, for which the three dimensional structure is known. Their analysis showed that the residues conserved between the *N. crassa* and the hexameric GDH's are clustered in the three-dimensional structure and suggested that the active site structures are similar for both tetrameric (large subunit) GDH's and hexameric GDH's. Tetrameric GDH's with small subunits (~50K) (certain eubacterial, archaebacterial, algal and plant GDH's fall into this category) were not included in this study.

The vertebrate GDH's form a homologous group with respect to subunit structure, amino acid sequence (there is a 95% identity between bovine and chicken GDH's (Moon *et al.*, 1973)), coenzyme specificity and regulatory properties. The C-termini of vertebrate GDH's have an amino acid sequence which is distinctly different from that of non-vertebrate GDH's. Among the non-vertebrate enzymes, plant GDH's (which display metal ion activation) and NAD-linked fungal GDH's appear to be relatively homogeneous subsets. Although only a few archaebacterial GDH's have been studied, with respect to coenzyme specificity and subunit structure, they do not appear to form a closely related group. The relationship between archaebacterial GDH's and eubacterial and eukaryotic GDH's will be a fruitful area for further study.

The Km values for glutamate and ammonia for a wide variety of NAD-specific, NADP-specific and dual coenzyme specific GDH's are presented in Table 5. It has been suggested by some authors that the relative Km values of the substrates of oxidative deamination and reductive amination are evidence of a particular *in vivo* reaction direction. However, induction by ammonia or repression by glutamate, or visa versa, is probably a more reliable indication of the *in vivo* reaction direction, as in most cases the inducer will be an *in vivo* substrate and the repressor an *in vivo* product. Such inductions and repressions are indicated in Table 5. The cofactor specificity of a GDH is also an indication of its *in vivo*, reaction direction. An inspection of Table 5, however, reveals several cases where Km's for glutamate and ammonia are similar, and several cases where the relative magnitudes of these Km's are not what would be expected on the basis of coenzyme specificity and/or on the basis of induction/repression data. Hence it is probably not valid to cite Km data as "proof" of a particular *in vivo* reaction direction, especially

since the common occurrence of nonlinear kinetics for ammonia is a further complicating factor. There are cases, however, where the Km for one substrate is so high that it is most unlikely it ever acts as a substrate *in vivo*: for example, it is unlikely that the NAD- and NADP-specific GDH's from the archaebacterium *Halobacterium halobium* catalyse reductive amination or oxidative deamination, respectively, *in vivo*, since the former has a very high Km for ammonia while the latter has an extremely high Km for glutamate (see Table 5).

It is notable that the activation of the isolate AN1 GDH by divalent metal ions is unidirectional (only the oxidative deamination reaction is activated), therefore may play a part in determining the *in vivo* reaction direction of this enzyme. Divalent metal ions may also play a part in determining the *in vivo* reaction direction of plant GDH's (Itagaki *et al.*, 1988). Since higher cellular levels of magnesium ions represent lower energy charge (due to ADP being a poorer chelator of magnesium ions than ATP), the response of some GDH's to metal ions may be a simple mechanism by which these enzymes respond to energy charge. The allosteric ADP-activation seen with vertebrate GDH's is a more complicated means of enzyme response to energy charge (it requires the development of allosteric sites), and probably evolved relatively recently.

The *in vivo* reaction directions of GDH's in archaebacteria are of particular interest, since high levels of GDH have been found in two archaebacterial species: *Pyrococcus furiosus* (16% of the soluble cell protein) (Consalvi *et al.*, 1991b) and isolate AN1 (~10% of the soluble cell protein) (Hudson *et al.*, 1993), both of the family *Thermococcaceae*. Unfortunately, the inability of these organisms to grow on defined media hampers induction/repression experiments. Whatever the *in vivo* reaction direction of these archaebacterial GDH's, considering the very high levels present, it seems likely that they play an important role in these organisms. It is hoped that future experiments (such as the inactivation GDH *in vivo* through the use of a specific reagent or genetic engineering) will shed light on what this role may be.

TABLE 5

Km Values for Glutamate and Ammonia[a]

Source	Km for glutamate (mM)	Km for ammonia (mM)	Reference
Dual specific GDH's			
Bovine liver	1.8	3.2	Smith *et al.*, 1975
Dogfish liver	84	4.5	Corman *et al.*, 1967
Frog liver	1.8	0.5	Fahien *et al.*, 1965
Human brain	17.7		Hussain *et al.*, 1989
Rat brain	3.6	18.3	Colon *et al.*, 1986
Lemna minor[b]	12	15, 27	Ehmke and Hartmann, 1976
	2.5	29	Stewart and Rhodes, 1977
Chlamydomonas reinhardtii[c]			
isozyme 1	0.64	36, 30 (I)	Moyano *et al.*, 1992a
isozyme 2	3.52	53, 13	"
isozyme 3	3.00	41, 44	"
Azospirillum brazilense[d]	10	0.38, 100; 66 (I)	Maulik and Ghosh, 1986
Bacteroides distasonis		2.4	Yamamoto *et al.*, 1987
Bacteroides fragilis		1.7, 5.1	"
Bacteroides ovatus		2.9	"
Bacteroides thetaiotaomicron[e]	3.7, 12.3	5.0, 15	Glass and Hylemon, 1980
Bacteroides vulgatus		1.5	Yamamoto *et al.*, 1987
Mycoplasma laidlawii[f]	32.5, 20	5.5, 30	Yarrison *et al.*, 1972
Sulfolobus solfataricus	2.5 (R)	4.2, 34	Consalvi *et al.*, 1991a
Pyrococcus furiosus	0.6	6, 27	Consalvi *et al.*, 1991b
NAD-specific GDH's			
Pisum sativum	9.3	52.6	Kindt *et al.*, 1980
Brassica rapa	28.6	22.2	Itagaki *et al.*, 1988
Lupin nodules	5+/-2	86, 800, 1010	Stone *et al.*, 1980
Stichococcus bacillarius naeg.		33	Ahmad and Hellebust, 1986
Symbiodinium microadriaticum	39	5, 35	Dudler and Miller, 1988
Apodachyla	7.3 (I)	21	Price and Gleason, 1972
Candida utilis[g]	20, 128		Hemmings, 1980
Neurospora crassa	5.5	17	Smith *et al.*, 1975
Phycomyces blakesleeanus (spores)	10.7	10.8	van Laere, 1988
Trypanosoma cruzi	11	18	Cazzulo *et al.*, 1979
Bacillus stearothermophilus	18	15	Mantsala, 1985
Clostridium botulinum 13B	5.3	243	Hammer and Johnson, 1988
Clostridium SB_4	1.8	0.32	Winnacker and Barker, 1970
Peptostreptococcus assacharolyticus	6 (I)	184	Hornby and Engel, 1984
Pseudomonas aeruginosa		15	Janssen *et al.*, 1980
Thiobacillus novellus	13.3	0.5	Le John *et al.*, 1968
Halobacterium halobium	4	450	Bonete *et al.*, 1986
NADP-specific GDH's			
Acropora formosa	18, 81	9.2, 416	Catmull *et al.*, 1987
Anthopleura xanthogrammica	4.7	61	Male and Storey, 1983
Chlorella sorokiniana			
alpha-isozyme	38.2	3.3 (I, low NH_4^+)	Bascomb and Schmidt, 1987
beta-isozyme	32.3	77 (I, high NH_4^+)	"
Euglena gracilis		0.4	Javed and Merrett, 1986
Stichococcus bacillarius naeg.		1.6	Ahmad and Hellebust, 1986

Symbiodinium microadriaticum		<1, 200	Dudler and Miller, 1988
Cenococcum graniforme		2, 8	Martin *et al.*, 1983
Laccaria laccata	26	5	Brun *et al.*, 1992
Neurospora crassa	45 (R)	10 (I)	Smith *etal.*, 1975
		0.6-1.11	Wootton, 1983
		5.3-11.9	"
Saccharomyces cerevisiae	10	11	Smith *et al.*, 1975
Sphaerostilbe repens	5.5	2.6, 21.2	Botton and Msatef, 1983
Plasmodium lophurae	0.467	0.0510	Sherman *et al.*, 1971
Trichomonas vaginalis	1.2		Turner and Lushbaugh, 1988
Trypanosoma cruzi	1.5	8.7	Cazzulo *et al.*, 1977
	2.0	1.7, 66	Juan *et al.*, 1978
	1.3	2.8	Walter and Ebert, 1979
	2.5	0.42	Caldas *et al.*, 1980; Carneiro and Caldas, 1983
Bacillus fastidiosus	44	6.5, 20	op den Camp *et al.*, 1989
Bacillus licheniformis	39 (R)	5.5	Phibbs and Bernlohr, 1971
Bacillus megaterium	29	22 (I)	Hemmila and Mantsala, 1978
a thermophilic *Bacillus* sp.	11	21	Epstein and Grossowicz, 1975
Escherichia coli	2.2 (R)	36 (I)	Lin and Reeves, 1991b
Hyphomicrobium X	10.2	0.5, 3.3	Duchars and Attwood, 1987
Lactobacillus fermentum	79	6.76	Misono *et al.*, 1985
Methylobacillus flagellatum	7	11.5	Kiriukhin *et al.*, 1992
Mycobacterium smegmatis	62.5	33	Sarada *et al.*, 1980
Nitrobacter agilis		6.3, 33	Kumar and Nicholas, 1984
Nitrosomonas europaea	6.7	16	Hooper *et al.*, 1967
Paracoccus denitrificans	1.5	14	Kremeckova *et al.*, 1992
Phormidium laminosum[h]	(I)	18.2 (I)	Martinez-Bilbao *et al.*, 1988
Pseudomonas aeruginosa		2	Janssen *et al.*, 1980
Pseudomonas sp. strain AM1	31.6	20.2 (I)	Bellion and Tan, 1984
Ruminococcus flavefaciens	62	19	Duncan *et al.*, 1992
Salmonella typhimurium	50	0.29	Coulton and Kapoor, 1973b
Streptomyces fraidiae	28.6	30.8	Vancurova *et al.*, 1989
Synechocystis PCC 6803		3.7 (I)	Florencio *et al.*, 1987
Thiobacillus novellus	35.5	7.5	Le John *et al.*, 1968
Halobacterium sp. of the Dead Sea	14.2	16.6	Leicht *et al.*, 1978
Halobacterium halobium	4600	3.1	Bonete *et al.*, 1987
Isolate AN1	9.12	15.5	Hudson *et al.*, 1993

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a: No distinction was made here between true and apparent Km's, the majority being apparent Km's. Where 2 Km values are given, they apply to low and high concentrations of substrates (nonlinear kinetics are implied), unless otherwise stated. An I or R in brackets after a Km value indicates that the substrate in question induces or represses the enzyme, respectively.

b: The 2 Km's given for ammonia were determined with NADPH (first value) and NADH (second value). The Km for glutamate was determined with NAD^+.

c: The 2 Km's given for ammonia were determined with NADPH (first value) and NADH (second value). The Km's for glutamate were determined with NAD^+.

d: Values of 0.38 and 100 mM were determined as the apparent Km's for ammonia with NADPH as the cofactor; the value of 66 mM was with NADH as cofactor. The value for glutamate was determined with $NADP^+$.

e: These values are $[S]_{0.5}$ values. The first value given for glutamte was determined with $NADP^+$ as cofactor, the second was with NAD^+ as cofactor. The values for ammonia were determined with NADPH (nonlinear kinetics).

f: The 2 Km's given for both glutamate and ammonia were determined with NADP(H) (first value) and NAD(H) (second value).

g: The first Km value is for the dephospho form, the second for the phospho form of the enzyme.

h: The kinetics for ammonia were nonlinear. The value of 18.2 is an $[S]_{0.5}$ value obtained from a Hill plot.

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CHAPTER 2

GLUTAMATE DEHYDROGENASE FROM THE EXTREMELY THERMOPHILIC ARCHAEBACTERIAL ISOLATE AN1

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Abbreviations Used: EPPS, N-[2-hydroxyethyl]-piperazine-N'-[3-propane-sulfonic acid]; CHES, 2-[N-cyclohexylamino]-ethanesulfonic acid; B.S.A., bovine serum albumin; MOPS, 3-[N-morpholino]-propane-sulfonic acid.

ABSTRACT

Glutamate dehydrogenase (L-glutamate: $NADP^+$ oxidoreductase, deaminating and transaminating, EC 1.4.1.4) was purified to homogeneity from the extremely thermophilic archaebacterial isolate AN1 (a member of the Thermococcales). The enzyme comprised a large proportion of the soluble cell protein (11%) and was purified in high yield. The Mr of the native enzyme was 204,000, while the subunit Mr was 47,000, indicating a tetrameric structure.The enzyme is specific for NADP(H) rather than NAD(H) by a factor of greater than 1000, as judged by Vmax/Km. Glutamate synthase activity was about 50% of the glutamate dehydrogenase activity. Activity was markedly enhanced by calcium, magnesium and manganese ions. The enzyme was highly thermostable with $t_{1/2}$ values of 12.5 h and 47 min at 90°C and 103°C respectively.

INTRODUCTION

Glutamate dehydrogenases are important enzymes in that they provide a link between carbon and nitrogen metabolism, and they have been extensively studied in both eukaryotes and eubacteria. However, few glutamate dehydrogenases have been studied from the archaebacterial kingdom. Bonete *et al.* [1,2] and Leicht *et al.* [3] studied glutamate dehydrogenases from *Halobacterium halobium* and a *Halobacterium* isolated from the Dead Sea respectively. Glutamate dehydrogenases from *Sulfolobus solfataricus* and *Pyrococcus furiosus* [4,5,6] are the only glutamate dehydrogenases from extremely thermophilic archaebacteria which have been described to date.

Isolate AN1 is an extremely thermophilic, sulphur-reducing archaebacterium which was isolated from a geothermal spring in Rotorua, New Zealand [7]. It has a temperature optimum for growth of 75-80°C and is a member of the Thermococcales [8,9].

This work describes the purification to homogeneity and some properties of a glutamate dehydrogenase from this organism.

MATERIALS AND METHODS

Growth of Bacteria

Isolate AN1 was grown anaerobically under nitrogen with shaking at 75°C in 1 or 2 l Schott bottles. The medium contained (g/l) trypticase peptone 8, sodium chloride 2.5, dipotassium hydrogen phosphate 1.5, magnesium chloride hexahydrate 0.3, sodium thioglycollate 1 and elemental sulphur 7.5. The pH of the medium was adjusted to 7.4 at room temperature. Reduction was indicated by the inclusion of 1 ml/l of a 0.1% resazurin solution.

The cells were harvested by centrifugation at 13,000 x g (r av. 11.9 cm) for 15 min. Cells were stored at -70°C until required.

Preparation of Cell-Free Extract

The cells (41g wet weight) were re-suspended in 164 ml of 0.1M-EPPS-NaOH pH_{20} 7.85 and sonicated on ice for 3 periods of 5 minutes at 80% relative output using a Dynatech Sonic Dismembranator fitted with a 1/2 inch titanium tip. The cell debris was removed by centrifugation at 150,000 x g for 1.5 h at 4°C in an MSE Prepspin 50 centrifuge.

Purification of Glutamate Dehydrogenase

All steps were performed at room temperature (approximately 20°C) unless otherwise stated.

Reactive Red-120 was linked to Sepharose CL-6B by the method of Scopes [10].

The dye matrix column (16.5 x 5 cm) was equilibrated with 0.1M-EPPS-NaOH pH_{20} 7.85. Cell-free extract (600 ml containing 1.2 mg/ml protein) from 41 g wet weight cells was loaded onto the column which was then washed with 650 ml buffer and then eluted using buffer containing 0.1 mM-$NADP^+$. Active fractions were pooled and ultrafiltered in order to remove the $NADP^+$ and reduce the volume to 35 ml.

Concentrated enzyme was reloaded onto the 16.5 x 5 cm Reactive Red Sepharose CL-6B column, again equlibrated with 0.1M-EPPS-NaOH pH_{20} 7.85. The glutamate dehydrogenase activity was this time eluted with a 2 l linear salt gradient (0-0.7M-NaCl in 0.1M-EPPS-NaOH pH_{20} 7.85). Active fractions were pooled and stored at -70°C for 1 month. This material was then thawed and dialysed against 2x5 l 0.01M-NaH_2PO_4-Na_2HPO_4 pH_{20} 6.8 for 24 h at 4°C. The non-diffusible material was concentrated by ultrafiltration and loaded onto a 16 x 2.5 cm hydroxyapatite column equilibrated with 0.01M-NaH_2PO_4-Na_2HPO_4 pH_{20} 6.8. Glutamate dehydrogenase activity was eluted with a 400 ml linear gradient of 0.01-0.4M-NaH_2PO_4-Na_2HPO_4 . Active fractions were pooled and stored at -70°C in 50mM-EPPS-NaOH pH_{20} 7.9.

Enzyme Assays

Glutamate dehydrogenase was routinely assayed at 80°C in 0.1M-NaH_2PO_4-$Na_2HPO_4$$pH_{80}$ 7.54 by measuring the absorbance of NADPH at 340 nm for 1 minute. The assay mixture for oxidative deamination contained 0.3 mM-$NADP^+$ and 100 mM-monosodium L-glutamate, while that for reductive amination contained 0.2 mM-NADPH, 20 mM-2-ketoglutarate and 150 mM-ammonium chloride.The assay volume was 1.5 ml. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol NAD(P)(H) per minute under the assay conditions. The literature values for the millimolar extinction coefficients of the reduced cofactors (6.22 for NADPH; 6.2 for NADH) were used in all activity calculations.

Assays to monitor the glutamate dehydrogenase levels during the purification were performed similarly, but at 60°C in 0.1M-EPPS-NaOH buffer, pH_{60}7.36, containing 0.3 mM-$NADP^+$ and 20 mM-monosodium L-glutamate. Assays which contained metal ions used the same method with a buffer of 10 mM-MOPS-NaOH pH_{60}7.1, in order to prevent the formation of metal phosphates or hydroxides. Sodium chloride was used for the ionic strength controls.

The pH optima were determined at 60°C rather than 80°C in order to minimise the autodegradation of NADPH at high temperature and acidic pH. The buffers used were 90 mM-NaH_2PO_4-Na_2HPO_4 (pH 5.75-8.25), 90 mM-HEPES-NaOH (pH 6.22-7.85) and 90 mM-CHES-NaOH (pH 7.76-10.05). The pH of the buffers was measured at 60°C in the presence of 20 mM-monosodium L-glutamate (for oxidative deamination) or 0.1 M-ammonium chloride and 20 mM-2-ketoglutarate (for reductive deamination). The cofactor (0.3 mM-$NADP^+$ or 0.2mM-NADPH) was added immediately prior to starting the reaction with enzyme.

Control assays were performed to check for activity in the absence of enzyme, and, where unpurified enzyme preparations were used, for activity in the absence of glutamate. Reaction rates were corrected for such activities.

Thermostabilities

Samples of glutamate dehydrogenase (30µl) at a protein concentration of 0.069 mg/ml were sealed in glass capillary tubes. The tubes were incubated submerged under water for temperatures less than 100°C or under silicone oil for higher temperatures. Duplicate tubes were removed at the required time intervals and immediately cooled in ice/water. Enzyme activity remaining was determined by assaying at 60°C.

Purity and Mr Determinations

Gel electrophoresis was performed using the Pharmacia Phast System Gel (Pharmacia, Uppsala). Sample preparation and silver staining followed procedures recommended by the manufacturer. Subunit Mr was determined by SDS-PAGE on 10-15% gradient gels. IEF was performed using IEF 3-9 gels.

The native Mr of the glutamate dehydrogenase was determined by FPLC on a Superose 6 HR 10/30 column. The standard proteins were apoferritin, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase and cytochrome c. The void volume was determined with blue dextran and the column buffer was 20 mM-bistrispropane pH 6.8 containing 0.15 M-sodium chloride.

RESULTS

Enzyme Purification

Glutamate dehydrogenase was purified 7-fold with about 90 % yield (Table 1). No increase in specific activity was achieved in the final step, but it removed minor contaminating proteins. After hydroxyapatite chromatography no contamination could be detected on silverstained polyacrylamide or IEF gels loaded with 0.03 µg of protein. Glutamate dehydrogenase was found to constitute 11% of the soluble cell protein (mean of 4 experiments), and about 0.1% of the wet cell weight, accounting for the low degree of purification.

The enzyme had specific activities at 80° C of 464 and 2940 units/mg for .the oxidative deamination and the reductive amination reaction, respectively.

Coenzyme Stability

Isolate AN1 grows optimally at 75-80°C, but NADH has been reported [11] to be unstable at this temperature.

In 0.1M-Na_2HPO_4-NaH_2PO_4 pH_{80}7.54, the degradation rate of 0.03mM-NADPH was 0.96 or 1.71 nanomoles/min, in a 1.5 ml volume, at 80 and 90°C respectively (measured by monitoring the decrease in A_{340} over a 10 minute period). The degradation rate was not slowed by the presence of enzyme, nor by 2mM-dithiothreitol. The end product of thermal decomposition is apparently not the oxidised form of the coenzyme, since attempts to regenerate NADPH after thermal decomposition using glutamate dehydrogenase plus glutamate were unsuccessful.

TABLE 1

PURIFICATION OF GLUTAMATE DEHYDROGENASE FROM AN1

Purification Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Overall purification (fold)
1) Cell-free extract	719	5237	7.28	100	1
2) Red Sepharose ($NADP^+$ Elution).	110	5115	46.5	98	6.4
3)Red Sepharose (NaCl elution).	91	4975	54.7	95	7.5
4) Hydroxyapatite	91	4677	51.4	89	7.1

Experimental details are given in the text. The enzyme activity is that assayed with $NADP^+$ as cofactor at 60°C. At 80°C, in 0.1M Na_2HPO_4-NaH_2PO_4 pH_{80}7.54, the specific activity of the enzyme from step 4 is 464 units/mg protein.

Physical Properties

The subunit Mr of glutamate dehydrogenase was found by SDS PAGE to be 47,000. The native Mr was determined to be 204,000 by gel filtration on a FPLC Superose 6 column, indicating a tetrameric structure. The isoelectric point was found to be approximately 4.0.

Other Properties

The pH optima were approximately 8.25 for both oxidative deamination and reductive amination. The pH profiles obtained are shown in Figure 1.

The presence of 1 mM AMP, ADP, ATP or GTP had no effect on the activity of the glutamate dehydrogenase in either reaction direction, when the concentration of reactants was saturating. When the concentration of reactants was subsaturating, (5 mM-monosodium-L-glutamate and 0.1 mM-$NADP^+$ or 3 mM-2-ketoglutarate, 10 mM-NH_4Cl and 0.1 mM-NADPH), 1 mM-ATP produced a 10% increase in the activity measured for oxidative deamination, while 1 mM-ADP or ATP stimulated the activity of the reductive amination reaction by 10%. 1 mM-GTP, NAD, AMP or PEP did not effect the activity measured in either reaction direction with a subsaturating concentration of reactants.

The activity of the glutamate dehydrogenase was markedly affected by increasing ionic strength. For the oxidative deamination reaction, this effect was more pronounced in zwitterionic buffer (10 mM-MOPS-NaOH pH 7.1) than in phosphate buffer (10 mM-Na_2HPO_4-NaH_2PO_4 pH 7.54). In the former buffer, the activity reached a maximum

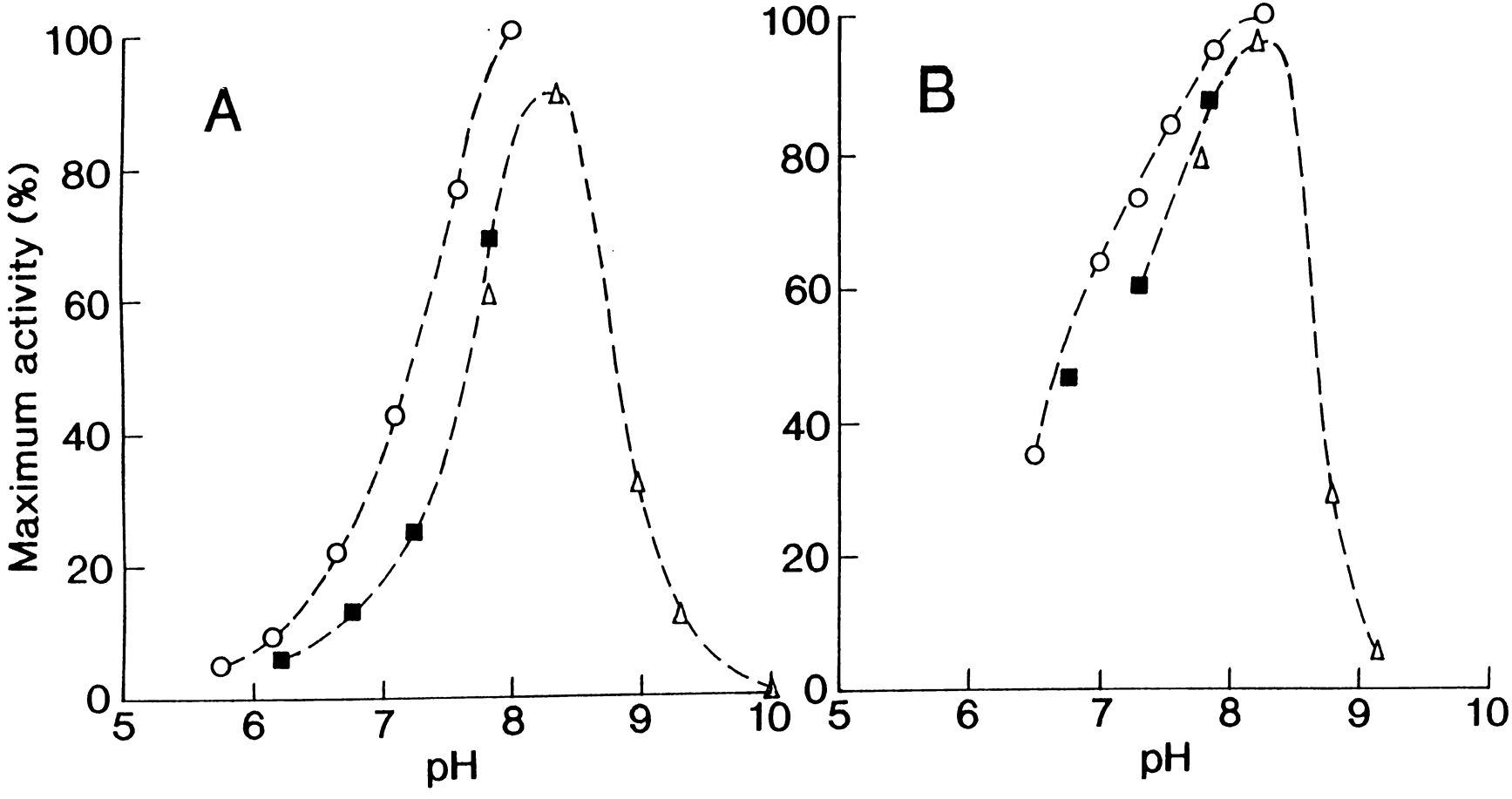

Fig. 1. The pH profile of the glutamate dehydrogenase from isolate AN1. **A**: oxidative deamination, **B**: reductive amination. Circles: Na_2HPO_4-NaH_2PO_4 buffer; squares: HEPES-NaOH buffer; triangles: CHES-NaOH buffer.

(approximately 430% of the activity of the control solution which did not contain NaCl) at 0.3-0.4 M-NaCl. The effect of KCl was indistinguishable from that of NaCl.

The effects of metal ions on the oxidative deamination reaction are summarised in Table 2. A low ionic strenghth buffer (10 mM MOPS-NaOH) and a lower concentration of monosodium L-glutamate (20 mM) were used in these experiments in order to minimise the concentration of sodium ions in the assay mixture. $CaCl_2$, $MgCl_2$ and $MnCl_2$ also activated the enzyme when 0.1 M-MOPS-NaOH buffer containing 0.1 M monosodium L-glutamate was used (data not shown), but higher concentrations of the metal ions were required to produce an effect. The chelating agents EDTA, 8-hydroxyquinoline, sodium azide and *o*-phenanthroline, at a concentration of 5 mM, had no effect on enzyme activity.

The effects of metal ions on the activity of the enzyme in the reductive amination reaction were relatively small.

Substrate Specificity

The glutamate dehydrogenase was specific for NADP(H). Although some activity with NAD(H) was also found, the apparent Km values were very high compared to those for NADP(H) (see Table 3). A comparison of kinetic parameters showed that at 80°C, the values of Vmax/apparent Km (units/mg/mM) were 17270, 0.605, 48660 and 159 for $NADP^+$, NAD^+, NADPH and NADH respectively (the Vmax values used in these

TABLE 2

EFFECT OF METAL IONS ON THE GLUTAMATE DEHYDROGENASE

Metal Salt	Concentration (mM)	% Activity
$HgCl_2$	1.0	0
$CuCl_2$	1.0	82
$NiCl_2$	1.0	85
VCl_3	1.0	85
$CaCl_2$	1.0	138
$CaCl_2$	5.0	235
$CaCl_2$	10.0	291
$CaCl_2$	20.0	300
$MgCl_2$	1.0	127
$MgCl_2$	5.0	204
$MgCl_2$	10.0	276
$MgCl_2$	20.0	293
$MnCl_2$	1.0	188
$MnCl_2$	5.0	350
$MnCl_2$	10.0	398
$MnCl_2$	20.0	337

Values for activity are those compared to control (100%). The following had no effect on activity: 1 mM $CdCl_2$, LiCl and $ZnCl_2$. The following precipitated out of solution: 1 mM $La(NO_3)_3$ and $CrCl_3$. Cu^{2+} formed a blue complex with glutamate, but this was soluble and did not absorb at 340 nm.

calculations were obtained from the Lineweaver-Burk plots for the respective cofactors). The enzyme did not reduce FAD or FMN (0.25 mM).

When DL-norvaline (0.1M) replaced L-glutamate, the glutamate dehydrogenase showed 3% of the oxidative deamination activity displayed with L-glutamate. Twenty other amino acids were unable to replace glutamate as substrates of the glutamate dehydrogenase. These comprised (L-forms) alanine, arginine, aspartate, cysteine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, serine, valine, ornithine and glutamine, (DL forms) norleucine and homoserine, and D-glutamate.

When pyruvate or 2-ketobutyrate (20 mM) replaced 2-ketoglutarate, the glutamate dehydrogenase showed 4% of the oxidative deamination activity displayed with 2-ketoglutarate. The enzyme showed negligible activity (less than 1% of the activity with 2-ketoglutarate) with 2-ketoisovalerate and 2-ketoisocaproate.

When methylamine (0.15 M) replaced ammonium, the enzyme showed 1% of the activity displayed with ammonium. Hydroxylamine, glycine and asparagine (0.15 M) were not substrates of the enzyme in the reductive amination reaction.

L-glutamine could replace ammonium as a substrate, hence the enzyme displayed glutamate synthase (E.C. 1.4.1.13) activity. Glutamate synthase activity was 47% of the glutamate dehydrogenase activity (at 40 and 80°C) when the concentration of glutamine was 0.15 M. Both activities had identical half lives at 103°C (see below). Glutamine could not be detected as a product of the oxidative deamination reaction (less than 5% of the amount of ammonia formed).

Kinetic Properties

When the substrate saturation curves were analysed using double reciprocal plots, Michaelis-Menten kinetics were seen. The apparent Km values are given in Table 3. The concentrations of the non-varied substrates were 100, 0.3, 20, 0.2 and 150 mM for glutamate, $NADP^+$, 2-ketoglutarate, NADPH and NH_4Cl, respectively.

Temperature Effects

Thermal inactivation of the glutamate dehydrogenase followed first order kinetics. At 90°C, and a protein concentration of 0.069 mg/ml, the enzyme had a half life of 12.5 h in 0.1M-NaH_2PO_4-Na_2HPO_4. pH_{80} 7.54. The half life values at 103°C under a variety of conditions are shown in Table 4.

The half life of the glutamate dehydrogenase at a protein concentration of 0.5 mg/ml was 21 min at 103°C.

The activation energies calculated from the Arrhenius plots (Fig. 2) were 69.7 kJ/mol for oxidative deamination and 40.9 kJ/mol for reductive amination.

TABLE 3

APPARENT Km VALUES FOR THE SUBSTRATES IN 0.1M NaH_2PO_4 -Na_2HPO_4 pH 7.54

SUBSTRATE	APPARENT Km (mM)		
	40°C	60°C	80°C
OXIDATIVE DEAMINATION			
Glutamate	1.39	1.93	9.12
$NADP^+$	0.014	0.027	0.038
NAD^+	ND	ND	28.6
REDUCTIVE AMINATION			
2-ketoglutarate	0.39	0.52	1.7
NADPH	0.023	0.036	0.066
NADH	ND	ND	0.32
NH_4Cl	5.7	5.7	15.5
Glutamine	ND	ND	26.9

ND=NOT DETERMINED

TABLE 4

$T_{1/2}$ VALUES OF AN1 GLUTAMATE DEHYDROGENASE AT 103°C

BUFFER	ADDITIVE	$T_{1/2}$ (MIN)
0.1 M NaH_2PO_4 Na_2HPO_4, pH_{80} 7.54	NIL	17.0
"	10%(W/V) GLYCEROL	30.5
"	2mM DITHIOTHREITOL	15.5
"	1% (W/V) TRITON X-100	9.0
"	0.1M SODIUM L-GLUTAMATE	57.5
"	5mM EDTA	26.5
"	0.5 mg ml^{-1} BSA	11.5
0.1 M NaH_2PO_4 Na_2HPO_4, pH_{80} 6.13	NIL	15.4
0.1 M NaH_2PO_4- Na_2HPO_4, pH_{80} 4.5	NIL	<5.0
0.1 M EPPS-NaOH pH_{60} 7.36	NIL	46.8
0.1 M EPPS-NaOH pH_{60} 7.36	5 mM $CaCl_2$	39.0
0.1 M EPPS-NaOH pH_{60} 7.36	50 mM $CaCl_2$	6.8
0.1 M EPPS-NaOH pH_{60} 7.36	5 mM $MgCl_2$	49.5
0.1 M EPPS-NaOH pH_{60} 7.36	50 mM $MgCl_2$	<5

The concentration of glutamate dehydrogenase was 0.069 mg/ml.

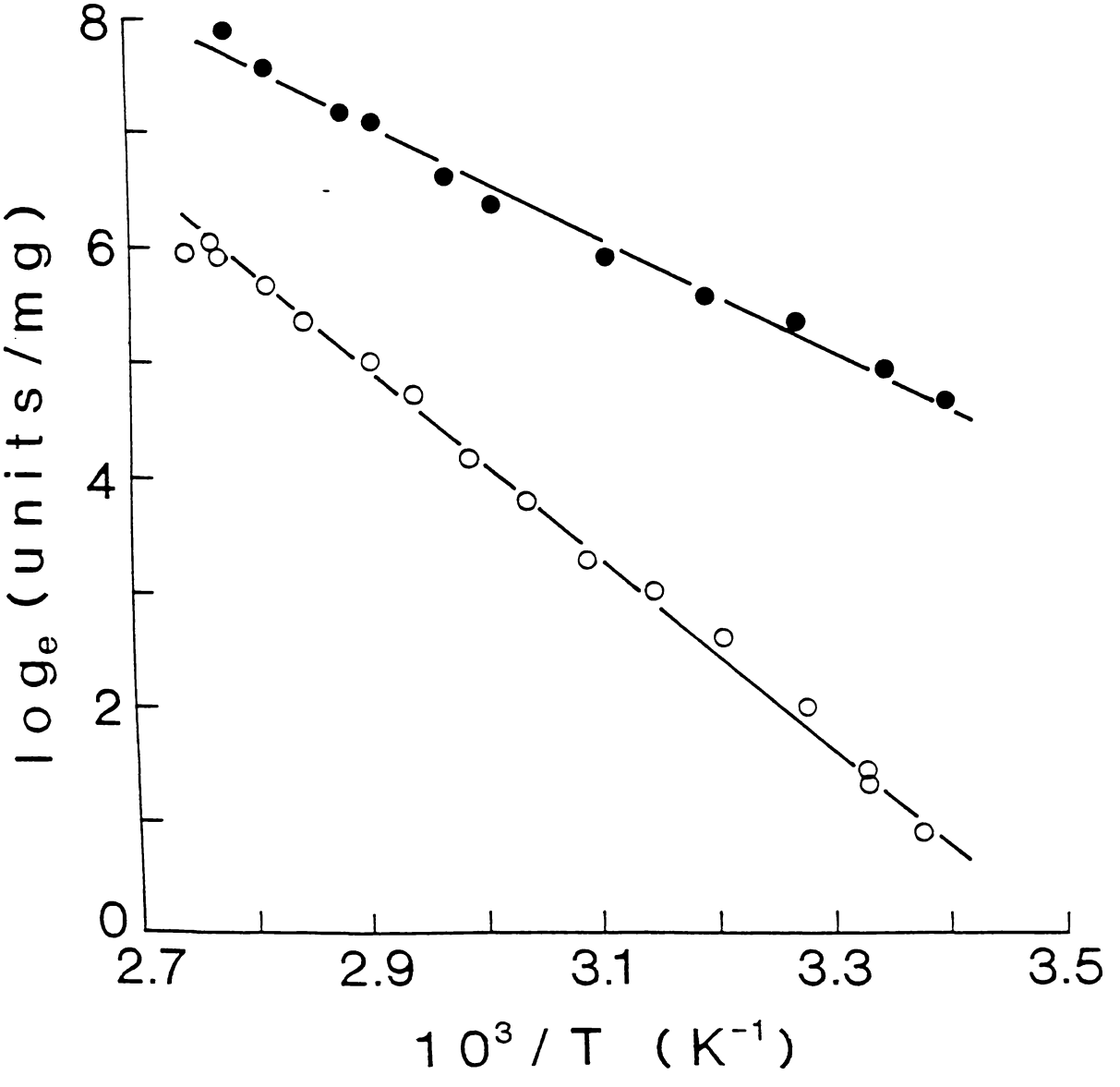

Fig. 2. Arrhenius plots. Open circles: oxidative deamination; closed circles: reductive amination.

DISCUSSION

Glutamate dehydrogenase from the archaebacterial isolate AN1 constitutes a large proportion of the soluble cell protein (11%). High levels are also found in the extremely thermophilic archaebacterium *Pyrococcus furiosus* (Consalvi *et al.* [6] have reported that glutamate dehydrogenase represents 20% of the total soluble protein in this organism, but Klump *et al.* [12] report a figure of 1-2% of the soluble protein) and in *Sulfolobus solfataricus* [5]. The aerobe *Peptostreptococcus asaccharolyticus* (previously known as *Micrococcus aerogenes* and *Peptococcus aerogenes*) also produces a high level of glutamate dehydrogenase (approx. 5-8% of the cell protein), when grown in medium containing glutamate [13,14].

Most glutamate dehydrogenase enzymes previously isolated have hexameric structures, including the enzymes from *Sulfolobus solfataricus* and *Pyrococcus furiosus* [5,6]. However, the glutamate dehydrogenase from isolate AN1 was found to be a tetramer, in common with the enzymes from *Synechocystis* sp. PCC 6803 [15], *Streptomyces fradiae* [16], *Pseudomonas* sp. strain AM1 [17], *Neurospora crassa* [18] and the archaebacterium *Halobacterium* sp. isolated from the Dead Sea [3].

Glutamate dehydrogenase from isolate AN1 had a pH optimum for the oxidative deamination of glutamate which was approximately the same as the pH optimum for the reductive deamination of 2-ketoglutarate. This is also the case for the pH optima for the glutamate dehydrogenases from *Mycoplasma laidlawii* (with NADP(H) as cofactor) [19] and *Pyrococcus furiosus* [6]. For most glutamate dehydrogenases, the pH optimum for reductive amination is more acidic than that for oxidative deamination [20].

The glutamate dehydrogenase was markedly activated by calcium, magnesium and manganese ions. It shares this property with the enzyme from peas (*Pisum sativum*) [21] and other higher plants [22,23] which are virtually inactive in the absence of divalent metal ions. However, the pea enzyme requires only 25 μM Ca^{2+} for maximal activation and is inactivated by chelating agents, whereas the AN1 enzyme was markedly activated only in the presence of millimolar concentrations of divalent metal ions and was unaffected by chelating agents. High concentrations of sodium ions (>0.1 M) reactivated the pea enzyme in the absence of other metal ions [21]. The AN1 enzyme is also markedly activated by high concentrations of sodium ions, which essentially "saturate" the enzyme, thus interfering with the effects shown by calcium, magnesium and manganese ions.

The apparent Km values of the substrates of glutamate dehydrogenase from isolate AN1 were found to vary markedly with temperature. In particular the apparent Km value for glutamate increased approximately 5-fold between 60 and 80°C; the increase in the Km with temperature was less marked for the substrates of the reductive deamination reaction. Wrba *et al.* [24] working with glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima*, also found that the apparent Km for the cofactor (NAD^+) increased markedly with temperature.These results emphasise the need to determine kinetic data near the growth temperature of the organism if the results are to be of any physiological significance.

The cofactor specificity of glutamate dehydrogenase from isolate AN1 resembled that of the enzyme from *Halobacterium* sp. from the Dead Sea [3] in that NADH supported only a fraction of the activity exhibited with NADPH. In contrast, *Halobacterium halobium* produces two glutamate dehydrogenases, one NAD^+-specific and the other $NADP^+$-specific [1,2]. The glutamate dehydrogenases from the

archaebacteria *Sulfolobus solfataricus* and *Pyrococcus furiosus* do, however, appear to have dual cofactor specificity, the specific activity with NADH being 30% of that with NADPH in the former case and 100% in the latter [5,6]. The possession of dual cofactor specific glutamate dehydrogenase is not limited to higher eukaryotes (e.g. mammals) and archaebacteria, as such enzymes have been reported in the eubacteria *Mycoplasma laidlawii* [19] and *Azospirillum brasilense* [25].

In common with some eukaryotic glutamate dehydrogenases (bovine liver glutamate dehydrogenase [20] and $NADP^+$-specific glutamate dehydrogenase from the sea anemone *Anthopleura xanthogrammica* [26]), the glutamate dehydrogenase from AN1 had glutamate synthase activity. Wootton *et al.* [27] found that the glutamate synthase activity previously reported [20] for the NADP-specific glutamate dehydrogenase from *Neurospora crassa* was an artifact caused by the contamination of glutamine solutions by ammonia. They also suggest that the apparent glutamate synthase activity claimed for the bovine glutamate dehydrogenase is also due to ammonium contamination of glutamine solutions. This effect may have increased the experimental error in the determination of the glutamate synthase activity of the AN1 enzyme, but our kinetic experiments found that the Km for glutamine (26.9 mM at 80°C) was less than twice the magnitude of the Km for ammonia (15.5 mM at 80°C). Thus at 27 mM glutamine more than 20% of the glutamine present would have to have decomposed to ammonia if all the apparent glutamate synthase activity was due to this. The experiments of Ratcliffe and Drozd [28] indicate that the thermal degradation of glutamine at the temperature and times used in our experiments would have been relatively small (they found a 10% degradation of a glutamine solution held at 76°C for 30 min), and this has been confirmed by our own estimations.

Glutamate dehydrogenase from AN1 is one of the most thermostable glutamate

dehydrogenases isolated to date. It retained 41% of its initial activity after incubation for 1h at 103°C in 0.1 M-EPPS-NaOH pH_{60},7.36, similar to that found for the enzyme from *Sulfolobus solfataricus* which retained 20% of its initial activity after incubation for 1h at 100°C [4]. The glutamate dehydrogenase from *Pyrococcus furiosus* is even more stable, having half lives at 100 and 110°C (12h and 25 min) [6] which are similar to those found for the ANI enzyme at 90 and 103°C, respectively.

Consalvi *et al* (1991) [5] found that the thermostability of the glutamate dehydrogenase from *S. solfataricus* was strongly dependant on the protein concentration. Robb *et al.* [29] report that the thermostability of the enzyme from *P. furiosus* is also dependent on protein concentration (they reported half lives at 100°C of 10h when the enzyme concentrtion was 1.06 mg/ml and 2.3h when the protein concentration was 20-fold lower), while Consalvi *et al.* [6] report the opposite. This discrepancy may be due to adsorption of enzyme to the walls of the vessel in the experiments of Robb *et al.*; this would have been avoided in the experiments of Consalvi *et al.* as they added glycerol to their buffer. The thermostability of the glutamate dehydrogenase from AN1 appeared to be independent of protein concentration, since increasing the enzyme concentration 7-fold (to 0.5 mg/ml), or adding 0.5 mg/ml BSA, had little effect on the half life at 103°C.

The activation energies calculated from the Arrhenius plots for the glutamate dehydrogenase from AN1 were similar to those calculated for the glutamate dehydrogenases from *S. solfataricus* (97.8 and 44.3 kJ/mol for glutamate oxidation and biosynthesis, respectively) [5] and *P. furiosus* (79.3 and 52.3 kJ/mol) [6].

Acknowledgements

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CHAPTER 3

STEADY STATE KINETICS OF THE GLUTAMATE DEHYDROGENASE FROM AN ARCHAEBACTERIAL EXTREME THERMOPHILE, ISOLATE AN1

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Key Words: Glutamate dehydrogenase; Kinetic mechanism; Steady state kinetics; Archaebacterium; *Thermococcales*; Thermophile.

Abbreviations used: K, Michaelis constant; K_i, dissociation constant; K_{ii}, inhibition constant for the intercept; K_{is}, inhibition constant for the slope; Vmax, maximum velocity; glu, glutamate; 2kg, 2-ketoglutarate; glut, glutarate; RR, Reactive Red 120; I-linear, inhibition in which the replot of the Lineweaver-Burk intercepts gives a straight line; I-parabolic, inhibition in which the replot of the Lineweaver-Burk intercepts gives a parabola; S-linear, inhibition in which the replot of the Lineweaver-Burk slopes gives a straight line; S-parabolic, inhibition in which the replot of the Lineweaver-Burk slopes gives a parabola.

SUMMARY

A steady state kinetic study was carried out with the glutamate dehydrogenase from the thermophilic archaebacterial isolate AN1. Initial velocity studies of the oxidative deamination reaction showed the mechanism is sequential and indicated that the order of substrate addition is random, while inhibition studies with products and substrate analogues suggested a strong preference for $NADP^+$ to bind first. Initial velocity studies of the reductive amination reaction showed that the mechanism is sequential and indicated that the order of substrate addition is random, while product inhibition studies, and the effect of substrate saturation on the initial velocity, suggested that the preferred order of substrate addition is NADPH, 2-ketoglutarate, ammonia.

INTRODUCTION

Glutamate dehydrogenases (E.C. 1.4.1.2-4.) catalyse the reversible oxidative deamination of L-glutamate to 2-ketoglutarate, hence are involved in both carbon and nitrogen metabolism. Kinetic studies have been carried out with glutamate dehydrogenases from a variety of sources, with the bovine liver enzyme receiving the most attention. Early studies with the bovine liver glutamate dehydrogenase indicated a compulsory order mechanism (Frieden, 1959), but later studies using more sensitive methods disproved this. The enzyme is now known to follow a random order rapid equilibrium mechanism for the oxidative deamination reaction[1] (Smith *et al.*, 1975; Rife and Cleland, 1980), and is thought to follow a partially ordered mechanism, in which ammonia can bind only after 2-ketoglutarate, in the reverse direction (Rife and Cleland, 1980; Adams, 1987).

Several studies on glutamate dehydrogenases from microorganisms (Le John *et al.*, 1968; Coulton and Kapoor, 1973; Sarada *et al.*, 1980) have indicated compulsory order mechanisms on the basis of initial velocity and/or product inhibition studies. However, more rigourous treatments, including statistical analysis of data, are required in order to provide an unequivocal understanding of a mechanism. An initial velocity study with the NAD-linked glutamate dehydrogenase from *Clostridium symbiosum* (the only Glutamate dehydrogenase with a known structure from X-ray diffraction studies), which included statistical analysis of the data, indicated that the reductive amination reaction follows a random order rapid equilibrium mechanism. The oxidative deamination reaction was shown to be sequential but the order of substrate addition could not be deduced (Syed *et al.*, 1991).

There is no reason *per se* to assume that all glutamate dehydrogenases will follow the same mechanism. Indeed, the kinetic study by Le John *et al.* (1968) of the NAD- and NADP-linked glutamate dehydrogenases from *Thiobacillus novellus* suggested that the mechanisms followed by these two enzymes are different (sequential and compulsory

[1]At pH 8.8 the oxidative deamination is thought to be ordered with cofactor leading (Silverstein, 1974), and it is thought to be ordered with glutamate leading when high concentrations of ADP are present (Hornby *et al.*, 1984).

order with cofactors leading in both cases, but with different orders of addition of ammonia and 2-ketoglutarate).

As yet, there has not been steady state kinetic study undertaken with an archaebacterial glutamate dehydrogenase. The glutamate dehydrogenase from the thermophilic archaebacterium isolate AN1, belonging to the order *Thermococcales* (Zillig *et al.*, 1987), displays Michaelis-Menten kinetics with all of its substrates (Hudson *et al.*, 1993), unlike the glutamate dehydrogenases from *Sulfolobus solfataricus* and *Pyrococcus furiosus* (Consalvi *et al.*, 1991a and b), hence is a convenient enzyme with which to carry out a steady state kinetic study.

EXPERIMENTAL PROCEDURES

Chemicals

Monosodium L-glutamate was from BDH (Poole, U.K.), ammonium chloride was from Merck (Darmstadt, Germany), glutaric acid was from Riedel-de Haen (Seelze, Germany), and all other chemicals, including $NADP^+$ and NADPH, were from Sigma (St Louis, Mo., U.S.A.).

Enzyme Assays

Reaction rates were monitored at 340 nm using a Perkin Elmer Lambda 3B spectrophotometer fitted with a Perkin Elmer C 570-0701 digital temperature controller. The "PECSS for Lambda 3 (C695-0002)" software package was used in conjunction with the spectrophotometer and facilitated the accurate measurement of reaction rates even when the absorbance changes were small. All assays were performed at 60°C in 0.1 M Na_2HPO_4-NaH_2PO_4 buffer pH_{60}7.6 (this is a similar buffer system to that used by Engel and Dalziel (1969 and 1970) and Engel and Chen (1975)). The assay volume was 1.5 ml, and the assays were conducted in duplicate or triplicate. For the oxidative deamination reaction, 0.18-0.37 µg enzyme was added to start the reaction; 0.02-0.05 µg was used for the reductive amination reaction. The substrate and inhibitor concentrations were as indicated in the figures. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 umol NADP(H) per minute under the assay conditions.

Glutamate dehydrogenase purified to homogeneity from cell free extracts of isolate AN1 was used in this work. The growth of the organism and the purification procedure has been described previously (Hudson *et al.*, 1993).

Experimental Design and Data Analysis

Initial velocity experiments for the oxidative deamination reaction were performed by varying the concentration of one substrate at varying fixed levels of the other. A similar approach was used to determine the effect of substrate saturation on the reductive amination reaction: one substrate was held at a saturating level while the other two were varied as described above. Inhibition experiments were performed by varying the concentration of one substrate (holding the others at fixed, non-saturating concentration) at several concentrations of inhibitor (including 0). All these approaches are described by Cleland (1970).

The data for the above experiments were first manually plotted to check for linearity of the Lineweaver-Burk plots and to determine the initial velocity/inhibition pattern represented by sets of such plots. The data were then fitted to the appropriate equation[2] (see below) using SigmaPlot (Version 5.01) in conjunction with a 80386DX computer (Unitron). The equations (written using the nomenclature of Cleland (1963)) for sequential initial velocity (two substrate reaction), competitive inhibition, noncompetitive inhibition, uncompetitive inhibition, competitive inhibition (S-linear), noncompetitive inhibition (I-linear, S-parabolic), noncompetitive inhibition (I-parabolic, S-parabolic) and uncompetitive inhibition (I-parabolic) are equations I, II, III, IV, V, VI, VII and VIII, respectively, below:

(I) $v = VAB/ (K_{ia}K_b + K_aB + K_bA + AB)$

(II) $v = VA/ (K_a(1+I/K_{is}) + A)$

(III) $v = VA/ (K_a(1+I/K_{is}) + A(1+I/K_{ii}))$

(IV) $v = VA/ (K_a + A(1 + I/K_{ii}))$

(V) $v = VA/ (K_{ia}(1+I/K_{is}+I^2/K_{is}K_{ii}) + A)$

(VI) $v = VA/ (K_{ia}(1+I/K_{is}+I^2/K_{is}K_{ii}) + A(1+I/K_{ii}))$

(VII) $v = VA/ (K_{ia}(1+I/K_{is}+I^2/K_{is}K_{ii}) + A(1+I/K_{ii}+I^2/K_{ii}K_{is}))$

(VIII) $v = VA/ (K_{ia} + A(1+I/K_{ii}+I^2/K_{ii}K_{is}))$

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[2] For the inhibition by NADP+ with respect to 2-ketoglutarate at high (0.1 mM) NADPH concentration, linear regession was used to fit the individual lines to the Lineweaver Burk plot. The replot of slopes was fitted by nonlinear regression (order =2), while the replot of intercepts was not fitted by regression since it was convex.

where v is initial velocity, A and B are substrate concentrations, I is the concentration of inhibitor, K_a and K_b are Michaelis constants, K_{ia} is the dissociation constant for substrate A (the variable substrate) and K_{ii} and K_{is} are the inhibition constants for the intercept and slope, respectively. The estimates of kinetic parameters yielded by these equations were used to draw the lines (superimposed on real data points) shown in the figures.

The initial velocity patterns for the reductive amination reaction were determined by the method suggested by Rudolph and Fromm (1979) in which one substrate is varied while the other two are held at a fixed ratio at different levels. This is repeated so that each substrate is used as the variable substrate. Double reciprocal plots were constructed (a Hanes plot was used where NADPH was the variable substrate) as were replots of the slopes and intercepts. Non-linear regression (order = 2) was used to fit parabolas to these replots and thus determine the kinetic parameters.

Inhibition of the Glutamate Dehydrogenase by Pyridoxal 5'-Phosphate

Samples of glutamate dehydrogenase (0.112 mg/ml) were exposed to 2 mM pyridoxal 5'-phosphate in 0.1 M Na_2HPO_4-NaH_2PO_4 buffer pH_{20}7.68. $NADP^+$ (3 mM), sodium glutarate (0.1 M), NADPH (2.5 mM), 2-ketoglutarate (50 mM) and ammonium chloride (0.1 M) were added to some samples. The controls did not contain pyridoxal 5'-phosphate. After 15 h at room temperature, enzyme samples were assayed in the oxidative deamination direction as described above (samples were diluted 300-fold in the assay mixture which contained 0.1 M glutamate and 0.3 mM $NADP^+$).

RESULTS

Initial-Rate Studies of the Oxidative Deamination Reaction

When the concentration of $NADP^+$ was varied at a number of fixed concentrations of glutamate, the double reciprocal plots were intersecting (see Fig. 1), indicating a sequential mechanism. The calculated parameters were: Vmax 353+/-9 μmol/min/mg; K_{NADP+} 0.086+/-0.005 mM; K_{glu} 2.0+/-0.1 mM; K_{iNADP+} 0.054+/-0.007 mM; K_{iglu} 1.3+/-0.2 mM.

The relationship $K_{iNADP+}K_{glu} = K_{iglu}K_{NADP+}$ holds true; this suggests a random order mechanism (Cleland, 1970). Furthermore, $VmaxK_{iNADP+}/K_{NADP+} = VmaxK_{iglu}/K_{glu}$ = 220 units/mg. This value is considerably less than any of the estimates of the maximal rate for the reductive amination reaction (see below, Table1), hence an ordered mechanism and a Theorell-Chance mechanism must be ruled out (Dalziel relationships, or maximum rate tests, for these mechanisms are not satisfied (Engel,1981)).

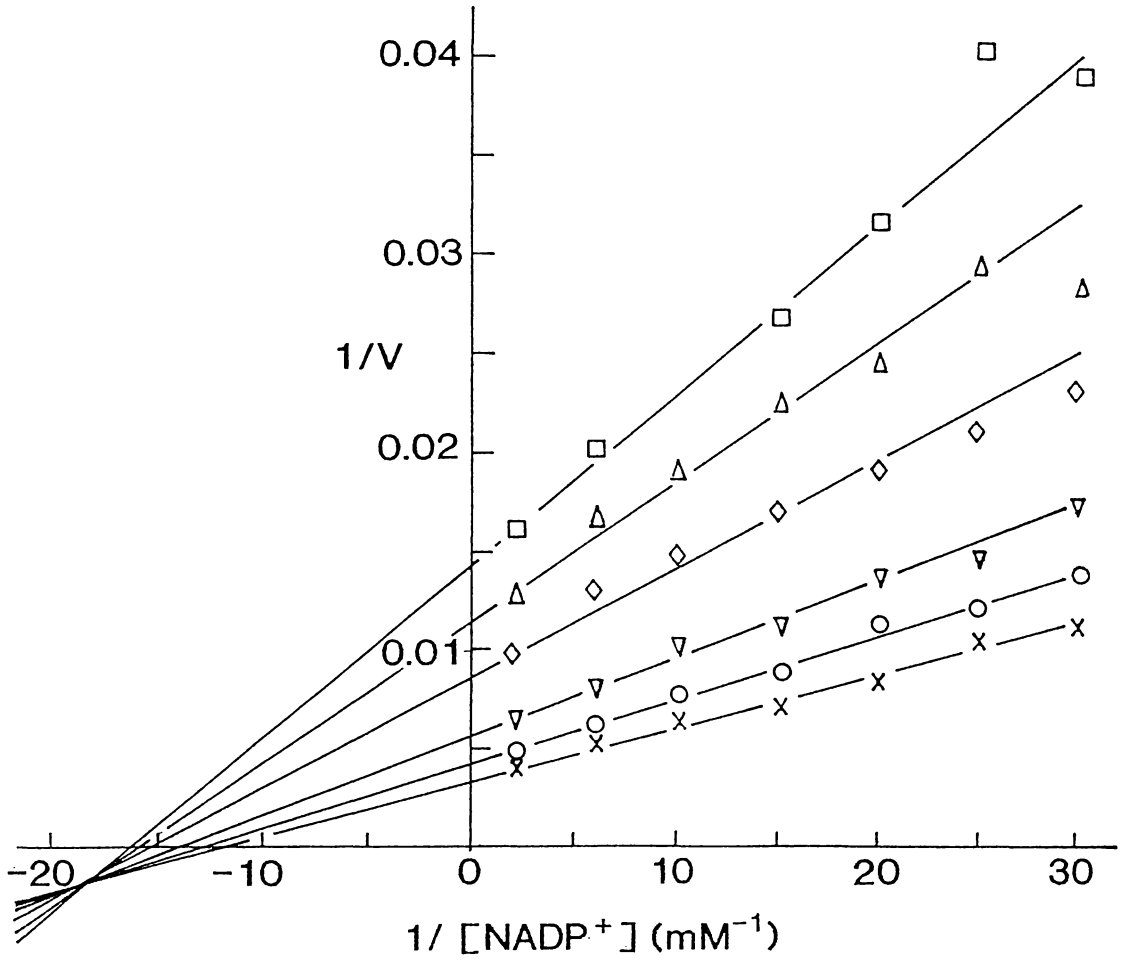

Fig. 1. Double reciprocal plot of initial reaction velocity (units/mg) for the oxidative deamination reaction vs $NADP^+$ concentration, at changing fixed levels of glutamate. The concentrations of glutamate used were: 10.0 mM (cross), 4.0 mM (circle), 2.0 mM (inverted triangle), 1.0 mM (diamond), 0.6667 mM (triangle), 0.5 mM (square).

Initial-Rate Studies of the Reductive Amination Reaction

The Lineweaver-Burk plots of the initial rate data for the reductive amination reaction are shown in Figures 2 and 3 (where 2-ketoglutarate and ammonia are the variable substrates, respectively). A Hanes plot of the initial rate data (NADPH as the variable substrate) is shown in Fig. 4. None of the slope and intercept replots (Figs. 5-10) intercept the axes at the origin, indicating that the mechanism is sequential and either completely random or completely ordered. The kinetic constants for the reductive amination reaction are presented in Table 1. The concentrations of NADPH used were always greater than the Km for NADPH; this was necessary because data at low concentrations of NADPH tended to be unreliable due to the short period of linearity, especially at higher concentrations of 2-ketoglutarate and ammonia.

TABLE 1

KINETIC PARAMETERS FOR THE REDUCTIVE AMINATION REACTION[a]

Variable Substrate	Vmax[b] (units/mg)	Km[c] for variable substrate (mM)	Ki[d] for variable substrate (mM)
NADPH	2830	0.0145	0.0182
2-ketoglutarate	1490	0.712	1.79
ammonia	1760	10.7	8.03

a The slopes and intercepts of the Lineweaver-Burk or Hanes plots for the initial rate data were computer fitted by nonlinear regression (parabolic, order = 2). The correlation coefficients were >0.99 in all cases.

b Vmax is equal to the reciprocal of the intercept of the intercept replot (or slope replot where a Hanes plot was used).

c The Km for the variable substrate is equal to the intercept of the slope replot (or intercept replot where a Hanes plot was used) multiplied by Vmax.

d The replot data was fitted to a parabola (described by $y = a + bx + cx^2$). Thus 2cx is the slope of a tangent to the parabola at point x. The c value for the slope replots divided by the c value for the intercept replots (or visa versa where a Hanes plot was used) gives a value for the Ki of the variable substrate.

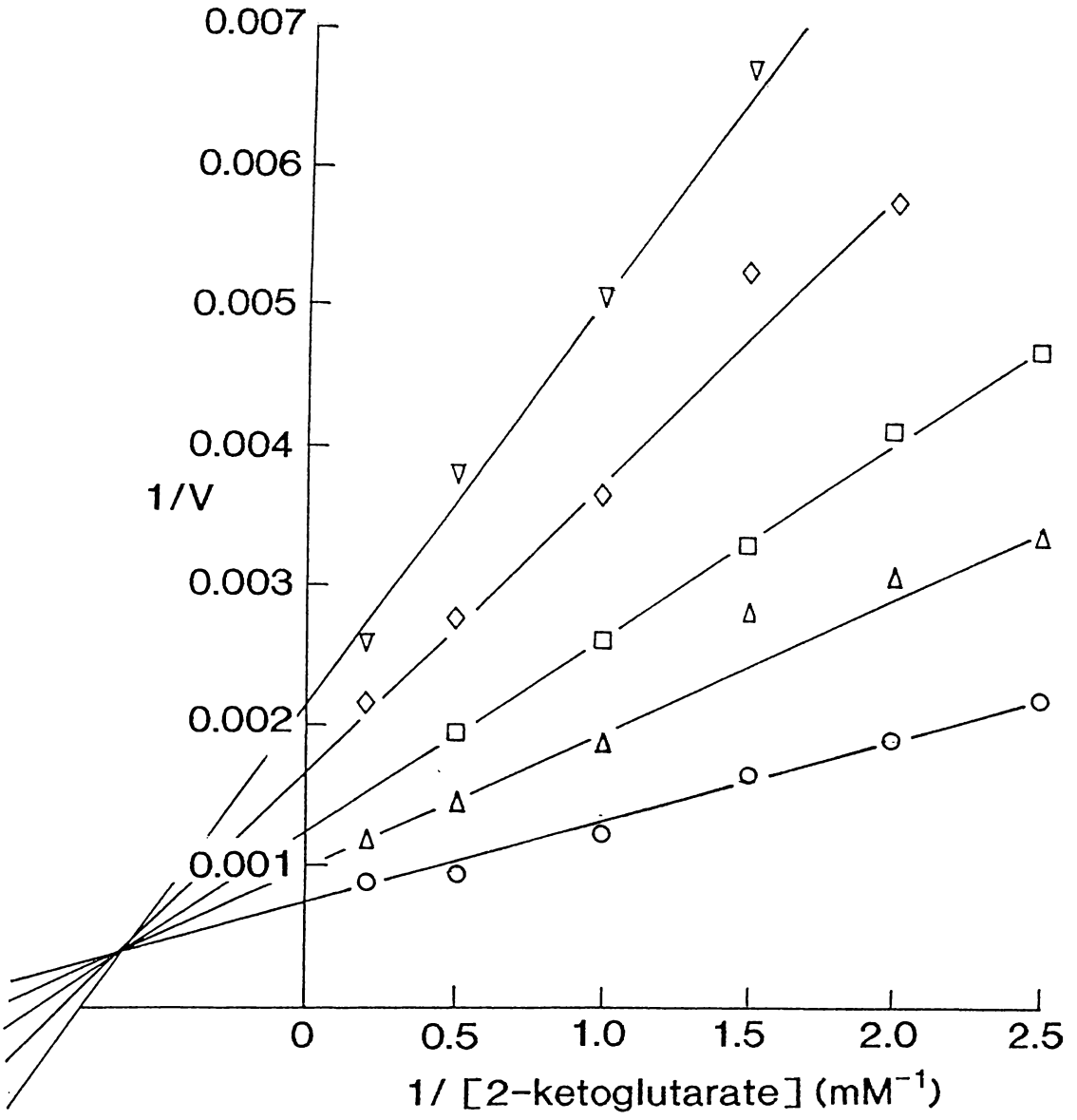

Fig. 2. Double reciprocal plot of initial reaction velocity (units/mg) for the reductive amination reaction vs 2-ketoglutarate concentration, at changing fixed levels of NADPH and NH_4Cl in constant ratio. The concentrations of NADPH used were: 0.15 mM (circle), 0.06 mM (triangle), 0.03 mM (square), 0.02 mM (diamond), 0.015 mM (inverted triangle). The concentrations of NH_4Cl used were: 40 mM (circle), 16.0 mM (triangle), 8.0 mM (square), 5.333 mM (diamond), 4.0 mM (inverted triangle).

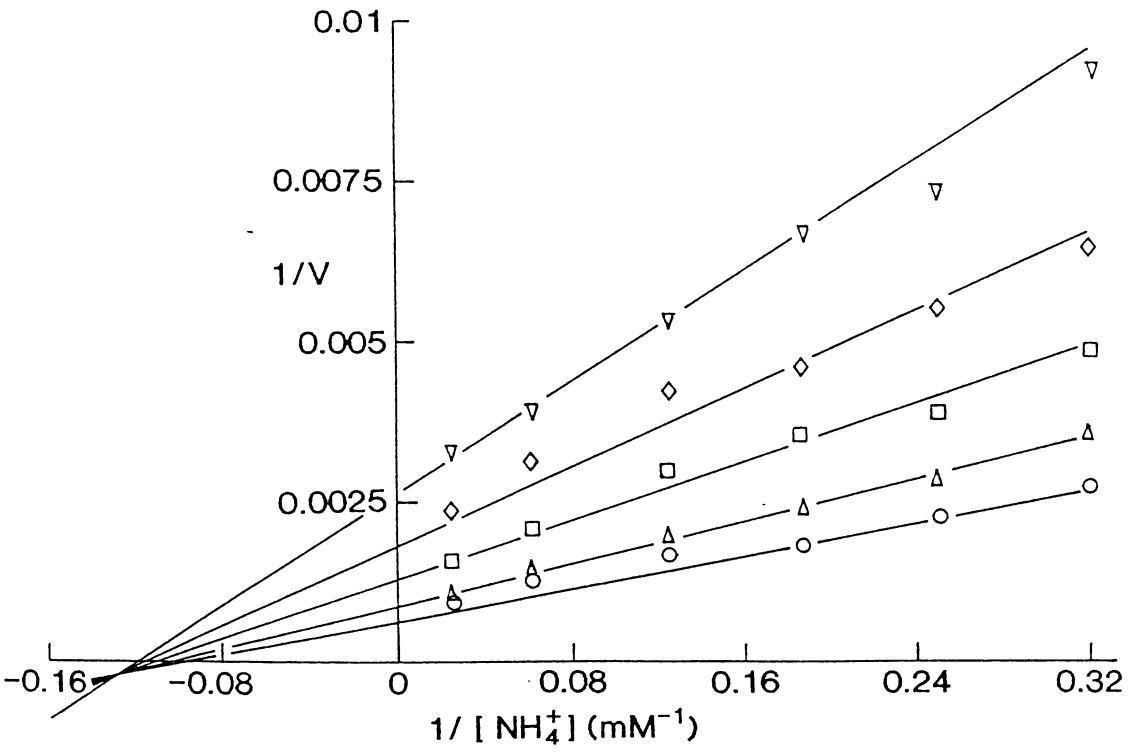

Fig. 3. Double reciprocal plot of initial reaction velocity (units/mg) for the reductive amination reaction vs NH_4Cl concentration, at changing fixed levels of NADPH and 2-ketoglutarate in constant ratio. The concentrations of NADPH used were: 0.15 mM (circle), 0.06 mM (triangle), 0.03 mM (square), 0.02 mM (diamond), 0.015 mM (inverted triangle). The concentrations of 2-ketoglutarate used were: 5.0 mM (circle), 2.0 mM (triangle), 1.0 mM (square), 0.6667 mM (diamond), 0.5 mM (inverted triangle).

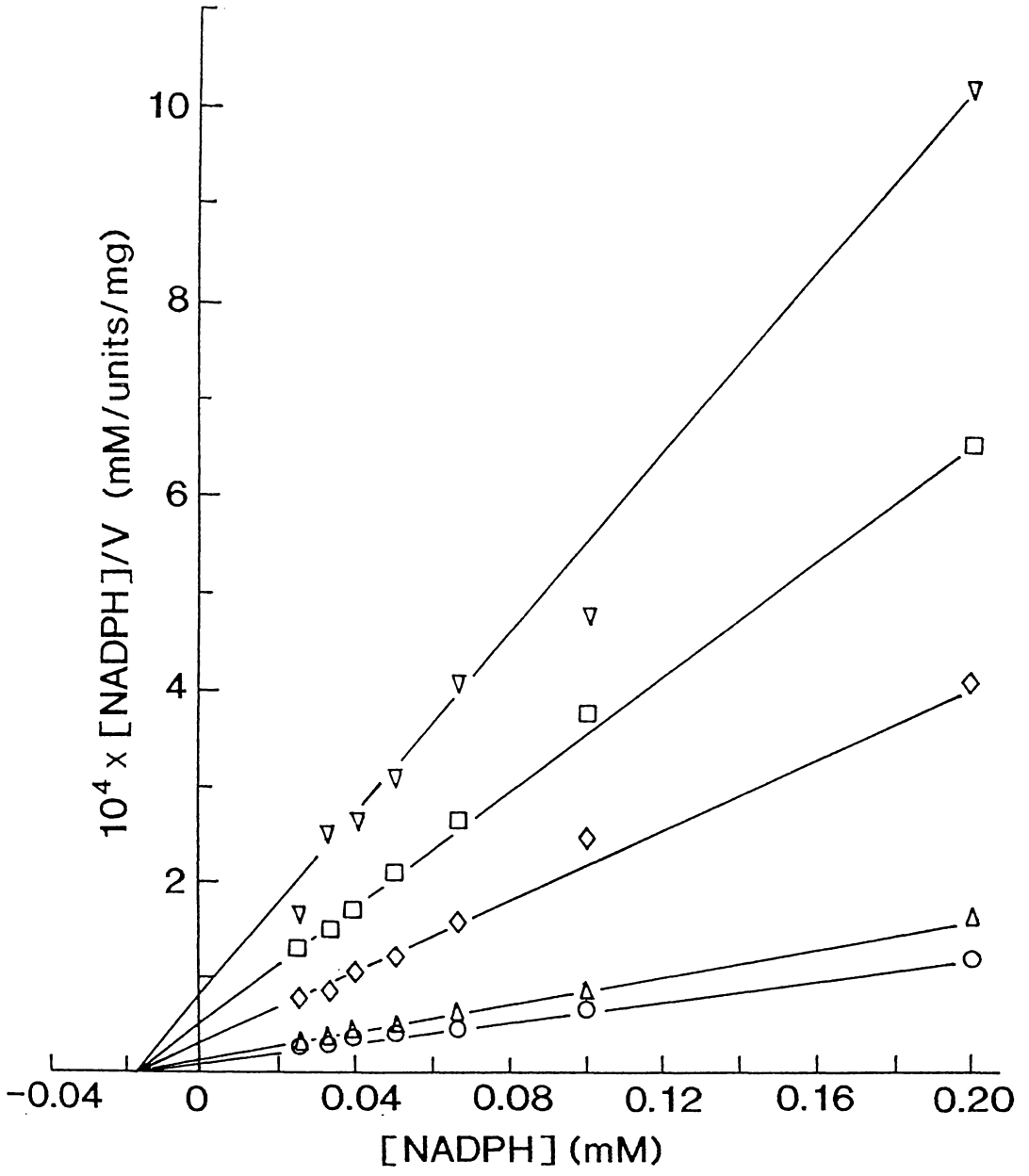

Fig. 4. Hanes plot of [NADPH]/(initial velocity of reductive amination reaction) vs [NADPH], at changing fixed levels of 2-ketoglutarate and NH_4Cl in constant ratio. The concentrations of 2-ketoglutarate used were: 5.0 mM (circle), 2.0 mM (triangle), 1.0 mM (diamond), 0.6667 mM (square), 0.5 mM (inverted triangle). The concentrations of NH_4Cl were 10x those of 2-ketoglutarate.

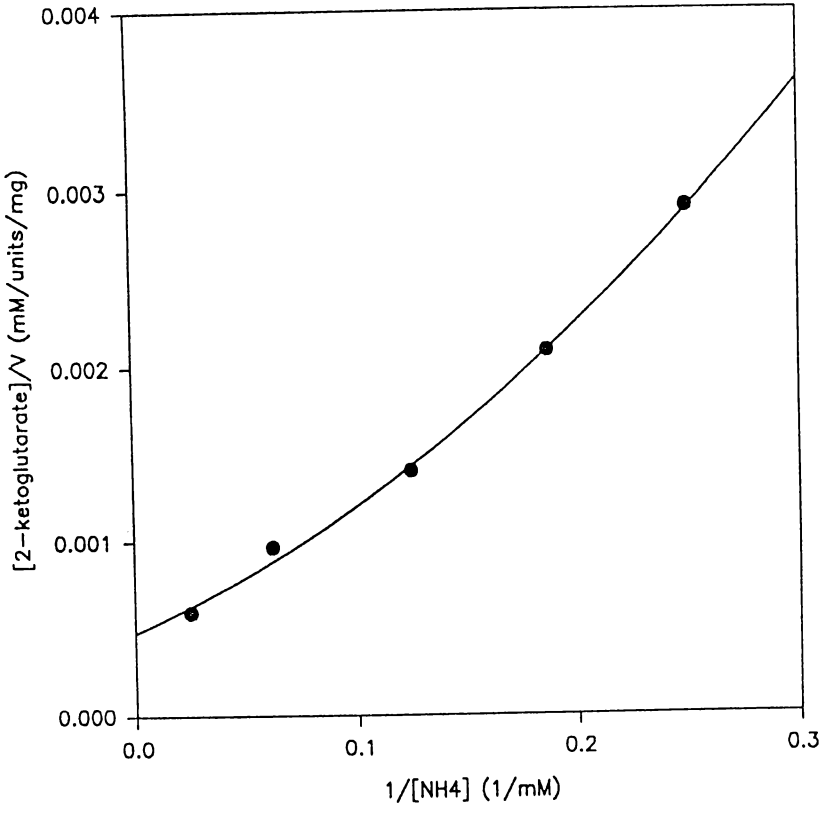

Fig. 5. Replot of the slopes from Fig. 2.

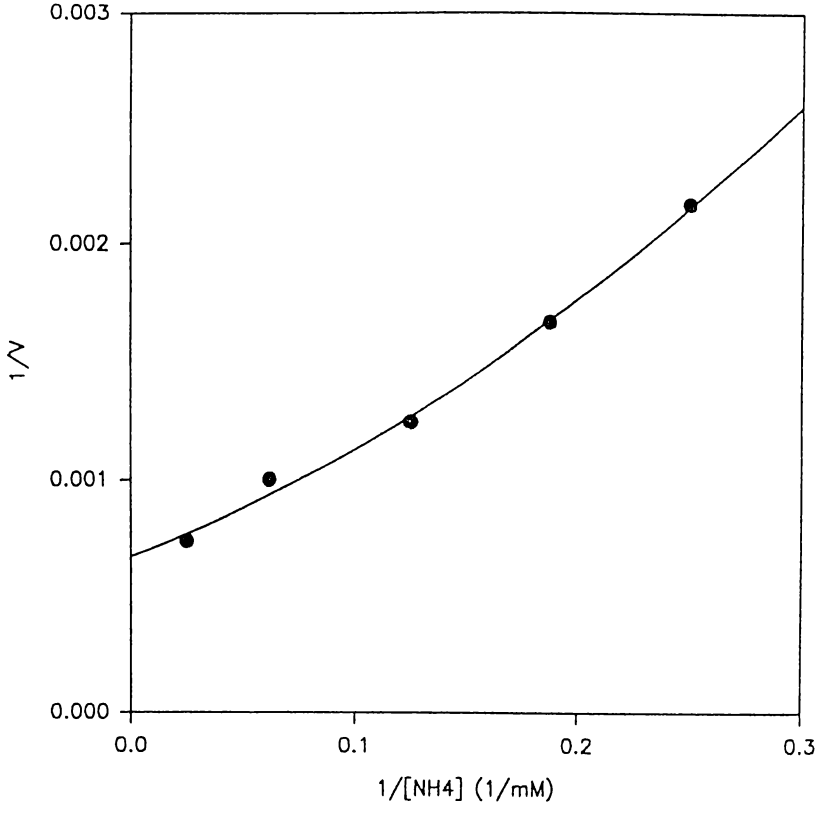

Fig. 6. Replot of the intercepts from Fig. 2. The velocity is expressed as units/mg.

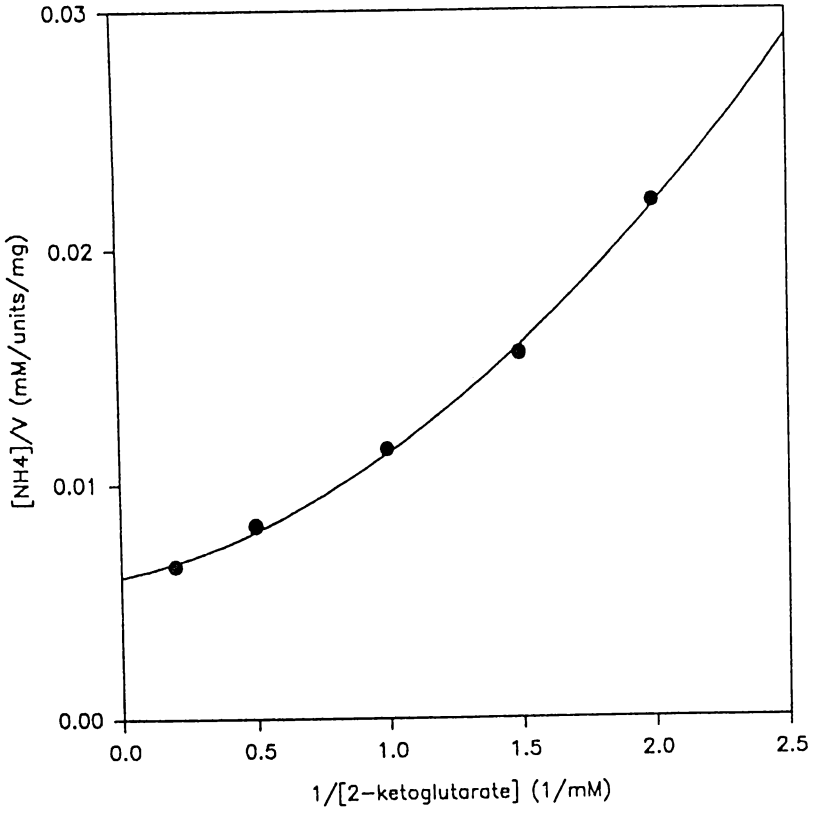

Fig. 7. Replot of the slopes from Fig. 3.

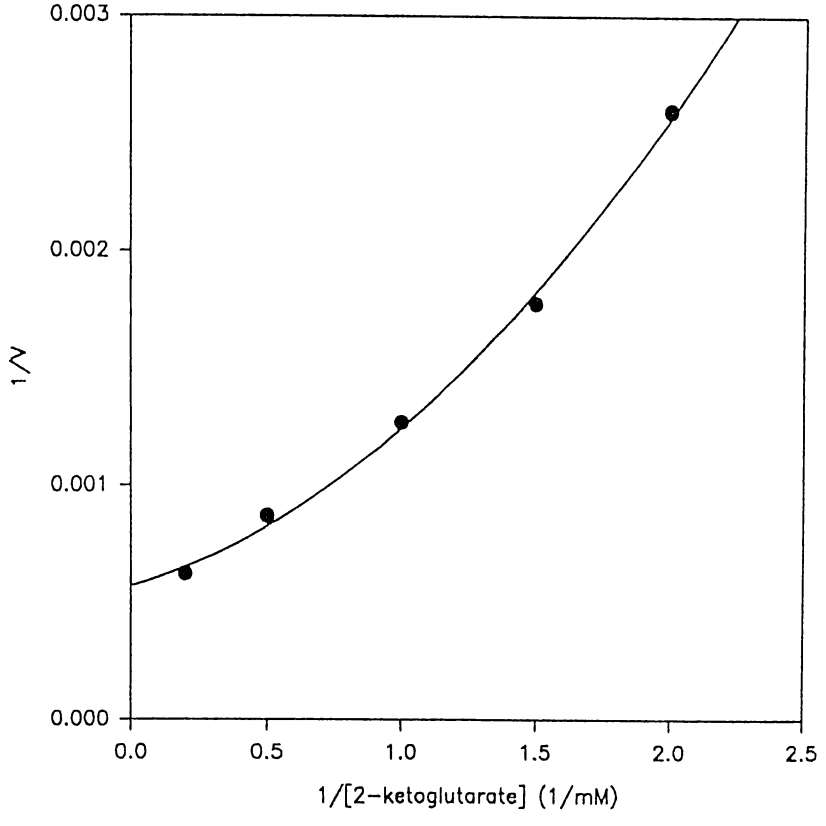

Fig. 8. Replot of the intercepts from Fig. 3. Velocity is expressed as units/mg.

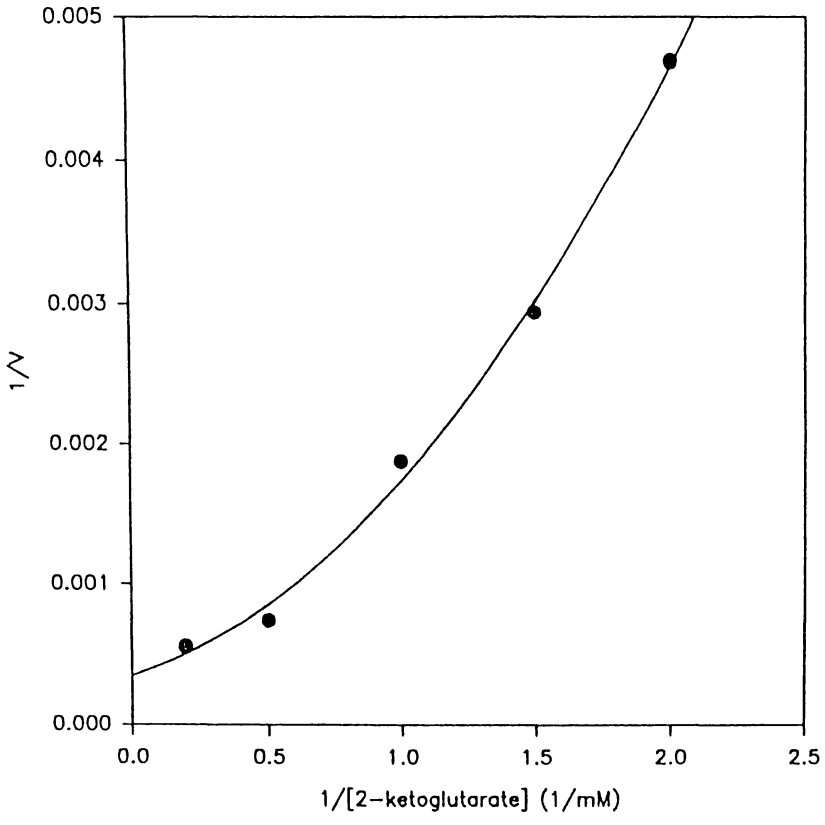

Fig. 9. Replot of the slopes from Fig. 4. Velocity is expressed as units/mg.

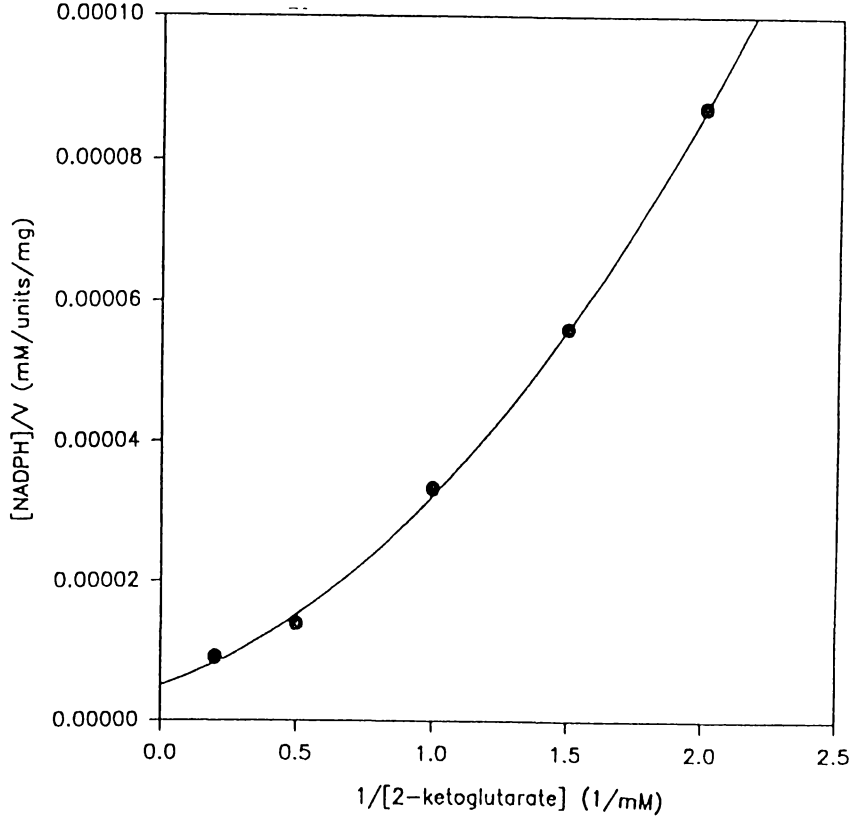

Fig. 10. Replot of the intercepts from Fig. 4.

The x coordinates of the Lineweaver-Burk plots (Figs. 2 and 3) were approximately equal to $-1/K_{i2kg}$ and $1/K_{iNH4+}$, respectively, while the x coordinate of the point of convergence of the Hanes plot (Fig. 4) was approximately equal to $-K_{iNADPH}$ (which is the equivalent of $-1/K_{iNADPH}$ in a Lineweaver-Burk plot). The y coordinates of the point of convergence of the Lineweaver-Burk plots (Figs. 2 and 3) were approximately equal to $(1-K_{2kg}/K_{i2kg})/Vmax$ and $(1-K_{NH4+}/K_{iNH4+})/Vmax$, respectively. These coordinates were those expected for a random mechanism (extrapolated from the coordinates predicted for a 2 substrate random mechanism by Cleland (1970)). The exact y coordinate of the point of convergence of the Hanes plot could not be accurately determined; it appeared to be equal to zero because the values of the K_{NADPH} and K_{iNADPH} were very similar. The Km's and Ki's are related as follows: $K_{iNADPH}/K_{NADPH} = K_{2kg}K_{i2kg} = K_{NH4+}/K_{iNH4+} = 1.3$.

Initial-rates for the reductive amination reaction were also determined with saturating concentrations of either 2-ketoglutarate or ammonia (see Lineweaver-Burk plots Figs. 11 and 12, respectively). When 2-ketoglutarate was saturating (Fig. 11: 70x K_{2kg}), the lines in the Lineweaver-Burk plot appeared nearly parallel, but the slopes did slightly increase with increasing NADPH concentration and this data was able to be fitted reasonably well to the equation for sequential initial velocity. When ammonia was saturating (Fig. 12: 50x K_{NH4+}), the double reciprocal plots clearly intersected, suggesting that ammonia was not the second substrate to bind the enzyme under these conditions. However, in adding 0.5 M ammonia to the assays we have considerably altered the ionic strength and thus this experiment is not strictly comparable with the others. We note that the concentration of the saturating substrate should be at least 100x its Km in order to be truly saturating (not practicable here because ammonia has a strong influence on the ionic strength and both 2-ketoglutarate and NADPH absorb at 340 nm) and also that the saturating substrate approach was not successful in ascertaining the mechanism of the reaction catalysed by bovine liver glutamate dehydrogenase (Smith *et al.*, 1975).

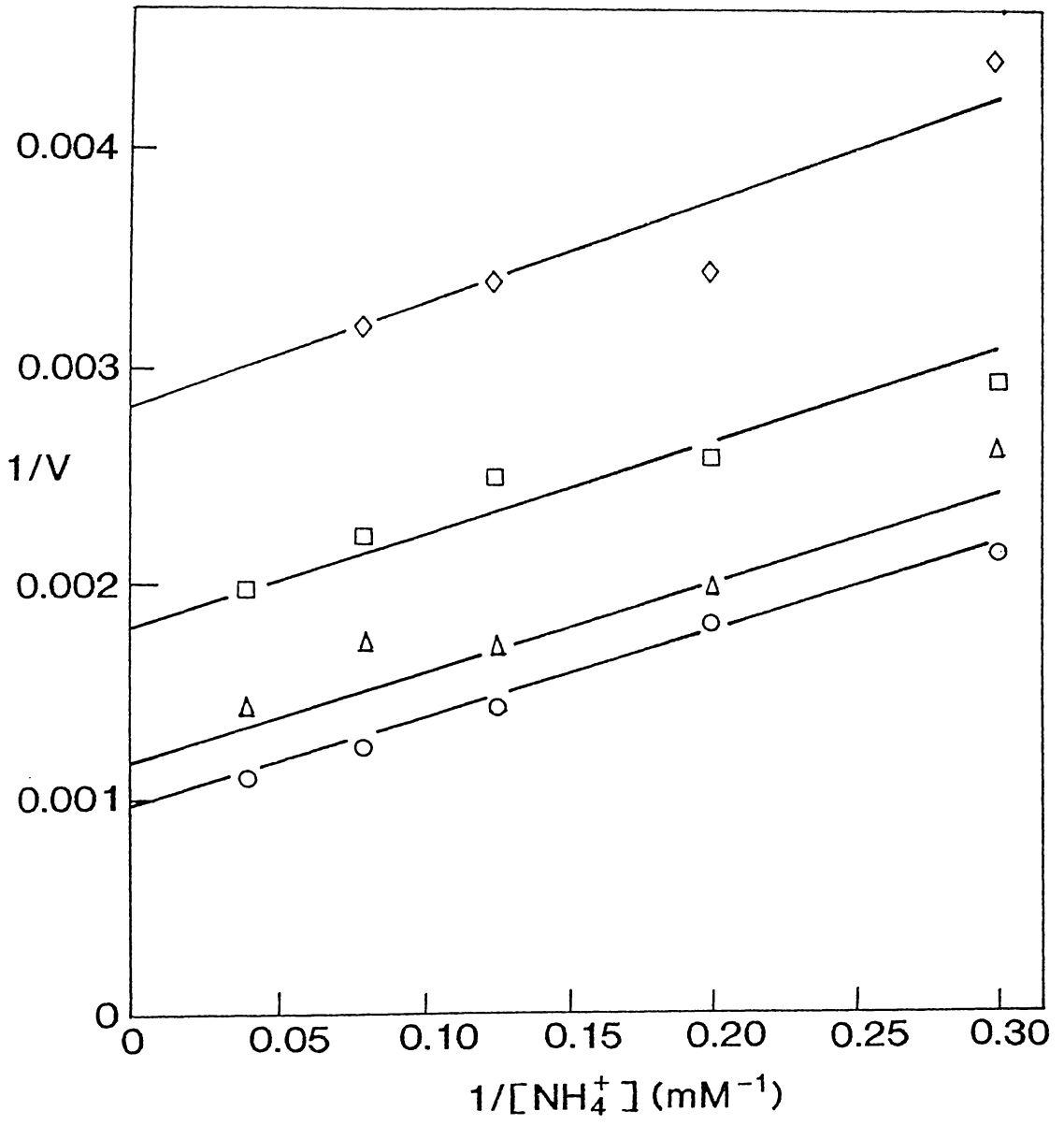

Fig. 11. Double reciprocal plot of initial reaction velocity (units/mg) for the reductive amination reaction vs NH_4^+ concentration, at changing fixed levels of NADPH. The concentration of 2-ketoglutarate was saturating at 50 mM. The concentrations of NADPH used were: 0.1 mM (circle), 0.05 mM (triangle), 0.02 mM (square), 0.01 mM (diamond).

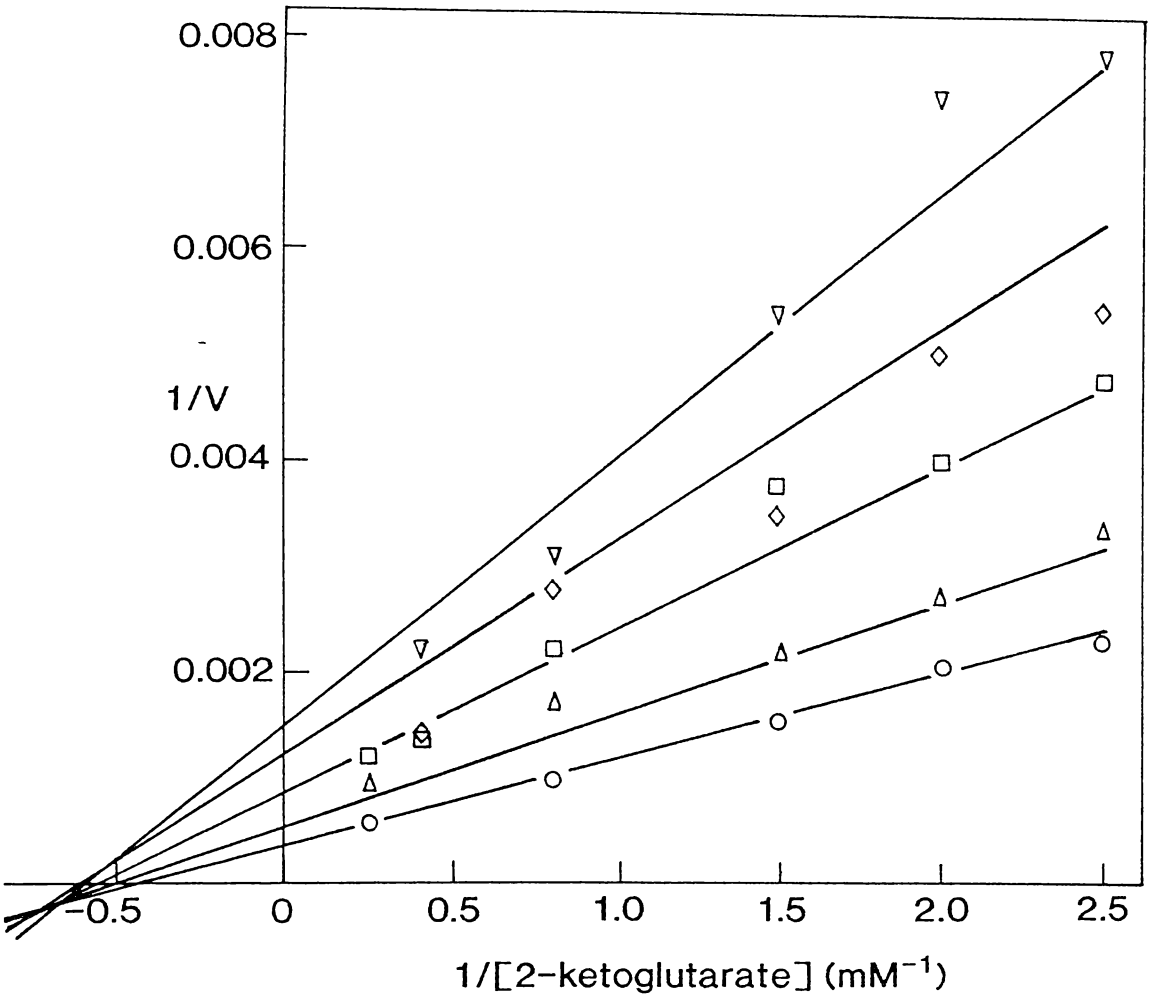

Fig. 12. Double reciprocal plot of initial reaction velocity (units/mg) for the reductive amination reaction vs 2-ketoglutarate concentration, at changing fixed levels of NADPH. The concentration of NH_4Cl was saturating at 0.5 M. The concentrations of NADPH used were: 0.1 mM (circle), 0.05 mM (triangle), 0.025 mM (square), 0.01667 mM (diamond), 0.0125 mM (inverted triangle).

Product Inhibition Studies

The Lineweaver-Burk plots showing the product inhibition of the forward and reverse reactions of the glutamate dehydrogenase are shown in Figs. 13-27. The competitive inhibition shown by NADPH against $NADP^+$ (Fig. 13) (and visa versa (Fig. 14)) indicates that these substrates bind to the free enzyme and suggests that they are the leading substrates if the mechanism is ordered. With the exception of the uncompetitive inhibition (I-parabolic) shown by $NADP^+$ with respect to NH_4^+ at high (0.1 mM) NADPH concentration (Fig. 21), other product inhibition patterns were noncompetitive, and in several cases the replots were parabolic (Figs. 15, 16, 19 and 20). The product inhibition relationships between the glutamate dehydrogenase substrates are summarised in Table 2.

The kinetic constants for the inhibition of the oxidative deamination and reductive amination are also summarised in Table 2.

Substrate Analogue Inhibition of the Oxidative Deamination Reaction

Glutarate and Reactive Red 120 both inhibited competitively with respect to glutamate (Figs. 31 and 33). Reactive Red 120 inhibited noncompetitively with respect to $NADP^+$ (although the distinction between noncompetitive and uncompetitive inhibition was small) (Fig. 32). The nature of the inhibition of glutarate with respect to $NADP^+$ (Fig. 30) was uncertain as the data could be fitted to the equations for both noncompetitive inhibition (equation III) and uncompetitive inhibition (equation IV) equally well. The inhibition constants are summarised in Table 2.

We were unsuccessful in finding a competitive inhibitor for $NADP^+$; AMP (which was competitive against oxidised and reduced cofactor for the glutamate dehydrogenase from lupin nodules (Stone et al. 1980a and b)) was completely ineffective as an inhibitor of the isolate AN1 glutamate dehydrogenase even at a concentration of 10 mM.

TABLE 2

PRODUCT AND SUBSTRATE ANALOGUE INHIBITION OF THE ISOLATE AN1 GLUTAMATE DEHYDROGENASE

Inhibitor	Varied Substrate	Type of Inhibition[a]	Apparent K_i values (mM)	
			K_{is} (slope)	K_{ii} (intercept)
NADPH	$NADP^+$	C (l)	0.016+/-0.001	
	glutamate	NC (I-l, S-p)	0.04+/-0.01	-[b]
2-ketoglutarate	$NADP^+$[c]	NC (l)	6.7+/-0.8	7.2+/-0.5
	$NADP^+$[d]	NC (l)	30+/-20	3.1+/-0.3
	glutamate	NC (l)	2.8+/-0.9	14+/-12
NH_4^+	$NADP^+$	NC (I-p, S-p))	12+/-4	12+/-3
	glutamate	NC (I-l, S-l/S-p)[e]	4.8+/-0.5	60+/-20
$NADP^+$	NADPH	C (l)	0.052+/-0.005	
	2-ketoglutarate[f]	NC (l)	0.46+/-0.09	0.18+/-0.01
	2-ketoglutarate[g]	NC (I-nl,S-p)[h]	-	-
	NH_4^+[i]	NC (I-p, S-p)	1.4+/-0.4	0.15+/-0.02
	NH_4^+[j]	UC (I-p)	1.5+/-0.9	0.23+/-0.02
glutamate	NADPH	NC (I-l, S-p)	40+/-20	21+/-2
	2-ketoglutarate	NC (l)	7.7+/-0.7	70+/-20
	NH_4^+	NC (l)	25+/-2	19+/-1
Glutarate	$NADP^+$	NC/UC (l)[k]		4.6+/-0.3
	glutamate	C (l)	3.1+/-0.2	
Reactive Red	$NADP^+$	NC (l)	0.09+/-0.08	0.0051+/-0.0002
	glutamate	C (S-p)	0.0038+/-0.0004	0.090+/-0.007

a: Abbreviations for types of inhibition: C, competitive; NC, noncompetitive; UC, uncompetitive; l, linear; nl, nonlinear; p, parabolic; I-l, intercept replots linear; S-l, slope replots linear; I-p, intercept replots parabolic; S-p, slope replots parabolic.

b: A dash indicates the standard error for the K_i value was larger than the value itself or was unable to be determined.

c,d: 5mM and 2 mM glutamate, respectively.

e: The data fitted similarly to the equations for linear noncompetitive inhibition and I-linear, S-parabolic noncompetitive inhibition.

f,g: 0.05 mM and 0.1 mM NADPH, respectively.

h: The intercept replot was convex when [NADPH] was 0.1 mM. K_i values were not determined.

i,j: 0.05mm and 0.1 mM NADPH, respectively. This inhibition was uncompetitive when NADPH was present at 0.1 mM.

k: This experiment did not clearly distinguish between noncompetitive and uncompetitive inhibition.

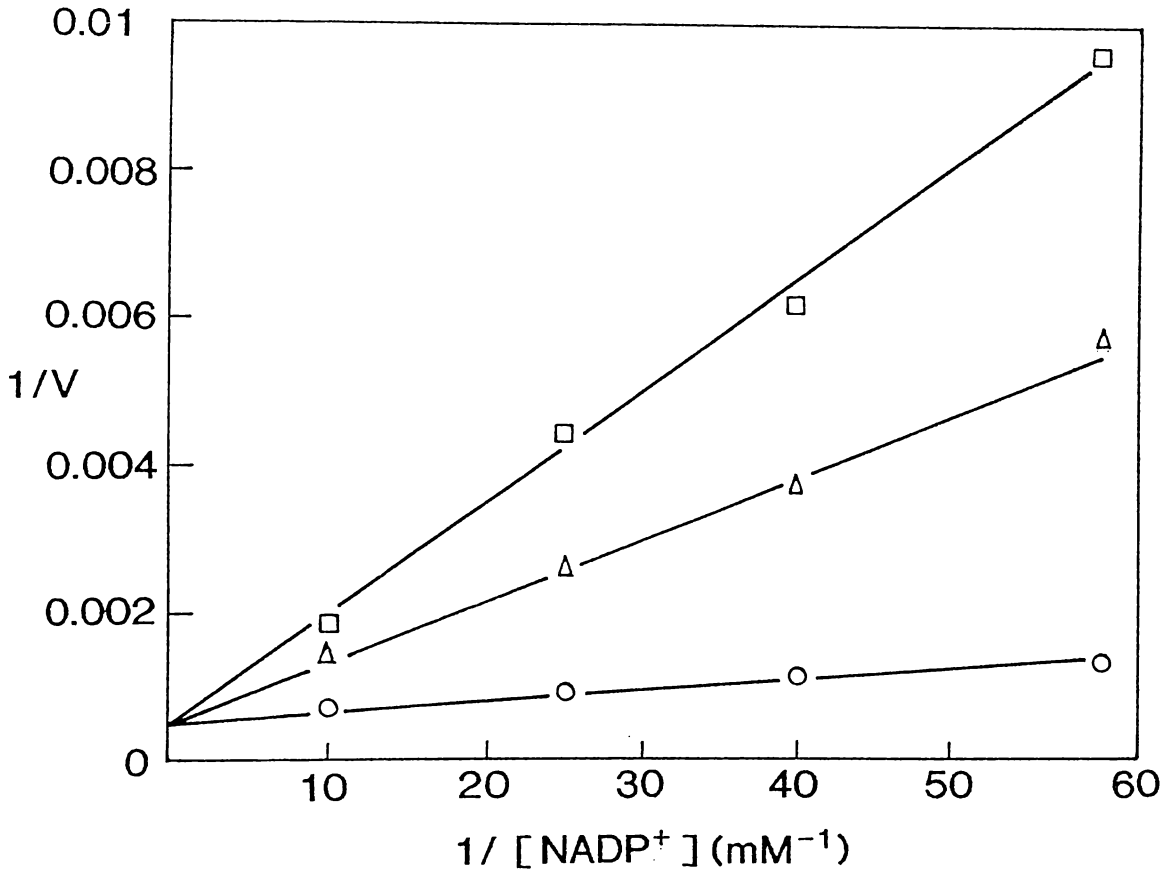

Fig. 13. Double reciprocal plot representing the inhibition by NADPH with respect to $NADP^+$. The concentration of glutamate was fixed at 5 mM. The velocities are expressed as units/mg. The concentrations of NADPH used were: zero (circle), 0.03 mM (triangle), 0.06 mM (square). The data were fitted to equation II.

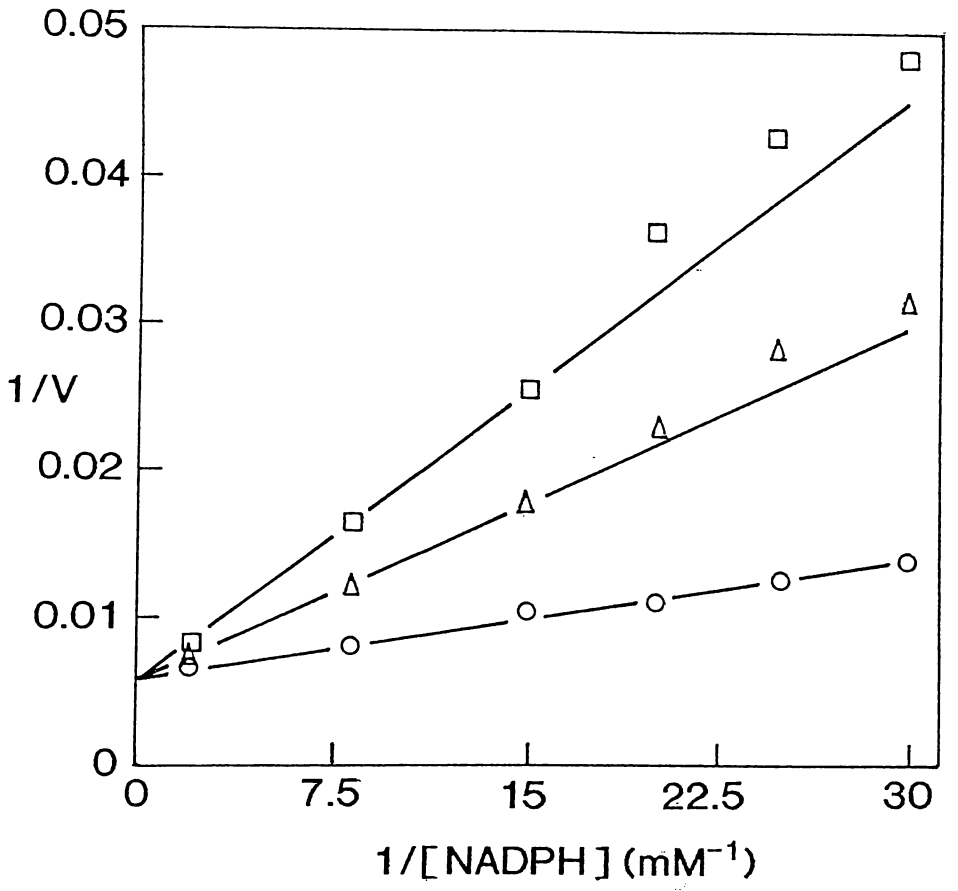

Fig. 14. Double reciprocal plot representing the inhibition by $NADP^+$ with respect to NADPH. The concentrations of NH_4Cl and 2-ketoglutarate were fixed at 10 mM and 3 mM, respectively. The velocities are expressed as units/mg. The concentrations of $NADP^+$ used were: zero (circle), 0.25 mM (triangle), 0.50 mM (square). The data were fitted to equation II.

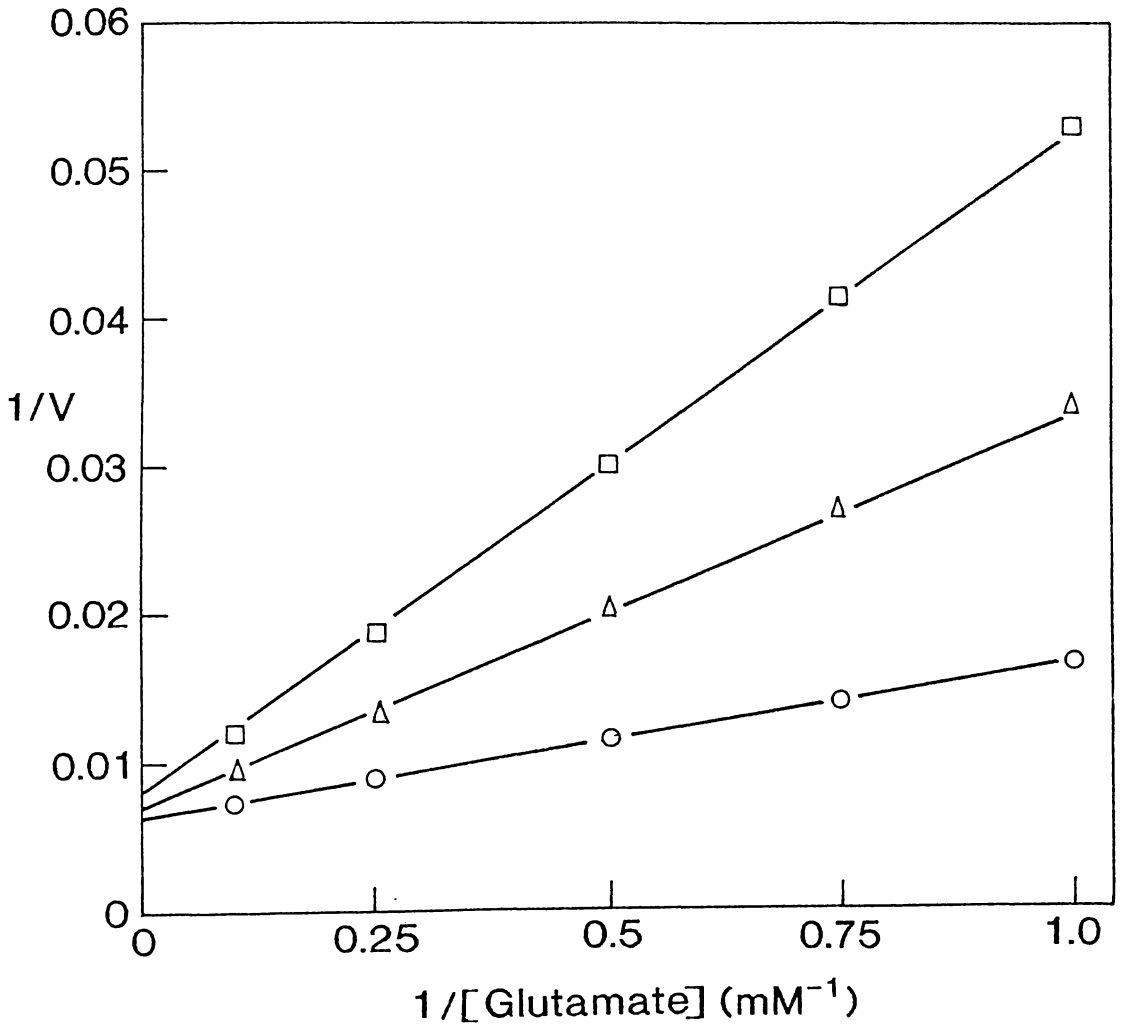

Fig. 15. Double reciprocal plot representing the inhibition by NADPH with respect to glutamate. The concentration of $NADP^+$ was fixed at 0.2 mM. The velocities are expressed as units/mg. The concentrations of NADPH used were: zero (circle), 0.06 mM (triangle), 0.125 mM (square). The data were fitted to equation VI.

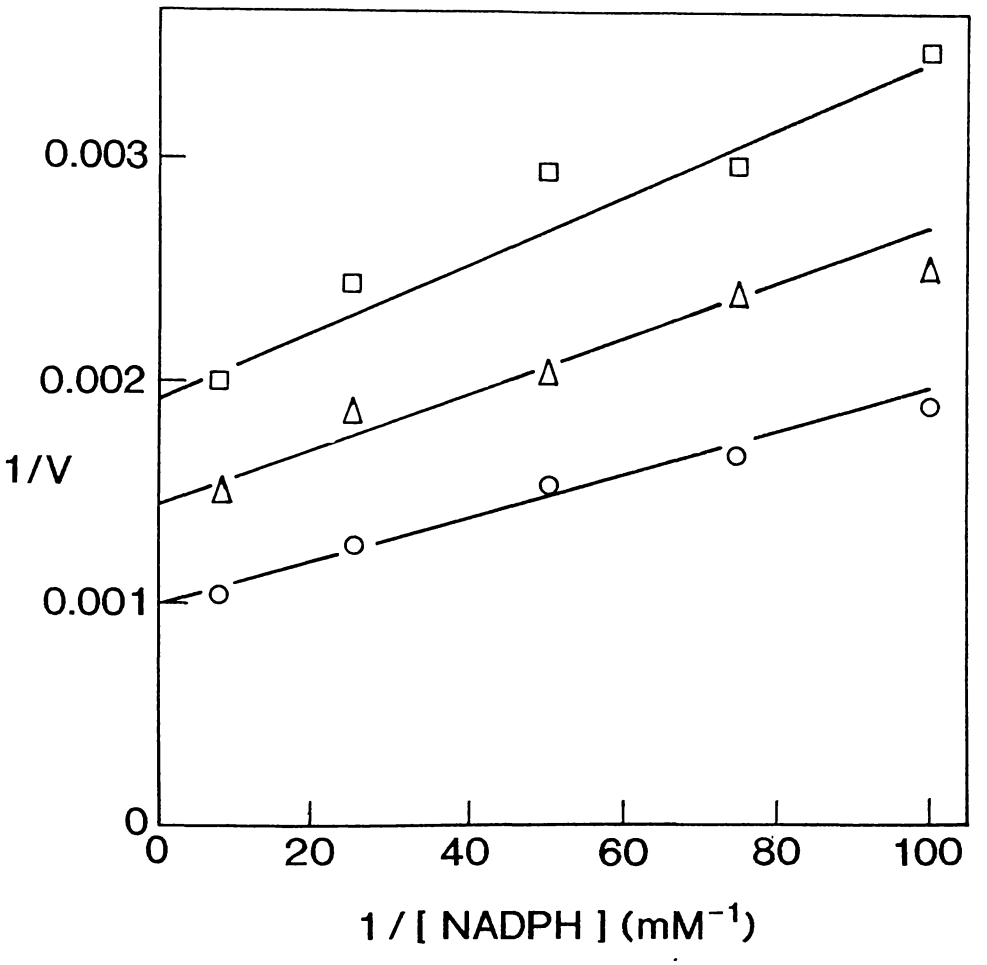

Fig. 16. Double reciprocal plot representing inhibition by glutamate with respect to NADPH. The concentrations of NH_4Cl and 2-ketoglutarate were fixed at 10 mM and 3 mM, respectively. The velocities are expressed as units/mg. The concentrations of glutamate used were: zero (circle), 10.0 mM (triangle), 20.0 mM (square). The data were fitted to equation VI.

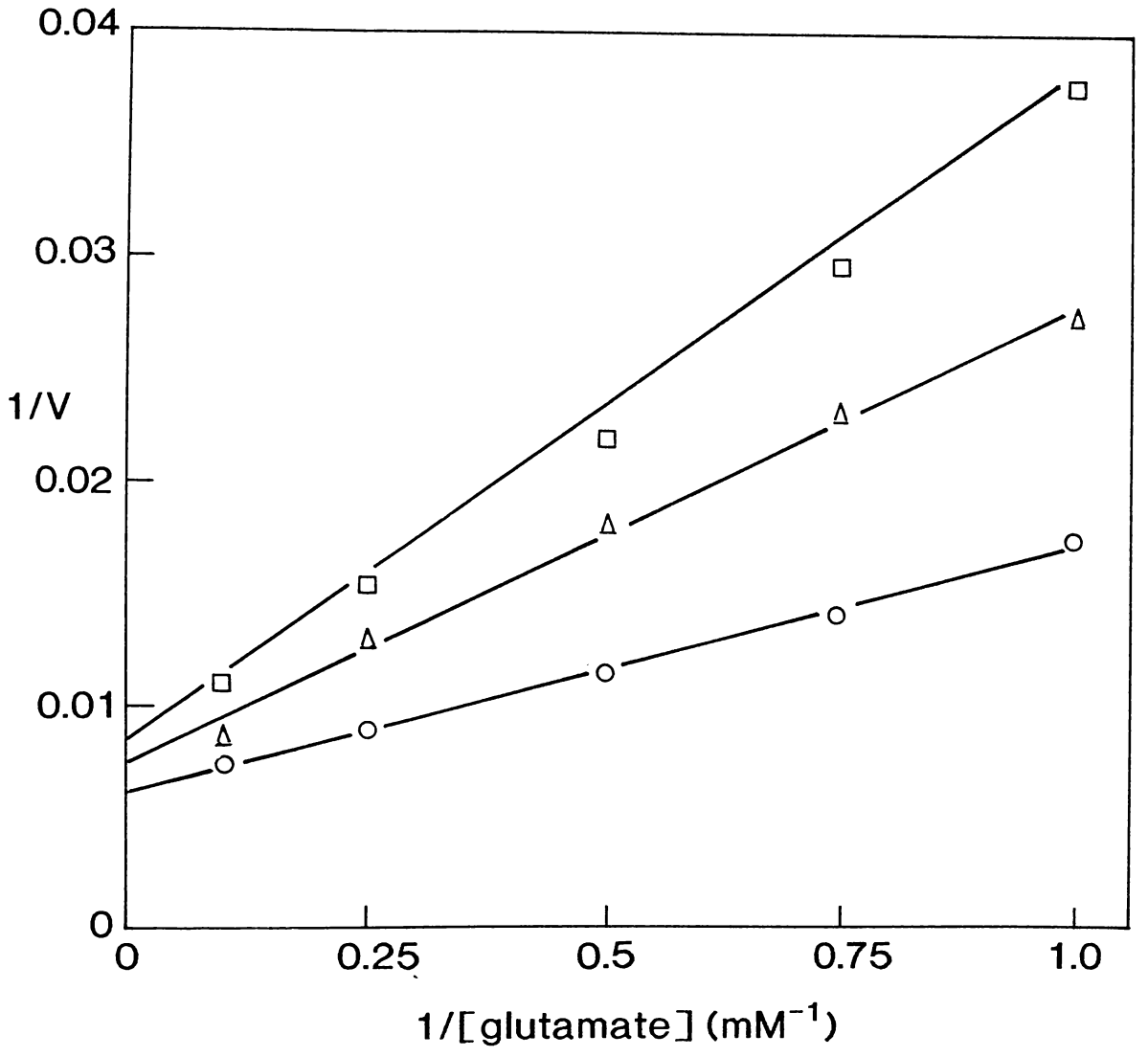

Fig. 17 Double reciprocal plot representing inhibition by 2-ketoglutarate with respect to glutamate. The concentration of $NADP^+$ was fixed at 0.2 mM. The velocities are expressed as units/mg. The concentrations of 2-ketoglutarate used were: zero (circle), 2.5 mM (triangle), 5 mM (square). The data were fitted to equation III.

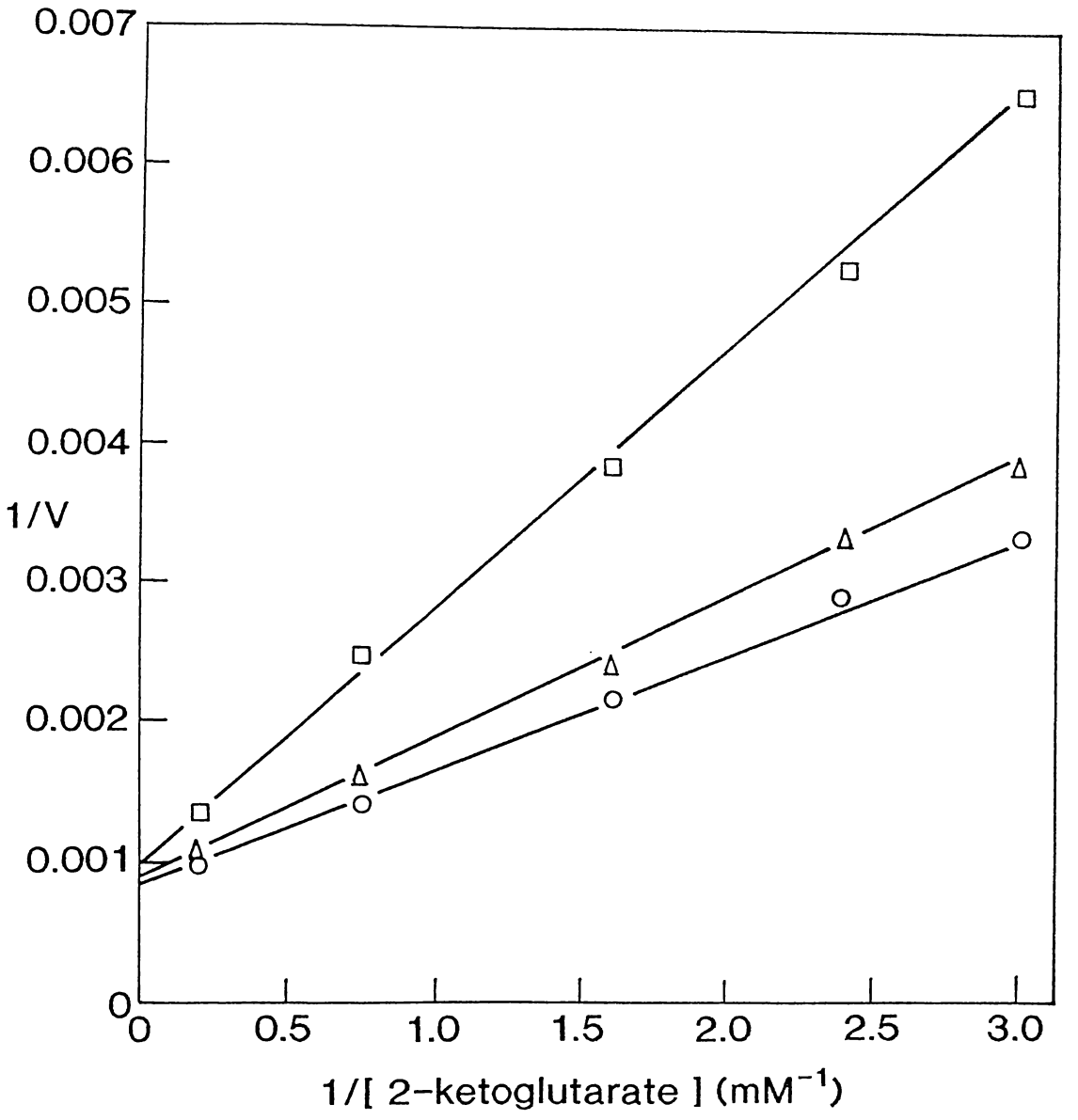

Fig. 18. Double reciprocal plot representing inhibition by glutamate with respect to 2-ketoglutarate. The concentrations of NADPH and NH_4Cl were fixed at 0.1 mM and 10 mM, respectively. The velocities are expressed as units/mg. The concentrations of glutamate used were: zero (circle), 2 mM (triangle), 10 mM (square). The data were fitted to equation III.

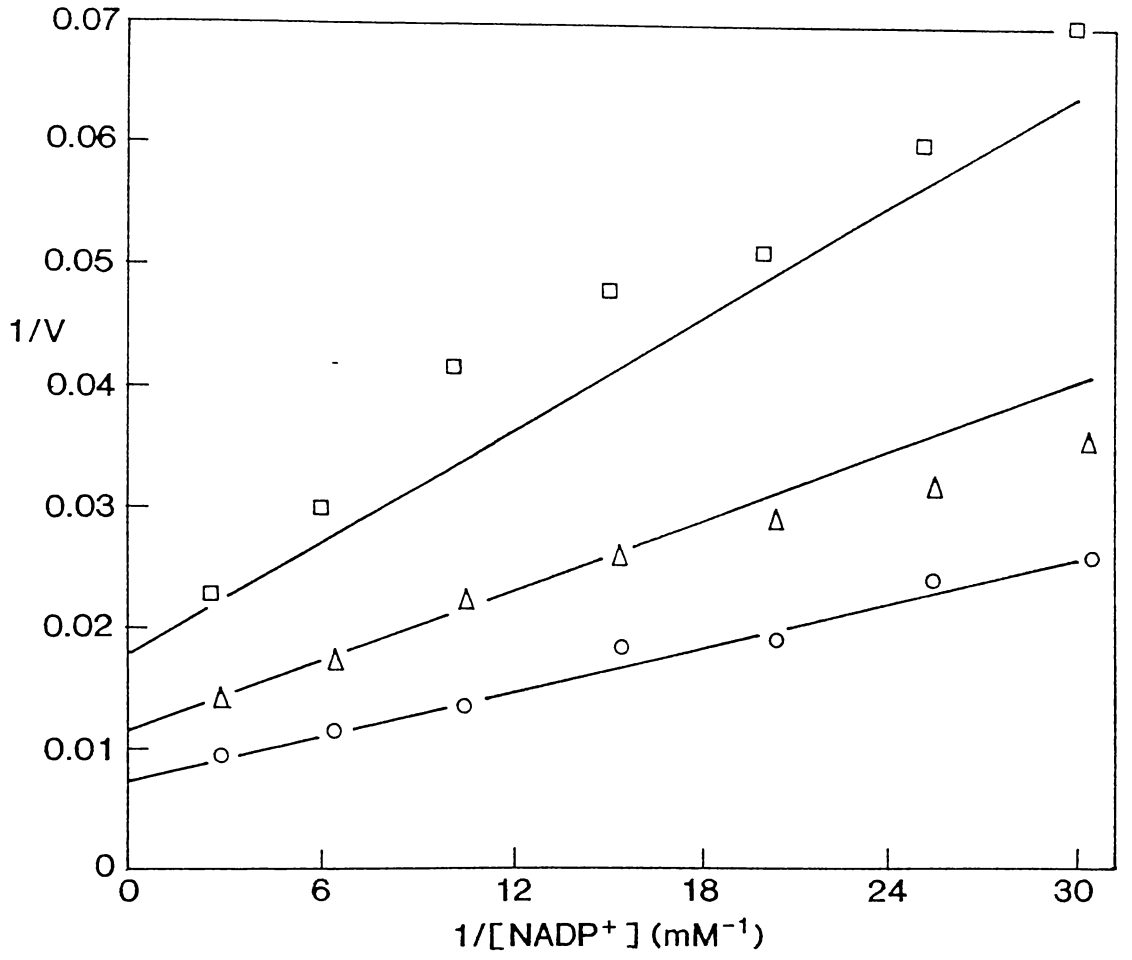

Fig. 19. Double reciprocal plot representing inhibition by NH_4Cl with respect to $NADP^+$. The concentration of glutamate was fixed at 5 mM. The velocities are expressed as units/mg. The concentrations of NH_4Cl used were: zero (circle), 5 mM (triangle), 10 mM (square). The data were fitted to equation VII.

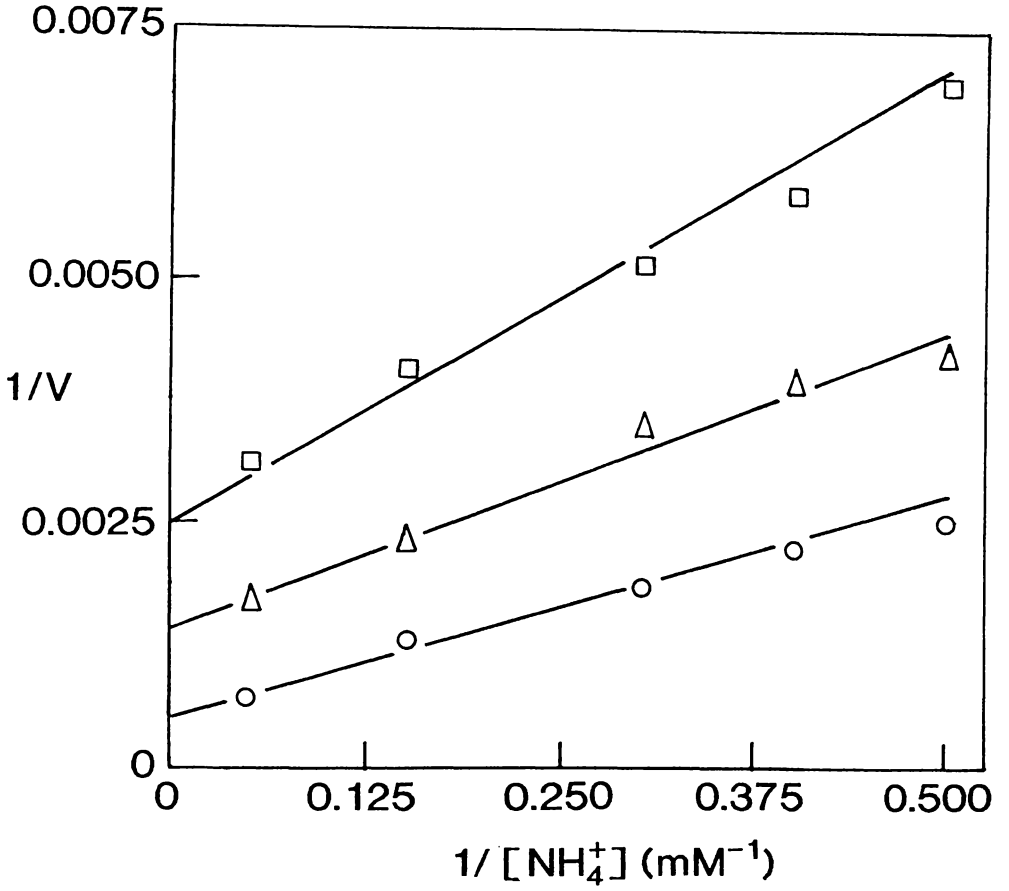

Fig. 20. Double reciprocal plot representing inhibition by $NADP^+$ with respect to NH_4Cl at a low concentration of NADPH (0.05 mM). The concentration of 2-ketoglutarate was fixed at 3 mM. The velocities are expressed as units/mg. The concentrations of $NADP^+$ used were: zero (circle), 0.2 mM (triangle), 0.4 mM (square). The data were fitted to equation VII.

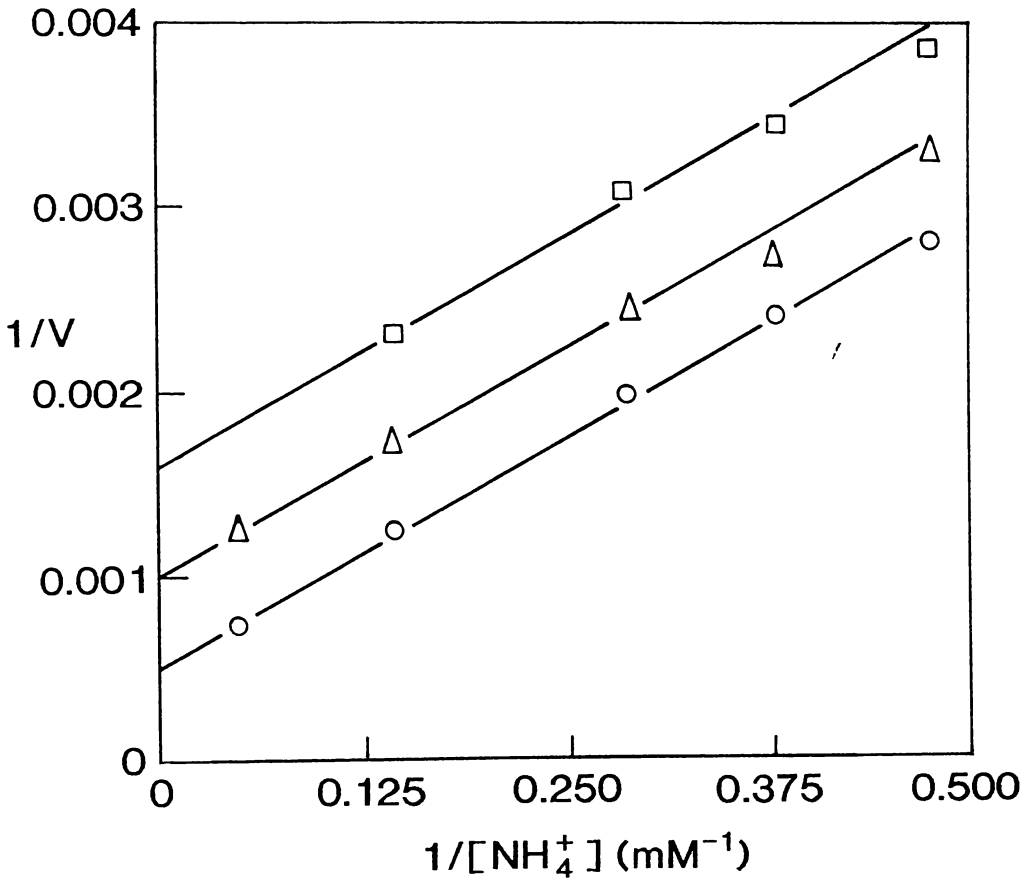

Fig. 21. Double reciprocal plot representing inhibition by $NADP^+$ with respect to NH_4Cl at a high concentration of NADPH (0.1 mM). The concentration of 2-ketoglutarate was fixed at 3 mM. The velocities are expressed as units/mg. The concentrations of $NADP^+$ used were: zero (circle), 0.2 mM (triangle), 0.4 mM (square). The data were fitted to equation VIII.

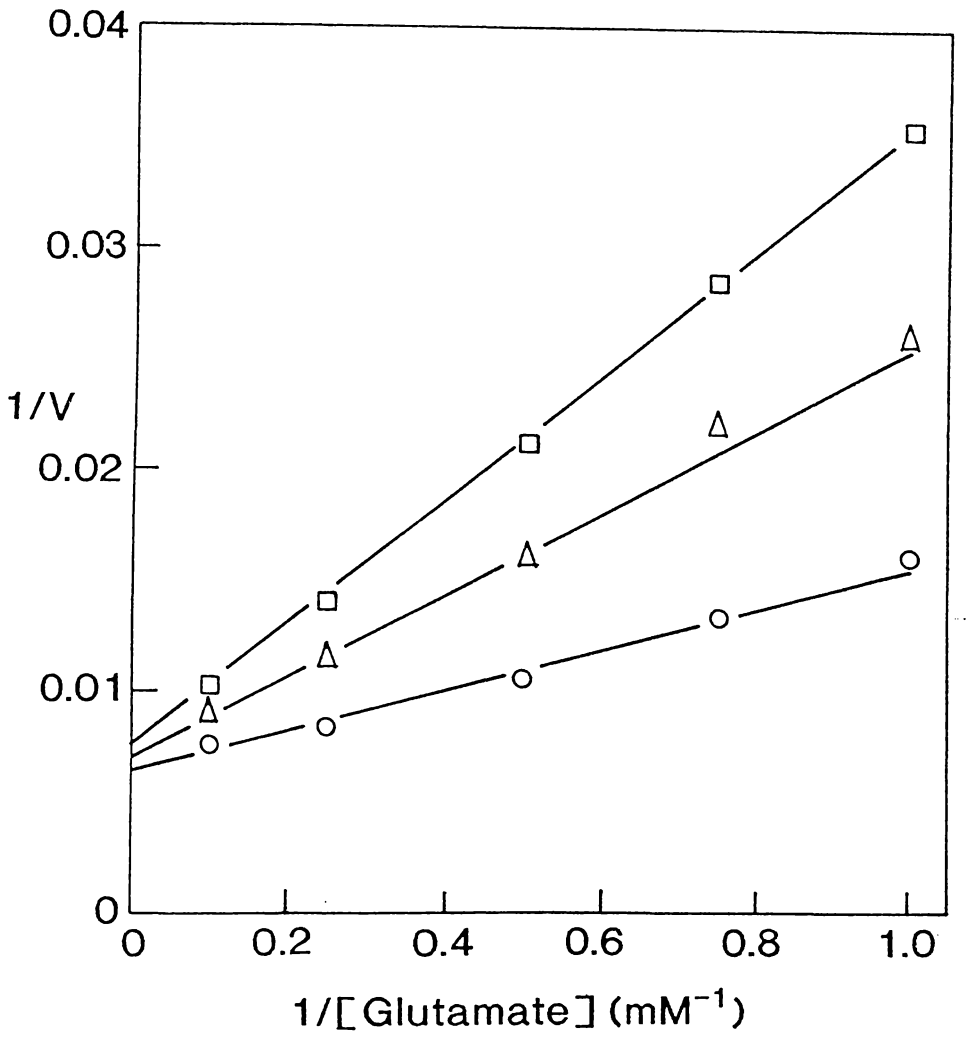

Fig. 22. Double reciprocal plot representing inhibition by NH_4^+ with respect to glutamate. The concentration of $NADP^+$ was fixed at 0.2 mM. The velocities are expressed as units/mg. The concentrations of NH_4^+ used were: zero (circle), 5 mM (triangle), 10 mM (square). The data were fitted to equation III.

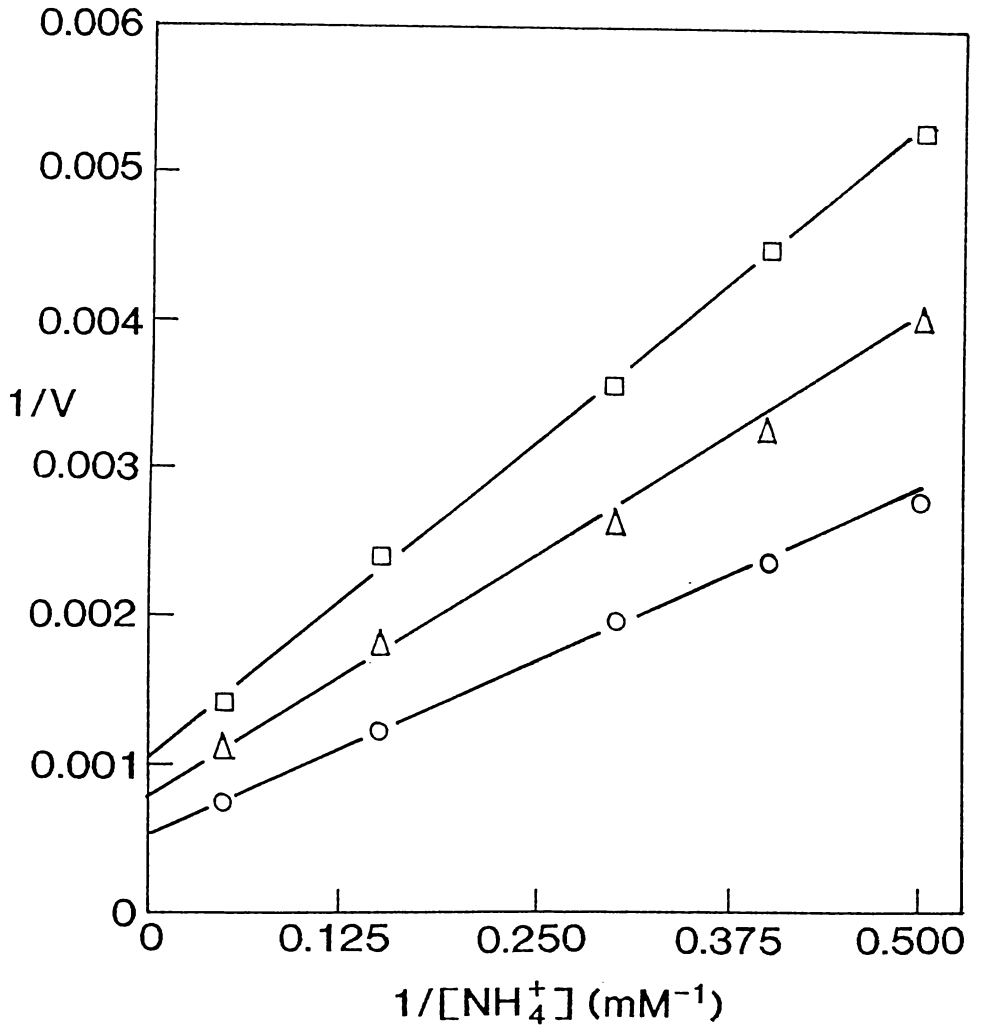

Fig. 23. Double reciprocal plot representing inhibition by glutamate with respect to NH_4^+. The concentrations of NADPH and 2-ketoglutarate were fixed at 0.1 mM and 3 mM, respectively. The velocities are expressed as units/mg. The concentrations of glutamate used were: zero (circle), 10 mM (triangle), 20 mM (square). The data were fitted to equation III.

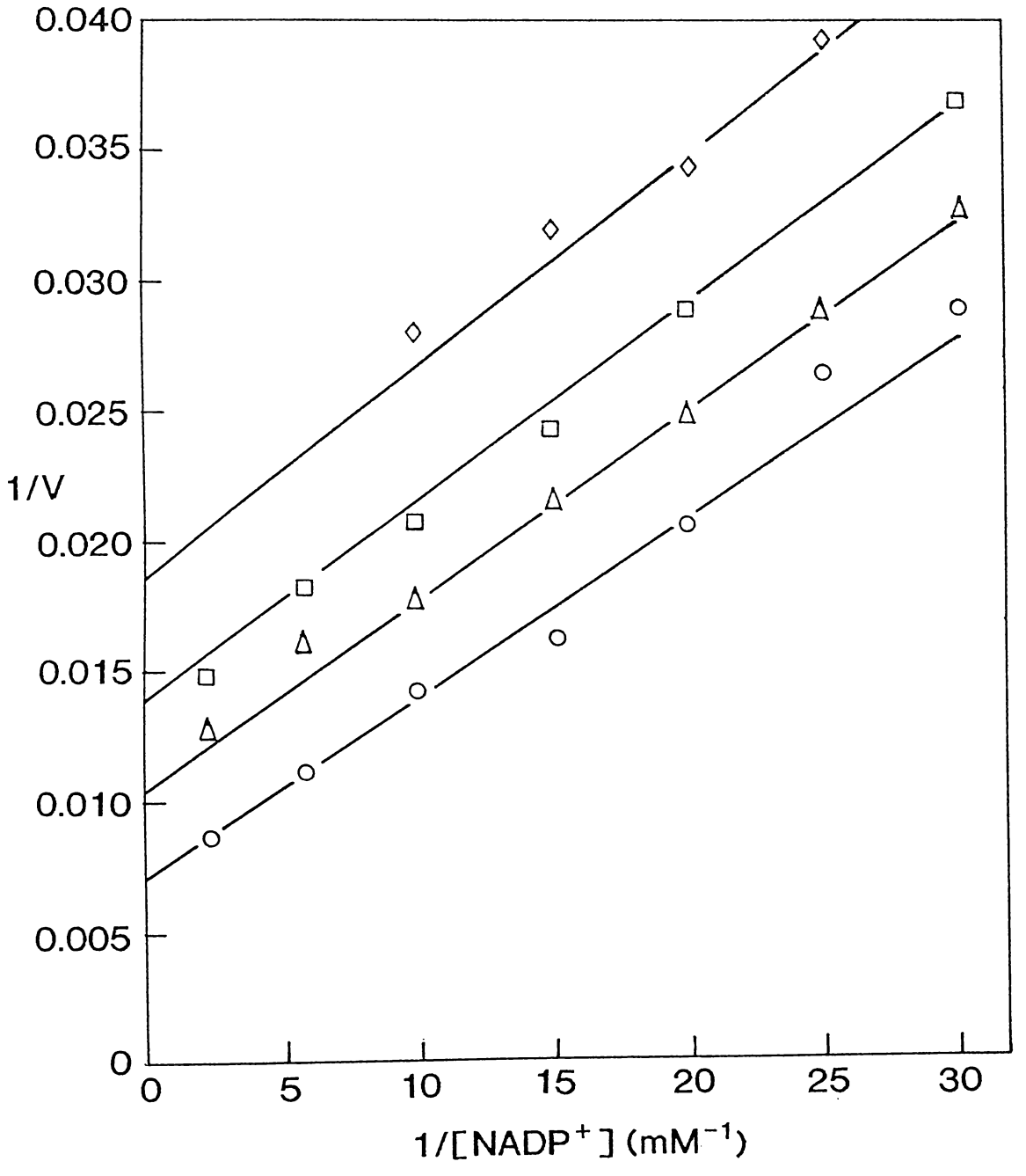

Fig. 24. Double reciprocal plot representing inhibition by 2-ketoglutarate with respect to $NADP^+$. The concentration of glutamate was fixed at 2 mM. The velocities are expressed as units/mg. The concentrations of NH_4Cl used were: zero (circle), 1.5 mM (triangle), 3 mM (square), 5 mM (diamond). The data were fitted to equation III.

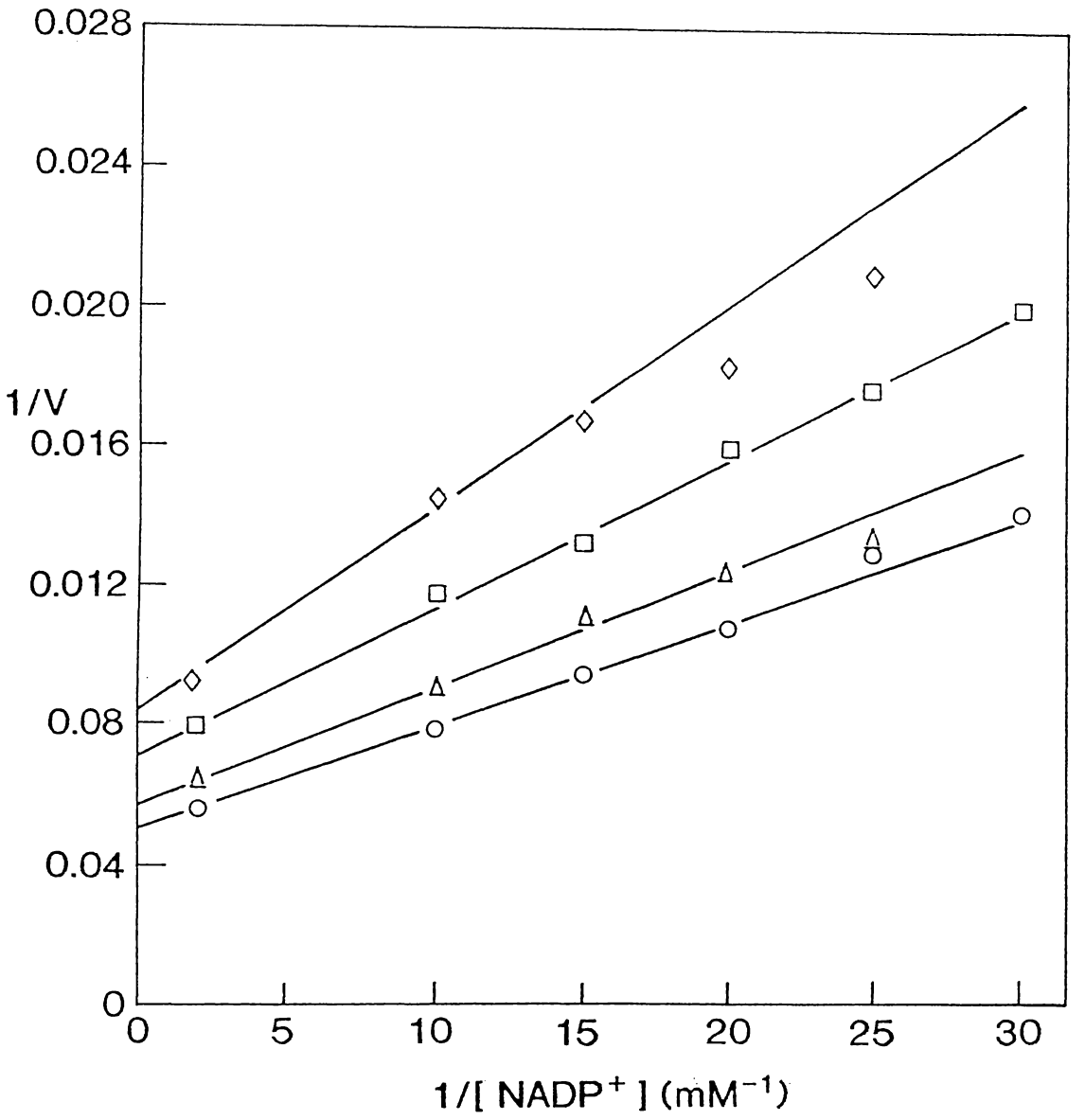

Fig. 25. Double reciprocal plot representing inhibition by 2-ketoglutarate with respect to $NADP^+$. The concentration of glutamate was fixed at 5 mM. The velocities are expressed as units/mg. The concentrations of 2-ketoglutarate used were: zero (circle), 1 mM (triangle), 3 mM (square), 5mM (diamond). The data were fitted to equation III.

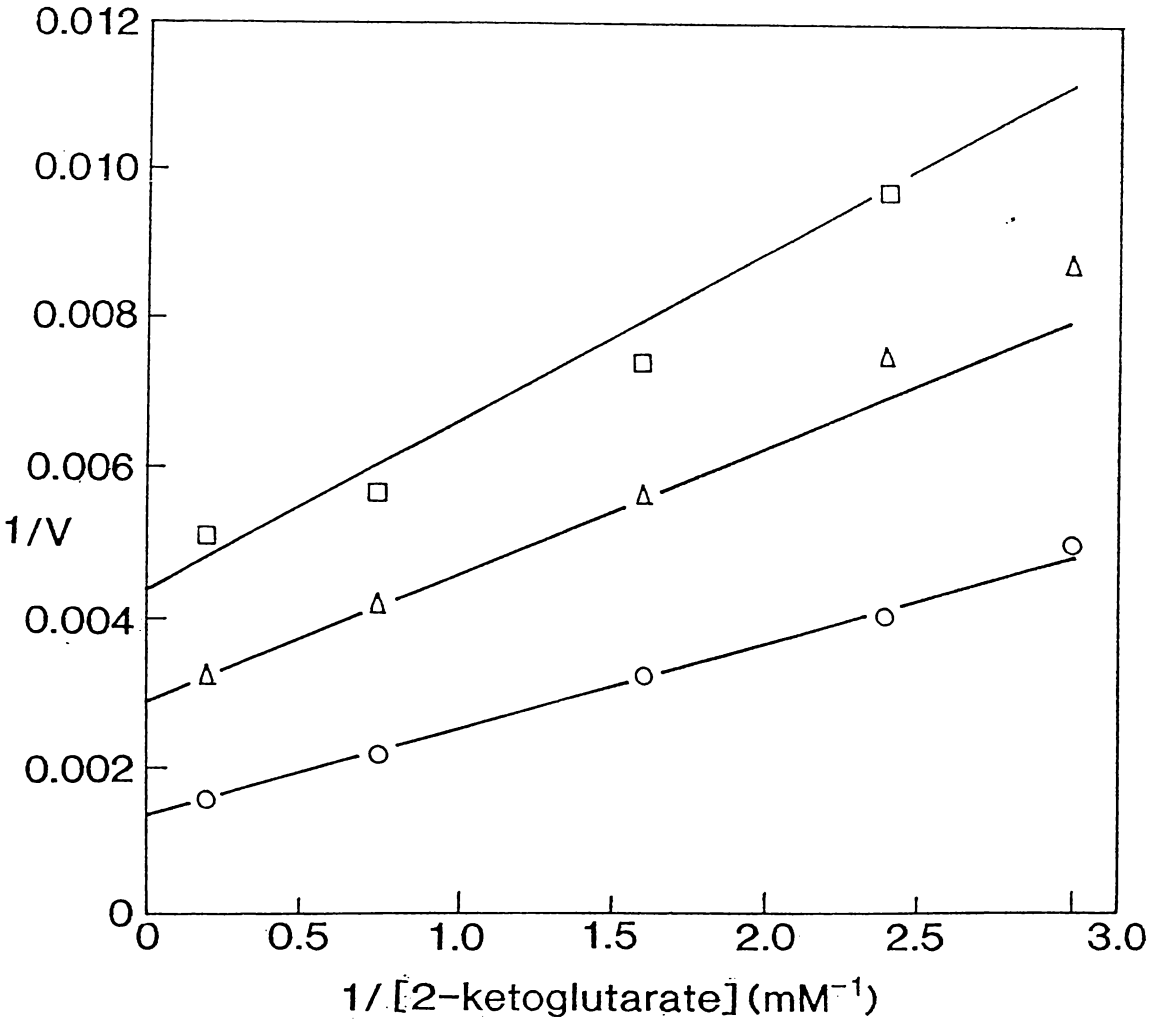

Fig. 26. Double reciprocal plot representing inhibition by $NADP^+$ with respect to 2-ketoglutarate at a low concentration of NADPH (0.05 mM). The concentration of NH_4Cl was fixed at 10 mM. The velocities are expressed as units/mg. The concentrations of $NADP^+$ used were: zero (circle), 0.2 mM (triangle), 0.4 mM (square). The data were fitted to equation III.

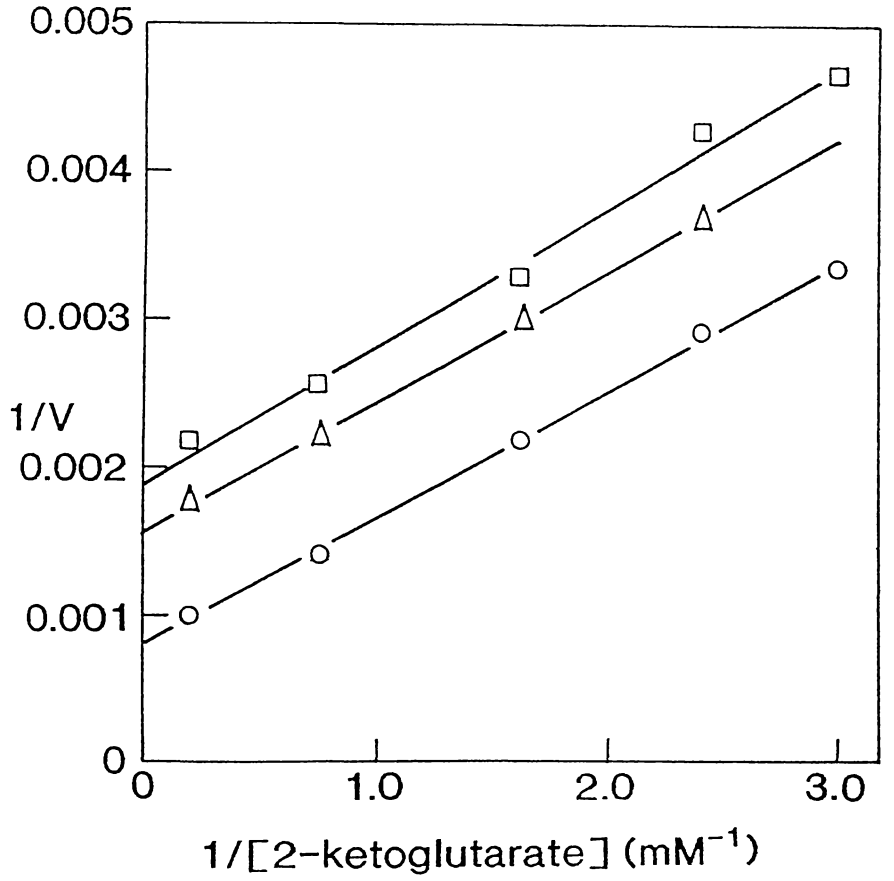

Fig. 27. Double reciprocal plot representing inhibition by $NADP^+$ with respect to 2-ketoglutarate at a high concentration of NADPH (0.1 mM). The concentration of NH_4Cl was fixed at 10 mM. The velocities are expressed as units/mg. The concentrations of $NADP^+$ used were: zero (circle), 0.2 mM (triangle), 0.4 mM (square).

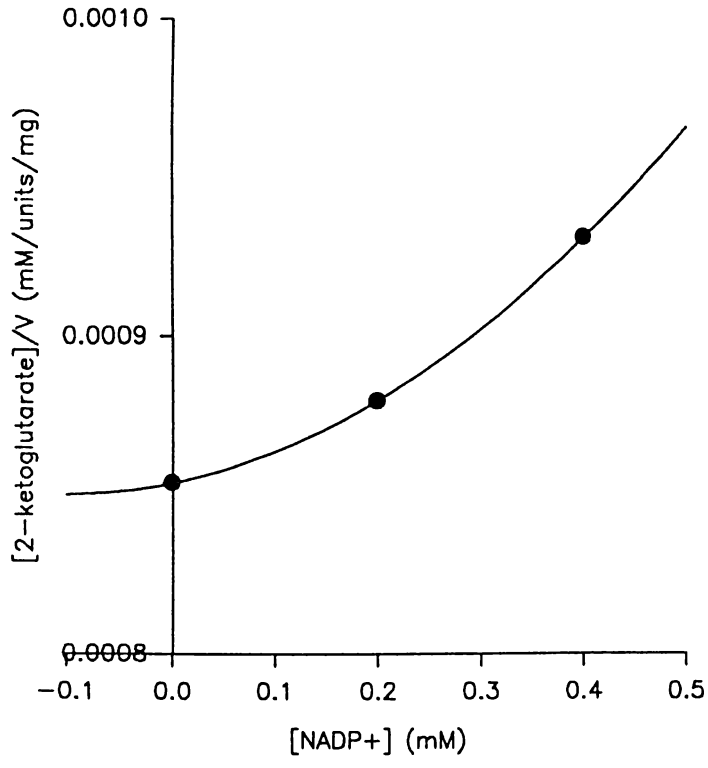

Fig. 28. Replot of the slopes of Fig. 27.

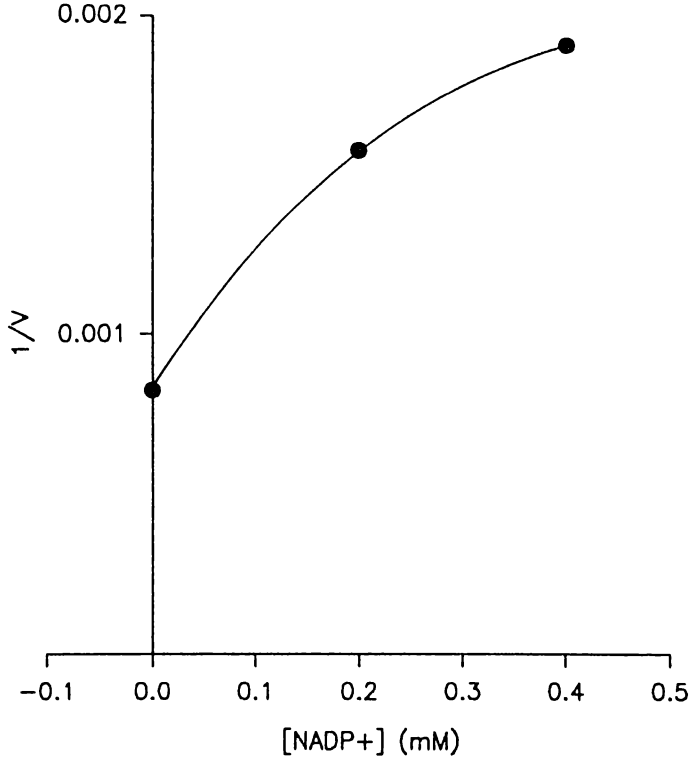

Fig. 29. Replot of the intercepts of Fig. 27.
The velocities are expressed as units/mg.

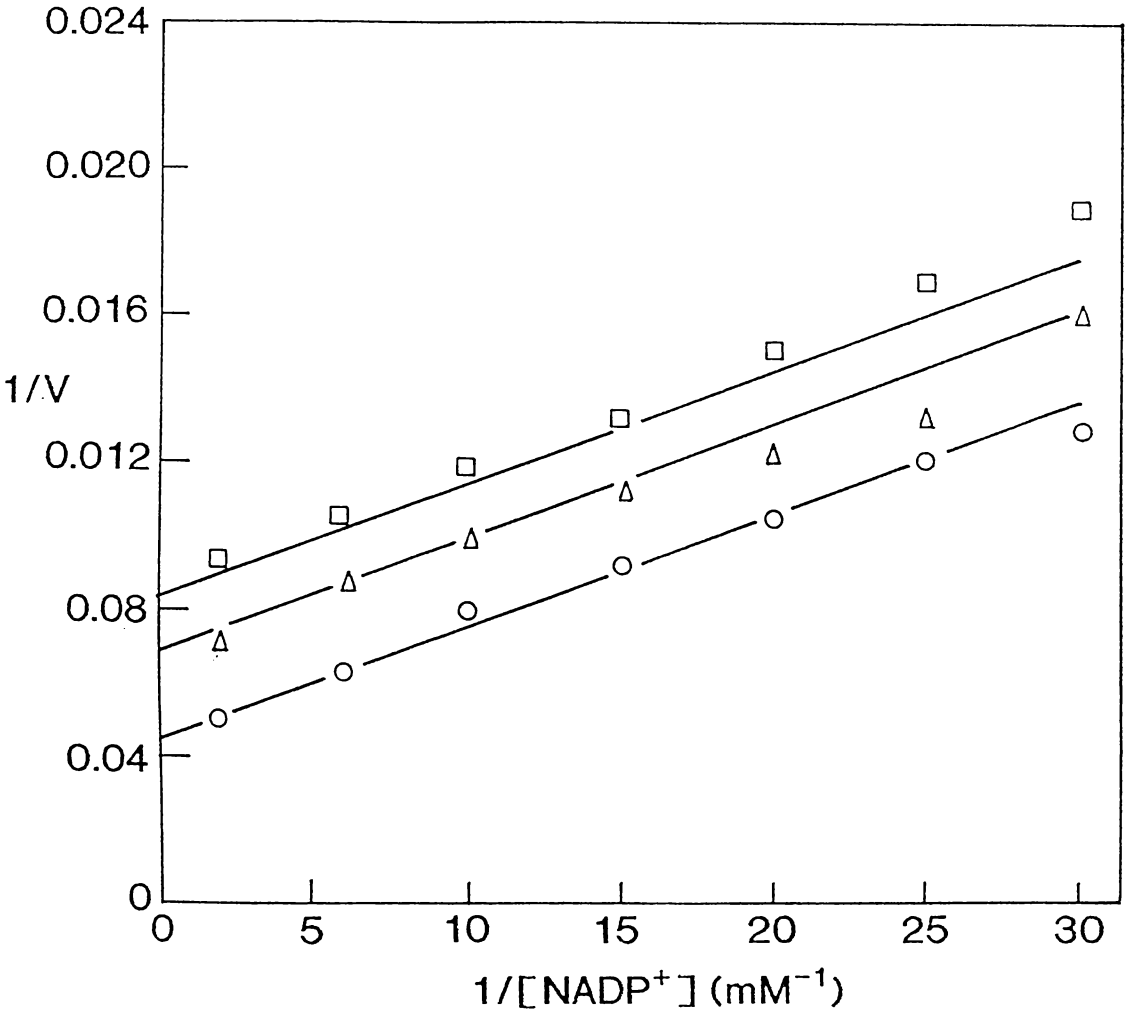

Fig. 30. Double reciprocal plot representing inhibition by glutarate with respect to $NADP^+$. The concentration of glutamate was fixed at 5 mM. The velocities are expressed as units/mg. The concentrations of glutarate used were: zero (circle), 2.5 mM (triangle), 4 mM (square). The data were fitted to equation IV.

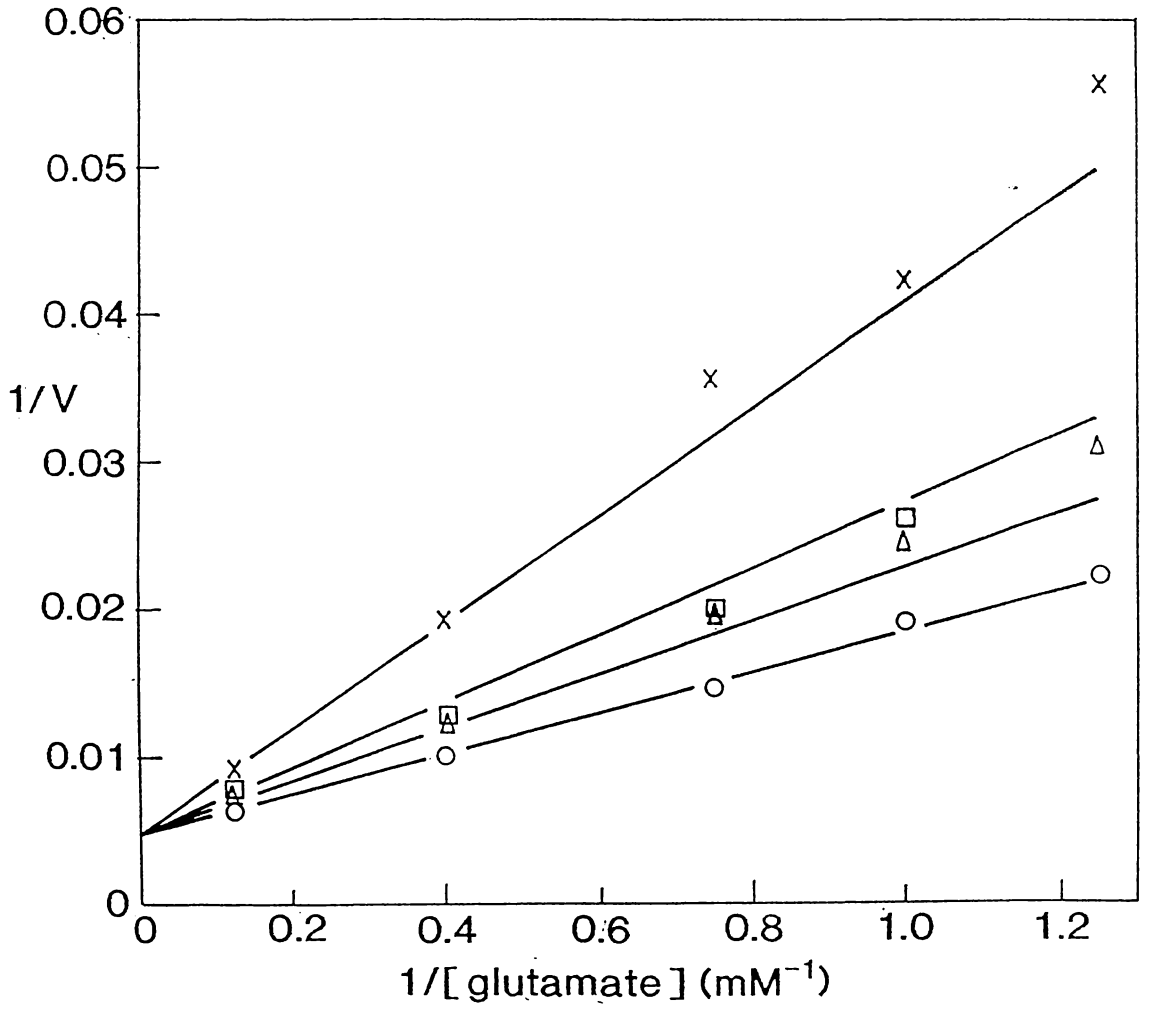

Fig. 31. Double reciprocal plot representing inhibition by glutarate with respect to glutamate. The concentration of $NADP^+$ was fixed at 0.2 mM. The velocities are expressed as units/mg. The concentrations of glutarate used were: zero (circle), 1 mM (triangle), 2 mM (square), 5 mM (diamond). The data were fitted to equation II.

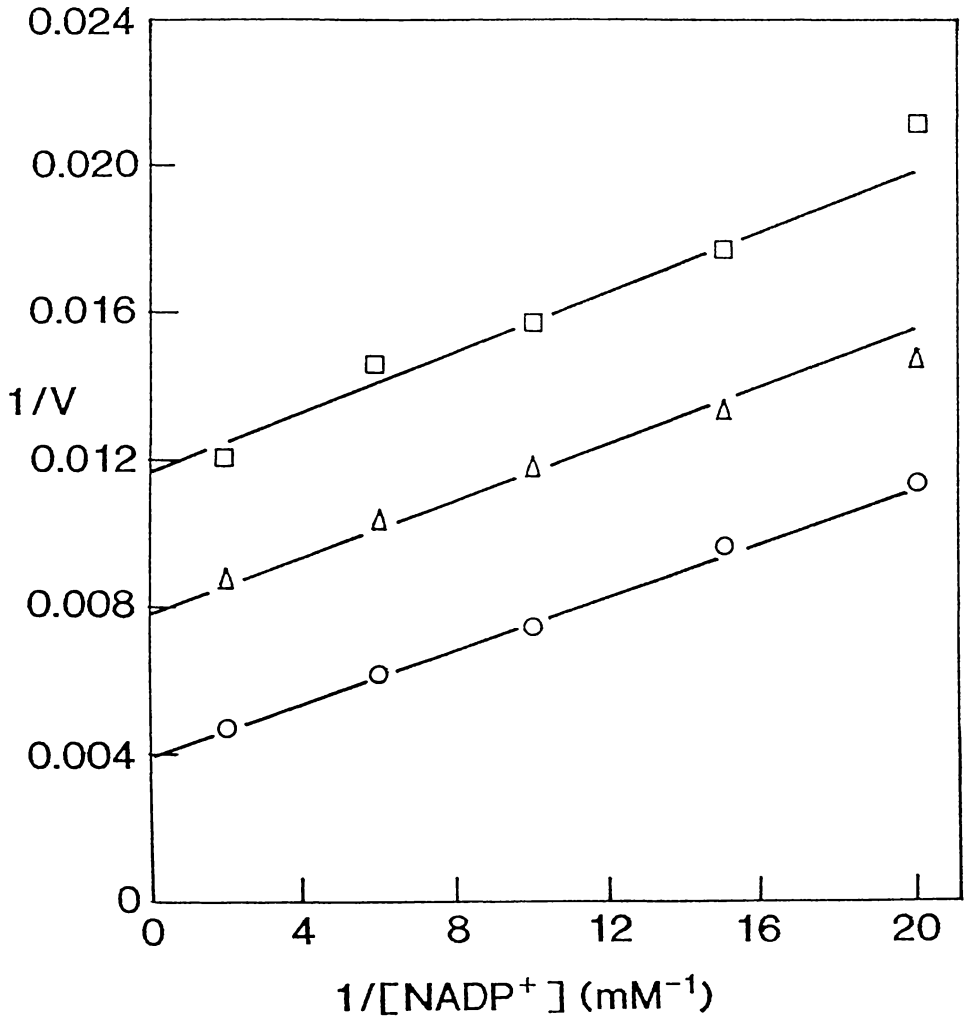

Fig. 32. Double reciprocal plot representing inhibition by Reactive Red 120 with respect to $NADP^+$. The concentration of glutamate was fixed at 5 mM. The velocities are expressed as units/mg. The concentrations of Reactive Red used were: zero (circle), 0.005 mM (triangle), 0.01 mM (square). The data were fitted to equation III.

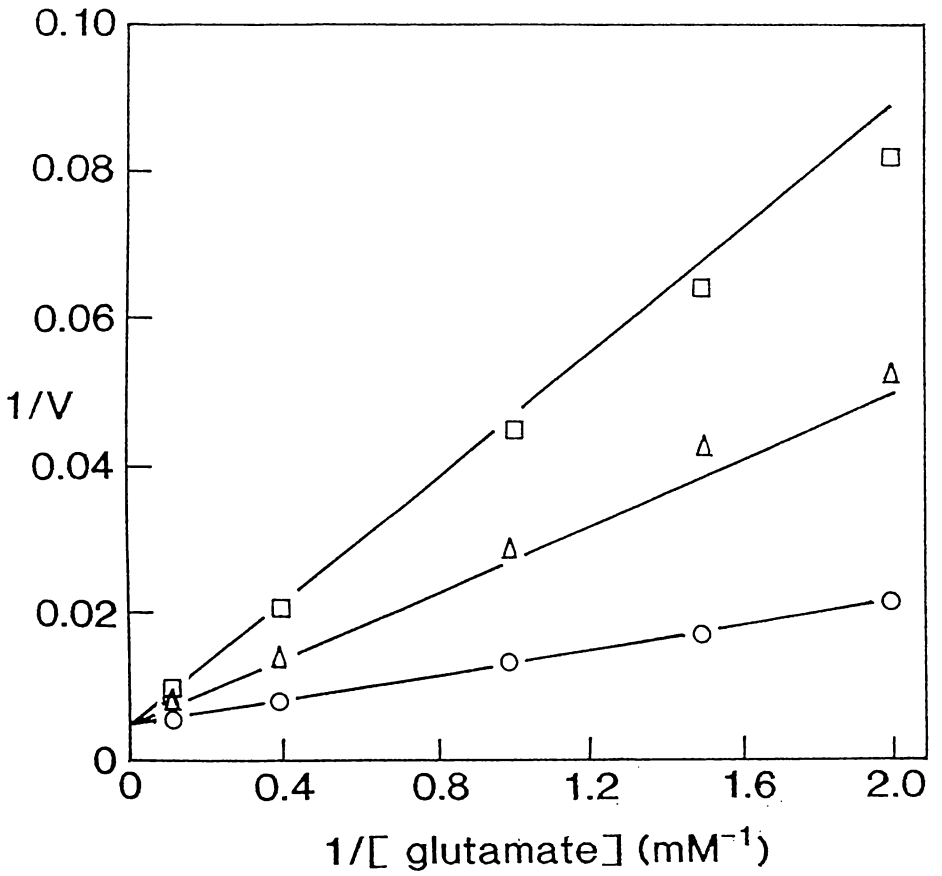

Fig. 33. Double reciprocal plot representing inhibition by Reactive Red 120 with respect to glutamate. The concentration of $NADP^+$ was fixed at 0.2 mM. The velocities are expressed as units/mg. The concentrations of Reactive Red used were: zero (circle), 0.005 mM (triangle), 0.01 mM (square). The data were fitted to equation V.

Inactivation of the Glutamate Dehydrogenase by Pyridoxal 5'-Phosphate

The glutamate dehydrogenase was inhibited 70% by 2mM pyridoxal 5'-phosphate in the absence of substrates. Inhibition of glutamate dehydrogenases by this reagent is known to be reversible (Engel, 1981). However, reactivation did not commence until the enzyme had been in the assay solution for approximately 50 sec, hence we were able to measure inhibited reaction rates. The effect of saturating concentrations of substrates in protecting the enzyme against inactivation by pyridoxal 5'-phosphate is summarised in Table 3. Glutarate, a competitive inhibitor of glutamate, was used in place of this substrate because glutamate is known to react with pyridoxal 5'-phosphate (Chen and Engel, 1974).

TABLE 3

INHIBITION OF ISOLATE AN1 GLUTAMATE DEHYDROGENASE BY PYRIDOXAL 5'-PHOSPHATE

Added Substrate or Inhibitor	% Inhibition
None	69
$NADP^+$	42
glutarate	70
$NADP^+$ + glutarate	13
NADPH	37
2-ketoglutarate	72
NH_4^+	77
NADPH + 2-ketoglutarate + NH_4^+	21

DISCUSSION

The initial velocity studies of the oxidative deamination reaction showed that the mechanism is sequential. A random substrate binding order is proposed for this reaction on the basis of the relationship between K_{NADP+}, K_{glu}, K_{iNADP+} and K_{iglu} ($K_{NADP+}K_{iglu} = K_{glu}K_{iNADP+}$), and the Dalziel relationship (VmaxKia/Ka must be greater than the Vmax(reverse direction) for a compulsory order mechanism, or equal to Vmax(reverse direction) for a Theorell-Chance mechanism). The initial velocity studies of the reductive amination reaction showed that it is also sequential. The relationship between the intersection points of the Lineweaver-Burk (or Hanes) plots and the K_i values determined from the secondary plots suggested a random order of substrate binding for this reaction. Since the initial velocity data for the reductive amination reaction were not computer-fitted, we realise that there is room for subjectivity in the plotting technique. However, since the data were linear and reliable, and parabolas were able to be computer-fitted to the replots with correlation coefficients of >0.99, this should not influence the interpretation of the data. Initial velocity studies of the reductive amination reaction catalysed by the bovine liver and *Clostridium symbiosum* glutamate dehydrogenases also indicated random binding[3] (Engel and Dalziel, 1970; Syed *et al.*, 1991), but initial velocity studies of the oxidative deamination reactions catalysed by these two enzymes did not clearly distinguish between ordered and random mechanisms (Engel and Dalziel, 1970; Syed *et al.*, 1991).

When the initial velocities of the reductive amination reaction were studied with saturating concentrations of either 2-ketoglutarate or ammonia, it was observed that the lines on the Lineweaver-Burk plot appeared almost parallel in the former case (Fig. 11), but not in the latter (Fig. 12). These results are the opposite to those of Frieden (1959) for the bovine liver glutamate dehydrogenase which led to a compulsory order of substrate addition (NADPH, ammonia, 2-ketoglutarate) being proposed for the reductive deamination reaction catalysed by this enzyme. In the light of the results from the (nonsaturating) initial velocity studies of the isolate AN1 glutamate dehydrogenase, we

[3] The study by Rife and Cleland (1980), which used initial velocity studies plus other methods, indicated a partially ordered mechanism.

propose that when ammonia is present in high concentration it is the first substrate to bind (outcompeting NADPH and 2-ketoglutarate). We also propose that 2-ketoglutarate may have a much higher affinity for the enzyme-NADPH complex than for the free enzyme, hence when it is present in high concentration ammonia is almost outcompeted for the binding of the enzyme-NADPH, therefore increasing the concentration of NADPH has very little effect on the slopes of the Lineweaver-Burk plots with ammonia as the variable substrate (and visa-versa).

The results of the product inhibition study were not compatible with either a compulsory order or a rapid-equilibrium random mechanism. To reconcile our product inhibition results with a compulsory order mechanism would require the formation of some unlikely or disallowed abortive complexes (such as enzyme-ammonia, which would be disallowed because in such a mechanism only $NADP^+$ and NADPH may bind the free enzyme, as indicated by their mutual competition). The product inhibition study of Engel and Chen (1975) was compatible with a rapid-equilibrium random mechanism for the bovine liver glutamate dehydrogenase, but did not exclude compulsory order mechanisms. With one exception, the product inhibition patterns for the bovine glutamate dehydrogenase were found to be linear (Engel and Chen, 1975). The product inhibition patterns for the glutamate dehydrogenase from lupin nodules were also found to be linear (Stone *et al.*, 1980a and b), and were almost identical to those found for the bovine liver enzyme (although a fully ordered mechanism was proposed for the lupin nodule enzyme). In contrast, many of the product inhibitions for the isolate AN1 glutamate dehydrogenase were parabolic: this may be explained by the combination of the inhibitor at more than one point in the reaction sequence (Cleland, 1970), assuming a steady state system. In a rapid equilibrium random mechanism (all enzyme substrate complexes in rapid equilibrium with one another) this does not hold true. Therefore the product inhibition pattern displayed by the isolate AN1 glutamate dehydrogenase was not compatible with a rapid-equilibrium random mechanism, but was compatible with a steady state random mechanism. The abortive complexes required to account for the product inhibition pattern shown by the isolate AN1 glutamate dehydrogenase (assuming a steady state random mechanism) are

presented in Table 4. Table 5 summarises the inhibition patterns expected for compulsory order, rapid-equilibrium random and steady state random mechanisms.

TABLE 4

EXPLANATION OF THE PRODUCT INHIBITION PATTERN FOR THE GLUTAMATE DEHYDROGENASE FROM ISOLATE AN1*

Observation*	Explanation	Abortive Complexes Required
1. Mutually competitive inhibition between $NADP^+$ and NADPH. (13, 14)	The unimolecular rate constants for the formation of the abortive complexes E-NADP+ and E-NADPH must be much lower than the rate constants for the formation of the ternary complexes E-($NADP^+$-glu) and E-(NADPH-2kg-NH_4^+).	E-$NADP^+$ E-NADPH
2. I-linear, S-parabolic inhibition by NADPH with respect to glutamate. (15)	Product inhibition has an S + I effect. The formation of E-NADPH also has an S + I effect, but the connection between the 2 I effects is broken by the addition of glutamate. Hence NC (I-l, S-p).	E-NADPH
3. I-linear, S-parabolic inhibition by glutamate with respect to NADPH. (16)	Product inhibition has an S + I effect. The formation of E-glu has an S effect. Hence NC (I-l, S-p).	E-glu
4. Linear noncompetitive inhibition by 2-ketoglutarate with respect to glutamate. (17)	Product inhibition has an S + I effect. There is presumably negligible formation of E-2kg (the rate constant for the formation of E-2kg must be much higher than that for the formation of E-(NADPH-2kg-NH_4^+)).	
5. Linear noncompetitive inhibition by glutamate with respect to 2-ketoglutarate. (18)	Product inhibition has an S + I effect. Presumably there is negligible formation of E-glu due to the high concentration of NADPH used (0.1 mM).	
6. I-parabolic, S-parabolic inhibition by NH_4^+ with respect to $NADP^+$. (19)	Product inhibition has an S + I effect. The formation of E-NH_4^+ or E-glu-NH_4^+ has an S effect, and the formation of E-$NADP^+$-NH_4^+ or E-NADP+-glu-NH_4^+ has an I effect. Hence NC (I-p, S-p).	E-NH_4^+ or E-glu-NH_4^+ E-$NADP^+$-NH_4^+ or E-$NADP^+$-glu-NH_4^+
7. I-parabolic, S-parabolic inhibition by $NADP^+$ with respect to NH_4^+ ([NADPH] = 0.05 mM). (20)	Product inhibition has an S + I effect. Formation of E-$NADP^+$ also has an S + I effect, but the connection between the 2 I effects is broken by the addition of NH_4^+. Formation of E-NH_4^+-$NADP^+$ or E-2-kg-NH_4^+-$NADP^+$ would give an I effect which is connected to the effect produced by product inhibition. Hence NC (I-p, S-p).	E-$NADP^+$ (or E-2kg-$NADP^+$) E-NH_4^+-$NADP^+$ or E-2kg-NH_4^+-$NADP^+$
8. Uncompetitive (I-p) inhibition by $NADP^+$ with respect to NH_4^+ ([NADPH] = 0.1 mM). (21)	NADPH at 0.1 mM (7xKm) outcompetes $NADP^+$ for binding of the free enzyme, and since there was no obvious slope effect, there must be a strong preference for glutamate to be the first product to be released in the reductive amination reaction. Therefore the product inhibition appeared UC. Formation of E-NH_4^+-$NADP^+$ or E-2kg-NH_4^+-$NADP^+$ has an intercept effect. Hence UC (I-p).	E-NH_4^+-$NADP^+$ or E-2kg-NH_4^+-$NADP^+$

9. Noncompetitive inhibition by NH_4^+ with respect to glutamate. (22)	Product inhibition has an S + I effect. Formation of E-NH_4^+would give an S + I effect (but connection between the 2 intercept effects is broken by addition of glutamate), and formation of E-$NADP^+$-NH_4^+ would give an S effect. Expect NC (I-l, S-p); the data actually fits similarly well to NC (I-l, S-l) and NC (I-l, S-p) equations.	
10. Linear noncompetitive inhibition by glutamate with respect to NH_4^+. (23)	Product inhibition has an S + I effect. Formation of E-glu would also produce an S effect, but presumably negligible E-glu forms due to the high concentration of NADPH used (0.1 mM). Hence NC (I-l, S-l).	
11. Linear noncompetitive inhibition by 2-ketoglutarate with respect to $NADP^+$. (24, 25)	Product inhibition has an S + I effect. Presumably negligible E-2kg forms (see 4). Hence NC (I-l, S-l).	
12. Linear noncompetitive inhibition by $NADP^+$ with respect to 2-ketoglutarate. ([NADPH = 0.05 mM). (26)	Product inhibition has an S + I effect. Formation of E-$NADP^+$ has an S + I effect, as does formation of E-NH_4^+-$NADP^+$ (but I effects are not connected to product inhibition I effect). Thus NC (I-l, S-p) inhibition is expected.	
13. Noncompetitive inhibition by $NADP^+$ with respect to 2-ketoglutarate (I-nl, S-p) ([NADPH] = 0.1 mM). (27)	Product inhibition should have an S+I effect (but see **7** above), although the high level of NADPH probably prevented formation of E-$NADP^+$. Formation of E-NH_4^+-$NADP^+$ has an S + I effect. The I replot was nonlinear, perhaps due to limitation of the product inhibition by NADPH. A parabolic S effect is expected and was observed.	E-NH_4^+-$NADP^+$

*The product inhibition patterns have been explained in terms of a steady state random order mechanism. The only effective product inhibitions are those which drive the equilibrium of the reaction towards the formation of substrates. Thus E-inhibitor binary complexes are abortive since the other products are released at zero concentration so cannot add to the binary complex to form the ternary complex needed to reverse the reaction. All true product inhibitions should be noncompetitive in a steady state random mechanism. A combination of product and dead-end inhibition will only result in a parabolic effect if the product inhibition increases the concentration of the enzyme species which reacts with the inhibitor to form the dead-end complex. The addition of the variable substrate represents an irreversible step with respect to intercept effects (at the y intercept the variable substrate is saturating). Rudolph (1979) summarises the rules for prediction of product inhibition patterns. The bracketed numbers in the observation column are the numbers of the figures to which the observations apply.

TABLE 5

PRODUCT INHIBITION PATTERNS PREDICTED FOR COMPULSORY ORDER, RAPID-EQUILIBRIUM RANDOM AND STEADY STATE RANDOM MECHANISMS[a]

Product Inhibitor	Variable Substrate	Inhibition Patterns for the Following Mechanisms:				
		Compulsory Order[b]	Compulsory Order[b] with formation of abortive complex E-$NAD(P)^+$-2-kg	Product inhibition pattern found for the Bovine Liver GDH[c] (Rapid-Equilibrium Random[d])	Steady State Random[f] in which the binary complex E-2kg is not significantly formed but abortive complexes containing both $NADP^+$ and NH_4^+ are formed. Terms in square brackets apply to the situation where [NADPH] is high.	Results of this study. Terms in Square Brackets Apply to the situation where [NADPH] was high.
NAD(P)H	$NAD(P)^+$	C	C	C	NC (S-p)	C[g]
	glu	NC	NC	NC	NC (S-p)	NC (S-p)
2kg	$NAD(P)^+$	UC	UC (p)	NC	NC	NC
	glu	UC	NC	C	NC	NC
NH_4^+	$NAD(P)^+$	NC	NC	NC/C[e]	NC (S-p, I-p)	NC (S-p, I-p)
	glu	NC	NC	NC	NC (S-p)	NC/ NC (I-p, S-p)
$NAD(P)^+$	NAD(P)H	C	C	C	NC (S-p)	C[g]
	2kg	NC	NC	NC	NC (S-p) [NC (S-p)]	NC [NC (I-nl, S-p)]
	NH_4^+	NC	NC	NC	NC (S-p, I-p) [NC (I-p)]	NC(S-p, I-p)[UC(I-p)]
glu	NAD(P)H	NC	NC	NC	NC (S-p)	NC (S-p)
	2kg	NC	NC	C	NC (S-p) [NC]	[NC]
	NH_4^+	NC	NC	NC	NC (S-p) [NC]	[NC]

a: All product inhibtions are linear unless otherwise Parabolic inhibition is indicated by "p". b Assuming that the order is $NADP^+$, glutamate for the oxidative deamination and NADPH, 2kg, NH_4^+ for the reductive amination.c Results from the study of Engel and Chen (1975). When interpreted in terms of a rapid-equilibrium random order mechanism, the following abortive complexes are required: E-(NAD^+-2kg), E-(NADH-glu), E-(NADH-NH_4^+-glu), E-(NAD^+-glu-NH_4^+). d: All product inhibitions are competitive for a rapid-equilibrium random mechanism without the inclusion of abortive complexes. e: The distinction between competitive and noncompetitive inhibition was not clear. f: All inhibitions should be noncompetitive (S-p) assuming that binary complexes form between the enzyme and all substrates and products, and that no other abortive complexes are formed. g: According to Rudolph (1979), competitive inhibition may be approximated in a steady state random mechanism if the unimolecular rate constants for abortive complex formation are much smaller than the outer rate constants.

The nonlinear inhibition (for the intercept effect) shown by $NADP^+$ with respect to 2-ketoglutarate at high (0.1 mM) NADPH concentration (see replots Fig. 28 and 29) requires further explanation. This phenomenon was also seen by Engel and Chen (1975) in their study of the bovine liver glutamate dehydrogenase when NAD^+ was the product inhibitor and 2-ketoglutarate was the variable substrate. Although this inhibition was noncompetitive, only the intercept replots were curved; the slope replots were linear. Engel and Chen found that the curvature of the intercept replot was less when the concentration of the fixed substrate ammonia was lowered, while we found that the inhibition became linear when the concentration of the fixed substrate NADPH was lowered. It was suggested that the nonlinear inhibition pattern may be due to an allosteric effect by NAD^+ in the case of the bovine enzyme (Engel and Chen, 1975). Since the isolate AN1 enzyme shows no other indications of allosterism, we propose that the cause of this nonlinearity is limitation of the $NADP^+$ inhibition by NADPH (NADPH almost certainly binds to the same site as $NADP^+$, and may therefore restrict its inhibition by limiting the extent to which it can bind the enzyme).

Dissociation constants for product inhibitors may be calculated from the results of product inhibition studies; this is explained by Engel and Chen (1975). The dissociation constants calculated from the product inhibition study of the bovine liver glutamate dehydrogenase on the basis of a rapid-equilibrium random order mechanism were in good agreement with independent estimates from initial velocity and direct binding studies (Engel and Chen, 1975). Stone *et al.* (1980a and b) also calculated dissociation constants for product inhibitors from product inhibition data, but on the basis of a compulsory order mechanism; the agreement between these values and those determined from initial velocity studies is poor. For the isolate AN1 glutamate dehydrogenase, the K_{is} value for inhibition by $NADP^+$ with respect to NADPH (0.052+/-0.006 mM) was in good agreement with the K_{iNADP+} value determined from initial velocity studies (0.054+/-0.007 mM). Likewise, the K_{is} value for inhibition by NADPH with respect to $NADP^+$ (0.016+/-0.001 mM) was in good agreement with the K_{iNADPH} value determined from initial velocity studies (0.018 mM). Such agreement is compatible with a compulsory order mechanism ($NADP^+$ and NADPH leading) or with a steady state random mechanism in which $NADP^+$ and

NADPH are the preferred leading substrates (i.e. the other substrates may also bind the free enzyme but the cofactors bind with the greatest affinity and the enzyme-cofactor complexes do not rapidly interchange with other binary enzyme-substrate complexes). Agreement between K_{is} for competitive inhibition by NADPH with respect to $NADP^+$ and K_{iNADPH} would be purely coincidental in a rapid-equilibrium random mechanism because inhibition by NADPH would affect the $K_{ia}K_b$ term as well as the K_aB term in the rate equation for initial velocity (equation I) (assuming NADP+ is designated as A). The argument is similar for inhibition by $NADP^+$ with respect to NADPH in a rapid-equilibrium random order mechanism.

Glutarate and Reactive Red 120 both inhibited the oxidative deamination reaction of the AN1 glutamate dehydrogenase and were competitive with respect to glutamate. The nature of glutarate inhibition with respect to $NADP^+$ was, unfortunately, not clearly defined. However, both uncompetitive and noncompetitive inhibition with respect to $NADP^+$ can be reconciled with a steady state random mechanism, depending on whether the inhibitor competes with glutamate for the binding of the free enzyme or for the binding of enzyme-$NADP^+$, or both. The inhibition by Reactive Red 120 (RR) was interesting, since it was linear parabolic with respect to glutamate and linear noncompetitive with respect to $NADP^+$. The most likely explanation for this behaviour is that both enzyme-RR and enzyme-$NADP^+$-RR were formed (the formation of the latter through reaction of $NADP^+$ with enzyme-RR or through reaction of Reactive Red with enzyme-$NADP^+$). The parabolic inhibition by Reactive Red with respect to glutamate is not compatible with a compulsory order mechanism unless two molecules of it combine with enzyme-$NADP^+$, which doesn't seem likely in view of its size (Mr = 1433.1).

Pyridoxal 5'-phosphate reversibly inactivates bovine liver glutamate dehydrogenase by reacting with lysine-126, which is essential for catalytic activity (Chen and Engel, 1974), but which is now thought not to be involved in the binding of glutamate (Singh *et al.*, 1993)[4]. Chen and Engel (1974) found that NADH, 2-ketoglutarate, and glutarate partially protected the bovine liver glutamate dehydrogenase against inactivation by

[4] The bovine liver glutamate dehydrogenase is now known to contain several active site lysine residues which are implicated in the catalytic reaction. Lysines 90 and 114 are thought to be involved in the binding of glutamate (Singh *et al.*, 1993).

pyridoxal 5'-phosphate, while NAD^+ alone provided no protection, but the protection provided by glutarate and 2-ketoglutarate was markedly enhanced by the presence of NAD^+. In the light of this information, we carried out similar experiments with the isolate AN1 glutamate dehydrogenase. As Table 3 shows, only $NADP^+$ and NADPH protected the enzyme against inactivation in the absence of other substrates. That the cofactors protected the isolate AN1 glutamate dehydrogenase against inactivation was not surprising, since product inhibition studies indicated that they bind the free enzyme. The failure of NAD^+ to protect against inactivation in the case of the bovine liver enzyme can be explained by the finding of Bayley and O'Neill (1980) (who studied the binding of NAD^+ to this enzyme using circular dichroism difference spectroscopy), that NAD^+ preferentially binds the adenine nucleotide regulatory site rather than the active site in the absence of dicarboxylates. Again in contrast to the findings with the bovine liver glutamate dehydrogenase, neither glutarate, 2-ketoglutarate nor ammonia protected the isolate AN1 glutamate dehydrogenase against inactivation when cofactor was not also present. These findings are more suggestive of an ordered mechanism than a random one. However, the residue or residues of the isolate AN1 glutamate dehydrogenase which react with pyridoxal 5'-phosphate are not necessarily the equivalent of lysine-126 of the bovine enzyme. The amino acid sequence of the isolate AN1 enzyme is not known. It is possible that the residue of the isolate AN1 enzyme which reacts with the reagent lies in a region of the active site which is only bound by the cofactors, or that is not tightly bound by the other substrates unless a cofactor is also present. The slight increase in the degree of pyridoxal 5'-phosphate inactivation which was seen in the presence of ammonia could perhaps be the result of increased accessibility of the pyridoxal 5'-phosphate binding site due to a change in enzyme conformation promoted by the binding of ammonia. Since the binding study was conducted at room temperature, and the glutamate dehydrogenase is from a thermophilic organism, it could be the case that dicarboxylates were not effectively bound at the active site due to a lack of flexibility in the enzyme structure. The isolate AN1 glutamate dehydrogenase appeared to be less susceptible to pyridoxal 5'-phosphate inactivation than the bovine enzyme: it still retained 30% of the control activity after 15h exposure to 2mM pyridoxal 5'-phosphate, whereas the bovine liver enzyme retained only

9% of its control activity when it was at equilibrium with this concentration of pyridoxal 5'-phosphate (Chen and Engel, 1974).

CONCLUSIONS

In summary, the mechanism which best explains our experimental results is a sequential, steady state random mechanism. Cleland (1963) suggested that such a mechanism would result in hyperbolic noncompetitive inhibition patterns, but according to Rudolph (1979), this should only occur if the initial rate behaviour is also nonlinear. The product inhibition studies and the effect of saturating substrate concentrations on the initial rates of the reductive amination reaction indicated that the preferred order of substrate binding is $NADP^+$, glutamate for the oxidative deamination reaction, and NADPH, 2-ketoglutarate, ammonia for the reductive amination reaction.

This steady state kinetic study was carried out at 60°C, 15-20°C lower than the optimum growth temperature of isolate AN1. The acquisition of reliable kinetic data at 75 or 80°C was precluded due to the instability of the reduced cofactor at these temperatures. We have previously observed marked increases in the apparent Km's for the substrates upon increasing the temperature from 60°C to 80°C (Hudson *et al.*, 1993), which indicates that they are more loosely bound at the higher temperature. It is also expected that the enzyme would have considerably more conformational flexibility at the higher temperature, and it is known that all chemical interactions proceed faster at higher temperatures. Therefore it is possible that the enzyme-substrate complexes would interchange rapidly at the growth temperature of the organism, thereby changing the mechanism to rapid-equilibrium random.

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CHAPTER 4

FINAL DISCUSSION

A Comparison of Glutamate Dehydrogenase from Isolate AN1 with Other Archaebacterial Glutamate Dehydrogenases

Isolate AN1 and the genus *Pyrococcus* belong to the family *Thermococcaceae*, which also includes *Thermococcus celer* (Zillig *et al.*, 1987). The *Thermococcaceae*, a family of considerable phylogenetic depth, is the only family in the order *Thermococcales*, which forms one of the 3 major divisions of the archaebacterial kingdom (see Figure 1) (halophiles + methanogens and *Thermoproteales* + *Sulfolobales* are the other major divisions) (Zillig *et al.*, 1987). Thus glutamate dehydrogenases have been characterised from all 3 major branches of the archaebacterial phylogenetic tree. An inspection of the phylogenetic tree of Zillig *et al.* (1987) (derived from DNA/rRNA cross-hybridisation velocities) (see Figure 1) reveals that the position of isolate AN1 is approximately equidistant from the positions of *P. woesei* (on the *Thermococcales* branch), *Sulfolobus solfataricus* (on the *Sulfolobales* + *Thermoproteales* branch) and *Halobacterium halobium* (on the halophiles + methanogens branch).

The glutamate dehydrogenase (GDH) from isolate AN1 was similar to the NADP-linked GDH from the *Halobacterium* sp. isolated from the Dead Sea (Leicht *et al.*, 1978) with respect to the molecular weight, number of subunits (4) and apparent Km's (see Table 1). The NADP-linked GDH from *Halobacterium halobium* (Bonete *et al.*, 1987), since it has a similar molecular weight to the isolate AN1 GDH, may also be tetrameric. It was somewhat surprising that the isolate AN1 GDH should have more in common with the halobacterial NADP-linked GDH's than with the hexameric GDH from *P. furiosus*, but when evolutionary distances are considered (see above), it is perhaps less so.

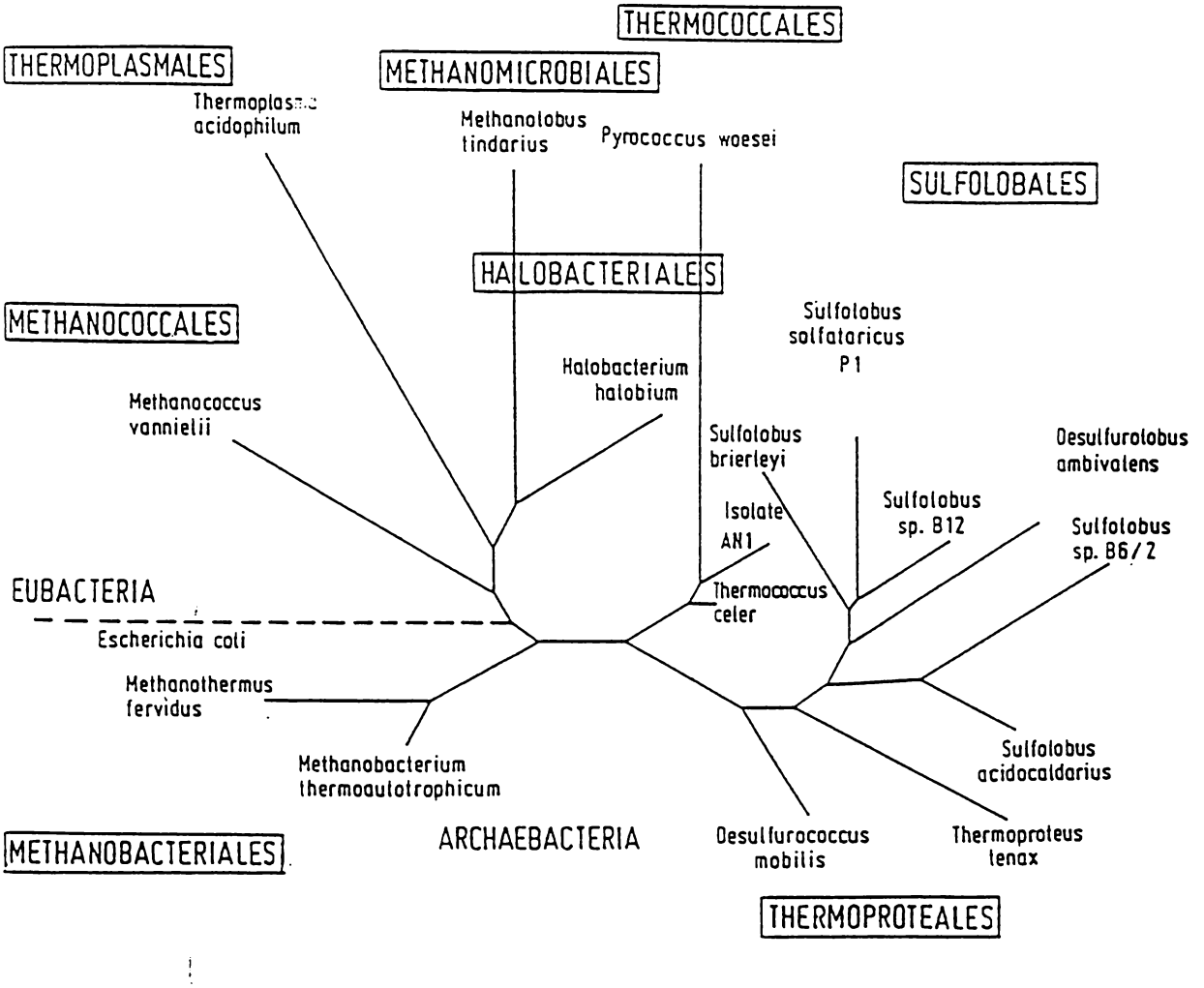


Fig. 1. Phylogenetic tree of representative species of archaebacteria derived from DNA/rRNA cross-hybridisation velocities. Distances correspond to calculated sequence distances. From Zillig *et al.* (1987).

TABLE 1

Some Properties of Archaebacterial Glutamate Dehydrogenases

	Halobacterium halobium[a,b] (NAD-GDH)	*Halobacterium halobium*[b] (NADP-GDH)	*Halobacterium* sp. of the Dead Sea[c] (NADP-GDH)	*Sulfolobus solfataricus*[d,e,f] (NAD(P)-GDH)	*Pyrococcus furiosus*[g,h] (NAD(P)-GDH)	Isolate AN1[i] (NADP-GDH)
M_r	148K	215K	212K	270K[d,e]	270-290K[g,h]	204K
Subunit M_r	-	-	53.5K	44-46K[d,e,f]	46[g]/48K[h]	47K
No. subunits	-	-	4	6[d,e]	6[g,h]	4
pH optima:						
amination	9	8-8.5	-	9[d]/7.6[e]	9[g]	8.25
deamination	8	10	-	10[d]	9[g]	8.25
pI	-	-	-	5.7[d]/6.2[e]	4.5[g]	4.0
Apparent Km (mM):						
NAD	0.3	-	-	-	0.53[h]	28.6
NADP	-	0.11	0.061	0.05, 0.25[d]*	0.2[g]/0.18[h]	0.038
glutamate	4	4600	14.2	2.5[d]	0.6[g]/1.6[h]	9.12
NADH	0.07	-	-	0.14[d]/0.007[e]	0.36[g]/0.98[h]	0.32
NADPH	-	0.077	0.024	0.01[d,e]	0.012[g]/0.56[h]	0.066
2-ketoglutarate	20.2	13.3	3.0	1.4[d]	0.33[g]/4.0[h]	1.7
ammonia	450	3.1	16.6	4.2, 34[d]*	6, 27[g]*	15.5

Refs. **a**: Bonete *et al.*, 1986; **b**: Bonete *et al.*, 1987; **c**: Leicht *et al.*, 1978; **d**: Consalvi *et al.*, 1991a; **e**: Schinkinger *et al.*, 1991; **f**: Maras *et al.*, 1992; **g**: Consalvi *et al.*, 1991b; **h**: Robb *et al.*, 1992; **i**: Hudson *et al.*, 1993. The apparent Km values given are those determined at 80°C.

*: Apparent Km depended on range of substrate concentration.

A summary of the levels of GDH found in the 5 archaebacterial species from which GDH has been characterised is presented in Table 2. The highest levels of archaebacterial GDH have been found in the extreme thermophiles isolate AN1, *Thermococcus* sp. and *P. furiosus*. Although the finding of very high levels of GDH is not unprecedented in microorganisms (some organisms which use the hydroxyglutarate pathway of glutamate fermentation, such as *Peptostreptococcus aerogenes* and *Clostridium symbiosum*, are also known to possess high levels of GDH (Hornby and Engel, 1984; Johnson and Westlake, 1972; Kew and Woolfolk, 1970; Rice *et al.*, 1985; Syed *et al.*, 1991)), the physiological reason for this in thermophilic archaebacteria remains a mystery.

TABLE 2

LEVELS OF GDH IN ARCHAEBACTERIAL SPECIES

Organism	% of Soluble Cell Protein Represented by GDH	mg GDH per g wet cells	Reference
Halobacterium halobium (NAD-linked GDH)*	0.8		Bonete *et al.*, 1986
Halobacterium sp. isolated from the Dead Sea	0.22	0.2	Leicht *et al.*, 1978
Sulfolobus solfataricus	1.1	0.5	Consalvi *et al.*, 1991a
Sulfolobus solfataricus	0.9	0.17	Schinkinger *et al.*, 1991
Pyrococcus furiosus	1.2	0.009	Robb *et al.*, 1992
Pyrococcus furiosus	16	16	Consalvi *et al.*, 1991
ES4	1-2		Di Ruggiero, Klump and Robb (pers. comm.)
Thermococcus sp.	~10		T. Kobayashi (pers. comm.)
Isolate AN1	11	2.5	This study.

* NAD-linked GDH was purified 261-fold from *H. halobium*, but the final preparation was not completely homogeneous; minor contaminants remained (Bonete *et al.*, 1986). An NADP-linked GDH has also been

purified from *H. halobium* (Bonete *et al.*, 1987), but the level of this enzyme was not reported, nor was a purification table given.

As shown in the table, the level of GDH reported in *P. furiosus* by Consalvi *et al.* (1991b) was an order of magnitude higher than that reported by Robb *et al.* (1992), even though Robb *et al.* used a higher g force (50 000 xg) in the preparation of the cell free extract than did Consalvi's group (30 000 xg). The growth conditions used by these groups were slightly different: Consalvi *et al.* used an artificial seawater medium (which contained 1.3 g/l ammonium sulphate) supplemented with 1 g/l yeast extract, 0.5 g/l cysteine and 0.2 g/l elemental sulphur, while Robb *et al.* used an artificial seawater to which 5 g/l maltose, 1.25 g/l ammonium chloride, 1 g/l yeast extract and vitamin and mineral supplements were added. Thus the major differences were that Consalvi *et al.* added sulphur but not maltose, while Robb *et al.* added maltose but not sulphur (Robb *et al.* grew the organism on a large scale; elemental sulphur was omitted because sparging with argon is required when it is used; titanium citrate was added as the initial reductant).

P. furiosus, *Thermococcus* sp. and isolate AN1 are all able to reduce elemental sulphur to hydrogen sulphide. Isolate AN1 and *Thermococcus* sp. possessed a high level of GDH, as did *P. furiosus* when it was grown on medium containing sulphur. This suggests that GDH may have some (indirect) involvement in the sulphur reduction process, perhaps in providing reducing potential or in control of cytoplasmic pH. However, the level of GDH present in the hyperthermophile ES4 (1-2%) (pers. comm. Di Ruggiero, Klump and Robb) sheds doubt on this theory, as this organism also reduces sulphur. The *in vivo* reaction direction of these enzymes is not known, but the preference of the isolate AN1 and *P.furiosus* GDH's for NADP(H) (the *P. furiosus* GDH is dual-coenzyme specific, but the Km's for $NADP^+$ and NADPH were an order of magnitude lower than that for NADH (Consalvi *et al.*, 1991b)) suggests an anabolic role for these enzymes.

Klages (1991) suggested that the GDH in AN1 has a role in recycling 2-ketoglutarate, which is a substrate of branched amino acid transferases (glutamate is one of the products). This would require that GDH acted predominantly in the oxidative deamination direction, thereby having a role in the elimination of excess nitrogen. AN1 grows best on trypticase peptone, or amino acid mixtures (especially branched chain amino acids) supplemented with a small amount of peptone (Klages, 1991), therefore it is

expected that the organism has an enzyme which is active in disposing of excess nitrogen. Whether or not this enzyme is the NADP-linked GDH is open to debate.

Influence of Metal Ions and Chelating Agents on the Glutamate Dehydrogenase from Isolate AN1

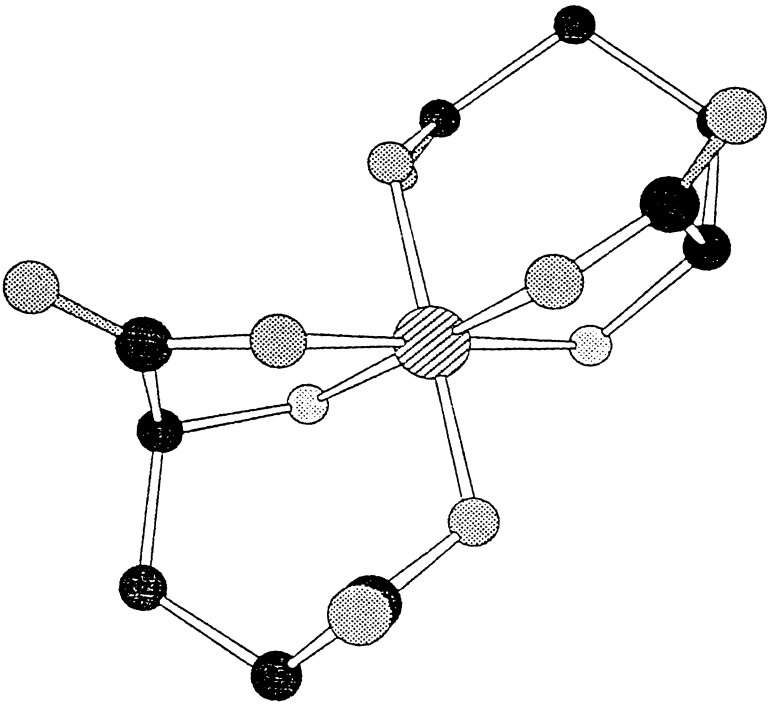
The oxidative deamination reaction catalysed by the isolate AN1 GDH was markedly activated by calcium, magnesium and manganese ions (see Table 2, Chapter 2). The fact that these metal ions have negligible influence on the activity of the enzyme in the reductive amination reaction (see Table 1 of Appendix 1), suggests that these metal ions may have an effect on one of the substrates particular to the oxidative deamination reaction, probably glutamate. Glutamate is a known chelating agent: it has electron pairs in its two carboxyl groups, and in its amino group, which are available to coordinate with suitable metal ions.

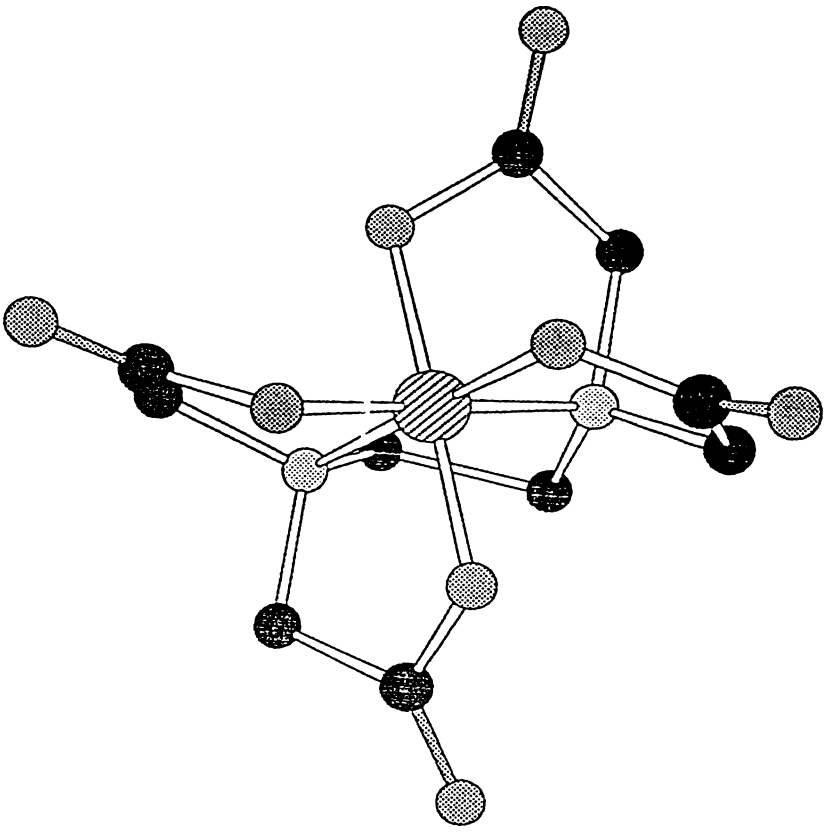
The effect of chelating agents on the oxidative deamination activity in the presence of metal ions was interesting, as the effect depended on the particular chelating agent used (see Table II of Appendix 1 for these results). In summary, the 3 chelating agents alone had only small effects, while divalent metals alone stimulated substantially. 1,10-Phenanthroline plus metal ion had approximately the same effect as the metal ion alone, EGTA plus metal ion had little or no effect, but EDTA plus Ca^{2+} or Mg^{2+} inhibited substantially (EDTA plus Mn^{2+} also inhibited, but since both metal ion and chelator were only present in 1 mM concentration, the effect was smaller (see Table II, Appendix 1)).

Since chelating agents had little effect on GDH oxidative deamination activity in the absence of metal ions (the greatest effect was given by EGTA with 13% inhibition), it seems unlikely that the GDH is a metalloprotein (ie. it is unlikely that GDH contains a metal ion as a prosthetic group). Glutamate was present at 18-20 mM concentration in these experiments (~10x the Km at 60°C), therefore it is unlikely that the activating effect is due solely to the lowering of the Km for glutamate. However, it is possible that glutamate chelates the metal ion and that the metal ion may make the glutamate molecule more reactive due to its electron-withdrawing (-I) properties. If this theory is correct, it is

probable that the metal ion-EDTA complex also enters the active site and hinders substrate access (this complex also has carboxyl groups coordinated to the metal ion, as in a metal ion-glutamate complex, see Fig. 2), which would account for the inhibition seen when EDTA and a metal ion were present together. Indeed, the metal ion-EDTA complex is of similar dimensions to the metal ion-glutamate complex, as evidenced by the superimposed computer-modelled structures seen in Fig 2. The metal ion-EGTA complex is probably too bulky to enter the active site, since the presence of EGTA plus metal ion did not cause significant inhibition, but the metal ion activation effect was not seen. The presence of 1,10-phenanthroline (3 mM) is unlikely to completely prevent the chelation of metal ion by glutamate (20 mM) (Table 3), and indeed metal ion activation was still seen.

Glutamate has an amine group as well as 2 carboxyl groups, but at the pH of these experiments (7.1) the amine group would have been positively charged and the carboxyl groups negatively charged (the $-NH_3^+$ groups has a pKa of 9.67 and the 2 -COOH groups have pKa's of 2.19 and 4.25 (Lehninger, 1975)). Therefore it might be expected that glutamate would act as a bidentate ligand, but this would result in an unstable 8-membered ring (see Fig. 2C). It is more likely that the amino group loses a proton and coordinates with the metal ion (a Lewis acid), such that glutamate acts as a tridentate ligand (see Fig. 2), forming smaller, more stable chelate rings (see below). The complex that glutamate forms with Mn^{2+} is more stable than those it forms with Mg^{2+} and Ca^{2+} (which are harder metal ions) (see stability constants in Table 3), which may explain why Mn^{2+} is the most effective activator. $EDTA^{4-}$ and $EGTA^{4-}$ are hexadentate ligands, therefore the complexes they form with metal ions are very stable (6 chelate rings formed) (Cotton and Wilkinson, 1980), as evidenced by the stability constants for the complexes they form with metal ions (see Table 3). 1,10-Phenanthroline (a bidentate ligand), because it has electron-rich aromatic rings (ie. it is a soft ligand), forms the most stable complexes with soft metal ions: Mg^{2+} and Ca^{2+} are hard metal ions, hence these metal ions form more stable complexes with glutamate (a harder ligand than 1,10-phenanthroline) than with 1,10-phenanthroline. Stability constants (see Table 3) indicate that 1,10-phenanthroline is a better ligand for Mn^{2+} than glutamate (Mn^{2+}, which has d-orbitals available, favours coordination with the soft ligand 1,10-phenanthroline), but since the Mn^{2+} activation





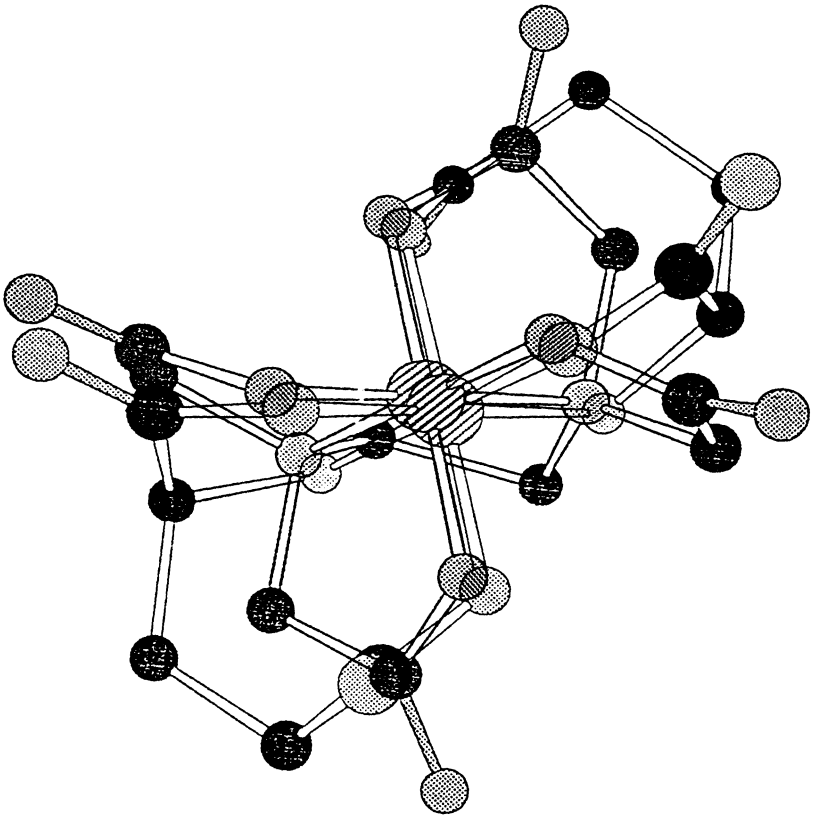


Fig. 2. A metal ion-EDTA complex (on the transparency) and a metal ion-glutamate complex (facing page) in which 2 molecules of glutamate (acting as tridentate ligands) are coordinated to the metal ion. The black spheres represent carbon atoms, the dark-speckled spheres oxygen atoms and the light-speckled spheres nitrogen atoms. The metal ion (Mn^{2+} in this case) is represented by the cross-hatched sphere in the centre of each complex. Hydrogen atoms have been omitted.

This figure was created with a molecular modelling program by Dr Rick Ede (University of Waikato).

effect was retained in the presence of 1,10-phenanthroline, it may be assumed that at least one molecule of glutamate was still able to coordinate (K_1 for glutamate is less than K_3 for 1,10-phenanthroline). Table 3 gives the formation constants of some metal ion-chelator complexes, including those formed between glutamic acid and Mg^{2+}, Ca^{2+} and Mn^{2+}.

TABLE 3

STABILITY CONSTANTS OF METAL ION-CHELATOR COMPLEXES

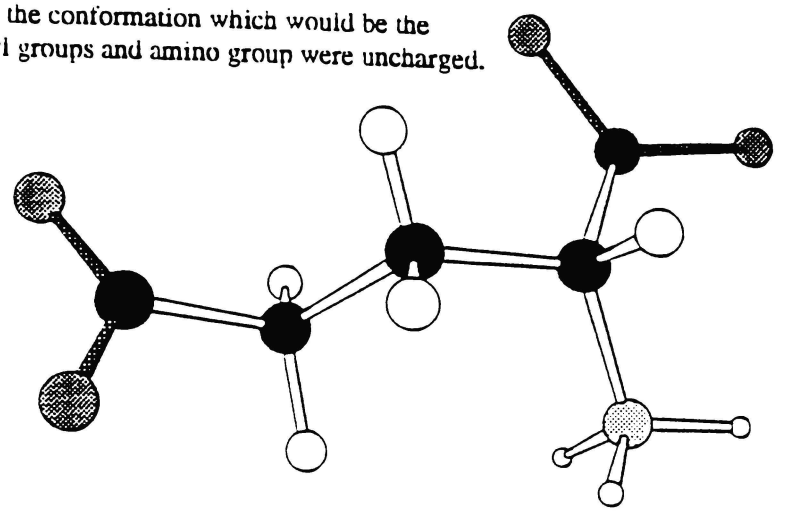
Chelator	Cation	Temperature	Log_{10} Equilibrium Constant[a]
glutamic acid[b]	Ca^{2+}	25°C	$K_1 = 1.43$ (2.05)[c]
"	Mg^{2+}	25°C	$K_1 = 1.9$
"	Mn^{2+}	20°C	$K_1 = 3.3$
2-ketoglutaric acid	Ca^{2+}	25°C	$K_1 = 1.29$[d]
EDTA	Ca^{2+}	25.3°C	$K_1 = 10.42$
"	Mg^{2+}	25.3°C	$K_1 = 8.64$
"	Mn^{2+}	25°C	$K_1 = 13.8$[e]
EGTA	Ca^{2+}	20°C	$K_1 = 10.97$
"	Mg^{2+}	20°C	$K_1 = 5.2$
"	Mn^{2+}	20°C	$K_1 = 12.11$
1,10-phenanthroline	Ca^{2+}	20°C	$K_1 = 0.7$
"	Mg^{2+}	20°C	$K_1 = 1.2$
"	Mn^{2+}	20°C	$K_1 = 4.13$, $K_2 = 3.48$, $K_3 = 2.7$

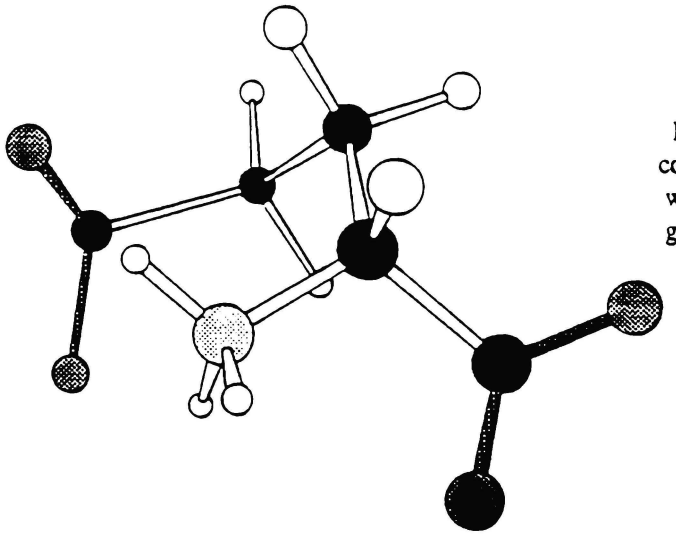
a: The constants were obtained from Sillen and Martell (1964 and 1971). The constants quoted here were determined by the same method, glass electrode (unless otherwise stated), and under similar conditions.
b: The ligand was described as "glutamic acid ($C_5H_9O_4N$)" in the reference used.
c: The value of 2.05 was determined by the sol method rather than the glass electrode method.
d: This value was determined by ion exchange in a medium with an ionic strength of 0.16 M.
e: This value was determined by emf with an Hg amalgam electrode.

Figure 3 shows a 3-dimensional representation of a complex formed between glutamate and a calcium ion, and of conformations of the free glutamate molecule. The open conformation of the glutamate molecule (Fig. 3A) is the most stable form if all the groups are uncharged. However, if the charges are taken into account, the most stable form of the molecule is shown in Fig 3B. A Ca^{2+}-glutamate complex, with glutamate acting as a bidentate ligand, is shown in Fig. 3C; this complex has an 8-membered ring, and is therefore inherently unstable. Thus glutamate most probably acts as a tridentate ligand (see Fig. 2), with the glutamate molecule in a conformation similar to that shown in Fig. 3B. The metal ion would have a strong electron withdrawing effect on the alpha-carbon atom of a glutamate molecule acting as a tridentate ligand, and probably reduces steric hindrance at the alpha-carbon atom, thus rendering it more susceptible to nucleophilic attack.

However, if the chemical mechanism for the GDH reaction proposed by Singh *et al.* (1993) on the basis of experimental results with the bovine liver enzyme and the active site structure of the *Clostridium symbiosum* enzyme is true also for the GDH from isolate AN1, the interaction of a metal ion-glutamate complex with the active site requires further explanation. In this mechanism, hydrogen bonding between the carboxyl groups of glutamate and lysine 90 and lysine 114 holds the glutamate molecule in the active site in extended conformation; hydride transfer occurs ($NADP^+$ is reduced and glutamate is converted to α -iminoglutarate), then a water molecule hydrogen-bonded to lysine 126 nucleophilically attacks the α -iminoglutarate to form an α -carbinolamine which loses ammonia to form 2-ketoglutarate. The nucleophilic attack would be aided by the -I effect of a metal ion, but if the carboxyl groups were coordinated to a metal ion at this point, the glutamate molecule would probably have to be held in the active site through coordination of a group (or groups) on the enzyme itself to the metal ion. It is indeed possible that the presence of a metal ion could change the mode of binding of the glutamate molecule, the reaction mechanism, or the rate limiting step (or combinations thereof). It may also be the case that the isolate AN1 GDH, an archaebacterial protein, has an active site structure which is not analogous to that of the *C. symbiosum* protein.

A. The glutamate molecule in the conformation which would be the most favourable if the carboxyl groups and amino group were uncharged.





B. The glutamate molecule in the conformation which is the most stable when the carboxyl groups and the amino group are charged.

C. A Ca^{2+}-glutamate complex, with glutamate acting as a bidentate ligand.

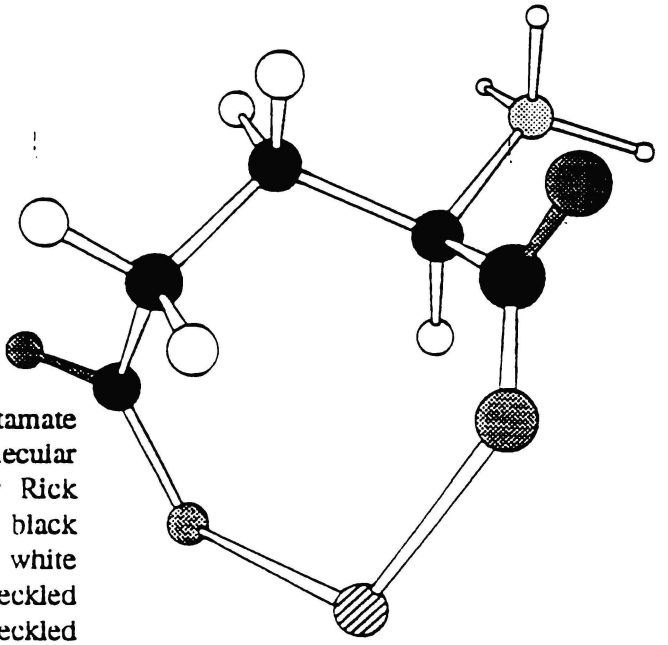


Fig. 3. Conformations of the glutamate molecule calculated via a molecular modelling program (courtesy of Dr Rick Ede, University of Waikato). The black spheres represent carbon atoms, the white spheres hydrogen atoms, the dark speckled spheres oxygen atoms and the light speckled spheres nitrogen atoms. The cross-hatched sphere represents a calcium ion. Sigma-bonds are represented by unshaded bars and pi-bonds by shaded bars.

Another apparent difficulty with the theory of metal ion activation via chelation of the metal ion by glutamate, is that 2-ketoglutarate also has two carboxyl groups and should also be able to act as a chelating agent, yet Ca^{2+}, Mg^{2+} and Mn^{2+} have no effect on the reductive amination reaction. However, the lack of a metal ion effect on the reductive amination reaction may be because the rate limiting step of this reaction does not involve release of product from enzyme-product ternary complexes (the rate limiting steps of the reductive amination reaction have not been well characterised (Rife and Cleland, 1980)) as is thought to be the case for the oxidative deamination reaction (Fisher, 1985). It is possible that metal ions activate the oxidative deamination reaction by chelating 2-ketoglutarate, hence increasing its dissociation from ternary enzyme-product complexes (this process is thought to be rate limiting for the bovine liver enzyme when the concentration of glutamate is low (Fisher, 1985)). Indeed, if the glutamate molecule undergoes reaction while coordinated to a metal ion (such that a metal ion-2-ketoglutarate complex is formed from a metal ion-glutamate complex), it is highly probable that this is the case. This theory is consistent with the observation that EDTA caused inhibition when it was present together with a metal ion, and with the observation that Mn^{2+} was the most effective activator. When the reaction was conducted in the reductive amination direction, a metal ion-2-ketoglutarate complex may have been the species to undergo reaction, but if the rate limiting step remained the same as that which occurred in the absence of divalent metal ions, no activation would be seen.

An interaction of metal ions with $NADP^+$ is possible as phosphate groups are available which may coordinate with a metal ion, but if this were the case, a metal ion effect on the reductive deamination reaction would be expected (since the only difference between $NADP^+$ and NADPH is the addition of hydride to the nicotinamide ring, and NADPH would be more likely to coordinate with a metal ion than $NADP^+$ since it does not have a positive charge in the nicotinamide ring). The results of the experiments where metal ions and chelating agents were present together also indicate that the effect is on glutamate rather than on $NADP^+$ (if a metal ion-$NADP^+$ complex enters the active site, a metal ion-EGTA complex should also be able to enter the active site). However, the lack of metal ion effects on the reductive amination reaction may be due to the rate limiting

step of this reaction having a different nature to that of the oxidative deamination reaction (see above). It is possible that a metal ion assists the removal of NADPH from product complexes, although Silverstein and Sulebele (1973) found that this step was only rate limiting for the bovine enzyme (which has a Km for glutamate similar to that of the isolate AN1 enzyme (Smith *et al.*, 1975)) when the concentration of glutamate was above 50 mM.

A simplified scenario which can explain the effects of divalent metal ions on the isolate AN1 GDH is presented in Fig. 4. This is by no means the only possible scenario, but considering the experimental evidence, and the information available for the bovine liver and *Clostridium symbiosum* enzymes, this scheme is one of the simplest explanations for the metal ion effect.

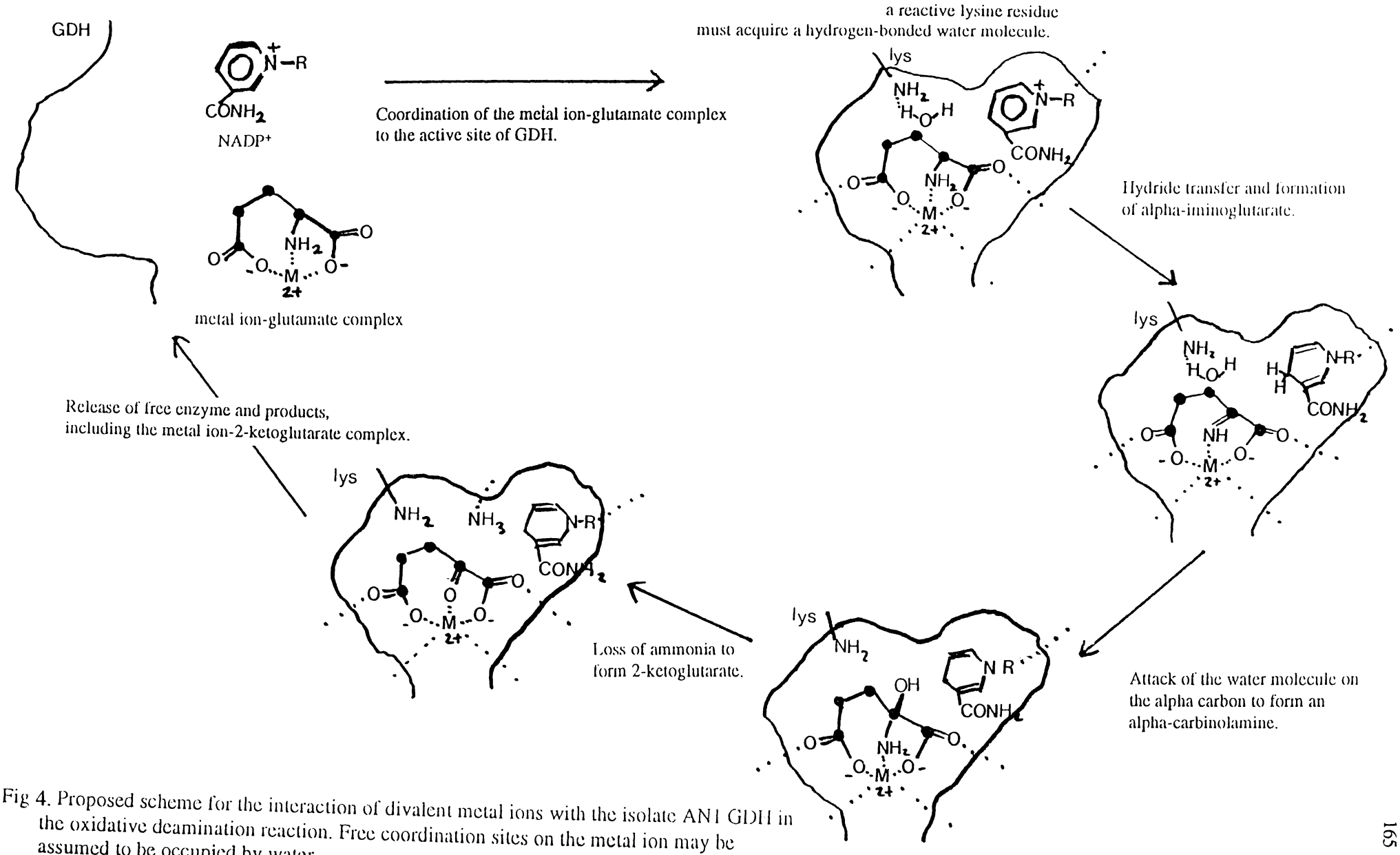


Fig 4. Proposed scheme for the interaction of divalent metal ions with the isolate AN1 GDH in the oxidative deamination reaction. Free coordination sites on the metal ion may be assumed to be occupied by water.

Calcium chloride and magnesium chloride at 5 mM had little effect on the thermostability at 103°C, but at 50 mM caused a marked decrease in the thermostability (see Table 4, Chapter 2). This decrease in thermostability at the higher concentration may have been due to a salting out effect.

Divalent cations, especially calcium, are known to activate higher plant GDH's (NAD-linked and dual coenzyme specific) (Kindt *et al.*, 1980; Srivastava and Singh, 1987). Calcium has also been found to activate the dual coenzyme specific GDH isozymes of the alga *Chlamydomonas reinhardtii* (Moyano *et al.*, 1992a). These enzymes, unlike the AN1 enzyme, are inhibited by EDTA (Srivastava and Singh, 1987; Moyano *et al.*, 1992a). The higher plant enzymes are thought to be metalloenzymes (Srivastava and Singh, 1987). In many cases, however, effects of metal ions on GDH's have not been reported or have not been determined. A range of chelating agents was found to have no effect on the GDH's from the thermophilic archaebacteria *Sulfolobus solfataricus* and *Pyrococcus furiosus* (Consalvi *et al.*, 1991a and b). The effect of metal ions appears not to have been determined.

As outlined in the Literature Review (Chapter 1), the role of NADP-linked GDH's is thought to be predominantly anabolic, although this is not always the case. The unidirectional activation by metal ions may represent a control mechanism which allows the isolate AN1 GDH to function effectively in the thermodynamically unfavourable oxidative deamination direction. It is known that ATP is a better chelator of magnesium ions than ADP, and therefore cellular magnesium concentrations should be higher when the energy charge is lower. Thus the metal ion activation effect may be a mechanism which allows the isolate AN1 GDH to respond to energy charge (the catabolic reaction direction is activated when the energy charge is low). This may be a primitive form of energy charge response. Bovine liver GDH is also activated by low energy charge (it is activated by ADP (Smith *et al.*, 1975)), the response being mediated by allosteric effectors.

Steady State Kinetic Studies

An attempt was made to study the kinetics of both the oxidative deamination and reductive amination reactions catalysed by the isolate AN1 GDH as thoroughly as possible. This is why the multi-faceted approach of initial rate studies, product inhibition studies, substrate analogue inhibition studies and a study of the protection offered by substrates against pyridoxal 5'-phosphate inactivation was adopted (although the latter is not strictly a kinetic study).

Initial Velocity Studies

The standard method explained by Cleland (1970) was used for the initial velocity studies of the oxidative deamination reaction. An examination of the primary plots obtained from such data will give little information apart from distinguishing between sequential mechanisms and the ping pong type. However, the relationship between the Km's and dissociation constants obtained by fitting the data to the equation for sequential initial velocity (equation I, Chapter 3), indicated a random mechanism (see Chapter 3 for the relationships).

In the initial velocity studies of the reductive amination reaction, the method explained by Rudolph and Fromm (1979) was used (this is detailed in Chapter 3), rather than the more common method of determining $\emptyset$ coefficients. The latter approach requires that a great deal of kinetic data to be acquired in order to construct primary, secondary and tertiary plots; there is also a good chance of errors accumulating in this process (Rudolph and Fromm, 1979). In contrast, the method of Rudolph and Fromm requires only 3 sets of kinetic experiments to be performed, in which each substrate in turn is used as the variable substrate. An inspection of the primary and secondary plots alone distinguishes between many different types of mechanism, but does not easily distinguish between completely random and completely ordered mechanisms (Rudolph and Fromm, 1979). However, an inspection of the intersection points of the primary (Lineweaver-Burk) plots obtained from the data indicated a random mechanism (Cleland (1970) discusses the predicted

intersection points of Lineweaver-Burk plots for 2 substrate mechanisms, which can be extrapolated to apply to 3 substrate mechanisms). It is obviously not ideal to base conclusions on something as subjective as a graphical intersection points, but product inhibition studies were used to further investigate the reaction, and also indicated a random mechanism.

Product Inhibition Studies

The inhibition studies with products were invaluable in this study, as they indicated that the mechanism was steady state random rather than rapid-equilibrium random[1]. A rapid-equilibrium random mechanism was suggested for the bovine liver GDH on the basis of initial velocity and product inhibition studies (Engel and Dalziel, 1969 and 1970; Engel and Chen, 1975), although later studies indicated that the order was only partially random (Rife and Cleland, 1980; Adams, 1987). It is possible that if the kinetics of the GDH from isolate AN1 could be studied at the growth temperature of the organism (75-80°C) a rapid-equilibrium random mechanism would also be indicated, as the enzyme-substrate complexes might be expected to interchange rapidly with each other at this higher temperature. Unfortunately, the instability of NADPH precluded the acquisition of highly accurate kinetic data at the growth temperature of isolate AN1, therefore a compromise was made by carrying out the kinetic study at 60°C. If a means of stabilising NADPH at 80°C could be found, a kinetic study at this temperature would be an interesting possibility, as it has already been found that an increase in temperature from 60 to 80°C has a profound effect on the apparent Km's for the substrates (see Table 3 of Chapter 2).

Product inhibition studies provide valuable insights into the regulation of enzyme activity by product levels, even though their interpretation is often complicated by abortive complex formation (Rudolph, 1979). Besides the binary enzyme-product abortive complexes, product inhibition studies with the isolate AN1 GDH indicated the formation of abortive complexes containing both $NADP^+$ and NH_4^+ (see Table 4 of Chapter 3).

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[1] Rapid-equilibrium mechanisms may still be studied by steady state kinetic investigations (Fromm, 1979). Fromm also notes that very few such mechanisms are truly rapid-equilibrium in both directions, although the condition will be approximated in the "slow" direction.

There was no suggestion of the abortive complexes enzyme-$NADP^+$-2-ketoglutarate or enzyme-NADPH-glutamate which the bovine liver enzyme is thought to form (Engel and Chen, 1975; Silverstein and Sulebele, 1973). Cleland (1970) notes that the products of the reaction as it occurs *in vivo* are more likely to give mixed dead-end and product inhibition than are the products of the reverse direction. Unfortunately, this gives no clue as to the *in vivo* direction of the isolate AN1 GDH, since both $NADP^+$ and NH_4^+ gave dead-end inhibition.

Inhibition by Triazinyl Dyes

The triazinyl dye, Reactive Red 120, which was used in the purification of the isolate AN1 GDH, was also found to be a potent inhibitor of this enzyme. The triazynal dye Cibacron Blue F3G-A is a competitive inhibitor with respect to NADH for the GDH from *Trypanosoma cruzi* (Orellano *et al.*, 1985), and has a 3-dimensional structure which is very similar to that of NAD (Scopes, 1987). Therefore the type of inhibition which these dyes gave towards $NADP^+$ was investigated as part of a search for a competitive inhibitor for this substrate. Cibacron Blue F3G-A, however, gave very weak inhibition with respect to $NADP^+$ compared to that given by Reactive Red 120 (results from preliminary experiments not shown; the type of inhibition could not be determined from the data), and inhibition by this dye was not investigated further. Reactive Red 120, surprisingly, gave linear noncompetitive inhibition with respect to $NADP^+$ and parabolically competitive inhibition with respect to glutamate. Reactive Red 120 (also known as Procion HE-3B) has a molecular weight even higher than that of Cibacron Blue F3G-A and has neither carboxyl nor primary amine groups (the structures of both dyes are given by Scopes (1987)), so it is surprising that it should act as a competitive inhibitor of glutamate. It may interact with the GDH active site in such a way that glutamate binding is prevented but $NADP^+$ binding is allowed. Since the inhibition with respect to $NADP^+$ had a much larger intercept effect than slope effect (see Fig. 32 and Table 2 in Chapter 2), Reactive Red presumably has a higher affinity for enzyme-$NADP^+$ than for the free enzyme.

Watson *et al.* (1978) found that Procion HE-3B (= Reactive Red 120) retarded a range of NADP-specific dehydrogenases more effectively than NAD-specific dehydrogenases, whereas the opposite was true for Cibacron Blue F3G-A. This suggests that these 2 groups of dehydrogenases have some fundamental structural difference in their active sites. An investigation of the mode of inhibition given by Reactive Red 120 towards NAD-linked, NADP-linked and dual coenzyme specific GDH's and other dehydrogenases would be an interesting exercise. It would be particularly interesting to know whether the dual-specific GDH's from the thermophilic archaebacteria *Sulfolobus solfataricus* and *Pyrococcus furiosus* were inhibited by this dye in the same way as the GDH from isolate AN1.

Summary

The GDH from isolate AN1 showed a number of interesting features: a tetrameric structure, activation by divalent metal ions, and an apparent steady state random kinetic mechanism. This enzyme is one of the few archaebacterial GDH's to have been characterised, and thus this study is an extension of the comparative enzymology of GDH enzymes. It is hoped that more archaebacterial GDH's will be characterised in the future, which will possibly allow correlations to be drawn between the type of GDH present (tetrameric or hexameric), the level of GDH present, and the type of archaebacterium which the GDH comes from. Such correlations are, at present, impossible to make. If more archaebacterial GDH's were characterised, it might also be possible to see relationships between particular types of archaebacterial GDH, and particular types of GDH from eukaryotes and eubacteria.

APPENDIX 1

DATA PERTAINING TO CHAPTER 2

Materials

Monosodium L-glutamate was from BDH (Poole, U.K.). $NADP^+$, NADPH, NAD^+, NADH, 2-ketocarboxylic acids, AMP, ADP, ATP, GTP, PEP, dithiothreitol, BSA, EPPS, MOPS, CHES, HEPES, EDTA (disodium salt), EGTA and Reactive Red 120 were from Sigma (St Louis, Mo., U.S.A.). Coomassie brilliant blue G-250 was from Pierce (Rockford, Il., U.S.A.). Standard proteins for molecular weight determination by gel filtration FPLC were from Sigma and United States Biochemicals (U.S.B.). Standard proteins for subunit molecular weight determination by SDS-PAGE and for isoelectric point determination were from Pharmacia (Uppsala, Sweden). Ammonium chloride was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Amicon (Lexington, MA., U.S.A.) YM10 membranes were used for all ultrafiltrations. Aqueous solutions were made up to volume with deionised reagent grade water from a reverse osmosis water purification system.

The Purification of the Glutamate Dehydrogenase

A number of dye-columns were tested on a trial scale (using a dye column test kit containing "Mimetic" dyes from Affinity Chromatography Ltd, Freeport, Ballasalla, Isle of Man, U.K.) for their effectiveness in binding the GDH from isolate AN1. The screening technique suggested by Scopes (1982), which uses an acidic pH (6-7), was adopted for these trials. Although the GDH bound all columns tested, the recovery was generally poor. However, when a sample of Reactive Red-Sepharose CL-6B (prepared in this lab by the method of Scopes (1982)) was tested using 0.1 M EPPS-NaOH buffer (pH 7.85), it was found to bind large quantities of the GDH, which was released upon the addition of NaCl to the buffer with negligible loss of activity. It appeared that the higher pH was necessary to achieve high recoveries of the GDH, since the test-kit column which contained

"Mimetic Red 2" (the equivalent dye to Reactive Red 120) gave an approximately 50% yield when used with MES-NaOH pH 6.5.

Figure 1 shows the Reactive Red-Sepharose CL-6B column used in the purification of the GDH. The silver-stained SDS-PAGE gels in Figures 2 and 3 show the GDH at various stages of purification. Track b of Figure 3 was loaded with 0.3 μg (greater than the maximum recommended loading of protein for silver-stained Phast-gels) GDH purified by Reactive Red-Sepharose CL-6B chromatography (2x) and hydroxyapatite chromatography.

Molecular Weight Determinations

The subunit molecular weight was determined by SDS-PAGE on 10-15% gradient gels using a Pharmacia Phast System (Pharmacia, Uppsala, Sweden). The molecular weight standards were α -lactalbumin (14.4K), soybean trypsin inhibitor (20.1K), carbonic anhydrase (30K), ovalbumin (43K), bovine serum albumin (67K) and phosphorylase b (94K). Figure 4 shows the calibration curve for molecular weight determination by SDS-PAGE. The SDS-PAGE gel in Figure 3 shows the standard proteins (tracks a and f) and purified GDH (track b).

The initial determination of native molecular weight was performed by Dr Tim Coolbear of the Dairy Research Institute (Palmerston North) using a Superose 6 HR 10/30 FPLC column. He observed a major peak of enzyme protein corresponding to a molecular weight of 204K, plus a minor peak corresponding to a molecular weight of 51K. It is not known whether this minor peak displayed GDH activity.

Gel filtration chromatography of the GDH was repeated in this laboratory using a Superose 12 Prep Grade HR 16/50 FPLC column. The molecular weight standards were glyceraldehyde-3-phosphate dehydrogenase (37K), alcohol dehydrogenase (150K), sweet potato β -amylase (200K) and jackbean urease (monomer 240K; dimer 480K) from Sigma, and cytochrome c (12.4K), adenylate kinase (32K), enolase (92K), lactate dehydrogenase (140K) and glutamate dehydrogenase (290K) from U.S.B. The buffer used was 0.1 M MOPS-NaOH pH 7.2. In additional experiments, the gel filtration of the GDH was performed with 20 mM monosodium L-glutamate, 50 mM ammonium chloride, 1 M

sodium chloride or 10 mM calcium chloride added to the buffer. The calibration curve is shown in Figure 5.

These experiments confirmed Dr Coolbear's result of 204K for the molecular weight of the native GDH. However, the protein peak corresponding to a molecular weight of 51K (presumably the monomeric form of the enzyme) observed by Dr Coolbear was not present in these experiments under any of the conditions used. Single protein peaks were observed when the GDH was chromatographed in MOPS buffer with or without monosodium glutamate, ammonium chloride, sodium chloride or calcium chloride, although the presence of the latter two additives slightly increased the elution volume.

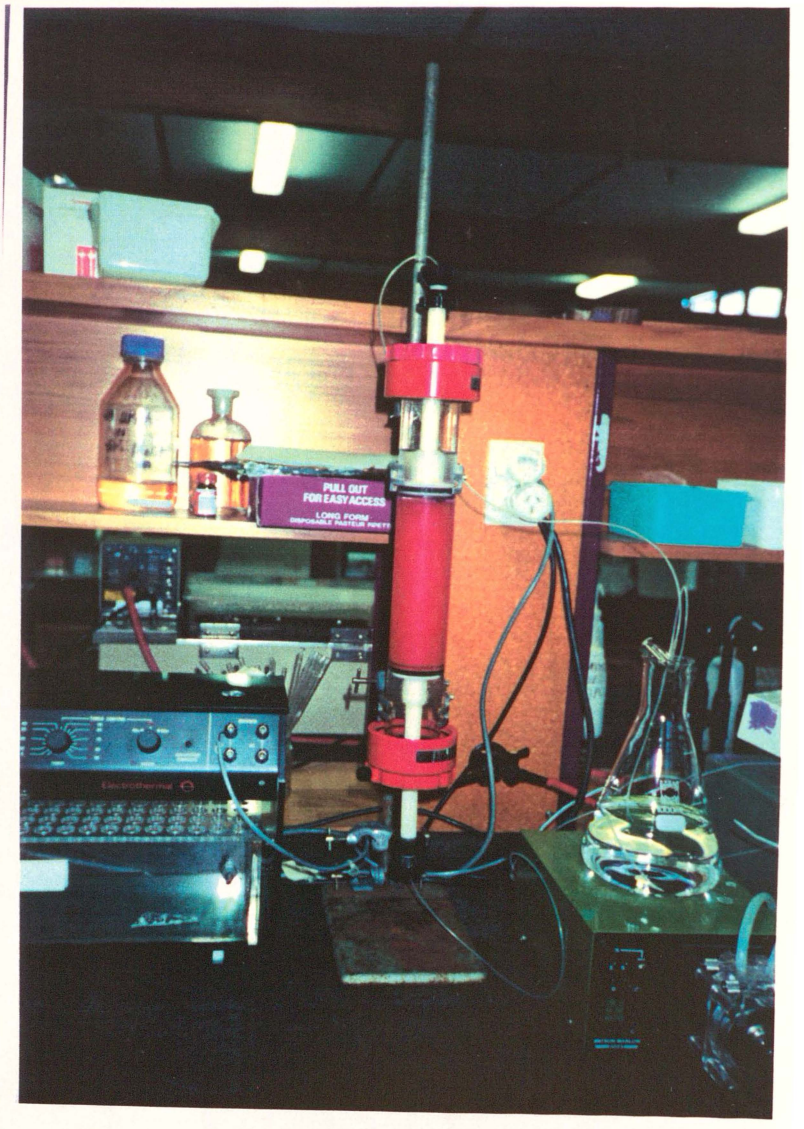
PULL OUT
FOR EASY ACCESS
LONG FORM

Fig. 1. The Reactive Red-Sepharose CL-6B column.

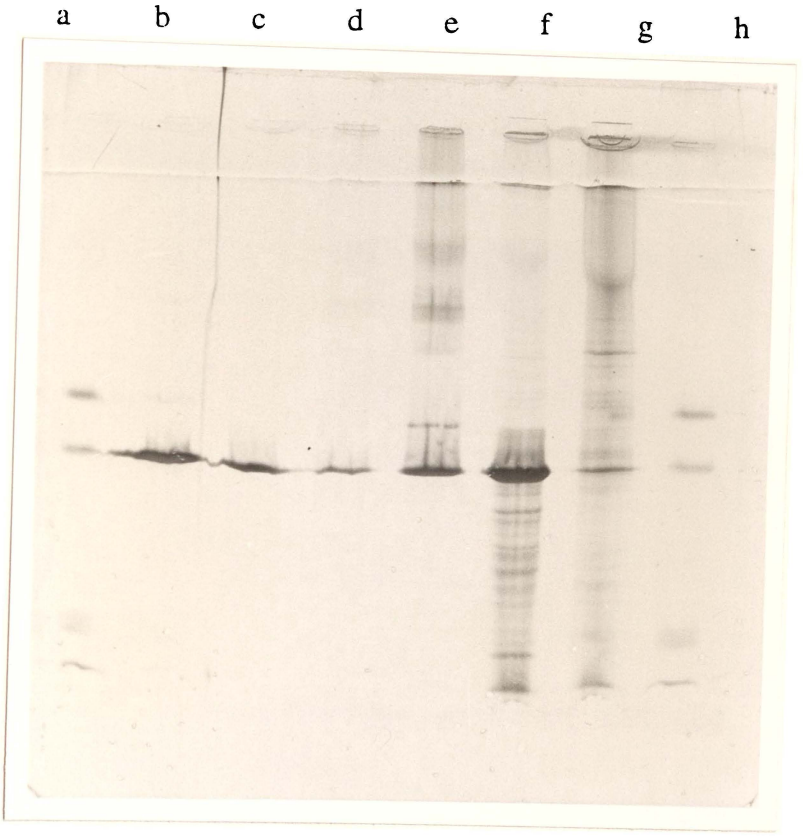

Fig. 2. SDS-PAGE gel showing various stages of GDH purification. The standard protein mixtures are in tracks a and h. Tracks b and c were loaded with material from a trial purification procedure. Tracks d and e were loaded with 0.093 and 0.24 µg, respectively, of material purified as far as the second passage through Reactive Red-Sepharose CL-6B. Track f was loaded with 0.50 µg material from the first purification step (one passage through Reactive Red-Sepharose CL-6B). Track g was loaded with 0.096 µg cell free extract.

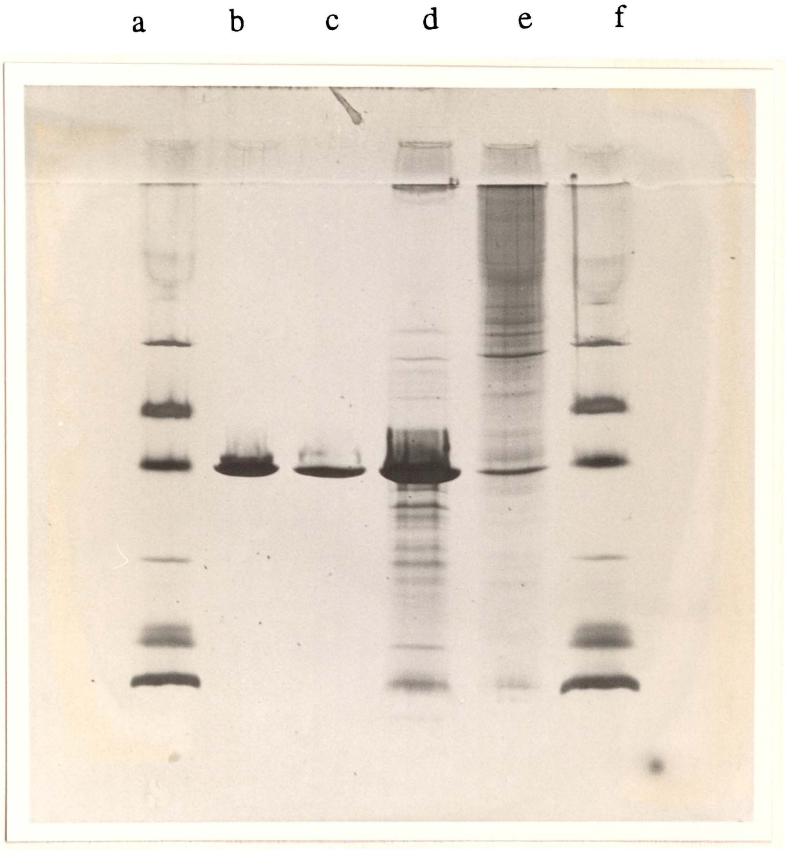

Fig. 3. SDS-PAGE gel showing various stages of GDH purification. The standard protein mixtures are in tracks a and f. Tracks b and c were loaded with 0.3 ug and 0.15 ug, respectively, pure GDH (after all three stages of purification). Track d was loaded with 0.5 ug material from the first purification step (one passage through Reactive Red-Sepharose CL-6B). Track e was loaded with 0.2 ug cell free extract.

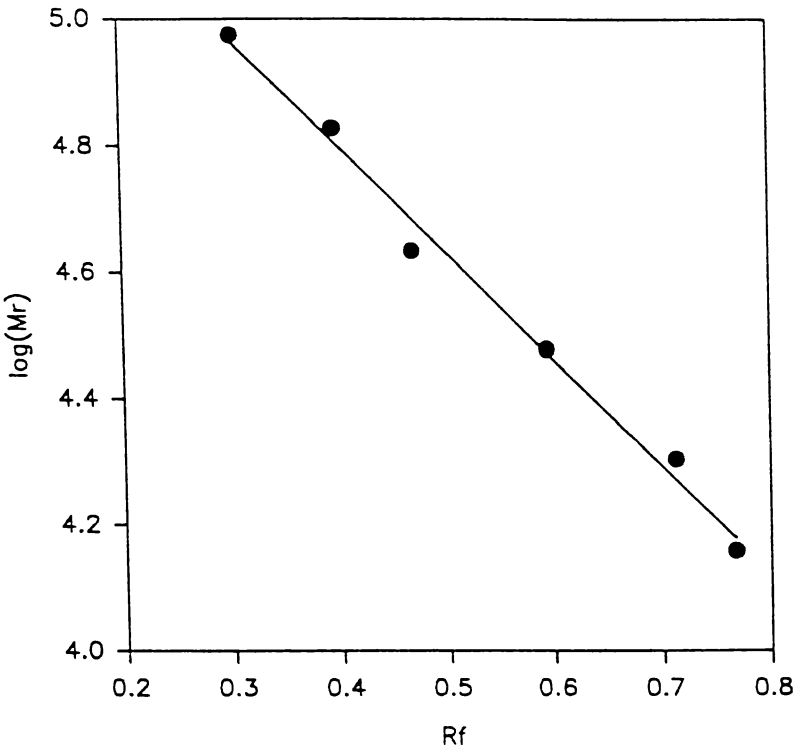

Fig. 4. Calibration curve for subunit molecular weight determination by SDS-PAGE.

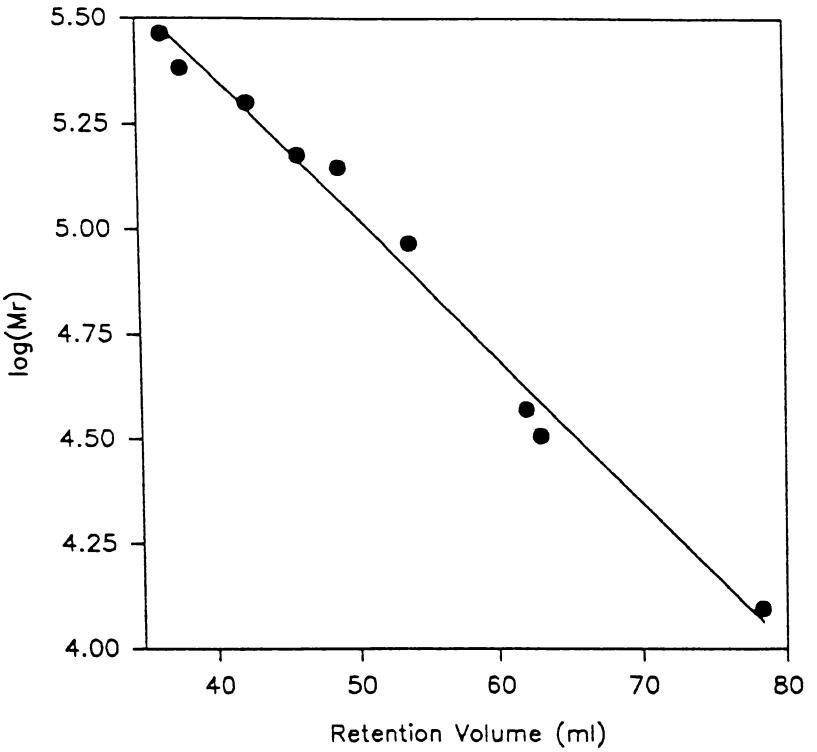

Fig. 5. Calibration curve for molecular weight determination by Superose 12 HR 16/50 gel filtration.

Isoelectric Point Determination

Isoelectric focussing (I.E.F.) of the GDH was performed using a Pharmacia Phast System and IEF 3-9 gels. The standard proteins were amyloglucosidase (pI 3.5), soybean trypsin inhibitor 9pI 4.55), β -lactoglobin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (pI 6.85 and 7.35) and lentil lectin (pI 8.15, 8.45 and 8.65). The calibration curve is shown in Figure 6. An example of an IEF gel showing the standard proteins (tracks a and d) and the purified glutamate dehydrogenase (0.2 μg) is shown in Figure 7.

Lineweaver-Burk Plots:

Figures 8-27 are the Lineweaver-Burk plots of the experiments performed to determine the Km values for the substrates at several temperatures. A linear regression program (SigmaPlot version 5.0) was used to calculate the Km values. Velocities are expressed as μmol/min/mg. The apparent Km values for $NADP^+$ were calculated as 0.014, 0.027 and 0.038 mM at 40, 60 and 80°C, respectively. The apparent Km values for glutamate were calculated as 1.39, 1.93, 3.66 and 9.12 mM at 40, 60, 70 and 80°C, respectively. The apparent Km values for NADPH were calculated as 0.023, 0.036 and 0.066 mM at 40, 60 and 80°C, respectively. The apparent Km values for 2-ketoglutarate were calculated as 0.39, 0.52 and 1.7 mM at 40, 60 and 80°C, respectively. The apparent Km values for ammonia were calculated as 5.7, 5.7 and 15.5 mM at 40, 60 and 80°C, respectively. At 80°C the apparent Km for glutamine was 26.9 mM, and at 60°C (in 0.1 M CHES-NaOH, pH 8.2) it was 37.8 mM. The apparent Km values for NAD^+ and NADH at 80°C were calculated as 28.6 and 0.32 mM, respectively.

The concentrations of the non-varied substrates were 100, 0.3, 20, 0.2 and 150 mM for glutamate, $NADP^+$, 2-ketoglutarate, NADPH and NH_4Cl, respectively. Unless otherwise stated, assays were performed under the standard conditions given in Chapter 2 (0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.54).

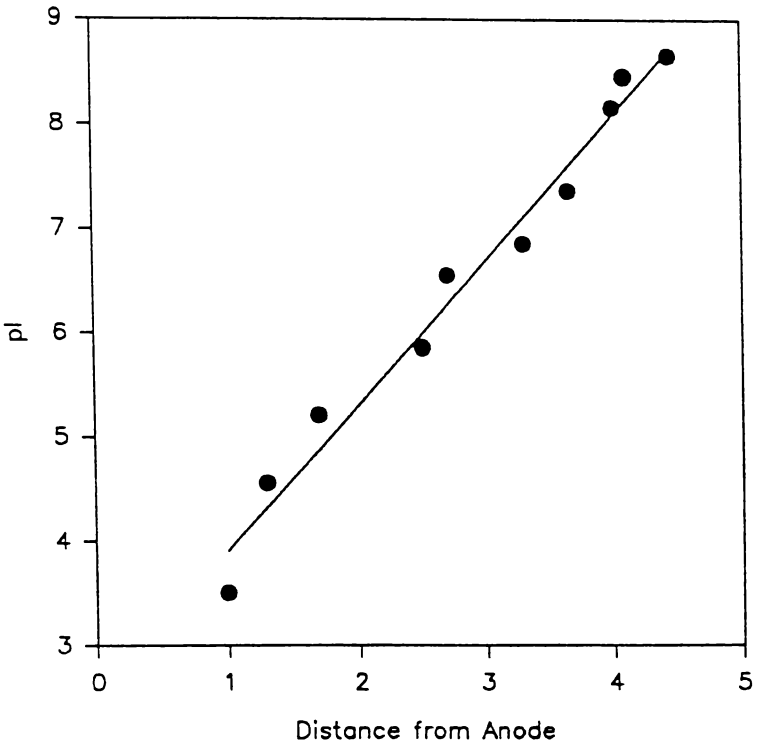

Fig. 6. Calibration curve for isoelectric point determination.

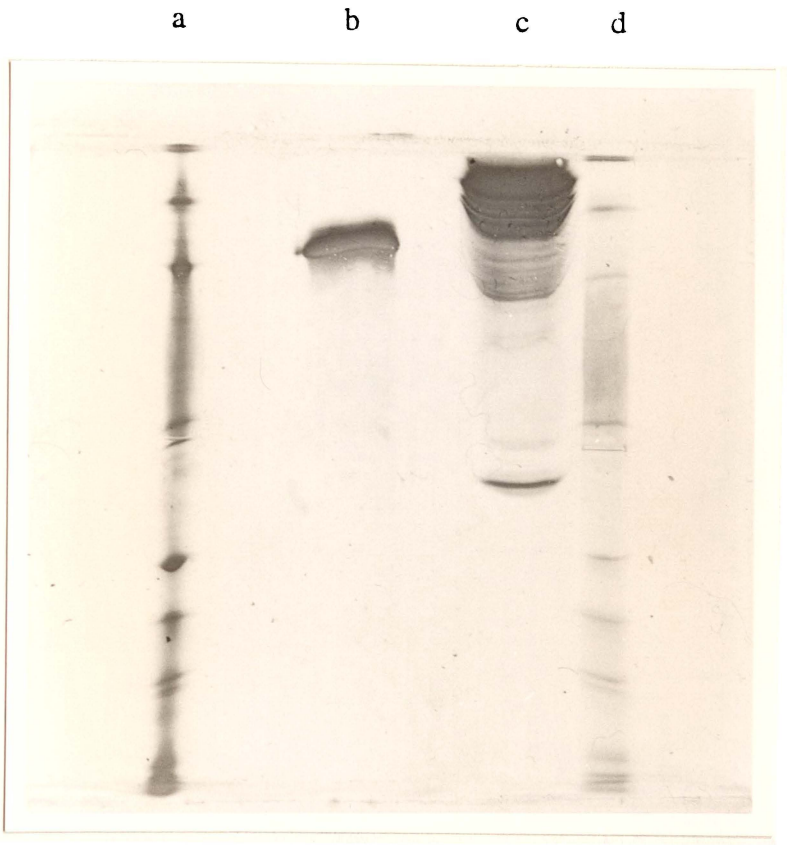

Fig. 7. An IEF 3-9 Phast gel. Tracks a and d were loaded with standard protein mixtures. Track b was loaded with 0.2 µg purified GDH. Track c was loaded with a colleague's sample.

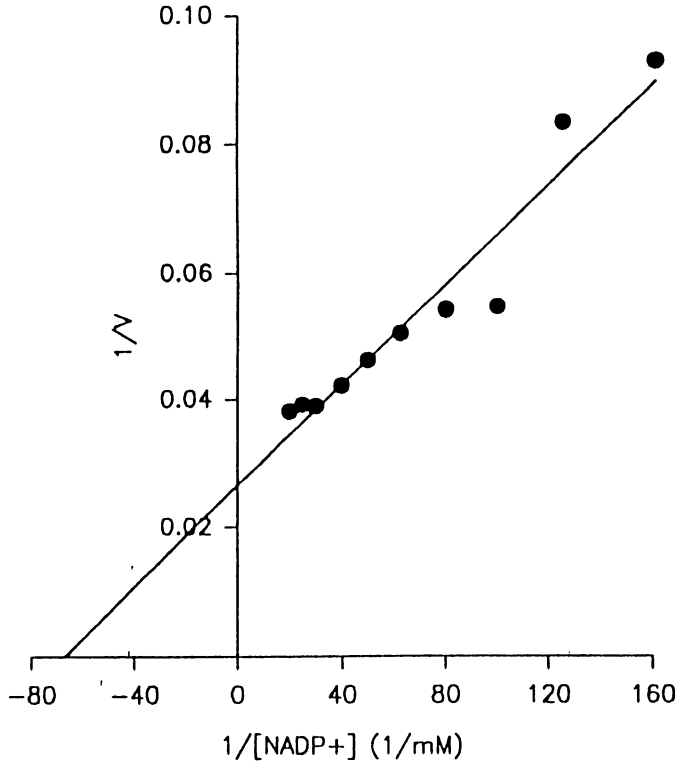

Fig. 8: Double reciprocal plot of velocity vs $NADP^+$ concentration at 40°C.

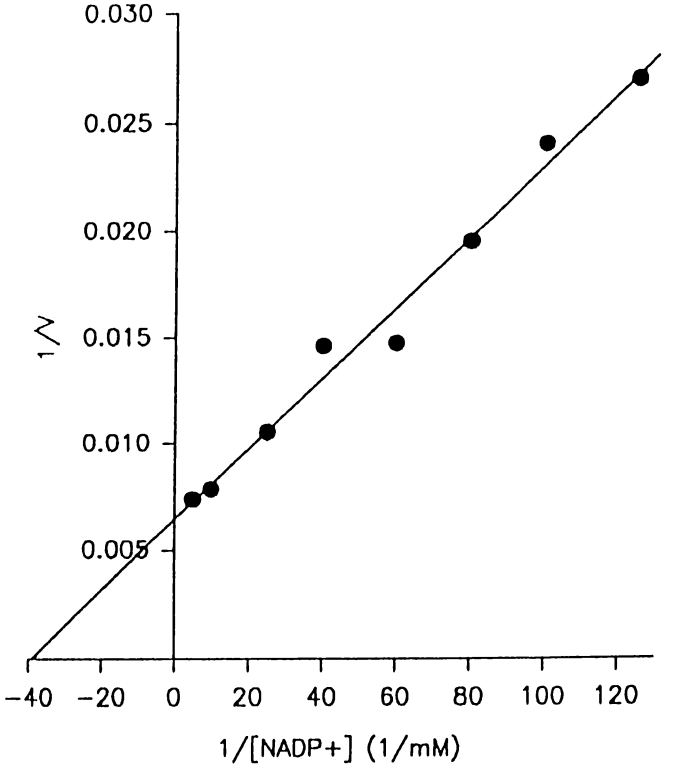

Fig. 9: Double reciprocal plot of velocity vs $NADP^+$ concentration at 60°C.

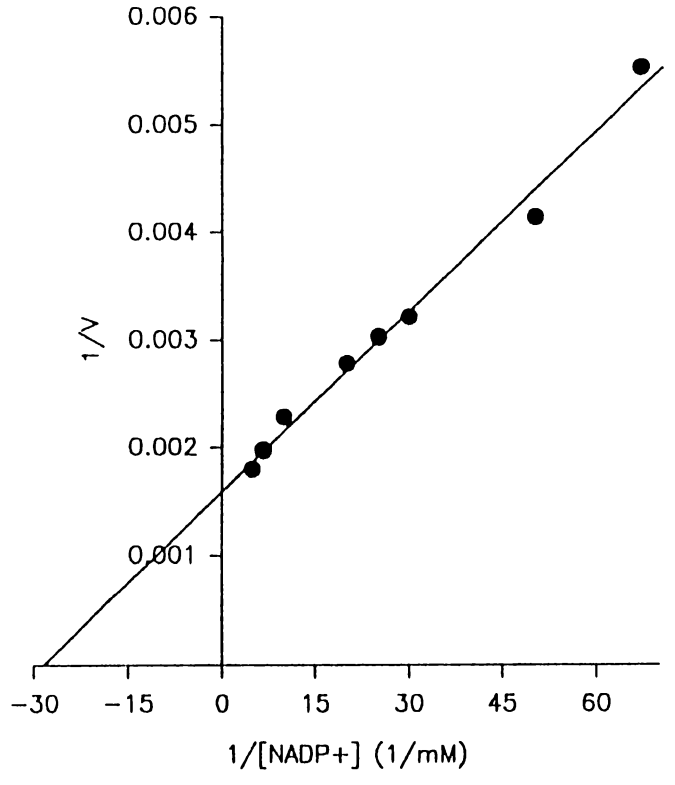

Fig. 10: Double reciprocal plot of velocity vs $NADP^+$ concentration at 80°C.

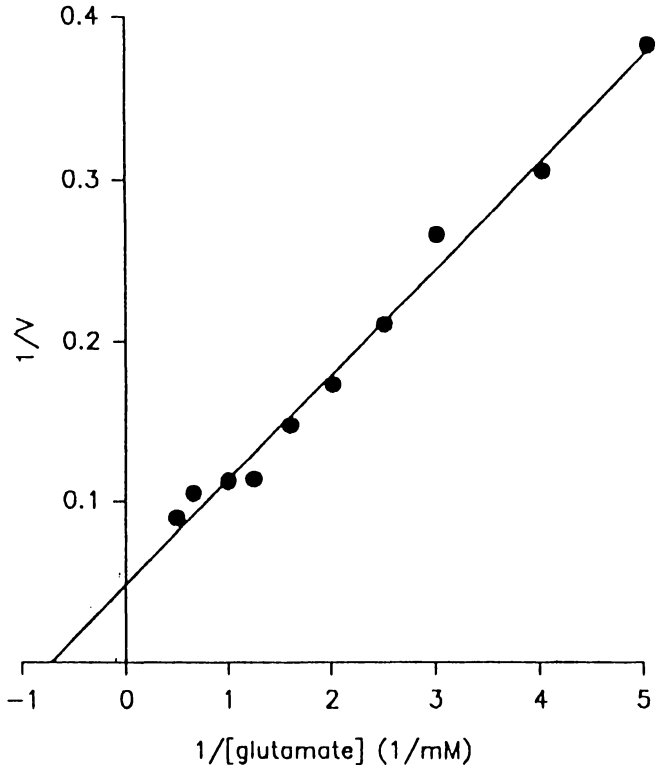

Fig. 11: Double reciprocal plot of velocity vs glutamate concentration at 40°C.

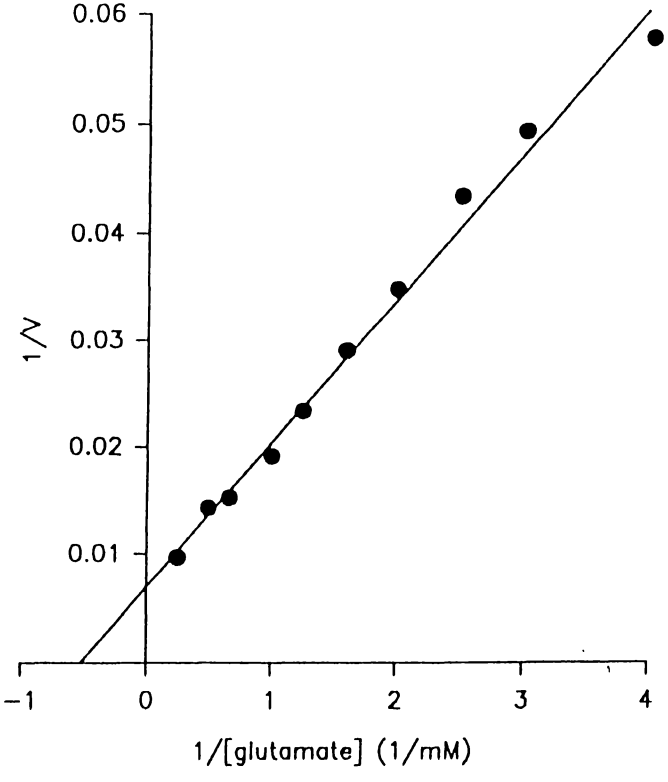

Fig. 12: Double reciprocal plot of velocity vs glutamate concentration at 60°C.

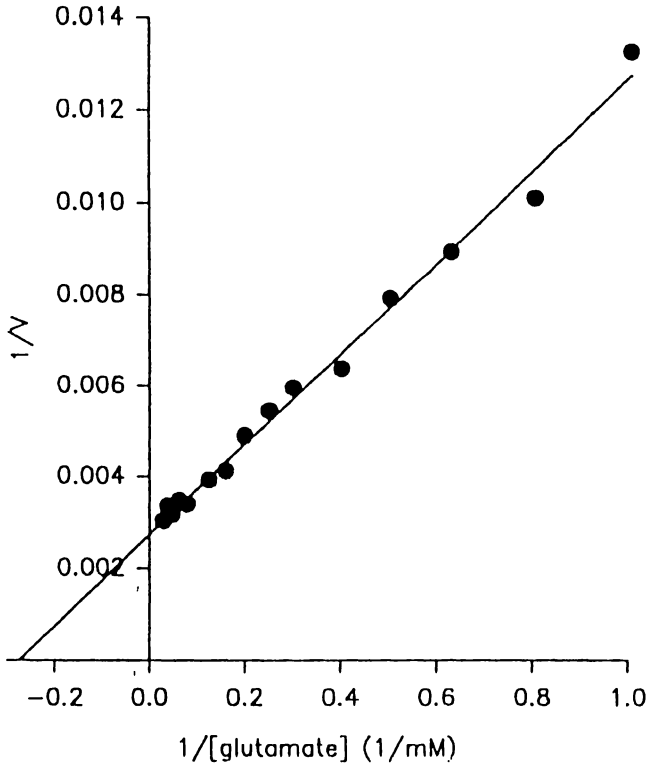

Fig.13 Double reciprocal plot of velocity vs glutamate concentration at 70°C.

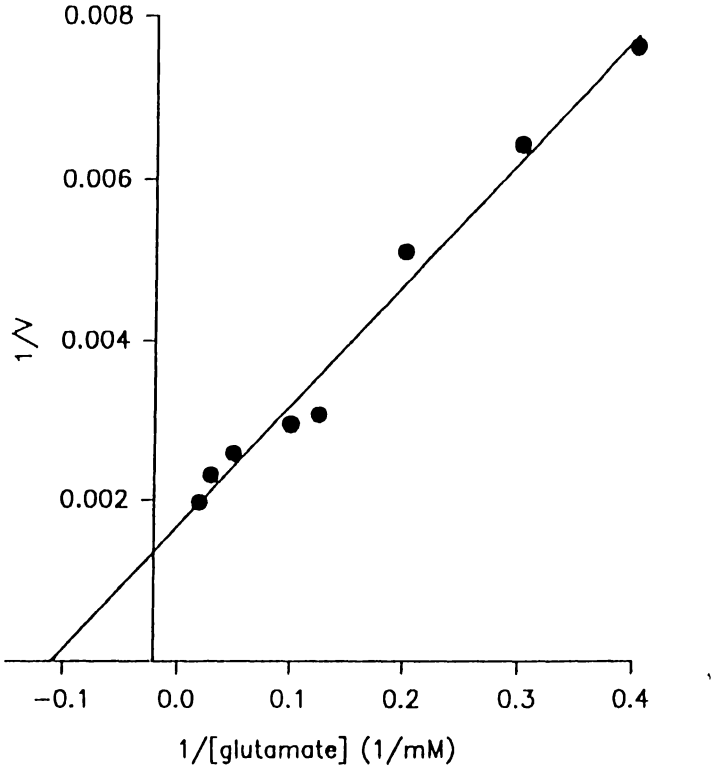

Fig. 14: Double reciprocal plot of velocity vs glutamate concentration at 80°C.

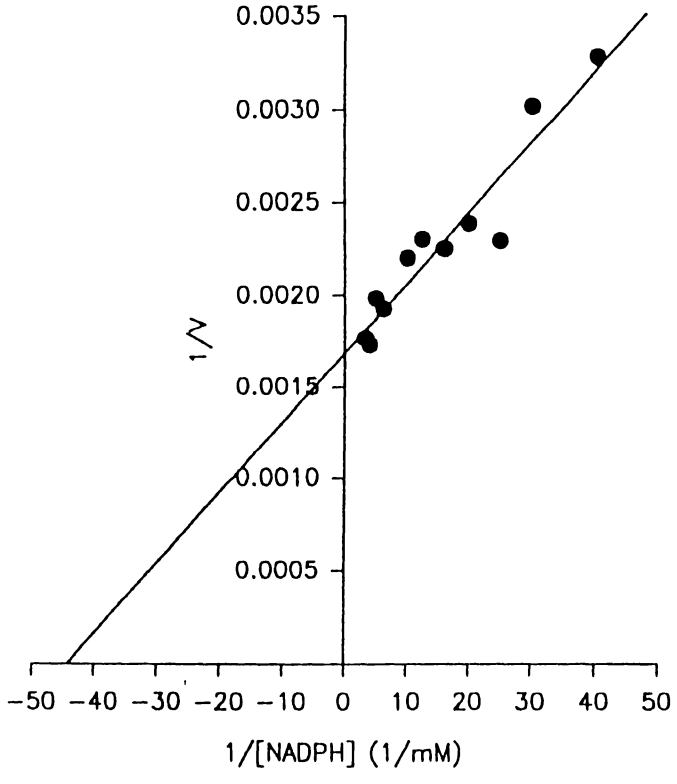

Fig. 15: Double reciprocal plot of velocity vs NADPH concentration at 40°C.

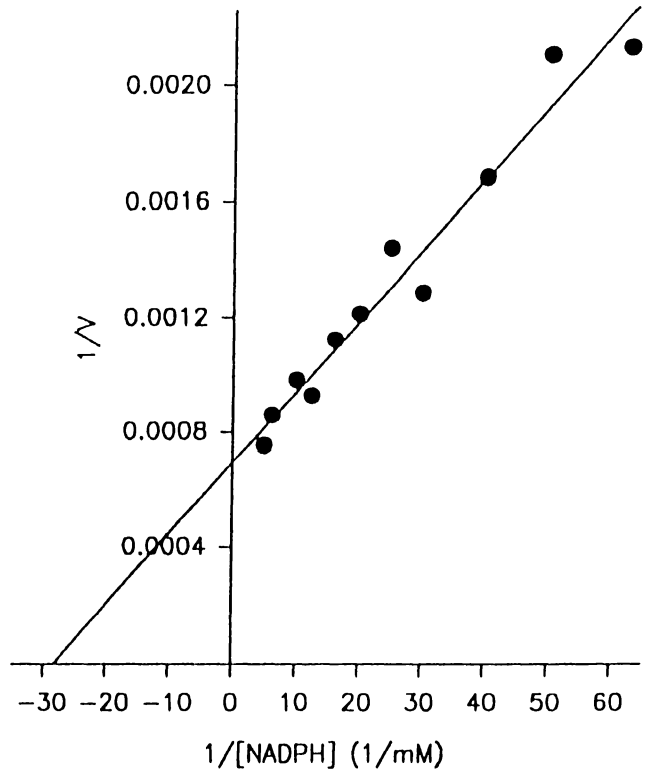

Fig. 16: Double reciprocal plot of velocity vs NADPH concentration at 60°C.

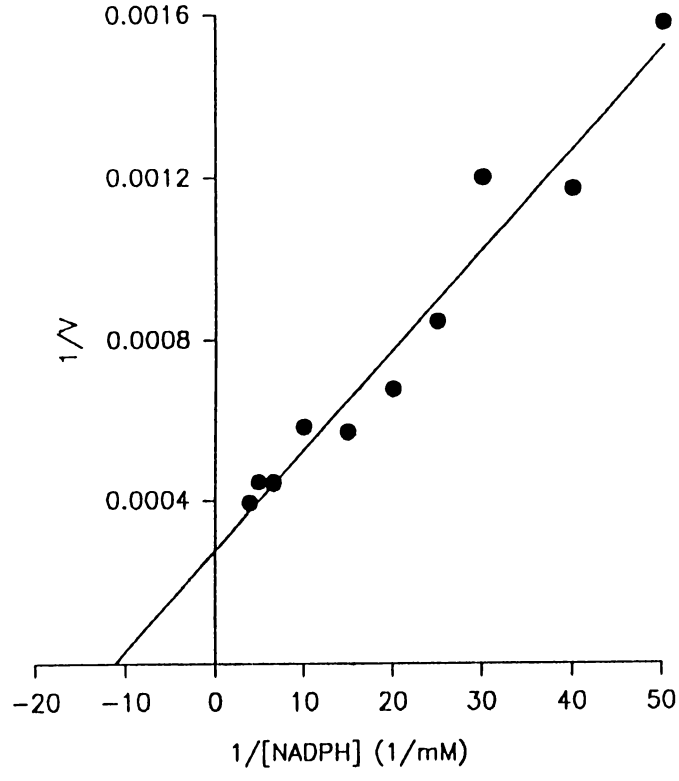

Fig. 17: Double reciprocal plot of velocity vs NADPH concentration at 80°C.

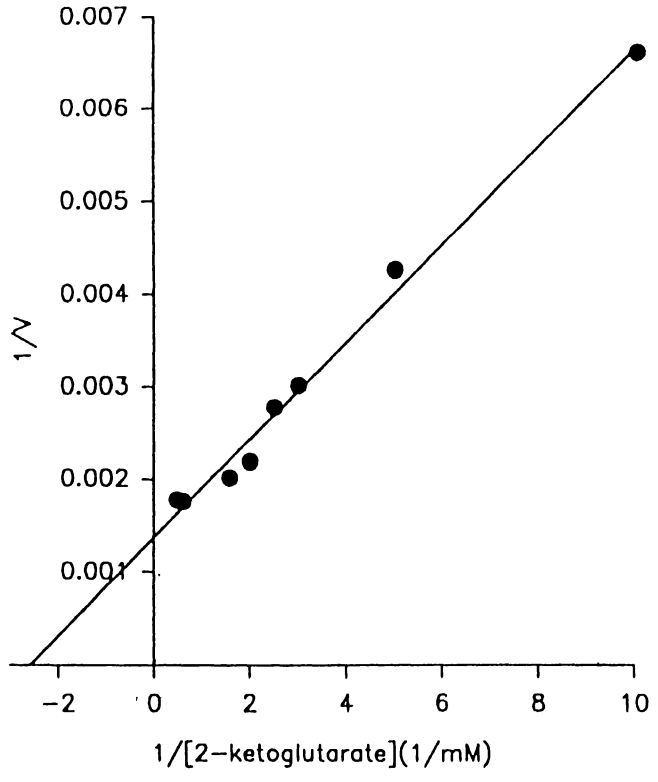

Fig. 18: Double reciprocal plot of velocity vs 2-ketoglutarate concentration at 40°C.

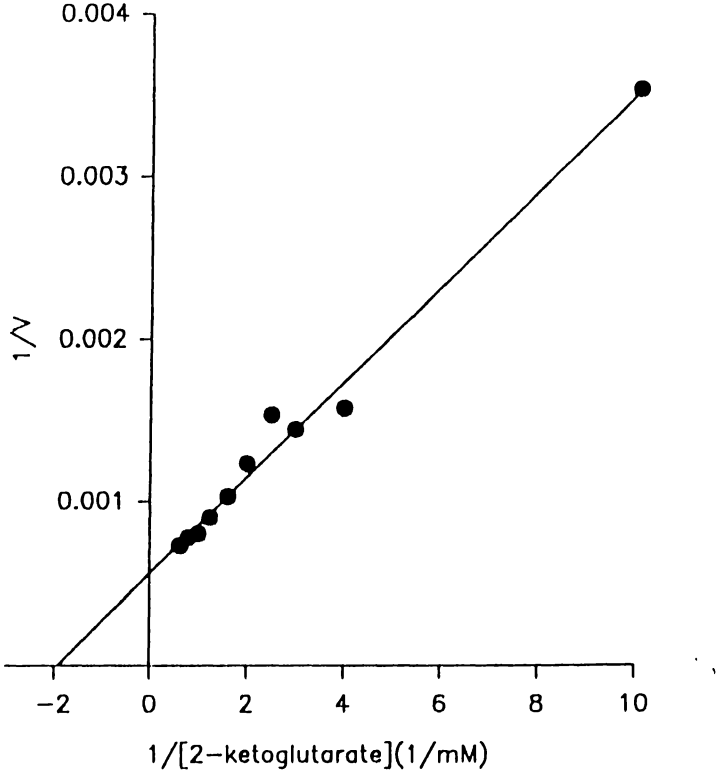

Fig. 19: Double reciprocal plot of velocity vs 2-ketoglutarate concentration at 60°C.

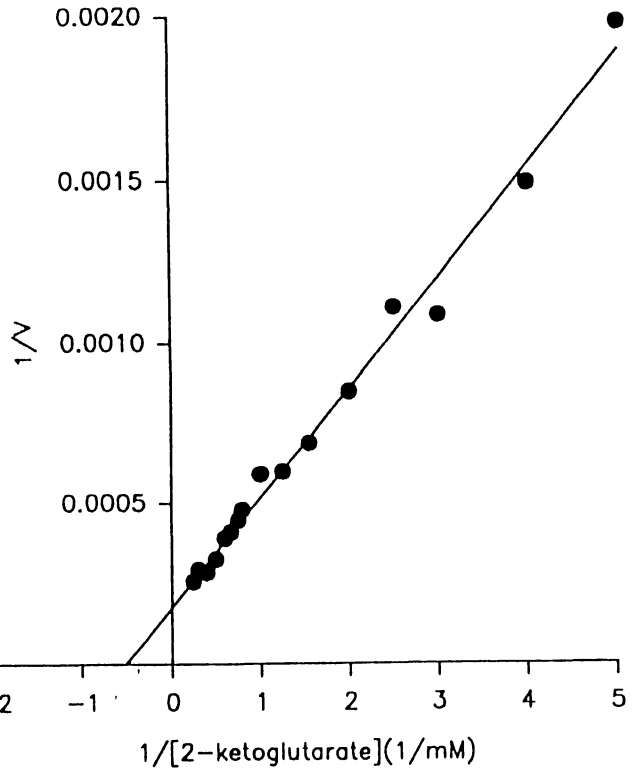

Fig. 20: Double reciprocal plot of velocity vs 2-ketoglutarate concentration at 80°C.

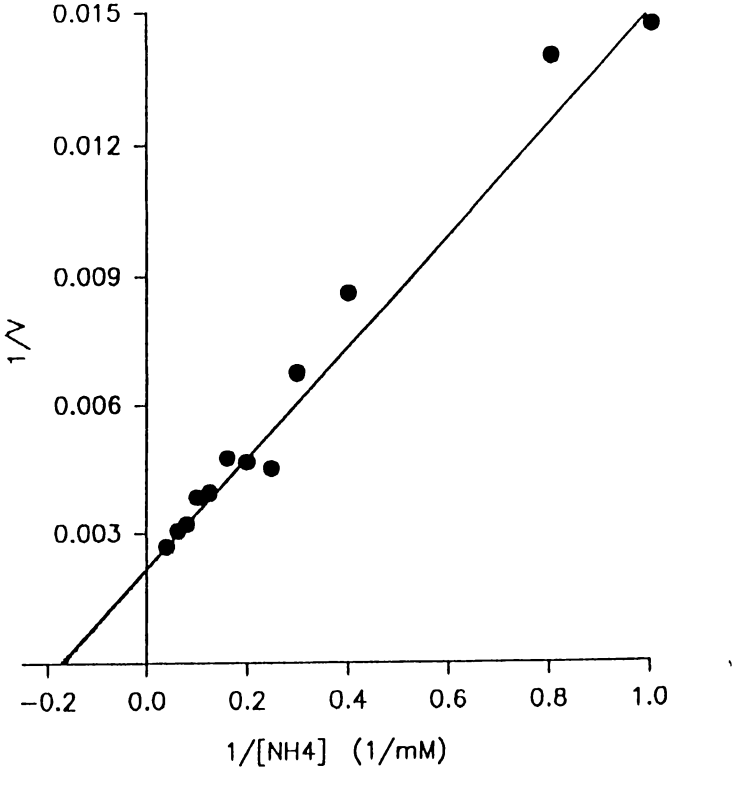

Fig. 21: Double reciprocal plot of velocity vs ammonium ion concentration at 40°C.

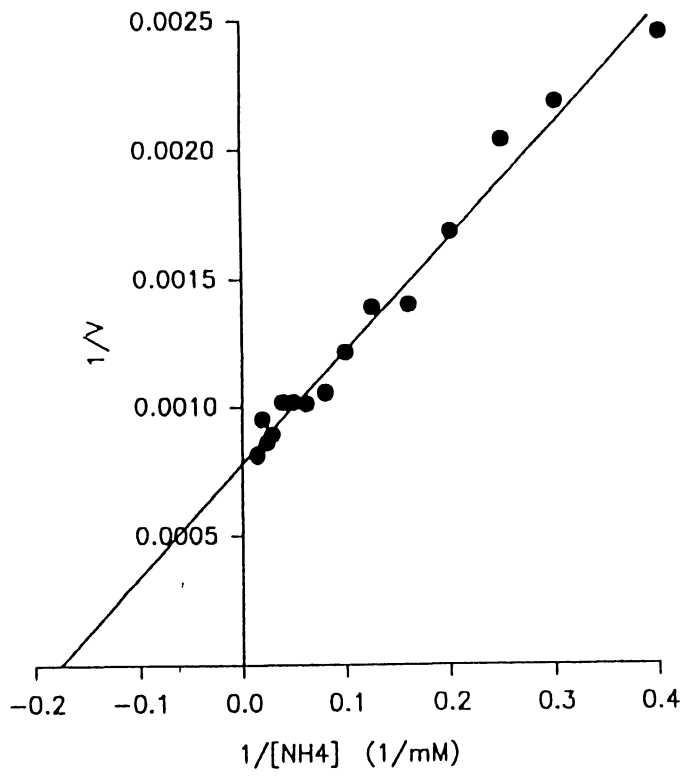

Fig. 22: Double reciprocal plot of velocity vs ammonium ion concentration at 60°C.

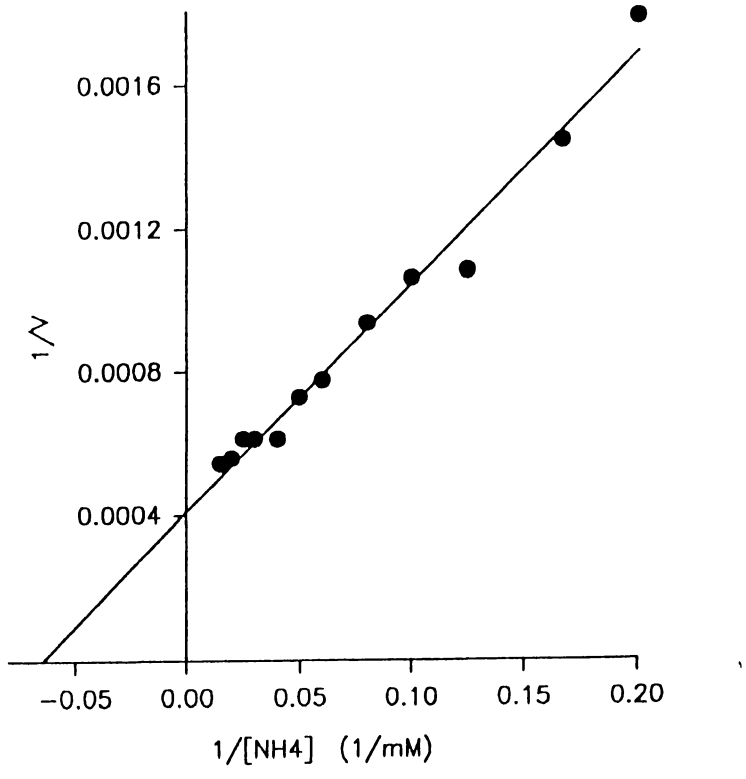

Fig. 23: Double reciprocal plot of velocity vs ammonium ion concentration at 80°C.

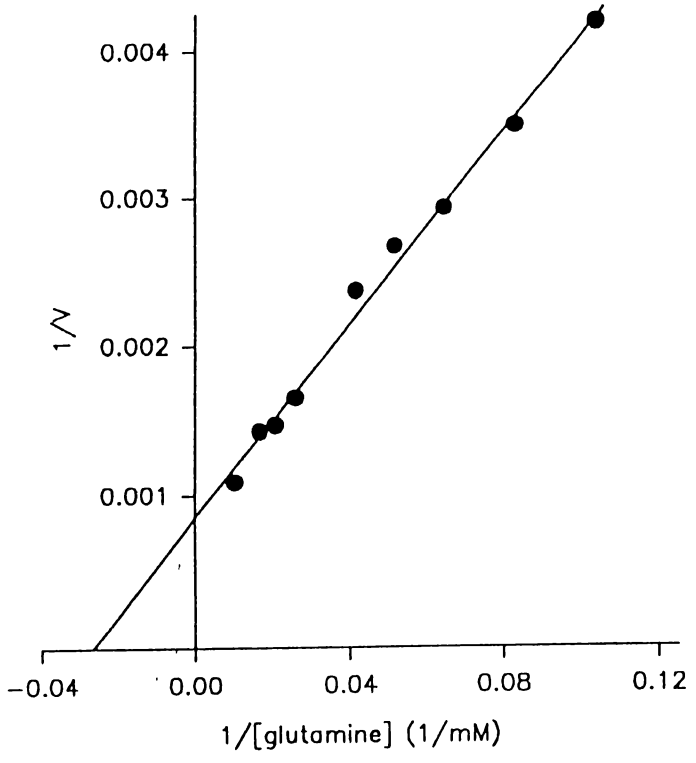

Fig. 25: Double reciprocal plot of velocity vs glutamine concentration at 60°C (pH 8.2).

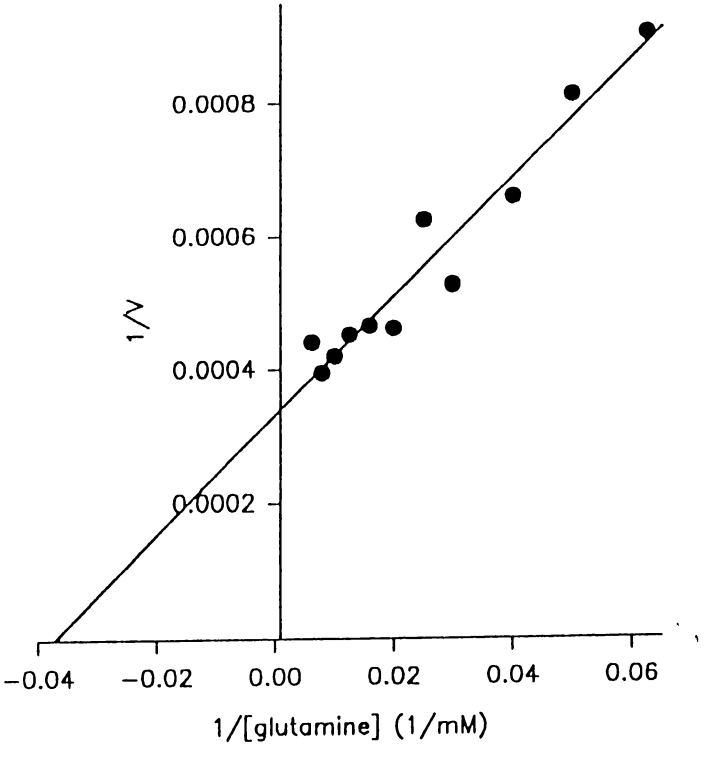

Fig. 24: Double reciprocal plot of velocity vs glutamine concentration at 80°C.

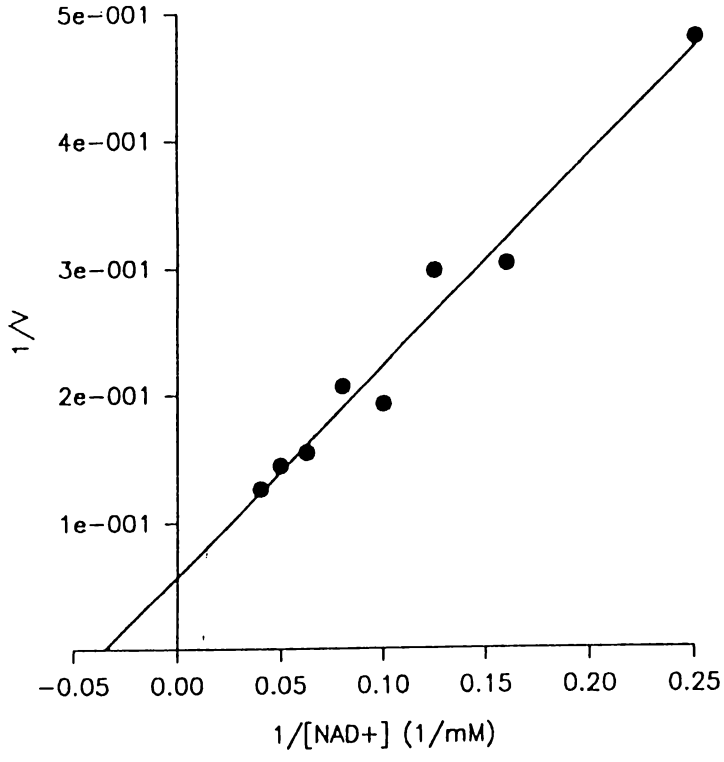

Fig. 26: Double reciprocal plot of velocity vs NAD^+ concentration at 80°C.

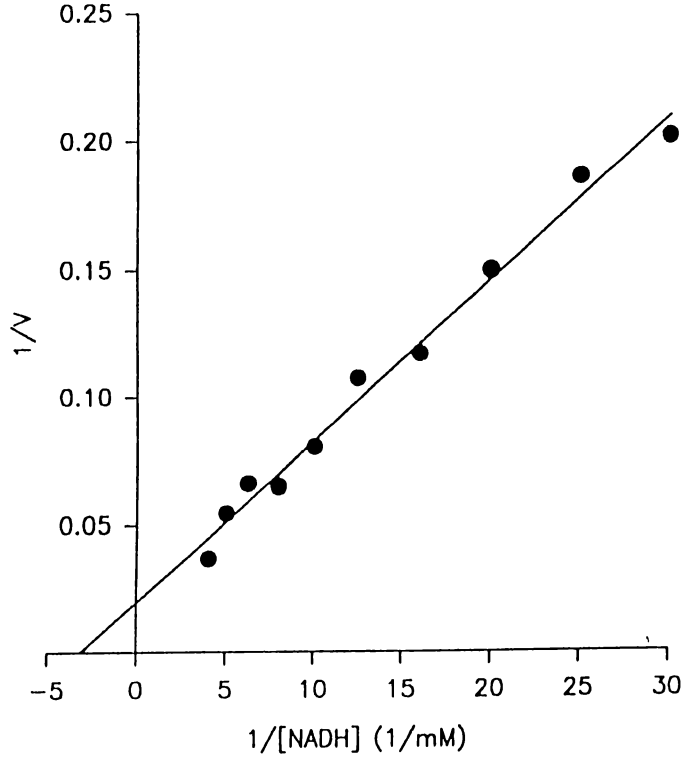

Fig. 27: Double reciprocal plot of velocity vs NADH concentration at 80°C.

The pH Profile for Glutamate Synthase Activity

The pH optimum for the glutamate synthase activity displayed by the glutamate dehydrogenase was determined at 60°C in 0.1 M buffers (Na_2HPO_4-NaH_2PO_4 for pH 6.1-7.8; CHES-NaOH for pH 7.7-9.8) containing 0.15 M glutamine, 20 mM 2-ketoglutarate and 0.2 mM NADPH. The pH values of the buffers were measured at 60°C in the presence of 0.15 M glutamine.

The pH profile is shown in Figure 28. The pH optimum for glutamate synthase activity was approximately 8.2. Thus the GDH had the same pH optima for glutamate dehydrogenase activity (in both oxidative deamination and reductive amination directions) and glutamate synthase activity.

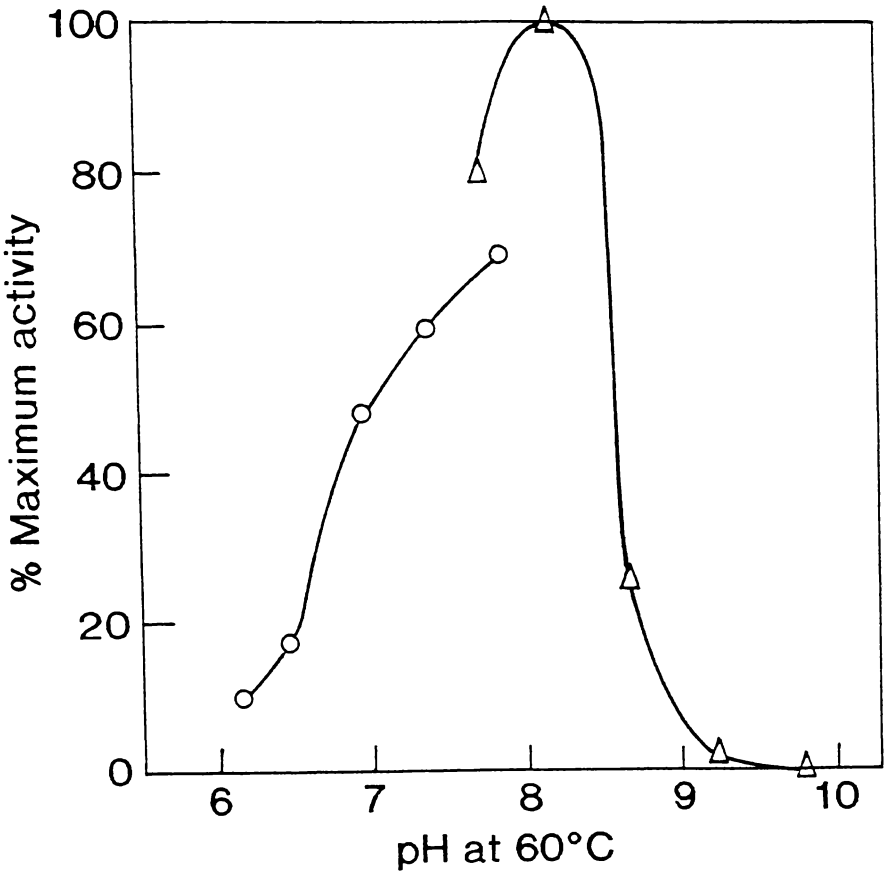

Fig. 28: The pH profile for glutamate synthase activity.

Circles: phosphate buffer; triangles: CHES buffer.

The Effect of Metal Ions on the Activity of Glutamate Dehydrogenase in the Reductive Amination Reaction

The effects of metal ions on the GDH in the reductive amination reaction are listed in Table I. In contrast to the large activation effected by calcium, magnesium and manganese ions in the oxidative deamination direction, metal ions, in most cases, had little effect on the reductive amination reaction. The apparent inhibition caused by copper ions was probably the result of NADPH depletion: copper ions promoted rapid degradation of NADPH, hence the enzyme probably wasn't saturated with substrate.

TABLE I

EFFECT OF METAL IONS ON THE REDUCTIVE AMINATION REACTION

Metal Salt	% Activity
$MnCl_2$	97
$MgCl_2$	96
$CaCl_2$	95
$CdCl_2$	91
LiCl	88
$NiCl_2$	88
$ZnCl_2$	84
VCl_3	57
$CuCl_2$*	10

The activities were determined at 60°C in 20 mM MOPS-NaOH buffer containing 20 mM 2-ketoglutarate, 40 mM NH_4Cl and 0.2 mM NADPH. The concentration of metal ions was 1 mM. Values for activity are those compared to control (100%). The following precipitated out of solution: $CrCl_3$, $La(NO_3)_3$ and $HgCl_2$.

* $CuCl_2$ markedly accelerated the autodegradation of NADPH.

The Effect of Chelating Agents on Glutamate Dehydrogenase Activity in the Presence of Metal Ions

Chelating agents had little effect on glutamate dehydrogenase activity in the absence of metal ions (see Chapter 2). However, when a chelating agent and a divalent metal ion were present together, the resultant effect depended on the particular chelating agent and metal ion. Table II summarises the results of these experiments, which were performed at 60°C under similar conditions to those outlined for the metal ion experiments in Chapter 2 (18 mM MOPS-NaOH pH_{60}7.13, 18 mM monosodium L-glutamate, 0.3 mM $NADP^+$; NaCl added to keep ionic strength constant; metal ions added as their chloride salts; EDTA and EGTA as sodium salts).

These results are further discussed in the Final Discussion (Chapter 4).

TABLE II

EFFECT OF METAL IONS AND CHELATING AGENTS ON THE OXIDATIVE DEAMINATION REACTION

Divalent Metal Ion	Chelating Agent	% of Control Activity
1 mM Ca^{2+}	nil	140
1 mM Ca^{2+}	1 mM EGTA	78
3 mM Ca^{2+}	nil	178
3 mM Ca^{2+}	3 mM 1,10-phenanthroline	202
5 mM Ca^{2+}	nil	230
5 mM Ca^{2+}	5 mM EDTA	34
5 mM Mg^{2+}	nil	219
5 mM Mg^{2+}	5 mM EDTA	45
1 mM Mn^{2+}	nil	240
1 mM Mn^{2+}	1 mM EGTA	91
1 mM Mn^{2+}	3 mM 1,10-phenanthroline	225
1 mM Mn^{2+}	1 mM EDTA	82
nil	3 mM 1,10-phenanthroline	114
nil	1 mM EDTA	93
nil	5 mM EDTA	95
nil	1 mM EGTA	87

APPENDIX 2

CRYSTALLISATION OF GLUTAMATE DEHYDROGENASE FROM THE ARCHAEBACTERIAL ISOLATE AN1

Preliminary Experiments

Preliminary crystallisation experiments were carried out by R.C.H. during a visit to the Crystallography Department at Massey University. Factorial solutions and the hanging drop method were used. The concentration of (homogeneous) GDH in the drops was 10 mg/ml. Crystals were obtained under the following conditions:

(1) 0.2 M ammonium acetate, 0.1 M Tris-HCl (pH 8.5), 30% PEG 4K

(3) 0.2 M magnesium chloride, 0.1 M HEPES-NaOH (pH 7.5), 15% PEG 400

(3) 0.1 M MES-NaOH (pH 6.5), 30% MPD.

Solution (1) gave the largest crystal, which was cuboid in shape. Solution (2) gave smaller crystals, which had the appearance of 4 feathers in a group with their quills converging. Solution (3) gave very small crystals.

None of theses crystals were of a large enough size to allow X-ray diffraction patterns to be determined. Further crystallisation work with the isolate AN1 GDH was carried out by Dr Gill Norris of the Crystallography Department at Massey University.

Continued Experiments

Dr Gill Norris obtained crystals with the sitting drop method under the following conditions:

(1) 45 mM calcium chloride, 0.1 M ammonium acetate (pH 5.5), 40% MPD, 10 mM glutamate, 10 mM $NADP^+$. Temp = ambient. Time taken to crystalise = 3 months.

(2) 45 mM calcium chloride, 0.1 M ammonium acetate (pH 5.1), 40% MPD, 10 mM $NADP^+$. Temp = ambient.

(3) 60 mM magnesium chloride, 0.1 M ammonium acetate (pH 5.5), 40% MPD, 10 mM $NADP^+$.

(4) 60 mM calcium chloride, 0.1 M ammonium acetate (pH 5.5), 40% MPD, 10 mM glutamate, 10 mM $NADP^+$.

The crystals obtained by method (1) are shown in Fig. 1. These crystals diffracted and the diffraction pattern is shown in Fig. 2. The picture was taken using a synchrotron radiation source at the Photon Factory, Japan (wavelength = 1.00 angstroms). The cell dimensions appear to be: a = 172.7, b = 148.6, c = 113.3 (Angstroms); alpha = 90.0°, beta = 91.0°, gamma = 90.0°. These dimensions indicate that the cell is monoclinic. However, the crystals diffracted poorly, giving a weak diffraction pattern, hence these results may not be reliable.

Fig. 3 shows the crystals obtained by method (2). These crystals did not diffract.

Needle-like crystals were obtained by method (3), in which magnesium chloride was used as the salt. These were bigger than those obtained with calcium chloride (Fig. 1, method (1)), and did diffract. A very large hexagonal crystal was obtained by method (3) (both glutamate and $NADP^+$ used as additives), but it did not diffract. No crystallisation occurred if manganese chloride (45 or 60 mM) was used as the salt.

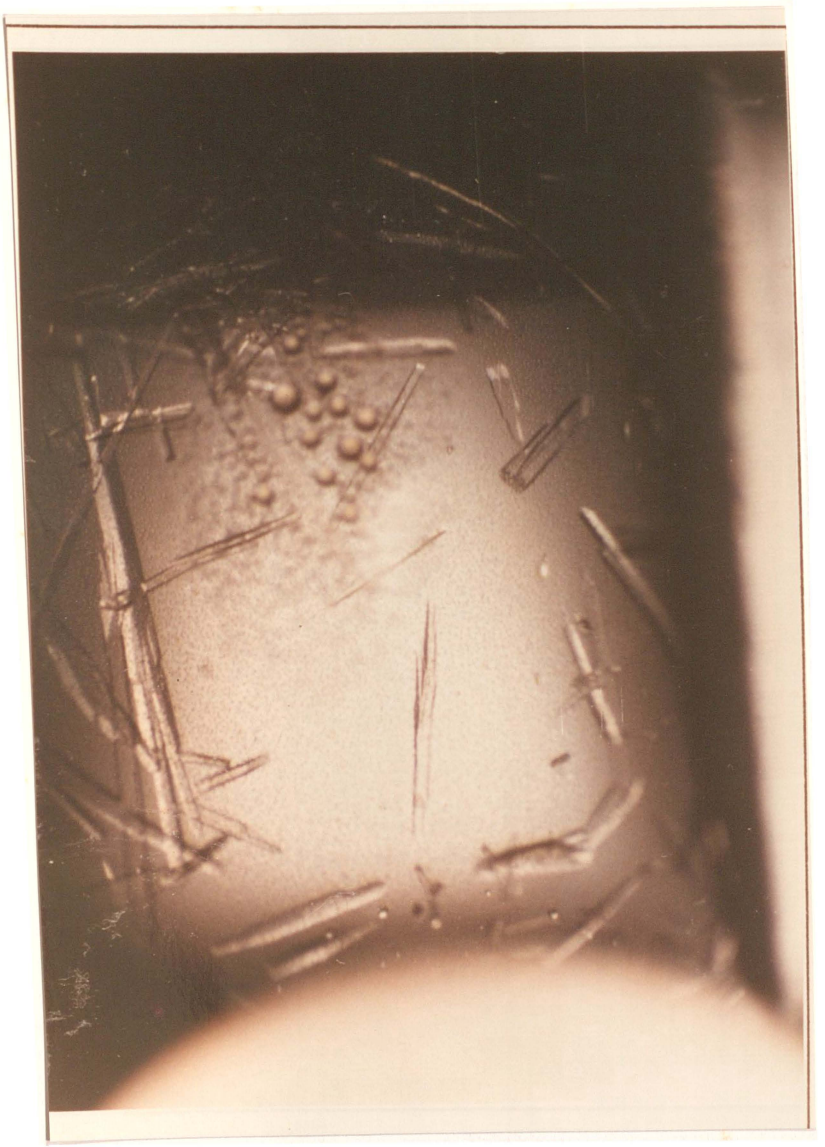

Fig. 1. Crystals of the isolate AN1 GDH obtained by method (1).

Fig. 2. X-Ray diffraction pattern of the crystals shown in Fig. 1. The x and y axes are approximately 176 and 150 angstroms, respectively.

Fig. 3. Crystals of the isolate AN1 GDH obtained by method (2).

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