THE UNIVERSITY OF CALGARY

# GLUTAMATE DEHYDROGENASE OF *SALMONELLA TYPHIMURIUM*: PURIFICATION, PROPERTIES, AND REGULATION

by

JAMES WILLIAM COULTON

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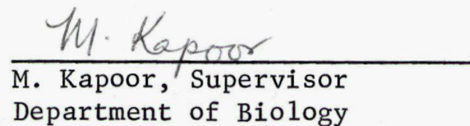
The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled GLUTAMATE DEHYDROGENASE OF SALMONELLA TYPHIMURIUM: PURIFICATION, PROPERTIES, AND REGULATION submitted by JAMES WILLIAM COULTON in partial fulfillment of the requirements for the degree of Master of Science.



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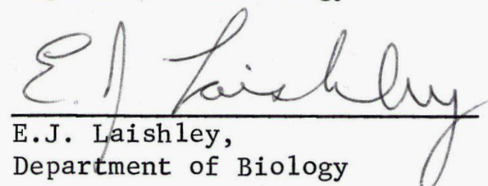

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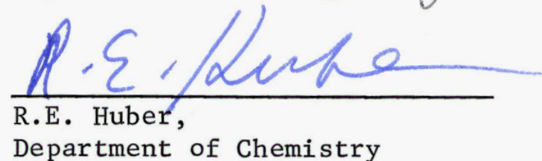

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# ABSTRACT

Glutamate dehydrogenase from Salmonella typhimurium was purified 190 - fold by heat treatment, ammonium sulphate fractionation, DEAE-Sephadex chromatography, reverse ammonium sulphate fractionation, and gel filtration. The enzyme proved to be stable to $55^{o}C$, and displayed a pH optimum at 8.6 in the amination reaction. The sedimentation coefficient of GDH, as determined by sucrose density gradient centrifugation, was approximately 10.3 S. From gel filtration chromatography, the molecular weight and Stokes' radius for the enzyme were estimated at 280,000 daltons and $54 \times 10^{-8}$ cm, respectively.

Kinetic parameters were determined for each of the three substrates in the forward reaction, NADPH, $NH_4^+$, and α-ketoglutarate, and for the substrates in the reverse direction, $NADP^+$ and glutamate. Based on an analysis of initial velocity patterns and product inhibition data, and employing Cleland's nomenclature, the mechanism was postulated to be an ordered Ter-Bi reaction. Of the several metabolites tested, only L-malate and L-glutamine exerted an appreciable effect on enzyme activity. ATP and AMP at 0.8 mM concentration were found to enhance GDH activity by 68 and 6 per cent, respectively. Unusual resistance was displayed by the enzyme to high concentrations of the protein denaturants, urea, SDS, and guanidine hydrochloride; the sulphydryl reagent, p-hydroxymercuribenzoate, was similarly required in high concentration to effect a loss of activity over a 60 minute interval.

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LIST OF ABBREVIATIONS

GDH - glutamate dehydrogenase

ADH - alanine dehydrogenase

LDH - lactate dehydrogenase

MDH - malate dehydrogenase

PK - pyruvate kinase

$NAD^+$ - nicotinamide adenine dinucleotide

NADH - nicotinamide adenine dinucleotide, reduced

$NADP^+$ - nicotinamide adenine dinucleotide phosphate

NADPH - nicotinamide adenine dinucleotide phosphate, reduced

AMP - adenosine 5'-monophosphate

ADP - adenosine 5'-diphosphate

ATP - adenosine 5'-triphosphate

cAMP - adenosine 3',5'-cyclic monophosphate

GMP - guanosine 5'-monophosphate

GDP - guanosine 5'-diphosphate

GTP - guanosine 5'-triphosphate

$\alpha$-KG - $\alpha$-ketoglutarate

PEP - phosphoenol pyruvate

OAA - oxaloacetic acid

SDS - sodium dodecyl sulphate

pHMB - p-hydroxymercuribenzoate

Tris - tris (hydroxymethyl) aminomethane

EDTA - ethylenediaminetetraacetate

DTT - dithiothreitol, Cleland's reagent

DEAE - diethyl amino ethyl

TCA - tricarboxylic acid

$K_m$ - the Michaelis constant

# CHAPTER I

## INTRODUCTION

Glutamate dehydrogenase (GDH) catalyzes the reversible amination of α-ketoglutarate to L-glutamate:

$$\text{L-glutamate} + \text{NAD(P)}^+ \rightleftharpoons \alpha\text{-ketoglutarate} + NH_4^+ + \text{NAD(P)H} + H^+$$

Classification of GDH is based on its coenzyme requirement; three types of enzyme are known — those specific for $NAD^+$, those specific for $NADP^+$, and those which utilize both coenzymes. The Enzyme Commission of the I.U.B. has classified these as follows (1961):

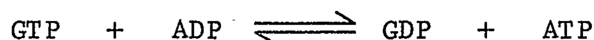
EC 1.4.1.2 L-glutamate:$NAD^+$ oxidoreductase (deaminating)

EC 1.4.1.3 L-glutamate:$NAD(P)^+$ oxidoreductase (deaminating)

EC 1.4.1.4 L-glutamate:$NADP^+$ oxidoreductase (deaminating)

Glutamate dehydrogenase plays a central role in metabolism. It is associated with the interconversion of α-ketoglutarate and glutamate, and consequently with all of the pathways in which α-ketoglutarate, or glutamate, or ammonium ion participate either directly or indirectly. In view of its metabolic position, therefore, one might expect this enzyme to be the target of a variety of control mechanisms regulating its behaviour. Three possible cellular regulatory signals which could exert an appreciable influence on the activity of glutamate dehydrogenase are described below.

A. Intra-cellular levels of α-ketoglutarate, generated by the operation of the TCA cycle, might be controlled by modulating the activity of GDH in the following manner — in the overall pathway from α-ketoglutarate to succinate, GDP is required for the conversion of succinyl-CoA to succinate, and GTP is produced. Furthermore, there is a nucleoside diphosphokinase in the cell which catalyzes the reaction:



$$GTP + ADP \rightleftharpoons GDP + ATP$$

The activity of these two enzymes is important in maintaining the balance between their respective products — GTP and ATP. These two compounds could provide a mechanism for controlling the rate of conversion of α-ketoglutarate to glutamate by interaction with glutamate dehydrogenase. Simultaneous regulation of the level of a crucial TCA cycle intermediate is, of course, a beneficial byproduct of this type of regulatory circuitry.

B. Glutamate serves as a precursor of glutamine, which, in turn, functions as an amide group donor to a variety of biosynthetic sequences. Glutamine synthetase has been demonstrated to be regulated by the end products of all such pathways, including the nucleotides originating from purine biosynthesis. However, if one considered α-ketoglutarate to be the starting point of this particular sequence of events culminating in nucleotide synthesis, it is logical to expect that the control mechanisms applicable to glutamine synthetase might concurrently exert a regulatory influence over glutamate dehydrogenase.

Feedback inhibition by adenine and guanine nucleotides could, therefore, serve to modulate the activity of GDH.

C. Since glutamate dehydrogenase is a major link between carbohydrate metabolism and amino acid metabolism, its activity could be altered by critical cellular metabolites to favour protein synthesis or energy production. In the living cell, $NAD^+$ is predominantly involved in catabolic reactions, while $NADP^+$ functions predominantly in biosynthesis. In considering the microbial sources of the enzyme, therefore, it is apparent that the $NAD^+$-dependent enzyme is responsible for the degradation of glutamate, and the $NADP^+$-dependent enzyme is involved in the synthesis of glutamate. By comparison, the mammalian enzyme reacts almost equally well with $NAD^+$ or $NADP^+$ as coenzyme. The bovine liver GDH has an NADH binding site distinct from the active site, which strongly affects enzyme activity, especially in the presence of purine nucleotides. Therefore, control could also be exerted by fluctuations in the quantity of reduced coenzyme available.

The following is a survey of the glutamate dehydrogenases with special emphasis on the enzyme from bacterial sources, in an attempt to explore their comparative regulatory mechanisms in terms of the preceding analysis.

Glutamate dehydrogenases non-specific with respect to coenzyme have been studied in various animal tissues, including rat liver (Sedgwick and Frieden, 1968), bovine liver (Frieden, 1959, 1965, Frieden and Colman, 1967), amphibian liver (Balinsky _et al_., 1970), and others. It has been demonstrated that cellular levels of GDH vary widely in mammalian tissues. Thus, liver, kidney, and brain contain

considerable amounts of the enzyme, while the heart has about 10 per cent of that amount, and skeletal muscle contains extremely low levels. Such observations tend to support the hypothesis that GDH is one of the key regulatory enzymes in these tissues.

In micro-organisms, glutamate dehydrogenase usually shows specificity toward either $NAD^+$ or $NADP^+$, that is, it requires as a substrate either one or the other cofactor. However, two distinct enzymes, one $NAD^+$-specific and the other $NADP^+$-specific, have been reported for the fungi Neurospora crassa (Sanwal and Lata, 1962), Schizophyllum commune (Dennen and Niederpruem, 1967), baker's yeast (Doherty, 1962), as well as the bacterial species Thiobacillus novellus (LéJohn and McCrea, 1968), and Hydrogenomonas H 16 (Krämer, 1970). Bacteria which possess only one type of GDH include Bacillus licheniformis and Bacillus cereus (Phibbs and Bernlohr, 1971), Nitrosomonas europaea (Hooper et al., 1967), Brevibacterium flavum (Shiio and Ozaki, 1970), Escherichia coli (Varrichio, 1969), Micrococcus aerogenes (Kew and Woolfolk, 1970, Johnson and Westlake, 1970), Clostridium SB 4 (Winnacker and Barker, 1970), and Rhodospirillum rubrum (Bachofen and Neeracher, 1968). A brief summary of the available information on the properties and regulation of the bacterial glutamate dehydrogenases is presented on the following pages.

In a survey of the distribution of L-alanine dehydrogenase and L-glutamate dehydrogenase activities in the genus Bacillus, Hong et al. (1959) reported that cell suspensions of wild type B. subtilis readily synthesized alanine from pyruvate and ammonia, but most of these organisms failed to form glutamate by the reductive amination of

α-ketoglutarate. Thus, B. subtilis, B. mycoides, B. mesentericus vulg., B. megatherium, and B. brevis showed an absence of detectible levels of glutamate dehydrogenase while the specific activities of alanine dehydrogenase were significantly high. Two notable exceptions were B. anthracoides which displayed twice the specific activity of GDH as compared to ADH, and an $am^+$ mutant of B. subtilis ($am^-$ indicating the inability of assimilating $NH_4^+$), in which no ADH was observed, but GDH was readily detectible. This work clearly demonstrated that the inability of the Bacilli to assimilate ammonia was not attributable to the absence of GDH, but that some other undefined metabolic defect was responsible.

More recently, Phibbs and Bernlohr (1971) have found high specific activities of $NADP^+$-specific GDH in cell free extracts of B. licheniformis and B. cereus. When ammonium ion served as the sole nitrogen source, maximum specific activities were observed in extracts prepared from cells harvested during the late logarithmic phase. Conversely, extremely low specific activities were encountered throughout the growth cycle when L-glutamate or Casamino acids were provided as the carbon and the nitrogen source. They determined the kinetic constants on a partially purified GDH preparation, but failed to observe sigmoidal saturation kinetics. Furthermore, the adenylates tested, AMP, ADP, ATP, cAMP, individually or in various combinations, did not appear to exert any influence on the kinetic parameters.

The kinetic constants determined in their experiments suggested a biosynthetic role for GDH, since the $K_m$ for glutamate was 5.8 - fold higher than that for α-ketoglutarate. As in the case of other

glutamate dehydrogenases to be discussed later, the _Bacillus_ enzyme did not appear to be a regulatory protein. In studying the control of enzyme synthesis, repression was evident during growth on L-glutamate or on Casamino acids; derepression could be achieved by addition of glucose (5 mM) or pyruvate (30 mM). Thus, synthesis of the GDH of _B. licheniformis_ was regulated by repression by the anabolic product, glutamate. Derepression of synthesis would ultimately follow as a consequence of a diminished intracellular glutamate level.

The partial purification of GDH from _Nitrosomonas europaea_, an ammonia-oxidizing chemoautotroph, has been reported by Hooper, Hansen, and Bell (1967). In this organism, ammonia is a possible participant in the sequence of reactions leading to the end product, hydroxylamine, thereby serving as a nitrogen source and an energy source. Using a 160 - fold purified enzyme, absolute specificity towards the substrates, α-ketoglutarate and glutamate, and the cofactor, NADPH, was demonstrated. Kinetic parameters were determined in the forward and reverse reactions, and only the $K_m$ of ammonium ion was found to be of an order of magnitude higher than the values reported for other organisms.

Based on observations of unusual inhibition by the nicotinamide adenine nucleotides, the authors postulated the presence of two binding sites on the _Nitrosomonas_ GDH:

(i) a catalytic site at which $NADP^+$ and NADPH bind; and

(ii) a regulatory site at which NADPH and NADH bind with approximately equal affinity and inhibit $NADP^+$ reduction and NADPH oxidation.

Hooper et al. arrived at the conclusion that in chemoautotrophs, glutamate served primarily as a substrate in the synthesis of proteins or other nitrogen compounds, and as an amine group donor, rather than as a source for the generation of α-ketoglutarate and reduced pyridine nucleotides. As the reverse reaction was demonstrated to be inhibited by NADPH and by $NH_4^+$, it is probable that in these organisms, glutamate formation is essentially unidirectional.

Regulation of the $NADP^+$-specific GDH from the glutamate producing bacterium, Brevibacterium flavum, was recently studied by Shiio and Ozaki (1970). With a 65 - fold purified enzyme, strict specificity for NADPH was observed, and kinetic data revealed homotropic interactions with respect to both α-ketoglutarate and L-glutamate. In the presence of KCl, however, homotropic interactions were completely lost. The enzyme was inhibited by its products, both in the forward and reverse direction. Tricarboxylic acid cycle intermediates and amino acids, on the other hand, showed practically no inhibition. Of the nucleotides tested, GMP inhibited the reverse reaction strongly, but none of the others affected the reaction in either direction. Control of activity by the above-mentioned product inhibition was shown to be the predominant regulatory mechanism of glutamate synthesis and degradation in this organism.

In Escherichia coli, the synthesis of $NADP^+$-specific GDH was found to be regulated by the glutamate level (Varrichio, 1969). In this study, glutamate dehydrogenase levels were compared after growth on (i) glycerol plus various nitrogen sources, and (ii) glycerol, ammonium ion, and a variety of amino acids. Low enzyme activities,

observed in cells grown on arginine or ornithine as sole nitrogen source, were attributable to the presence of glutamate originating from the catabolism of these amino acids. Glutamate is also a product of proline catabolism, but, surprisingly enough, growth on media containing glycerol and proline did not result in decreased levels of GDH. For *E. coli* grown on a medium containing glutamate, GDH activity was lower in the presence of glycerol alone, than it was in the presence of glycerol and ammonium ion. It is reasonable to conclude, therefore, that the cellular level of glutamate was also influenced by the availability of carbon and nitrogen. Repression of $NADP^+$-specific GDH under growth conditions leading to elevated glutamate levels supported the suggestion that in *E. coli*, the chief function of this enzyme is the biosynthesis of glutamate.

The facultative chemoautotrophic bacterium, *Thiobacillus novellus*, was reported by LéJohn and McCrea (1968) to possess two distinct types of glutamate dehydrogenase, one $NAD^+$-specific, and the other $NADP^+$-specific. Both of these enzymes were subject to metabolic regulation. The $NAD^+$-dependent enzyme was particularly interesting because it was shown to be specifically regulated by AMP which acted as an allosteric activator in the breakdown of glutamate, and an inhibitor in the formation of glutamate. In addition, the kinetic parameters were modified by pH shifts of the assay medium, such that the sigmoidicity of the substrate saturation curve was altered to a hyperbolic curve by a change from pH 9.5 to pH 8.0.

In a further communication, LéJohn *et al.* (1968) have presented detailed kinetic analyses on both the species of GDH. The $NAD^+$-specific

enzyme exhibited non-linear kinetics in the oxidative deamination assay, but linear kinetics in the opposite direction. Not only did the addition of AMP to the reaction system cause an alteration from sigmoidal kinetics to the classical Michaelis-Menten kinetics, but there was also a dramatic increase in the $K_m$ values for NADH, $NH_4^+$, and α-ketoglutarate. The Michaelis constants for $NAD^+$ and glutamate, however, remained unaltered in the presence of AMP. By comparison, the $NADP^+$-specific enzyme displayed neither sigmoidal kinetics nor an alteration in the mode of substrate binding upon the addition of AMP.

In the determination of reaction mechanism of the $NADP^+$-specific enzyme, the authors proposed that the order of binding is NADPH, followed by ammonium ion, and lastly α-ketoglutarate. However, with regard to the $NAD^+$-specific enzyme, an ordered binary-ternary mechanism was apparent only in the presence of AMP. In its absence, reasonable evidence was presented showing that the mechanism was allosteric. The possibility of a random mechanism in the absence of AMP was not excluded, but was considered unlikely.

The other bacterium in which two species of glutamate dehydrogenase have been reported is Hydrogenomonas H 16 (Krämer, 1970). Two chromatographically distinct forms of the enzyme which differed in their thermolability were isolated from this organism, one utilizing $NAD^+$ and the other $NADP^+$ as a cofactor.

Low specific activity of $NAD^+$-specific GDH was witnessed in cells grown with glutamate as sole nitrogen source, or in cells grown in a medium containing high levels of ammonium ion. In the presence of low concentrations of ammonium ion or in nitrogen-free medium, specific

activities of the $NAD^+$-specific enzyme increased. Concurrent with the rise in levels of $NAD^+$-GDH was the increased synthesis of glutamine synthetase; ratios of $NAD^+$-dependent GDH to glutamine synthetase activity differed only slightly with different nitrogen and carbon sources. By comparison, the $NADP^+$-dependent enzyme was found in high specific activities in cells grown in the presence of an excess of ammonium ion. This observation suggested that synthesis of $NADP^+$-specific GDH in Hydrogenomonas H 16 was induced by a high concentration of $NH_4^+$ and that it was primarily involved in the synthesis of glutamate. During growth of the cells on glutamate and a low level of ammonium ion, the synthesis of the enzyme ceased after exhaustion of both nitrogen sources.

Kew and Woolfolk (1970) observed unusually high specific activities of GDH in crude extracts of Micrococcus aerogenes. Evidence was presented showing that up to 10 per cent of total cellular protein consisted of this enzyme. It is remarkable that following a procedure which gave a final purification of 20 - fold, a homogeneous protein was obtained. The pure enzyme was demonstrated to utilize either NADH or NADPH in the reductive amination reaction, but they were able to demonstrate activity in the reverse direction only with $NAD^+$. Such a result could have been due to a requirement for activation of the enzyme not satisfied by the conditions of the $NADP^+$ assay. However, inclusion of $\alpha$-ketoglutarate, NADPH, and $NH_4^+$ in the assay system failed to activate the enzyme in the reverse direction. The NADH- and NADPH-linked activities displayed different pH optima, 6.5 and 9.5, respectively. Both were demonstrated to be inhibited by $NAD^+$, but not

by $NADP^+$. The authors concluded that the NADH and NADPH activities were exhibited by the same protein, since (i) the two activities were demonstrated to copurify following several purification schemes; (ii) identical thermal inactivation kinetics were observed; (iii) the two activities were not additive in the presence of saturating levels of NADH and NADPH; and (iv) the two activities cosedimented on sucrose gradients and coeluted from polyacrylamide gels during preparative electrophoresis.

In a brief note on the study of glutamic acid metabolism in *Peptococcus aerogenes*, Johnson and Westlake (1970) have shown that the conversion of glutamate to α-hydroxyglutarate proceeded via the intermediate, α-ketoglutarate. Two $NAD^+$-dependent enzymes which appeared to function in sequence were separated from cell extracts — the first was glutamate dehydrogenase, which catalyzed the reaction of glutamate to α-ketoglutarate, and the second enzyme reduced α-ketoglutarate to α-hydroxyglutarate. The two enzymes were partially purifed and were shown to be distinct proteins having different physical properties.

The isolation of an $NAD^+$-dependent GDH from *Clostridium* SB 4 has been reported by Winnacker and Barker (1970). The 250 - fold purified enzyme was found to be homogeneous by means of gel electrophoresis and ultracentrifugation. Although isolated from a strictly anaerobic organism, the enzyme was not sensitive to oxygen, and was stable to storage under various conditions, even in relatively high dilutions. Unlike the glutamate dehydrogenases isolated from other sources, the *Clostridium* enzyme did not appear to be a regulatory

protein. The presence of purine nucleotides AMP, ADP, or ATP did not influence the initial velocity patterns, the substrate inhibition by α-ketoglutarate and NADH, or the specificity characteristics.

Glutamate dehydrogenase from Rhodospirillum rubrum (Bachofen and Neeracher, 1967) showed a complete specificity for NADH as the hydrogen donor in the amination assay. In the direction of glutamate synthesis, however, low activities were detected using $NADP^+$ as a cofactor with the purified enzyme. Of the nucleotides tested, ADP, ATP, GDP, and GTP at 1.0 mM caused no significant alteration in initial velocity of the forward reaction. By comparison, when ATP was included at a concentration of 40 mM in the reverse reaction, GDH activity was inhibited to the extent of 87 per cent. Complete inhibition of GDH in both amination and deamination reactions was reported with 0.08 mM $HgCl_2$, but 1.0 mM $ZnCl_2$ inhibited it by 59 per cent only. The nitrogen source employed in the growth medium did not influence the synthesis of glutamate dehydrogenase. When Rhodospirillum rubrum was cultured in malate - $NH_4^+$ solution, crude extracts were shown to have five-fold higher levels of GDH as compared to those prepared from cells grown on glutamate as the carbon and nitrogen source.

The current study was undertaken due to the lack of comprehensive information concerning glutamate dehydrogenase from members of the Enterobacteriaceae. Salmonella typhimurium was selected for an exploration of the properties and regulatory mechanisms of this enzyme.

# CHAPTER II

# MATERIALS AND METHODS

## A. Culture, Media, and Growth Conditions

The strain LT-2 of Salmonella typhimurium, kindly provided by Dr. K.E. Sanderson, Department of Biology, University of Calgary, was used throughout this study; it is a wild type, $F^-$ bacterium. An inoculum was prepared by growing cells overnight at 37°C on a reciprocal water bath shaker (Research Model #6300, Eberbach Corporation) using a minimal salts medium of the following composition: 10.5 g $K_2HPO_4$; 4.5 g $KH_2PO_4$; 1.0 g $(NH_4)_2SO_4$; 0.42 g sodium citrate.$2H_2O$; 50 mg $MgSO_4$ (anhydrous); and distilled water to a final volume of one litre. L-glucose was employed as the sole carbon source at a concentration of 1.0 per cent. Fifty ml of the inoculum was introduced into a 14-litre capacity Modular Microferm Fermentor tank (New Brunswick), containing 10 litres of the minimal salts medium supplemented with 1.0 per cent glucose. The salts medium and the glucose solutions were sterilized separately at 121°C and 15 psi steam pressure; flasks were autoclaved for 15 minutes and the fermentor tanks for 120 minutes. Cells were grown under conditions of vigorous aeration, employing 5 litres of forced sterile air per minute and 200 rpm rotating paddle speed. A constant temperature of $37 \pm 0.5$°C was maintained. After approximately 7 to 10 hours of growth, when the culture had attained a turbidity reading of 600 units on a Klett-Summerson photoelectric colorimeter (blue filter, #42), cells were

chilled by the addition of ice, and harvested using a continuous flow centrifuge (Chemapec, Inc.) at a flow rate of 10 litres per hour and a speed of 30,000 rpm. The cell paste thus collected was scraped from the centrifuge cylinder, transferred to a beaker, and stored at $-20^{\circ}C$ until required.

B. Enzyme Preparation

1. Cell Breakage: Frozen bacterial cells were thawed and homogenized by continuous, manual stirring in a volume of cold extraction buffer 1.5 times the wet weight of the cells. Extraction buffer composition was 0.05 M Tris-HCl, pH 7.5, $10^{-4}$ M EDTA, $5 \times 10^{-4}$ M DTT. Cell breakage was carried out by two passages through a 40 ml capacity manual-fill, French pressure cell (#4-3398A, American Instrument Co.) mounted in a laboratory press, at a pressure not exceeding 20,000 psi. The lysate was collected in 50 ml capacity polycarbonate tubes and centrifuged at 27,000 x g for 15 minutes in a Sorvall RC-2 refrigerated centrifuge. The supernatant was decanted and treated as the crude extract. Unless otherwise stated, all subsequent centrifugations were carried out using the same instrument, speed, time, and temperature ($5^{\circ}C$).

2. Heat Treatment: The crude extract was transferred to a 2800 ml capacity Fernbach flask and subjected to heat treatment in a hot water bath. The extract was brought to $55^{\circ}C$ rapidly and maintained at a constant temperature for five minutes, with continuous swirling. The heat treatment resulted in the formation of a viscous suspension,

to which an additional quantity of cold extraction buffer was added to achieve a volume 2.5 times the original. Following centrifugation of the above mixture, the pellet was discarded and the supernatant fractionated by ammonium sulphate as described in the following section.

3. First Ammonium Sulphate Treatment: To the heat-treated supernatant, solid ammonium sulphate was added over a period of 15 minutes to a saturation of 0.30, under continuous stirring. After stirring the mixture for an additional 30 minutes at $4^{o}C$, the solution was centrifuged. The supernatant was retained and the precipitate discarded. Appropriate quantities of solid ammonium sulphate were then added to raise the saturation from 0.30 to 0.45, and the stirring and centrifugation steps repeated. The precipitate thus obtained was suspended in a small volume of 0.20 M Tris-HCl, pH 7.5, $10^{-4}$ M EDTA, $10^{-3}$ M DTT, henceforward referred to as the storage buffer.

4. Ion Exchange Chromatography: Enzyme preparations at this point were usually divided into two fractions, and treated separately for the following purification steps. The proteins were fractionated on a column (5 cm x 90 cm) of DEAE-Sephadex A-25, which had previously been equilibrated in a cold room against 10 litres of the extraction buffer. The enzyme was eluted with a four litre gradient of 0 to 0.5 M NaCl, prepared in the extraction buffer, at a flow rate of approximately 1.5 ml per minute. Ten ml fractions were collected using a Buchler fraction collector. Each of the fractions was scanned for absorbance at 280 nm in a Beckman-DU Spectrophotometer to locate

the protein peaks; individual fractions were also analyzed for GDH activity as outlined in the section on Enzyme Assays. High activity fractions containing GDH were pooled and the enzyme precipitated by addition of solid ammonium sulphate to a saturation of 0.60. The precipitate thus obtained was suspended in a small volume of storage buffer and frozen at -20°C.

5. Reverse Ammonium Sulphate Fractionation: The enzyme preparation from the preceding step was thawed and the volume increased to 10.0 ml by the addition of storage buffer. Precipitation of the protein material was carried out using solid ammonium sulphate to a saturation of 0.60. The pellet obtained, following centrifugation, was successively resuspended in 10.0 ml volumes of a given saturation of ammonium sulphate prepared in the storage buffer, stirred at 4°C for 30 minutes, and centrifuged. The supernatant was retained, and the precipitate again subjected to the same treatment. Saturations of ammonium sulphate solutions employed in this procedure were, respectively, 0.35, 0.30, 0.25, and 0.20. All supernatants were then analyzed for GDH, and those containing the highest activities were separately concentrated by precipitation with ammonium sulphate to a saturation of 0.50; the precipitate was dissolved in a small volume of storage buffer. All of the activity was usually recovered in two supernatants.

6. Gel Filtration on Sephadex G-200: Each of the enzyme suspensions from the above step was independently subjected to gel filtration through a column (2.5 cm x 95 cm) of Sephadex G-200,

equilibrated at 4°C with the extraction buffer. Elution was carried out at the rate of 10 to 12 ml per hour. Five ml fractions were collected and assayed for GDH activity. Only a minimum volume of the eluate, on either side of the enzyme activity profile, was pooled at this stage and precipitated by the addition of solid ammonium sulphate to a saturation of 0.60. The precipitate was suspended in less than 1.0 ml of the storage buffer.

C. Enzyme and other Assays

Assay procedures used for the determination of reaction rates of Salmonella glutamate dehydrogenase and of a variety of commercial enzymes, employed as molecular weight and sedimentation coefficient standards, are presented in the following.

1. Glutamate dehydrogenase: GDH activity was routinely determined by measuring the decrease in absorbance of NADPH at 340 nm (Schmidt, 1965). The assay mixture contained the following in a total volume of 3.0 ml: 200 μmoles Tris-HCl, pH 8.6; 0.36 μmoles NADPH; 50 μmoles $NH_4Cl$; 30 μmoles α-ketoglutarate, pH 7.45; 0.1 ml of enzyme preparation. The reaction rate was followed in 4.0 ml glass cuvettes of 1.0 cm light path by means of a Beckman-DU Spectrophotometer equipped with a Model 2000 Gilford multiple sample absorbance recorder.

A unit of activity is defined as the amount of enzyme which catalyzed the oxidation of 1 μmole of NADPH per minute, at 25°C, under the above mentioned assay conditions. Specific activities are given in units per mg protein.

2. Lactate dehydrogenase (L-lactate : NAD oxidoreductase, EC 1.1.1.27): LDH from beef heart was purchased as a crystalline suspension in ammonium sulphate. Prior to use, the enzyme was dialyzed against a 1000 volumes of the extraction buffer, and then diluted with the same buffer to a protein concentration of 25 µg/ml. LDH was assayed in a Beckman-DU Spectrophotometer by following the decrease in absorbance at 340 nm for one minute, in a reaction system containing 100 µmoles Tris-HCl, pH 8.0; 5.0 µmoles pyruvate; 0.12 µmoles NADH; and 0.1 ml enzyme in a total volume of 1.25 ml (Bergmeyer _et al._, 1965).

3. Catalase ($H_2O_2$ : $H_2O_2$ oxidoreductase, EC 1.11.1.6): Beef liver catalase was obtained as an aqueous ammonium sulphate suspension. The enzyme was dialyzed against the extraction buffer in a manner similar to that described for LDH and left undiluted at the original concentration. The assay for catalase was essentially identical to that recommended by Lück (1965). It consisted of two reagent solutions:

(a) 3.522 g $KH_2PO_4$ and 7.265 g $Na_2HPO_4 \cdot 2H_2O$, made up to one litre, giving a final phosphate concentration of M/15, pH 7.0; and

(b) 0.16 ml of a commercial, 30 per cent (w/v) $H_2O_2$ solution diluted to 100 ml with reagent (a), giving an optical density reading of approximately 0.500 at 240 nm.

The reaction was followed in 1.0 ml light path silica cuvettes by observing the decrease in absorbance at 240 nm of a system containing

2.9 ml of $H_2O_2$ - phosphate buffer plus 0.1 ml enzyme.

4. Malate dehydrogenase (L-malate : NAD oxidoreductase, EC 1.1.1.37): MDH from pig heart was dialyzed as before, and diluted appropriately prior to use. Assay for the enzyme utilized a reaction system of the following composition: 50 μmoles $K_2HPO_4$, pH 7.5; 0.12 μmoles NADH; 5.0 μmoles oxaloacetate, pH 7.0. Decrease in absorbance at 340 nm accompanying the oxidation of NADH was recorded spectrophotometrically over a one minute interval (Bergmeyer and Bernt, 1965).

5. Pyruvate kinase (ATP : pyruvate phosphotransferase, EC 2.7.1.40): PK from rabbit skeletal muscle was also obtained as a crystalline suspension in ammonium sulphate. It was assayed by means of a coupled reaction in the presence of LDH. The assay system contained 100 μmoles $K_2HPO_4$ - HCl buffer, pH 7.5; 0.03 μmoles PEP; 5.0 μmoles ADP; 25 μg of a dialyzed suspension of LDH; 0.12 μmoles NADH; and 0.1 ml enzyme, in a total volume of 3.0 ml. The activity was determined spectrophotometrically at 340 nm.

6. Urease (urea aminohydrolase, EC 3.5.1.5): Jack bean urease was purchased in a crystalline form. A stock solution of 10 mg/ml was prepared in the extraction buffer, and assayed, subsequent to elution, by determination of absorbance at 280 nm in a Beckman-DU Spectrophotometer.

7. Aldolase (fructose-1,6-diphosphate : D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.b): Aldolase from rabbit muscle was

purchased as a crystalline suspension in ammonium sulphate. For use as a molecular weight standard in Sephadex G-200 gel filtration, the position of the aldolase protein was determined by scanning the effluent at 230 nm.

8. Fumarase (L-malate hydrolyase, EC 4.2.1.2): Pig heart fumarase was obtained as a crystalline suspension, and to determine the activity of the enzyme, 2.9 ml of 0.05 M L-malic acid in 0.10 M $KH_2PO_4$, pH 7.4, was added to 1.0 cm light path silica cuvettes and the reaction started by the addition of the enzyme sample. Increase in absorbance at 240 nm was recorded spectrophotometrically.

9. Ovalbumin: A stock solution of 10 mg/ml was prepared from a lyophilized crystalline preparation, in the extraction buffer, and a 10 mg aliquot used for calibration of the G-200 column. The protein was identified by observing its absorbance at 230 nm in silica cuvettes of 1.0 cm light path.

## D. Protein Determination

Protein estimation of all samples was carried out according to the method of Lowry *et al*. (1951) with crystalline bovine serum albumin as a reference.

## E. Electrophoresis

The enzyme preparation was subjected to electrophoresis after different purification and fractionation steps, employing 7.5 per cent

polyacrylamide gels in a discontinuous system, according to the method of Davis (1964). Enzyme solutions containing approximately 100 μg protein in a volume of 50 μl were electrophoresed for 120 minutes using Tris-glycine buffer, pH 8.3, and a current of 3 mA per tube. Bromophenol blue was employed as the tracking dye. At the end of the electrophoresis, the gels were stained by using a non-specific protein stain of 1 per cent Amidoschwartz (Buffalo Black) prepared in 7 per cent acetic acid, for one hour, followed by electrophoretic destaining in 7 per cent acetic acid.

F. Sucrose Density Gradient Centrifugation

1. Preparation of gradients: Linear sucrose gradients were prepared according to the method of Martin and Ames (1961). Prior to each run, solutions of 5 per cent (w/v) and 20 per cent (w/v) sucrose (ultra pure) were made in 0.20 M Tris-HCl, pH 7.5, $10^{-4}$ M EDTA, $10^{-3}$ M DTT. A linear gradient was generated using a Buchler Gradient Mixer. Immediately following preparation, the tubes were transferred to a cold room and allowed to equilibrate for a minimum of 3 hours.

An appropriate dilution and volume of both the unknown sample, GDH, and one of the standards was made such that the final volume did not exceed 150 μl. Each was layered onto the gradient with a minimum of disturbance of the meniscus, and centrifuged at $4^{o}$C in a swinging bucket rotor (SB 405) in an International B-60 Ultracentrifuge, at 36,000 rpm, for 16 hours. A piercing apparatus (Buchler) was employed to punch a small hole in the bottom of the Lusteroid tubes, and 10-drop fractions were collected. Analysis of enzyme activity was

carried out immediately after sampling.

2. Standards: Three commercial standards of known sedimentation coefficient were used in order to obtain an estimate of the sedimentation coefficient for GDH. The quantities of each of these standards employed per gradient were as follows: beef heart lactate dehydrogenase (1.25 μg), beef liver catalase (12 to 24 μg), and pig heart malate dehydrogenase (12 to 30 μg).

G. Estimation of Molecular Weight and Molecular Size by Gel Filtration

Gel filtration techniques, as discussed by Andrews (1970), were employed to obtain an estimate of the molecular weight and size of GDH.

1. Preparation of the column: Sephadex G-200 was allowed to swell in 0.05 M Tris-HCl, pH 7.5, by boiling a 1:1 slurry with continuous stirring for a minimum of 5 hours. After cooling the mixture, the gel was introduced into a column (95 cm x 2.5 cm) and allowed to settle under a pressure head of not more than 15 cm. A final flow rate of between 10 to 14 ml per hour resulted. Five ml of a solution containing 50 mg bovine serum albumin was passed through the column in order to saturate any possible protein-binding sites prior to the calibration run.

2. Determination of the void volume: The upper exclusion limit of the gel was determined by the passage of 0.5 ml of a

suspension containing 2.0 mg of Dextran Blue 2000, of average molecular weight 2.0 x $10^6$ and specific extinction coefficient at 625 nm ($E^{1\%}_{1\ cm}$) of 2.0. As Dextran Blue has been observed to complex with a number of enzymes including pyruvate kinase and lactate dehydrogenase (Andrews, 1970), it was not included in the mixture of standards used for calibration of the column.

3. Standards: Enzymes of known molecular weight were selected as standards in order to establish an approximately linear calibration curve, encompassing the molecular weight range, appropriate for an accurate estimation of GDH molecular weight. The respective quantities of the standards were as follows: jack bean urease (4.0 mg), rabbit muscle pyruvate kinase (0.1 mg), pig heart fumarase (1.0 mg), rabbit muscle aldolase (5.0 mg), beef heart lactate dehydrogenase (2.0 mg), pig heart malate dehydrogenase (1.0 mg), and ovalbumin (10.0 mg). Assays for the standards were conducted as reported in a previous section.

## H. Reagents

The following reagents were purchased from Sigma Chemical Co.: α-ketoglutarate, L-glutamate, NADPH, NADH, $NADP^+$, pyruvate, PEP, OAA, L-malic acid, AMP, ADP, ATP, cAMP, GMP, GDP, GTP, acetyl CoA, L-glutamine, Trizma base, EDTA, DTT, urea, and pHMB. Sodium dodecyl sulphate from Matheson Coleman and Bell was used. All standard enzymes for calibration purposes were obtained from Sigma, with specifications as presented in Table I. Sephadex G-200, DEAE-Sephadex, and Dextran

Blue 2000 were products of Pharmacia Fine Chemicals. Enzyme grade ammonium sulphate, ultra pure sucrose, and ultra pure guanidine hydrochloride were purchased from Mann Research Laboratories. The remainder of the chemicals were obtained from standard commercial sources and were used without further purification.

Table I. Standard Enzymes for Molecular Weight Calibration and Sedimentation Coefficient Estimation.

| Enzyme | Source | Lot # | Protein concentration | Activity |
|---|---|---|---|---|
| LDH | beef heart | 101C-9560 | 9.4 mg/ml | 500 units/mg |
| catalase | beef liver | 49B-8020 | 20 mg/ml | 35,000 Sigma units/mg |
| MDH | pig heart | 98B-6370 | 3.0 mg/ml | 160 units/mg |
| urease | jack bean | 109B-5020 | - | 1830 units/g |
| PK | rabbit muscle | 31C-9510 | 9.7 mg/ml | 381 units/mg |
| fumarase | pig heart | 69B-1310 | 10.0 mg/ml | 420 units/mg |
| aldolase | rabbit muscle | 38B-1990 | 10.0 mg/ml | 8.6 units/mg |
| ovalbumin | | 62B-6190 | - | - |

# CHAPTER III

# RESULTS

## A. Growth Curve and Enzyme Induction

The use of the Microfermentor proved advantageous in the production of large batches of cells as starting material for enzyme purification. A typical growth curve for S. typhimurium (Fig. 1) in minimal salts medium containing 1.0 per cent glucose indicated the presence of a highly saturated culture after about 8 hours of growth. Doubling time during logarithmic phase was estimated at 42 minutes. A value of 0.99 was calculated for the specific growth constant by employing the expression

$$k = \frac{\log_e 2}{G.\ T.} \quad ;$$

where k is the rate constant at which a bacterial culture multiplies by a factor of 2, and

G. T. is the generation time during logarithmic growth.

Specific activity of crude extracts prepared from 50 ml samples at 30 minute intervals showed a steady increase in the level of GDH throughout the logarithmic phase, attaining a maximum value of 3.4 after about three hours into the stationary phase of growth. Following ten hours of growth, specific activities of the enzyme were observed to decline, and after eleven hours, the value was 2.5.

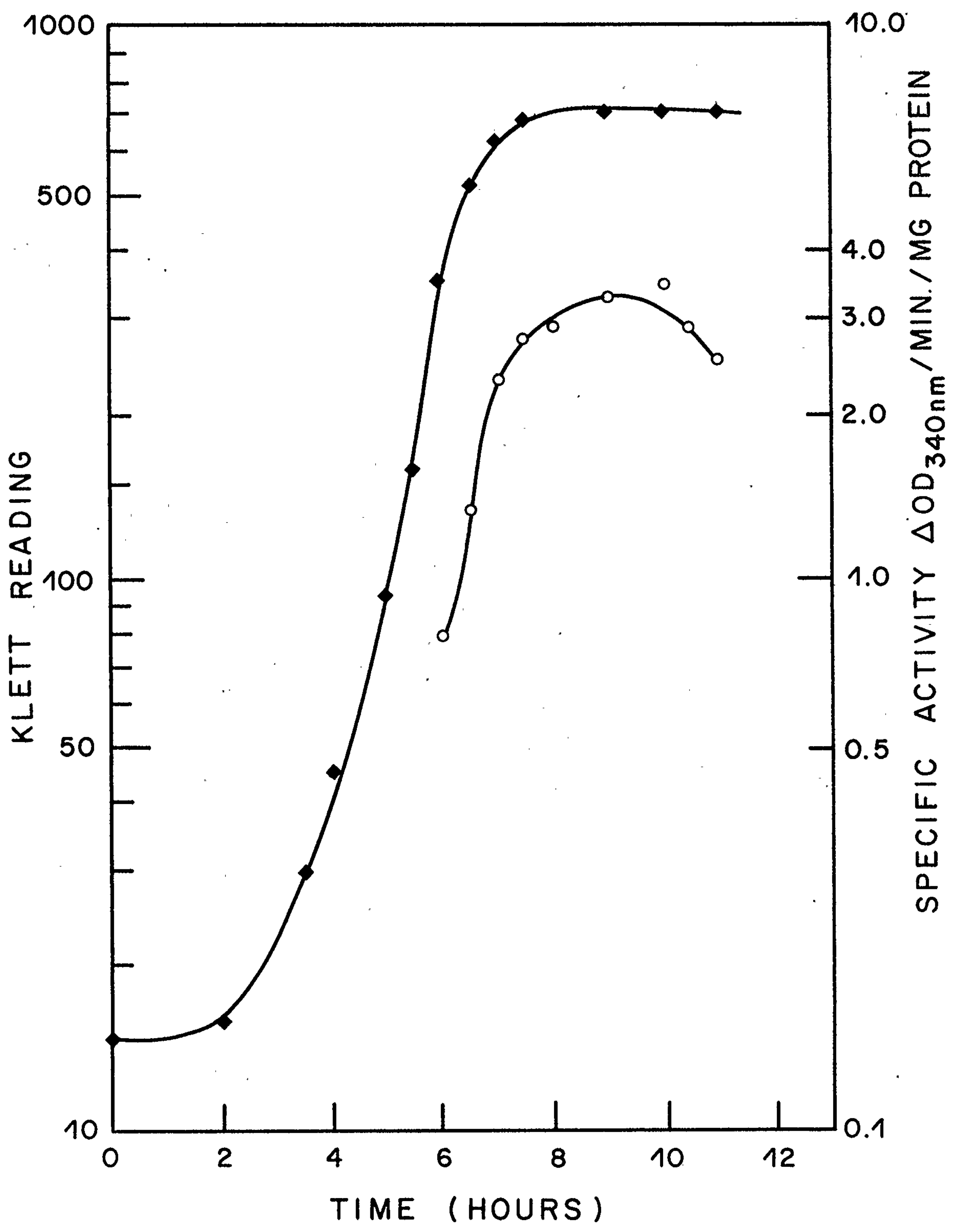
Harvesting of the cells was routinely carried out at late logarithmic phase when the culture had reached a Klett reading of 600.

The titre of cells at this saturation was determined to be $2.0 \times 10^9$ per ml, and the wet weight of Salmonella obtained from the combined contents of two ten litre fermentor tanks amounted to 110 to 120 g.

Figure 1. Profile of GDH specific activity during the growth of _S. typhimurium_ on minimal salts medium, supplemented by 1 per cent glucose. Crude cell free extracts were prepared from samples taken at 30 minute intervals; each value represents an average from duplicate determinations.

◆ Klett reading, #42 filter

o specific activity of GDH



1000
500
100
50
10
KLETT READING
10.0
4.0
3.0
2.0
1.0
0.5
0.1
SPECIFIC ACTIVITY ΔOD340nm/MIN./MG PROTEIN
0
2
4
6
8
10
12
TIME (HOURS)

B. Enzyme Purification

The results of purification of the enzyme using the scheme described in the Methods are presented in Table II. The specific activity of the crude extract employed was 1.79; however, depending on the individual preparation, the value for the specific activity of crude extracts was found to vary from 1.5 to 2.0. By use of the heat treatment step, a loss of approximately 80 per cent of the extraneous protein was witnessed, with essentially no loss of enzyme activity. Ammonium sulphate fractionation from 0.30 to 0.45 saturation resulted in a purification, following this step, of 11.2 - fold over the crude extracts, accompanied by yields averaging greater than 90 per cent of the initial activity.

Ion exchange chromatography led to another increase in purification of five-fold. The enzyme was eluted as a single, symmetrical peak at approximately 0.25 M NaCl. A considerable loss of GDH activity occurred invariably during ion exchange on DEAE-Sephadex. Numerous attempts were made to improve the efficiency of this particular step, including various modifications of the elution gradients, of buffer composition, and of the amounts of protein added to the column. However, none of these resulted in yields significantly greater than 55 per cent. In order to avoid overloading the column, the protein material from the first ammonium sulphate fractionation was divided into two equal fractions, and each of these treated separately on DEAE-Sephadex. The advantage of this procedure was an increase in the extent of purification obtained: an additional five-

Table II. Results from Enzyme Purification

| Fraction | Activity | Volume | Total Activity | Protein | Total Protein | Specific Activity | Fold Purification | Yield |
|---|---|---|---|---|---|---|---|---|
| | $\Delta OD_{340\ nm}$ /min/0.1 ml | ml | $\Delta OD_{340\ nm}$ /min* | mg/ml | mg | $\Delta OD_{340\ nm}$ /min/mg* | | per cent |
| Crude extract | 6.08 | 250 | 15,200 | 34.5 | 8,600 | 1.79 | - | 100 |
| heat treated supernatant (55°C) | 4.12 | 375 | 15,100 | 4.9 | 1,840 | 8.40 | 4.7 x | 99 |
| first ammonium sulphate ppt. | 72.0 | 21.0 | 15,100 | 36.0 | 756 | 20.0 | 11.2 x | 99 |
| DEAE chromatog ON | 72.0 | 10.5 | 7,550 | 36.0 | 378 | 20.0 | 11.2 x | 99 |
| OFF | 143.2 | 2.90 | 4,150 | 15.0 | 43.5 | 95.5 | 53.2 x | 55 |
| reverse ammonium sulphate (a) | 196.0 | 1.20 | 2,350 | 18.6 | 22.4 | 105.4 | 58.9 x | 31 |
| (b) | 108.8 | 1.20 | 1,300 | 10.8 | 13.0 | 100.7 | 56.3 x | 17 |
| G-200 chromatog (a) | 97.6 | 0.75 | 740 | 2.88 | 2.16 | 340 | 190 x | 10 |
| (b) | 15.2 | 0.50 | 71 | 0.72 | 0.36 | 214 | 120 x | 1 |

* 1 mμmole NADPH oxidized per minute is equivalent to a $\Delta OD$ of 0.00622 per minute.

fold increase over the ammonium sulphate step was achieved, with specific activities of the order of 90 to 100. Since GDH from other bacteria had been reported to yield two chromatographically different forms of the enzyme (Shiio and Ozaki, 1970), fractions from the DEAE-Sephadex column were carefully scanned for the possible presence of low levels of activity eluting at a salt concentration different from the main peak. However, even when all the fractions following the void volume were assayed, no additional enzymatically active species were detected.

After ion exchange chromatography on DEAE-Sephadex, reverse ammonium sulphate fractionation was introduced in order to achieve a further purification of the enzyme. The precipitate obtained from the pooled eluate from the DEAE-Sephadex step was treated successively with a series of solutions of progressively lower saturations of ammonium sulphate. After each treatment, the supernatant was analyzed for GDH. As anticipated, all of the GDH activity could not be extracted in a single solution spanning a range of 0.05 saturation. The enzyme activity usually appeared distributed in two successive ammonium sulphate fractions. Since such precipitations are generally dependent upon protein concentration, it was difficult to predict with certainty the saturations in which the enzyme would appear. Table II therefore shows two sets of data, (a) and (b), corresponding to the two above-mentioned GDH fractions. As a consequence of reverse ammonium sulphate treatment, specific activities greater than 100 were usually attained, with a purification of 55 - to 60 - fold over the crude extracts.

Sephadex G-200 gel filtration proved reasonably effective in removing proteins with molecular weights outside the range of GDH — 280,000 daltons — and led to an additional two - to four-fold increase in specific activity over the preceeding step. A large protein peak was eluted with the void volume, followed by glutamate dehydrogenase, which was also separated from the lighter protein material during this step (Fig. 3). Although the elution profile obtained was consistently symmetrical, usually an arbitrary decision was made with regard to the number of fractions that were finally pooled. Thus, conservation of the maximum quantity of enzyme often resulted in a lower extent of purification compared to that when a narrower range of GDH fractions was selected for the final ammonium sulphate precipitation. The final preparation showed a purification of 120 - to 190 - fold over the crude extracts with a total yield of from one to ten per cent. The maximum specific activity of the final prepration was 340.

The calculations for specific activity, fold purification, and yield were based on specific activity of the crude extract. Since it was necessary to divide the precipitate obtained from the first ammonium sulphate treatment into two equal fractions, corrections were made in the subsequent calculations accordingly.

Figure 2. Elution profile from DEAE-Sephadex column. Ten ml fractions were collected as described in the Methods. GDH appeared at approximately 0.25 M NaCl when eluted with a linear gradient from 0 to 0.50 M NaCl.

● GDH activity, $\Delta OD_{340\ nm}$/min/0.1 ml

○ $A_{280\ nm}$

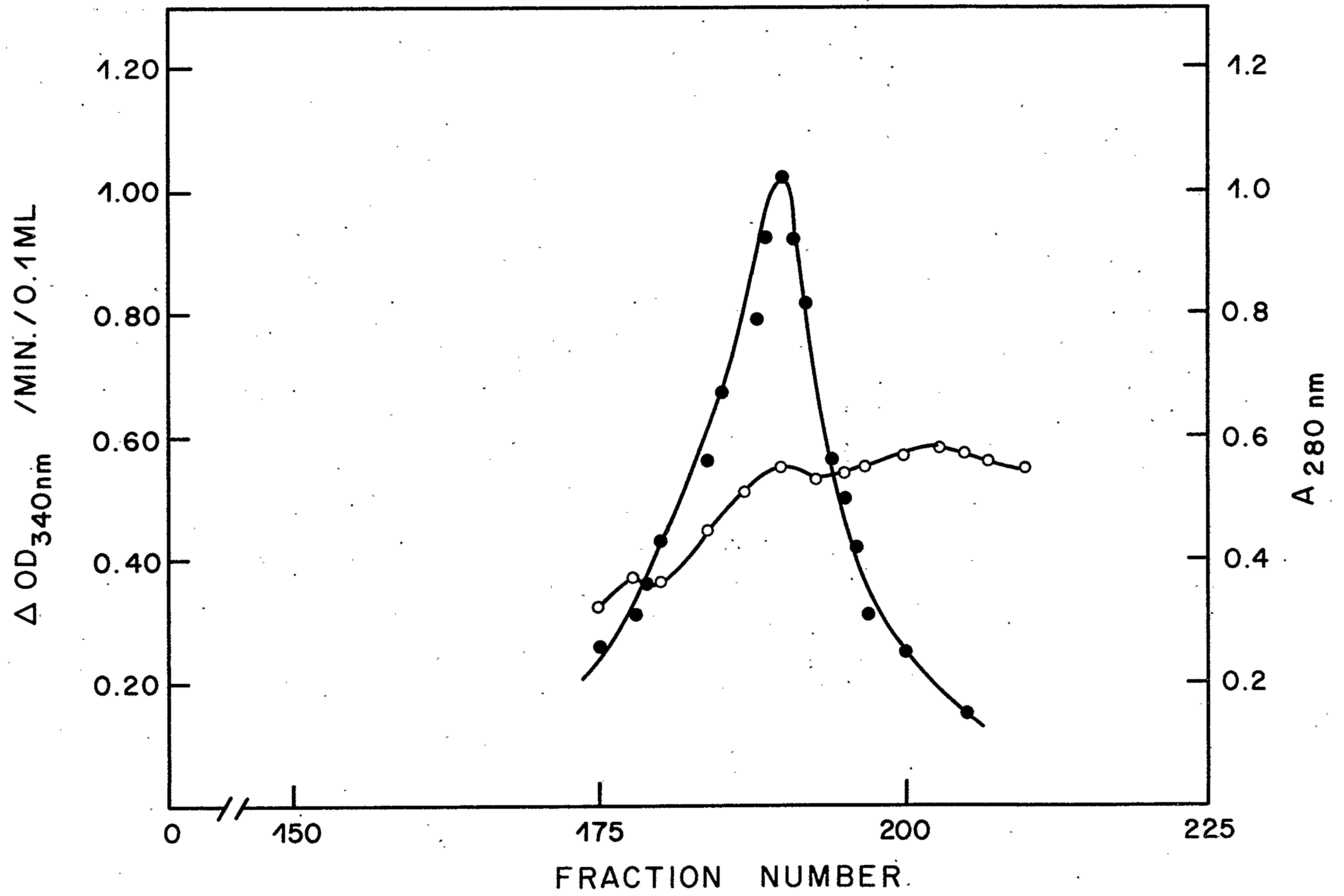
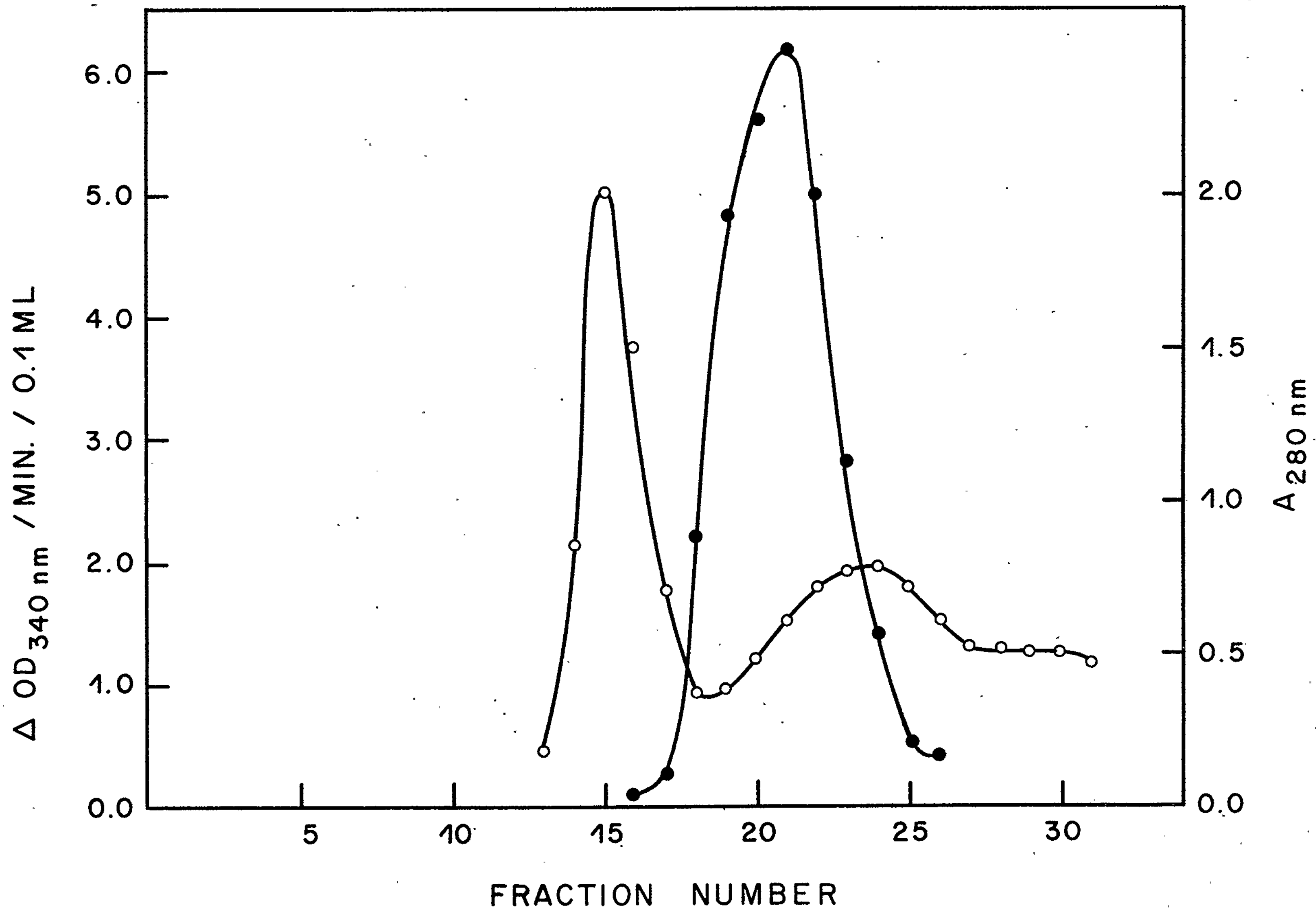

1.20
1.00
0.80
0.60
0.40
0.20
Δ $OD_{340nm}$ /MIN. / 0.1 ML
1.2
1.0
0.8
0.6
0.4
0.2
$A_{280\ nm}$
0
150
175
200
225
FRACTION NUMBER

Figure 3. Sephadex G-200 gel filtration of GDH.

● GDH activity

○ $A_{280\ nm}$, showing the general protein profile.


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Δ OD 340 nm / MIN. / 0.1 ML
2.0
1.5
1.0
0.5
0.0
A 280 nm
5
10
15
20
25
30
FRACTION NUMBER

C. Disc Gel Electrophoresis

A qualitative estimate of the degree of purification was obtained by disc gel electrophoresis of samples from the various fractionation steps. As illustrated in Figure 4 the crude extract contained a large number of unresolved bands. After electrophoresis of the supernatant resulting from the heat-treatment step, the first ammonium sulphate precipitate, the material obtained from the pooled DEAE fractions, and the reverse ammonium sulphate step, a progressive reduction in the number of protein bands was witnessed. Thus, the preparation from the reverse ammonium sulphate which was purified almost 60 - fold over the crude extracts revealed nine bands when stained with Amidoschwartz. As indicated in the purification procedure, division of the enzyme was necessary following both the first ammonium sulphate step and subsequent to reverse ammonium sulphate fractionation. Hence, from a single crude extract, four samples were run on the Sephadex G-200 column. After elution of the enzyme, the final precipitated material appeared to vary in the extent of purification from 120 - to 190 - fold. The number of bands from the protein-stained gels of these samples ranged from three to six (Fig. 5). Judging by the profile obtained from a densitometer scanning, one major band comprising 78 per cent of the total protein, and two relatively more cathodal bands of a lower intensity, were observed for the 190 - fold purified preparation.

Figure 4. A series of stained polyacrylamide gels, showing protein bands in enzyme samples at various stages of purification. Electrophoresis was carried out as described in the Methods.

A. Crude extract, 200 μg

B. Heat treatment supernatant, 150 μg

C. First ammonium sulphate ppt., 150 μg

D. DEAE-Sephadex ppt., 100 μg

E. Reverse ammonium sulphate ppt., 100 μg

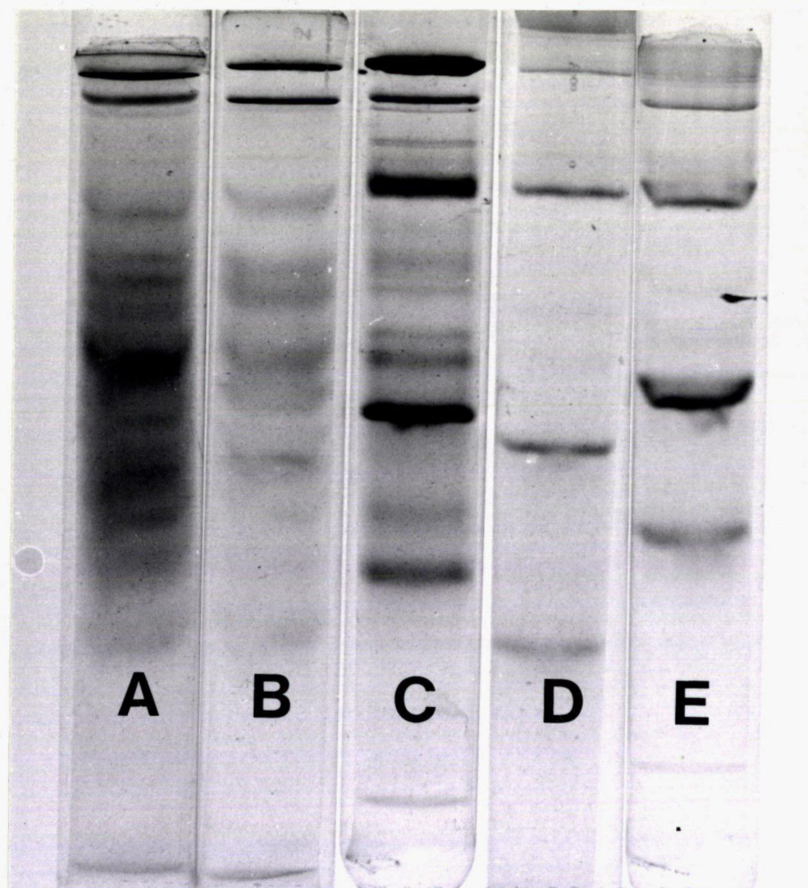
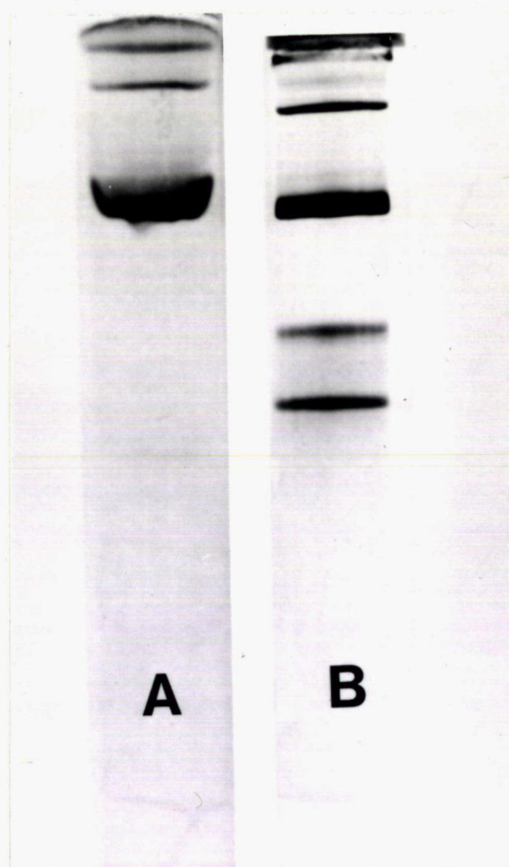

A
B
C
D
E

Figure 5. Polyacrylamide disc gels, showing samples obtained following G-200 gel filtration and precipitation with ammonium sulphate. Electrophoresis was conducted as described in Chapter II.

A. 190 - fold purification, 80 μg

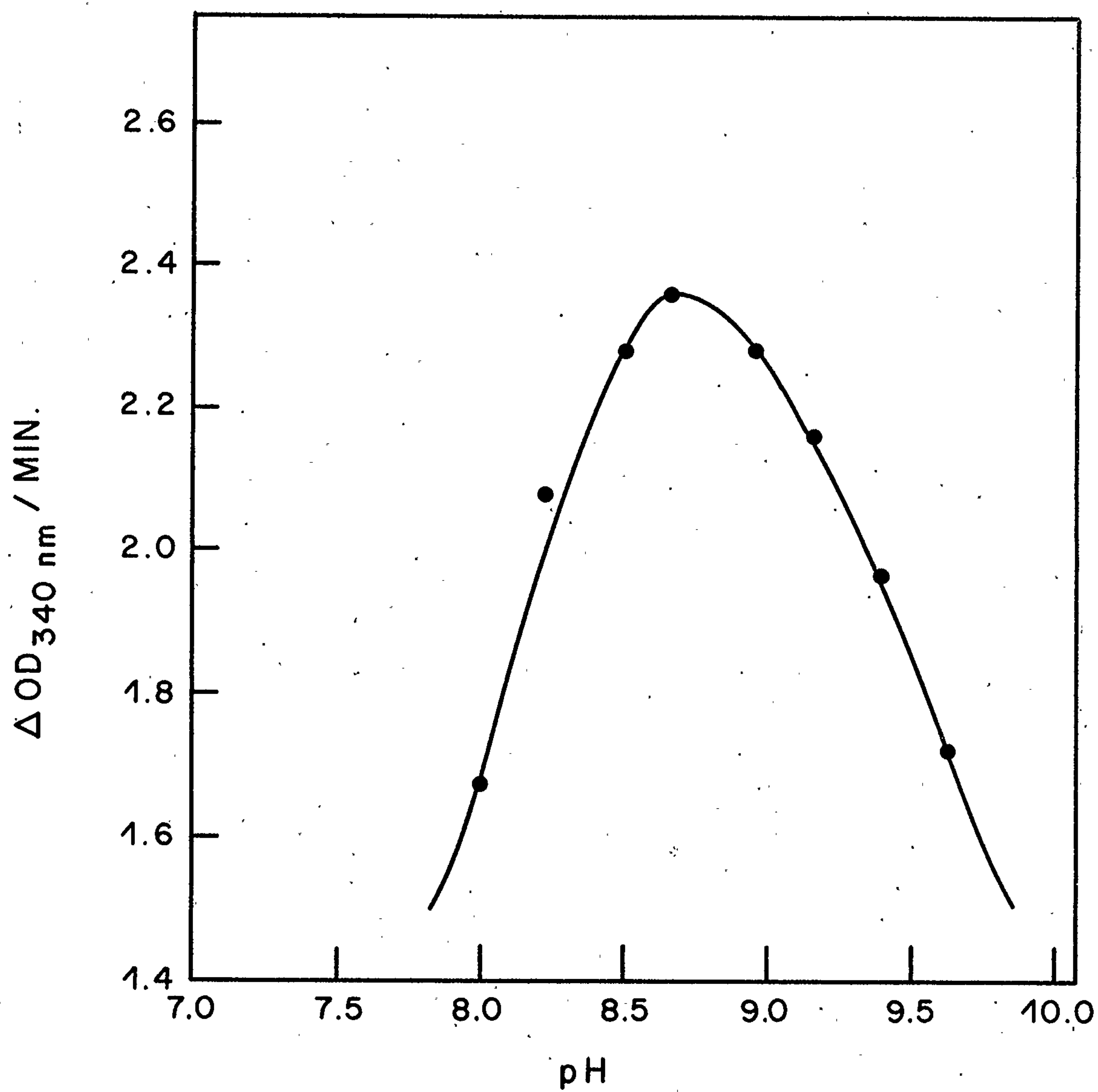
B. 120 - fold purification, 100 μg


A
B

D. Determination of pH Optimum

In order that the routine assay for GDH be carried out under conditions favouring maximum enzyme activity, the pH optimum was determined in 0.05 M Tris-HCl buffer. Solutions were prepared which varied by 0.20 to 0.25 pH units, over a range from 8.0 to 9.6, and enzyme samples containing 1.6 μg protein were tested for activities in these buffers. Initial velocities of the reductive amination reaction were recorded spectrophotometrically as outlined previously. Figure 6 indicates a value of 8.6 as the pH optimum for *Salmonella* glutamate dehydrogenase in the direction of glutamate synthesis.

Figure 6. Determination of pH optimum of GDH in the reductive amination reaction. Oxidation of NADPH was followed in the routine manner.


2.6
2.4
2.2
2.0
1.8
1.6
1.4
Δ OD 340 nm / MIN.
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

E. Thermostability of GDH

The activity of GDH was studied as a function of temperature by heat treatment of enzyme samples and measurement of residual activity. A stock solution of the following composition was made: 19.8 ml Tris-HCl, pH 7.5, containing $10^{-4}$ M EDTA, $10^{-3}$ M DTT, and 200 μl enzyme sample. Crude enzyme extracts (350 μg/ml) and preparations purified to the G-200 stage (40 μg/ml) were used in this experiment. Two ml of this solution was pipetted into a series of test tubes and allowed to equilibrate for 5 minutes in an ice bucket. Each of the tubes was then heated separately to a particular temperature in a hot water bath, and maintained at the same temperature for 5 minutes. Subsequently, the tube was chilled rapidly in an ice bath and enzyme activity of the sample assayed by the usual procedure. The range of temperatures selected was from 40 to 75°C.

Figure 7 shows the results of the thermostability studies conducted on Salmonella glutamate dehydrogenase. Exposure to temperatures of up to 50°C for 5 minutes resulted in virtually no enzyme inactivation in the crude or the partially purified preparation. While treatments at 55 and 60°C led to a loss of approximately 9 and 23 per cent of activity for the purified sample, respectively, a complete inactivation occurred at 65°C.

It is interesting to note that the purified GDH preparation was relatively more heat stable than the crude preparation. As evidenced by the data of Figure 7, greater than 95 per cent activity was lost in the crude extract upon exposure to 60°C, whereas at the same

temperature, more than 75 per cent of the initial activity was recovered in the purified preparation. The lesser heat stability of GDH in crude preparations may be explained by the binding of endogenous metabolites to the enzyme, thereby altering its conformation to a more heat-labile form.

Figure 7. The effect of temperature on GDH activity.

△ crude extract:

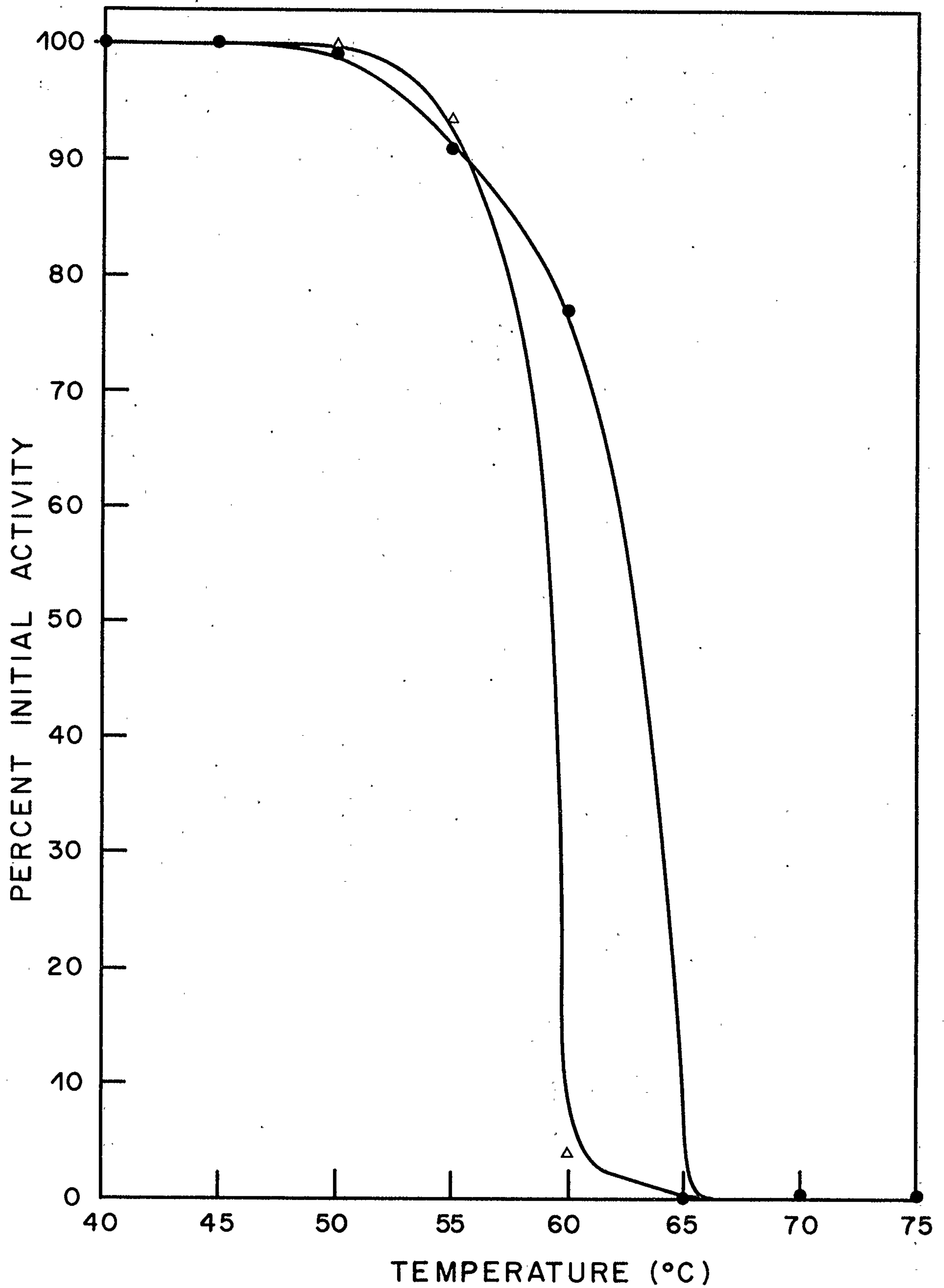
protein concentration, 350 μg/ml;

specific activity, 1.5.

● purified enzyme:

protein concentration, 40 μg/ml;

specific activity, 108.0.


PERCENT INITIAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
40
45
50
55
60
65
70
75
TEMPERATURE (°C)

F. Sucrose Density Gradient Centrifugation

Three sets of sucrose density gradients were used to obtain an estimate of the sedimentation coefficient for GDH, employing LDH, catalase, and MDH as internal standards. The calculations for sedimentation coefficient were made according to the method of Martin and Ames (1961) by using the relationship:

$$R = \frac{S_{20,w}\ (\text{unknown})}{S_{20,w}\ (\text{standard})}$$

where R is the ratio of

$$\frac{\text{distance travelled from meniscus by the unknown}}{\text{distance travelled from meniscus by the standard}}\ .$$

This calculation was applied to sucrose density gradient data using the three standards mentioned above, and the results are presented in Table III. Thus, with LDH as standard, the range of values obtained for sedimentation coefficient of GDH was 9.8 S to 10.4 S, with catalase, from 10.3 S to 10.8 S, and with MDH, from 7.9 S to 9.3 S. These data indicate the importance of selection of an appropriate internal standard for sucrose density gradient centrifugation, i.e., one with a sedimentation coefficient similar to that of the unknown. Thus, the use of MDH as a standard gave *s* values for GDH approximately 20 per cent lower than those derived by using LDH or catalase.

As pointed out by Martin and Ames, it is also possible to use the technique of sucrose density gradient centrifugation to obtain an estimate of the approximate molecular weight of an enzyme by employing the equation:

Table III. Estimation of Sedimentation Coefficient of GDH

| Standard | total # of fractions | peak fraction # of standard | $S_{20,w}$ of standard | peak fraction # of GDH | estimated $S_{20,w}$ GDH |
|---|---|---|---|---|---|
| a) LDH | 26.3 | 18 | 7.5 * | 13 | 9.8 |
| | 25.0 | 17 | | 12 | 10.4 |
| | 24.7 | 16 | | 11 | 10.2 |
| | 26.7 | 18 | | 13 | 10.2 |
| | 23.4 | 16 | | 12 | 10.0 |
| b) catalase | 24.0 | 12 | 11.4 * | 13 | 10.8 |
| | 26.3 | 12 | | 14 | 10.3 |
| | 27.1 | 13 | | 15 | 10.3 |
| c) MDH | 23.7 | 20 | 4.3 * | 12 | 9.3 |
| | 27.5 | 21 | | 11 | 7.9 |
| | 27.0 | 21 | | 11 | 8.3 |
| | 27.9 | 22 | | 12 | 8.3 |
| | 26.3 | 20 | | 10 | 8.1 |

*Values for the sedimentation coefficients and molecular weights of the standards used in these experiments were obtained from the following references:

(a) beef heart lactate dehydrogenase - Pesce <u>et al</u>. (1964)

(b) beef liver catalase - Samejima and Yang (1963)

(c) pig heart malate dehydrogenase - Thorne and Kaplan (1963)

$$\frac{S_1}{S_2} = \left[\frac{MW_1}{MW_2}\right]^{2/3}$$

where $S_1$ is the sedimentation coefficient of the standard;
$S_2$ is the sedimentation coefficient of the unknown;
$MW_1$ is the molecular weight of the standard; and
$MW_2$ is the molecular weight of the unknown.

A comparison of the sedimentation coefficient of catalase with the value derived for GDH of approximately 10.3 S is indicative of their close correspondence in molecular size. Based on a molecular weight of 250,000 daltons for catalase, the value of GDH was calculated to be 291,000.

G. Estimation of Molecular Weight and Molecular Size by Sephadex G-200 Gel Filtration

Gel filtration of glutamate dehydrogenase on Sephadex G-200 columns was carried out as described in the Methods. A pre-equilibrated column, with a known void volume, was used for calibration against seven standard proteins. Following elution from the gel matrix, effluent fractions were analyzed by means of enzymatic assays or protein determination to locate the standards and the unknown sample. After determination of the relative elution volumes of the standards, $V_e/V_o$, the ratio of elution volume to void volume, was calculated for each protein. A linear plot of $V_e/V_o$ versus $\log_{10} M$ (where M is the molecular weight of the standard protein) was utilized using the seven standard proteins to arrive at a molecular weight value of the GDH sample (Table IV). An extrapolated value for the molecular weight of GDH was shown to be 280,000 daltons (Fig. 8).

Another physical parameter of an enzyme which may be determined from Sephadex gel filtration is an estimation of molecular size. Stokes' radius, $a$, (in cm x $10^{-8}$) may be calculated from the formula:

$$a = kT/6\pi\eta D$$

where D is the diffusion coefficient;

k is the Boltzman constant;

T is the absolute temperature; and

$\eta$ is the viscosity.

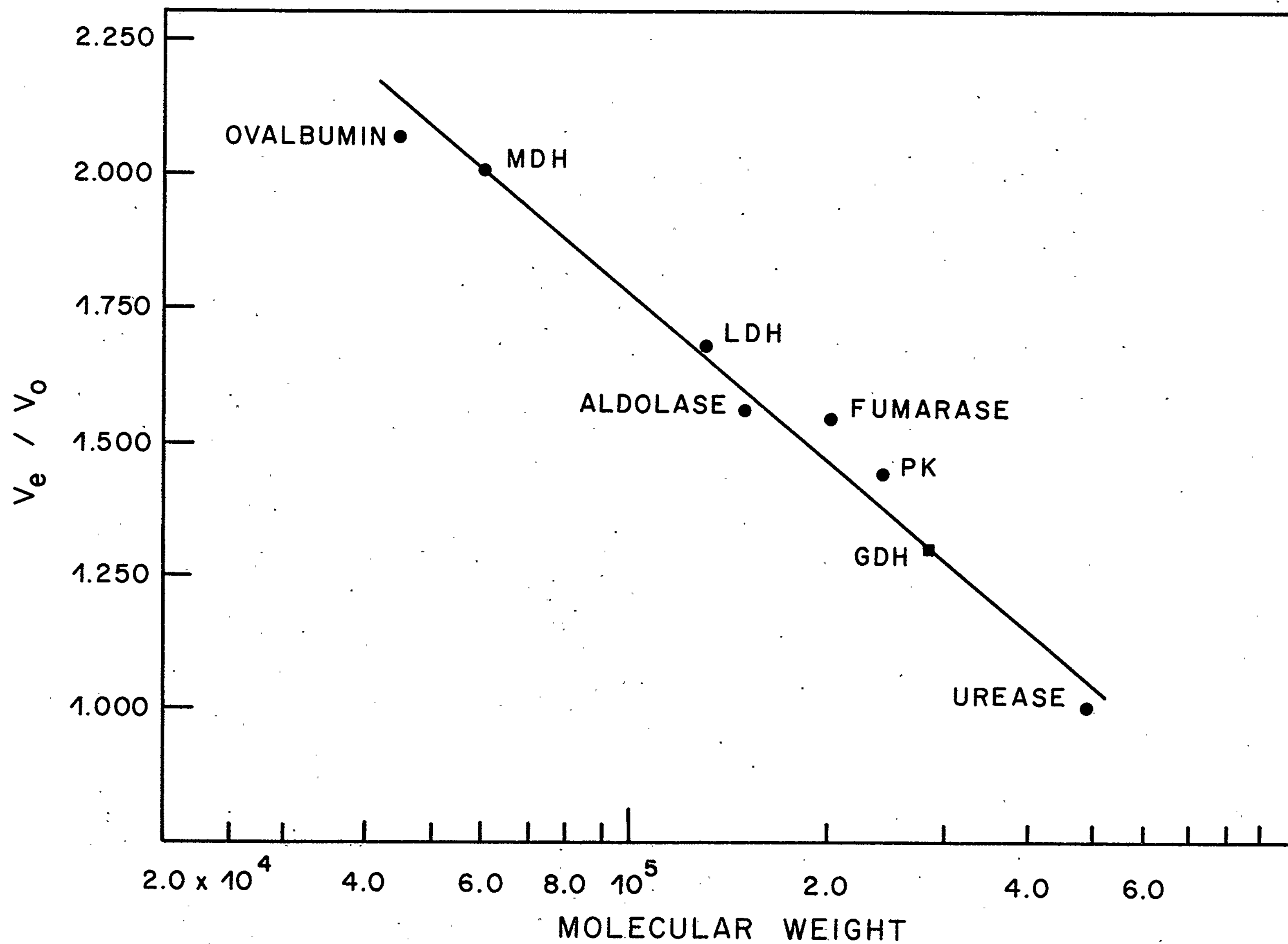
An empirical plot of the elution volume of the standards versus their

Table IV. Molecular Parameters of Protein Standards used for estimation of Molecular Weight and Size of GDH.

| Protein | $V_e$ ml | $V_e/V_o$ | Molecular Weight daltons | Stokes' radius cm x $10^{-8}$ | Reference |
|---|---|---|---|---|---|
| urease | 169 | 1.000 | 483,000 | 64 | Summer *et al.* (1934) |
| pyruvate kinase | 243 | 1.440 | 237,000 | 54 | Steinmetz and Deal (1966) |
| fumarase | 262 | 1.545 | 194,000 | 51 | Kanarek *et al.* (1964) |
| aldolase | 264 | 1.565 | 149,000 | 45 | Taylor and Lowry (1956) |
| LDH | 284 | 1.681 | 130,000 | 41 | Pesce *et al.* (1964) |
| MDH | 340 | 2.015 | 60,000 | - | Thorne and Kaplan (1963) |
| ovalbumin | 352 | 2.075 | 45,000 | 27 | Andrews (1970) |
| GDH | 221 | 1.310 | 280,000 | 55 | - |

Data for Stokes' radius of the standards were obtained from Andrews (1970).

Figure 8. Estimation of molecular weight by gel filtration on Sephadex G-200.


2.250
2.000
1.750
1.500
1.250
1.000
$V_e$ / $V_o$
OVALBUMIN
MDH
LDH
ALDOLASE
FUMARASE
PK
GDH
UREASE
2.0 x $10^4$
4.0
6.0
8.0
$10^5$
2.0
4.0
6.0
MOLECULAR WEIGHT

respective Stokes' radii yields either a straight line or a smooth curve. Hence, the derived value for $a$ of the unknown GDH was determined to be 54 x $10^{-8}$ cm (Fig. 9).

Verification of the value for the molecular weight of GDH has been obtained from a computer programme designed to test the efficacy of the plot $V_e/V_o$ versus $\log_{10}$ M in comparison to the other suggested methods of data analysis. The data included in Table IV were fitted into a straight line by the least squares method and values of M were determined from each of the following treatments (Andrews, 1970):

$K_d$ versus M;

$(K_d)^{1/3}$ versus $(M)^{1/2}$;

$V_e/V_o$ versus $\log_{10}$ M;

$V_e/V_o + (V_e/V_o)^{1/2}$ versus $\log_{10}$ M;

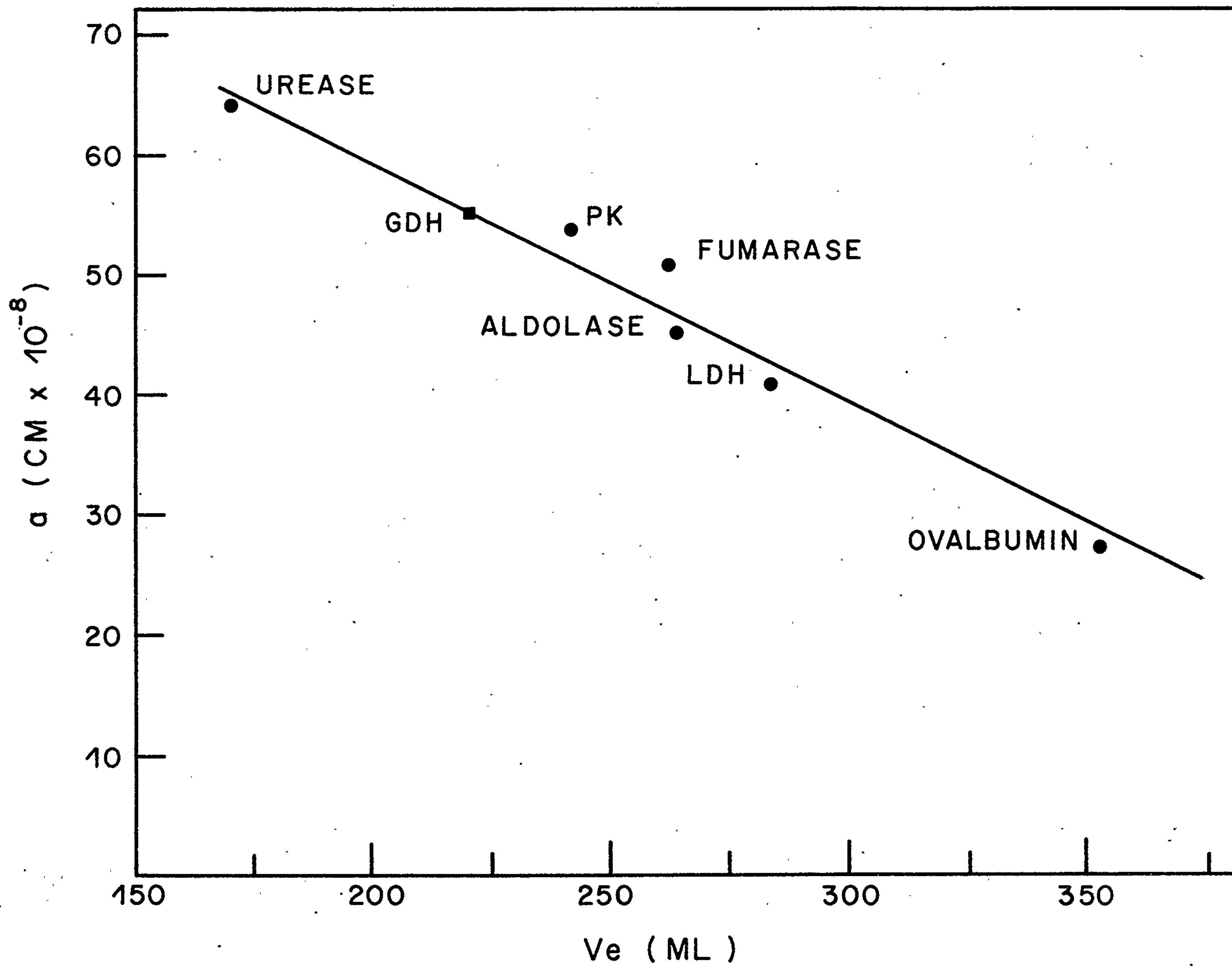
where $K_d = (V_e/V_o)/V_i$

$V_i$ = the internal volume of the column.

From the relationship $V_e/V_o$ versus $\log_{10}$ M, the value of 282,100 daltons was derived for GDH; and from $V_e/V_o + (V_e/V_o)^{1/2}$ versus $\log_{10}$ M, the calculated molecular weight was 279,800 daltons. Each of these was thus in close correspondence with the graphical analysis presented earlier.

Similarly, a least squares fit gave a value of 54.9 x $10^{-8}$ cm for the Stokes' radius of GDH.

Figure 9. Estimation of molecular size by gel filtration on Sephadex G-200, showing a plot of Stokes' radius, $a$, versus the elution volume, $V_e$. Values for the six standards were taken from Table IV. The extrapolated value of $a$ for the unknown, GDH, was determined as $55 \times 10^{-8}$ cm.


UREASE
GDH
PK
FUMARASE
ALDOLASE
LDH
OVALBUMIN
70
60
50
40
30
20
10
a (CM x 10$^{-8}$)
150
200
250
300
350
Ve ( ML )

## H. Kinetic Analysis and the Reaction Mechanism

A detailed kinetic analysis was undertaken in order to determine the mechanism for the multi-substrate reaction catalyzed by *Salmonella* glutamate dehydrogenase. Of the available methods employing steady state kinetics for distinguishing reaction mechanisms, the determination of initial velocity patterns are the simplest and, therefore, most commonly used. Initial velocity patterns enable one to distinguish unambiguously between sequential and ping pong mechanisms. End product inhibition studies, on the other hand, aid in the detection of central complexes and determination of the order of addition of reactants. Hence, a combination of initial velocity studies and end product inhibition patterns is an extremely useful tool in deducing the reaction mechanism. Cleland (1963a,b,c) has established a set of basic ground rules governing the relationship of the enzyme mechanism and the resultant initial velocity and end product inhibition patterns. By the use of Cleland's method, an attempt was made to elucidate the mechanism of the *Salmonella* glutamate dehydrogenase reaction.

The GDH reaction was considered in terms of the oxidative deamination as well as the reductive amination direction — the so-called reverse and forward reactions, respectively. Kinetic constants were determined for substrates in both directions. The results of this analysis are presented in the following pages.

1. The Oxidative Deamination Reaction: The oxidative deamination reaction involves the substrates L-glutamate and $NADP^+$, and the products α-ketoglutarate, $NH_4^+$, and NADPH. The assay system used

was as given in the Methods. All experiments were carried out at 25°C in 0.1 M Tris-HCl buffer, pH 8.6. A final concentration 7 µg/ml of the enzyme was employed in these studies. Since the equilibrium of the reaction lies in the direction of glutamate synthesis, it was necessary to use a relatively high amount of enzyme in order to drive the reaction toward α-ketoglutarate formation.

Lineweaver-Burk plots (1934) of reciprocal reaction velocity versus reciprocal glutamate concentration in the presence of 0.077 mM, 0.154 mM, and 0.308 mM $NADP^+$ are shown in Figure 10. A family of straight lines intersecting at the horizontal axis, on the left of the vertical axis, was obtained. As the plot did not show any parallel lines, it is evident that a reversible connection between the binding of glutamate and $NADP^+$ exists. This can be interpreted to indicate the formation of a ternary complex between the enzyme and the two substrates. Both $NADP^+$ and glutamate must bind to the enzyme prior to the release of any of the products. From the point of intersection of the three lines in Figure 10, the $K_m$ for glutamate was determined to be 50.0 mM.

A secondary plot (Florini and Vestling, 1957) of the apparent $1/V_{max}$ versus $1/NADP^+$ was derived from the intercepts of Figure 10, and is shown in Figure 11. The intersection on the negative abscissa of the straight line through these points gave a Michaelis constant for $NADP^+$ of 0.013 mM.

In the analysis of the oxidative deamination reaction presented above, the mechanism of addition of the substrates cannot be established in either the reverse or the forward direction.

Figure 10. Double reciprocal plot of initial reaction velocity against glutamate concentration, at changing fixed levels of $NADP^+$. Intersection at the unique point on the negative abscissa gave a value of $K_m$ for glutamate of 50.0 mM. Assays were performed in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. The concentrations of $NADP^+$ used were

△ 0.077 mM

● 0.154 mM

○ 0.308 mM

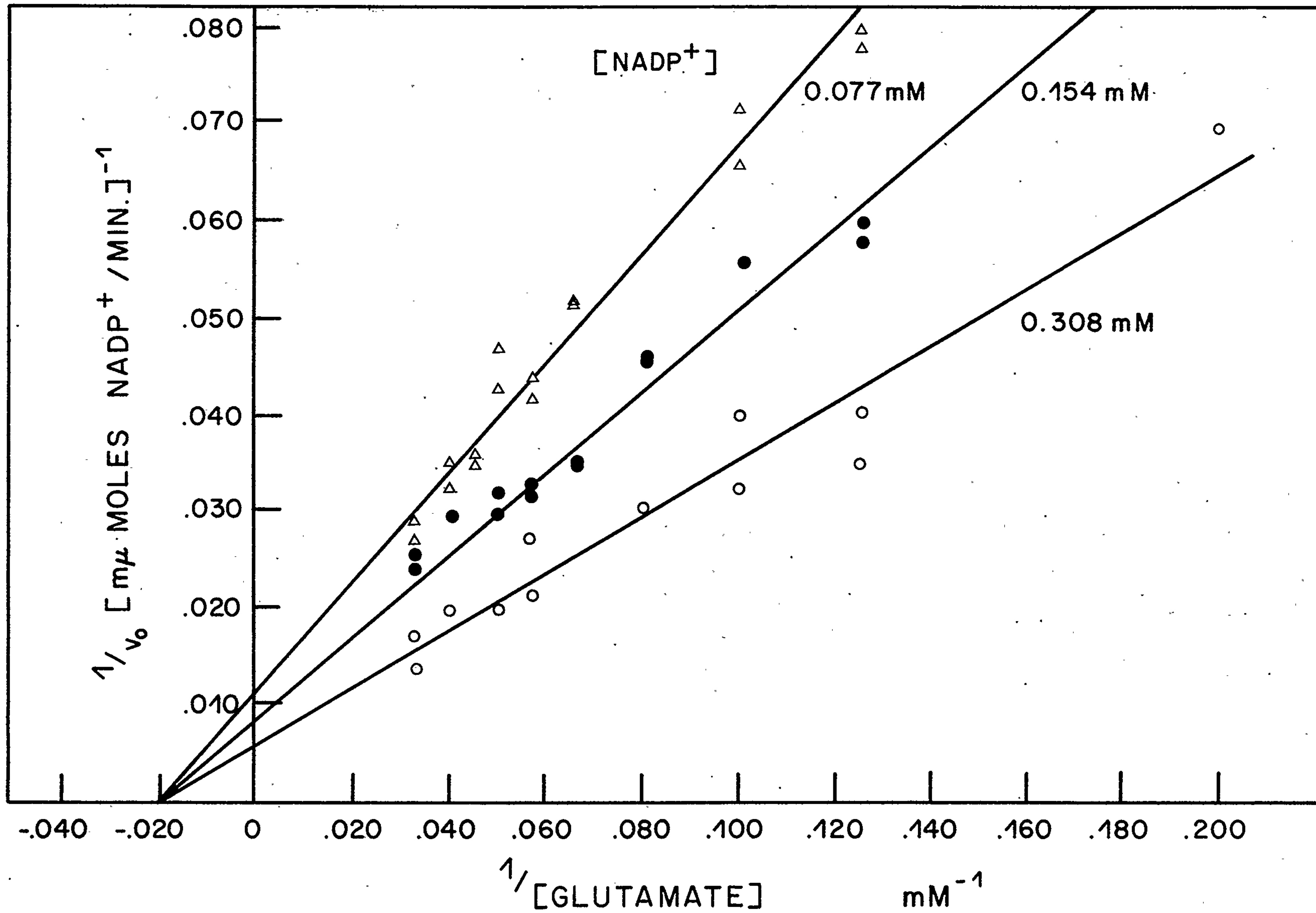
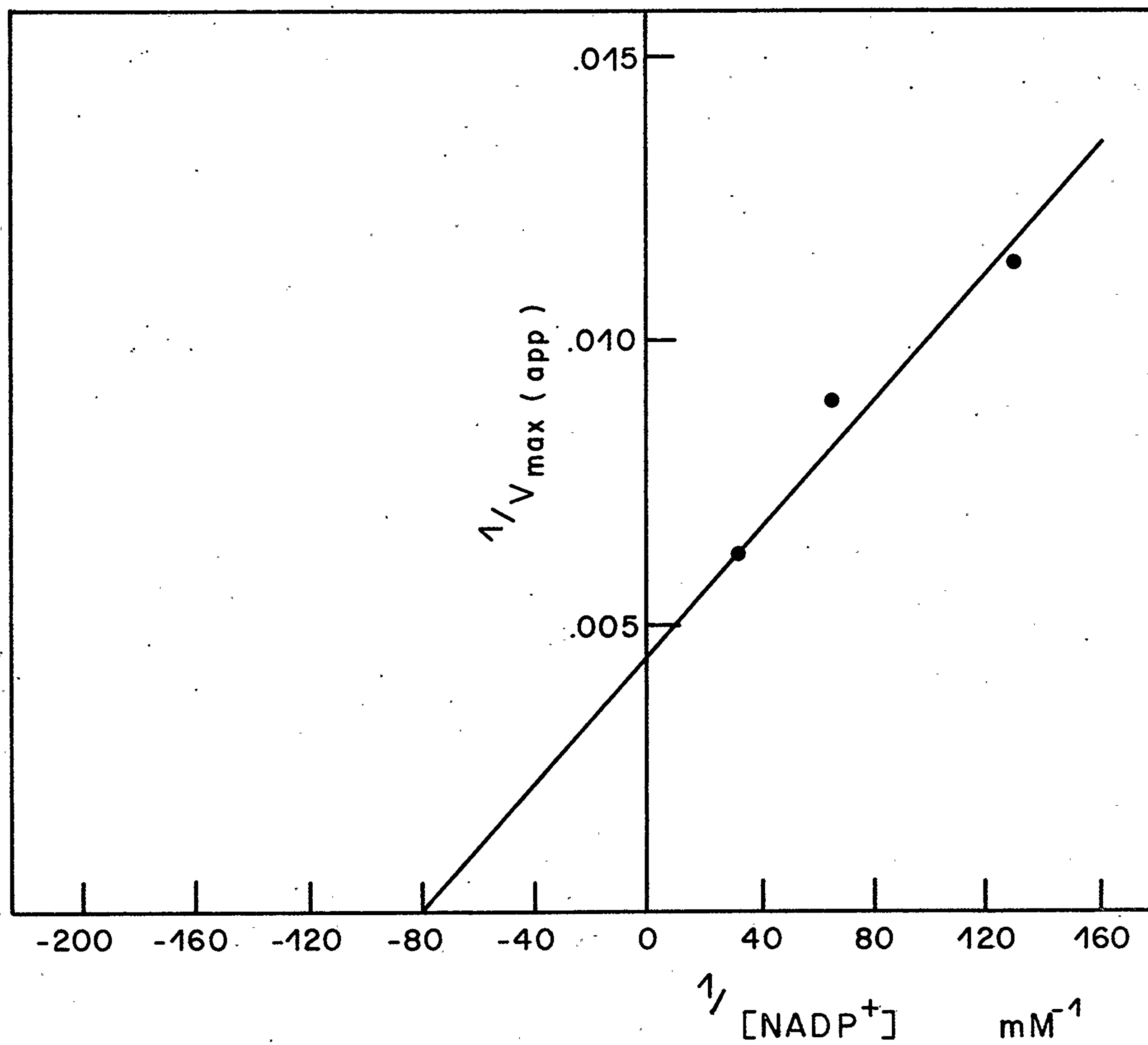

[NADP+]
0.077 mM
0.154 mM
0.308 mM
1/vo [mμ MOLES NADP+ / MIN.]-1
.080
.070
.060
.050
.040
.030
.020
.010
-.040
-.020
0
.020
.040
.060
.080
.100
.120
.140
.160
.180
.200
1/[GLUTAMATE] mM-1

Figure 11. Secondary plot of intercepts from Figure 10, showing apparent $1/V_{max}$ versus $1/NADP^+$. The point of intersection on the negative abscissa gave a $K_m$ for $NADP^+$ of 0.013 mM.


.015
.010
.005
1/Vmax (app)
-200
-160
-120
-80
-40
0
40
80
120
160
1/[NADP+] mM-1

2. The Reductive Amination Reaction: The substrates of GDH in the reductive amination reaction are α-ketoglutarate, ammonia, and NADPH. To determine the order of binding of the three substrates and their respective Michaelis constants, an analysis similar to that conducted by Frieden (1959) with the bovine liver enzyme, and by LéJohn *et al.* (1968) using the *Thiobacillus novellus* enzyme, was undertaken. All reaction velocity data were obtained at 25°C in 0.1 M Tris-HCl buffer, pH 8.6, and at a final protein concentration of 1.1 μg/ml. Initial velocity patterns were obtained by employing each of the substrates as the variable substrate, in combination with another as the changing fixed substrate, in the presence of the third at saturating levels. The data with regard to each substrate are now presented.

a. NADPH

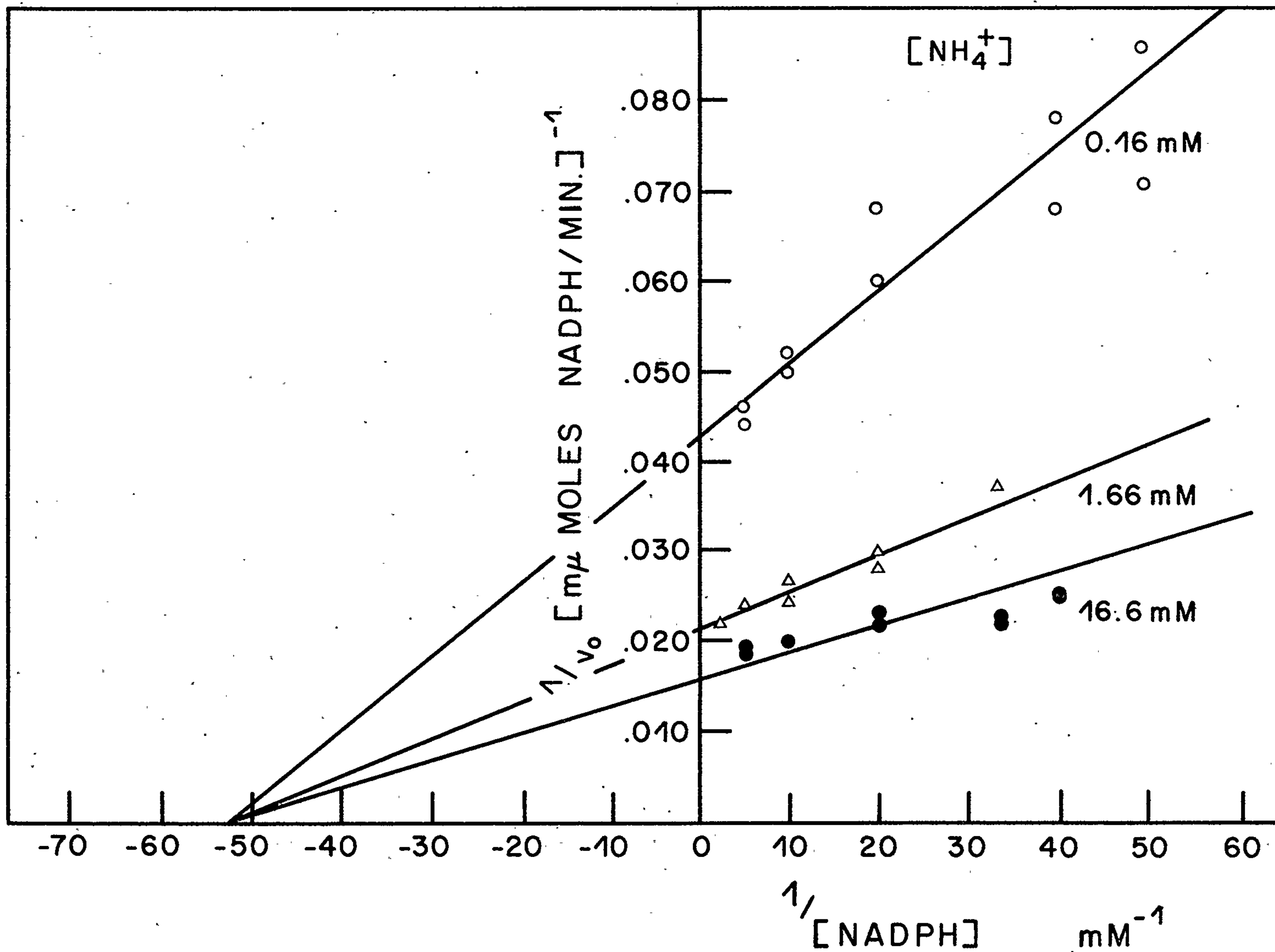
In the first instance, NADPH was used as the variable substrate, and ammonium chloride as the changing fixed substrate; α-ketoglutarate was maintained at a constant level of 30.0 mM. Figure 12 shows a plot of reciprocal initial velocity versus reciprocal NADPH concentration, at 0.16 mM, 1.66 mM, and 16.6 mM ammonium chloride. It is evident from these data that ammonium chloride changes the slope of the double reciprocal plot, indicating thereby that it and NADPH are connected in the reaction sequence by intervening reversible steps. The $K_m$ for NADPH of 0.019 mM was determined from the point of intersection on the horizontal axis.

Figure 12. Plot of reciprocal velocity versus reciprocal NADPH concentration at a constant, high level of α-ketoglutarate (30.0 mM). Assays were conducted in 0.1 M Tris-HCl buffer, pH 8.6 at 25°C. Concentrations of ammonium chloride were

○ 0.16 mM

△ 1.66 mM

● 16.6 mM


[NH$_4^+$]
0.16 mM
1.66 mM
16.6 mM
.080
.070
.060
.050
.040
.030
.020
.010
$^1/v_o$ [m$\mu$ MOLES NADPH / MIN.]$^{-1}$
-70 -60 -50 -40 -30 -20 -10 0 10 20 30 40 50 60
$^1/$[NADPH] mM$^{-1}$

The same experiment was repeated using a lower level of α-ketoglutarate. If the concentration of α-ketoglutarate were maintained at 1.0 mM, which is considerably lower than its $K_m$, a double reciprocal plot of initial velocity versus varying NADPH did not show a point of intersection on the negative abscissa (Fig. 13). Instead, the straight lines appeared to meet at a point to the left of the ordinate, above the horizontal axis, suggesting that the apparent Michaelis constant for NADPH was lower than the dissociation constant of 0.057 mM of the [enzyme-NADPH] complex.

Next, a study was made where NADPH concentrations were varied, and α-ketoglutarate was used as the changing fixed substrate. Figure 14 shows a double reciprocal plot of reaction velocities versus varying NADPH concentrations in the presence of 0.10 mM, 0.20 mM, and 1.00 mM α-ketoglutarate, and 2.76 mM ammonium chloride. A family of straight lines, intersecting at a point on the horizontal axis, appeared with these concentrations of α-ketoglutarate, again indicating a reversible connection between NADPH and α-ketoglutarate. The initial velocity studies, thus far, have established the existence of a reversible connection between NADPH and α-ketoglutarate, on the one hand, and between NADPH and ammonia on the other.

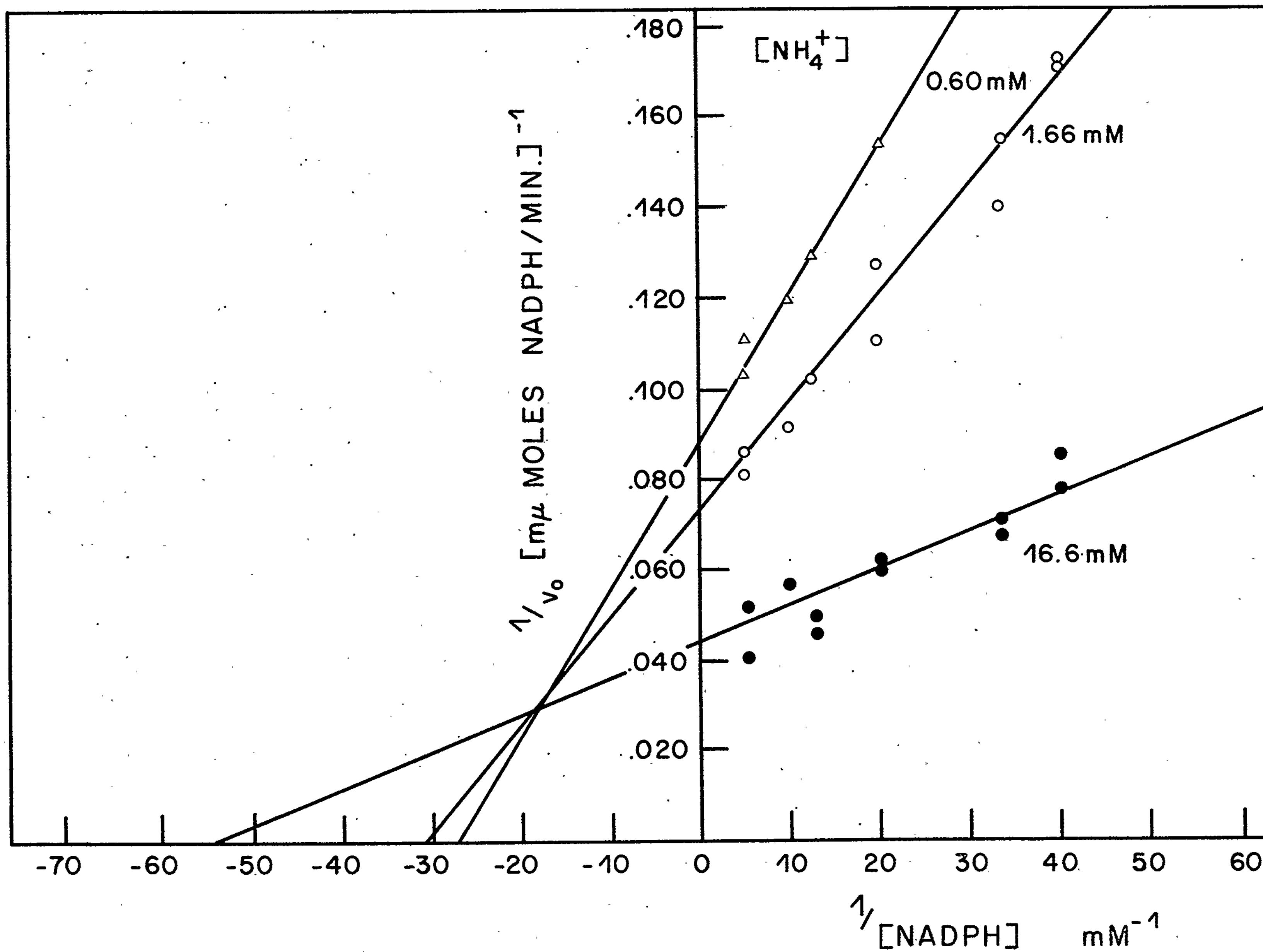
By comparison, if the above kinetic analysis were repeated at a higher level of ammonium chloride — 16.6 mM — in the presence of changing fixed amounts of α-ketoglutarate, 0.05 mM, 0.10 mM, 0.20 mM, and 0.50 mM, the double reciprocal plot yielded a group of straight lines which appeared to be near-parallel (Fig. 15). It was stated

Figure 13. Plot of reciprocal velocity versus reciprocal NADPH concentration at a constant, low level of α-ketoglutarate (1.0 mM). Assays were performed in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. Concentrations of ammonium chloride were

△ 0.60 mM

○ 1.66 mM

● 16.6 mM



[NH4+]
0.60 mM
1.66 mM
16.6 mM
1/vo [mμ MOLES NADPH/MIN.]-1
.180
.160
.140
.120
.100
.080
.060
.040
.020
-70
-60
-50
-40
-30
-20
-10
0
10
20
30
40
50
60
1/[NADPH] mM-1

Figure 14. Plot of reciprocal velocity versus reciprocal NADPH concentration at a constant, low level (2.76 mM) of ammonium ion. Assays were carried out in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. The concentrations of α-ketoglutarate used were

□ 0.10 mM

○ 0.20 mM

◆ 1.00 mM

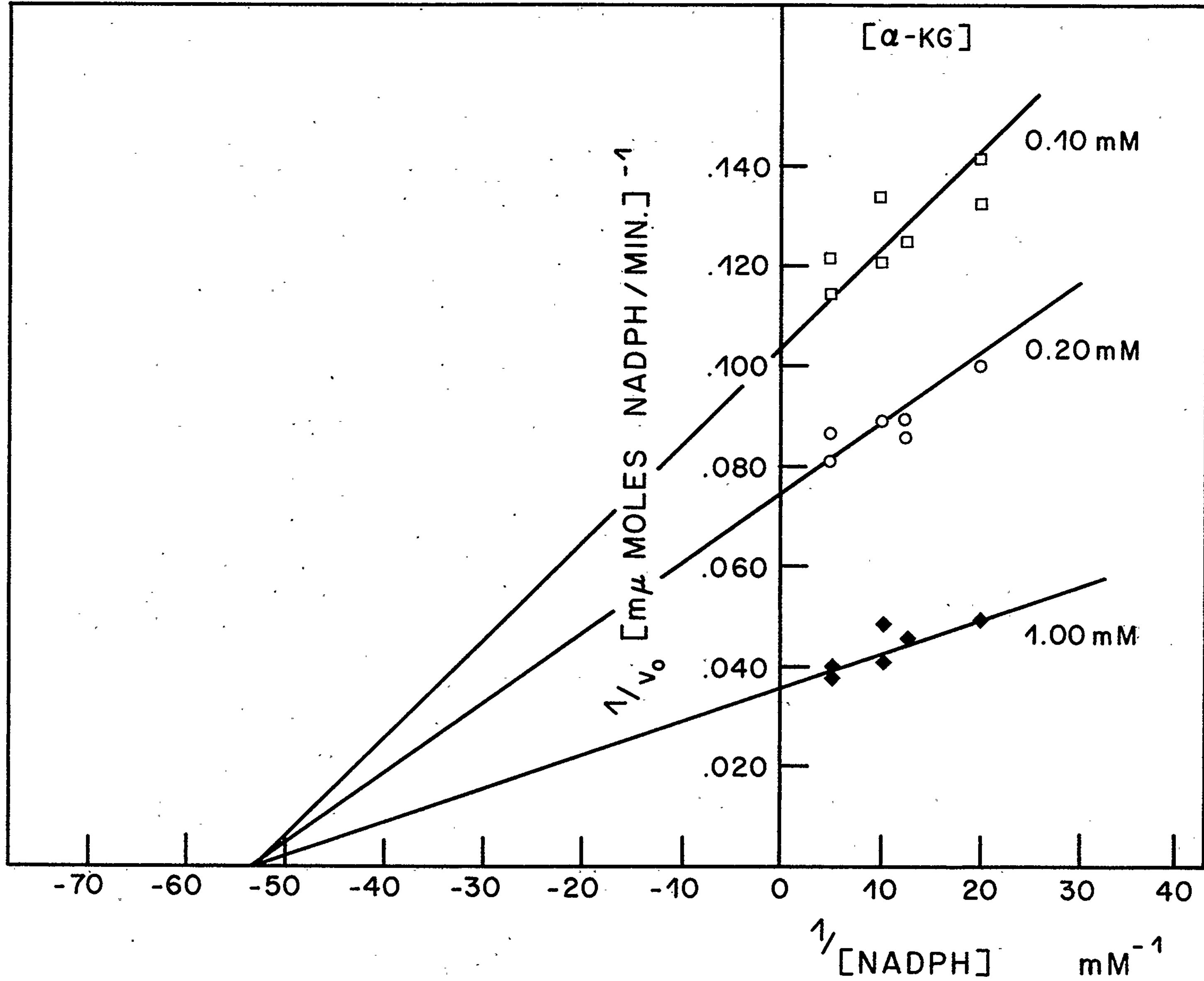

[α-KG]
0.10 mM
0.20 mM
1.00 mM
.140
.120
.100
.080
.060
.040
.020
1/v₀ [mμ MOLES NADPH/MIN.]⁻¹
-70
-60
-50
-40
-30
-20
-10
0
10
20
30
40
1/[NADPH] mM⁻¹

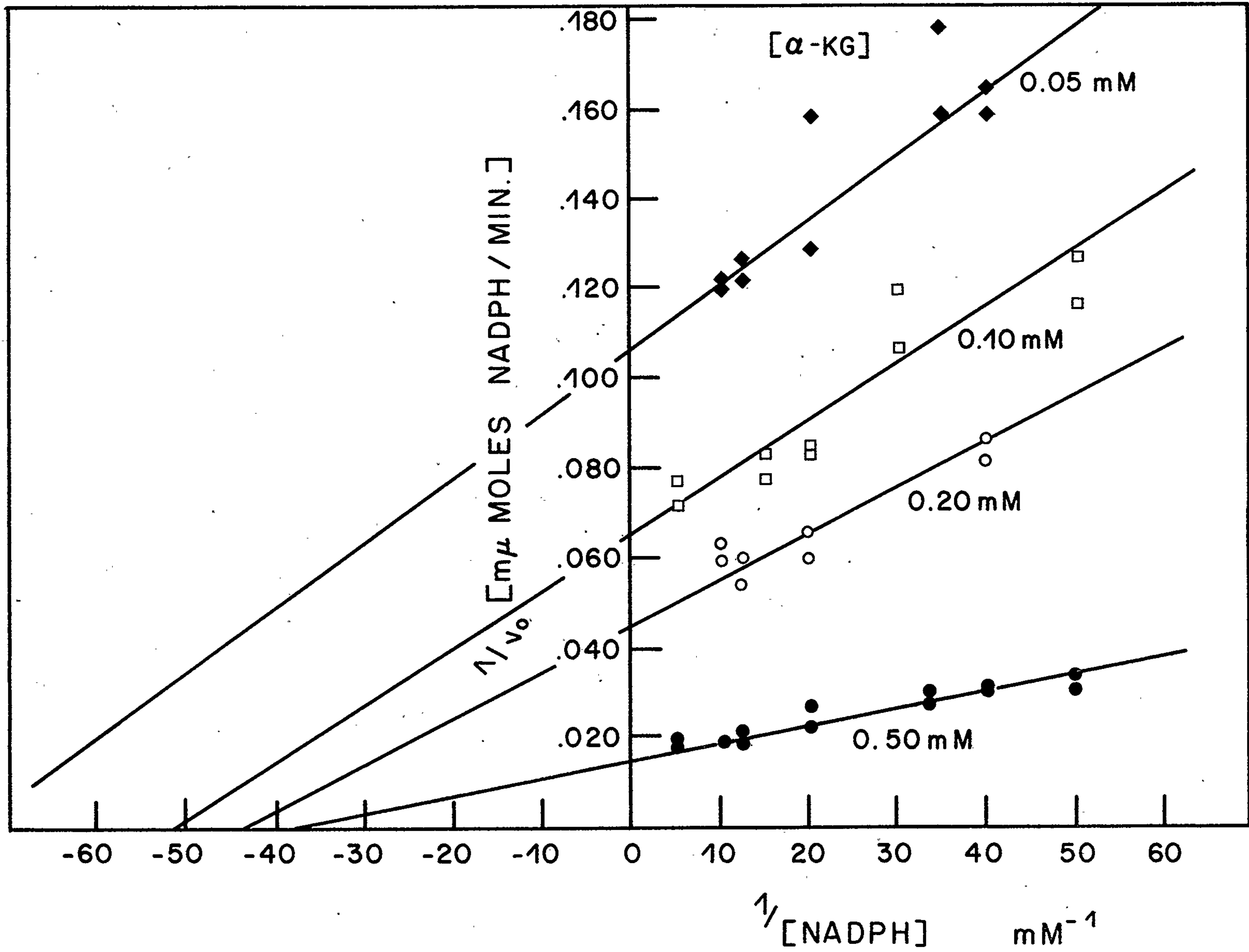
Figure 15. Plot of reciprocal velocity versus reciprocal NADPH concentration at a constant, high level (16.6 mM) of ammonium ion. The experiments were performed in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. The concentrations of α-ketoglutarate employed were

◆ 0.05 mM

□ 0.10 mM

○ 0.20 mM

● 0.50 mM



.180
.160
.140
.120
.100
.080
.060
.040
.020
[α-KG]
0.05 mM
0.10 mM
0.20 mM
0.50 mM
1/v₀ [mμ MOLES NADPH / MIN.]
-60
-50
-40
-30
-20
-10
0
10
20
30
40
50
60
1/[NADPH] mM⁻¹

earlier that a reversible connection between NADPH and α-ketoglutarate was demonstrated, based on the presence of intersecting straight lines in Figure 14. However, near-parallel lines in Figure 15 suggest an irreversible step between the binding of NADPH and α-ketoglutarate. According to Cleland (1963c), if the release of a product at zero concentration were to intercept the binding of the variable and changing fixed substrates, parallel lines will be observed in double reciprocal plots. Addition of a substrate, at saturating levels, in between the point of binding of the variable and the constant fixed substrates would also result in parallel lines. Since in the presence of high and low constant levels of ammonium chloride, α-ketoglutarate affects the intercept and the slope, respectively, of the double reciprocal plot, it may be concluded that ammonium chloride is second in the order of binding to the enzyme.

In Frieden's analysis (1959) of the order of substrate binding to bovine liver glutamate dehydrogenase, using NADPH as the cofactor, the differing plots of reciprocal velocity versus reciprocal NADPH concentration at a high and at a low level of $NH_4^+$ were correlated with kinetic equations to suggest that ammonium ion is the second substrate to bind to the enzyme.

b. Ammonium ion

The determination of the $K_m$ for ammonium ion was carried out essentially as for NADPH. Initial reaction velocity plots were prepared by using ammonium chloride as the variable substrate, α-ketoglutarate as the changing fixed substrate, and a constant level of NADPH. The latter was employed at 0.040 mM, as higher concentrations of the cofactor resulted in a decreased sensitivity of the assay. Lineweaver-Burk plots of reciprocal reaction velocity versus reciprocal $NH_4^+$ concentrations yielded straight lines with a single point of intersection on the negative abscissa (Fig. 16). The Michaelis constant for ammonia of 0.29 mM, obtained from this analysis, was demonstrated to be independent of α-ketoglutarate concentrations.

From the data of Figure 12, a secondary plot of intercepts, yielding apparent $1/V_{max}$ with varying NADPH concentrations, versus reciprocal changing fixed levels of $NH_4^+$, was prepared and is illustrated in Figure 17. The straight line drawn through these points was extended to the negative abscissa, and a value of 0.29 mM for the $K_m$ of ammonium ion was obtained.

Figure 16. Double reciprocal plot of velocity against ammonium chloride concentration at a fixed level of NADPH (0.040 mM) and constant varying α-ketoglutarate concentrations. Assays were conducted in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. The concentrations of α-ketoglutarate employed were

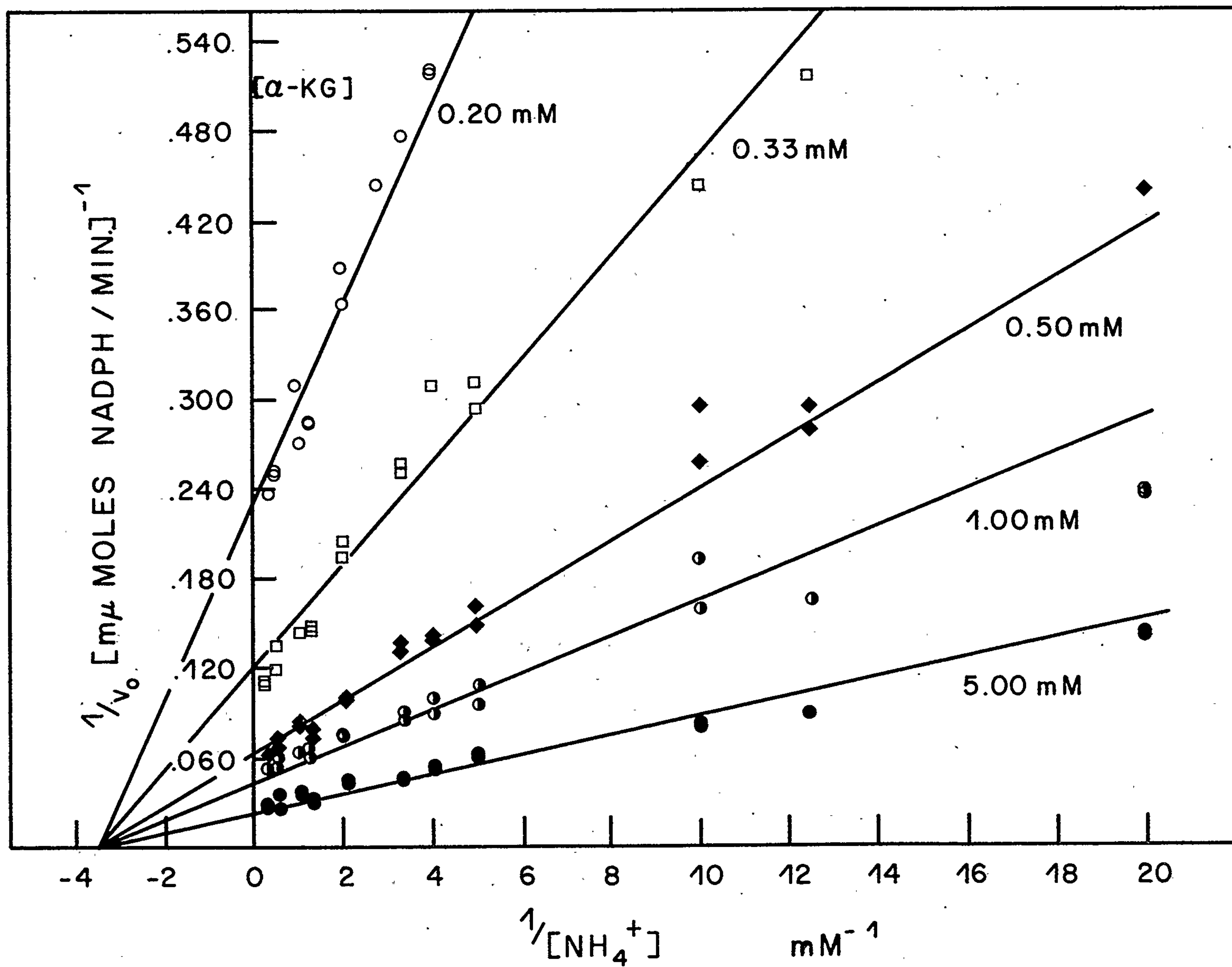
○ 0.20 mM

□ 0.33 mM

◆ 0.50 mM

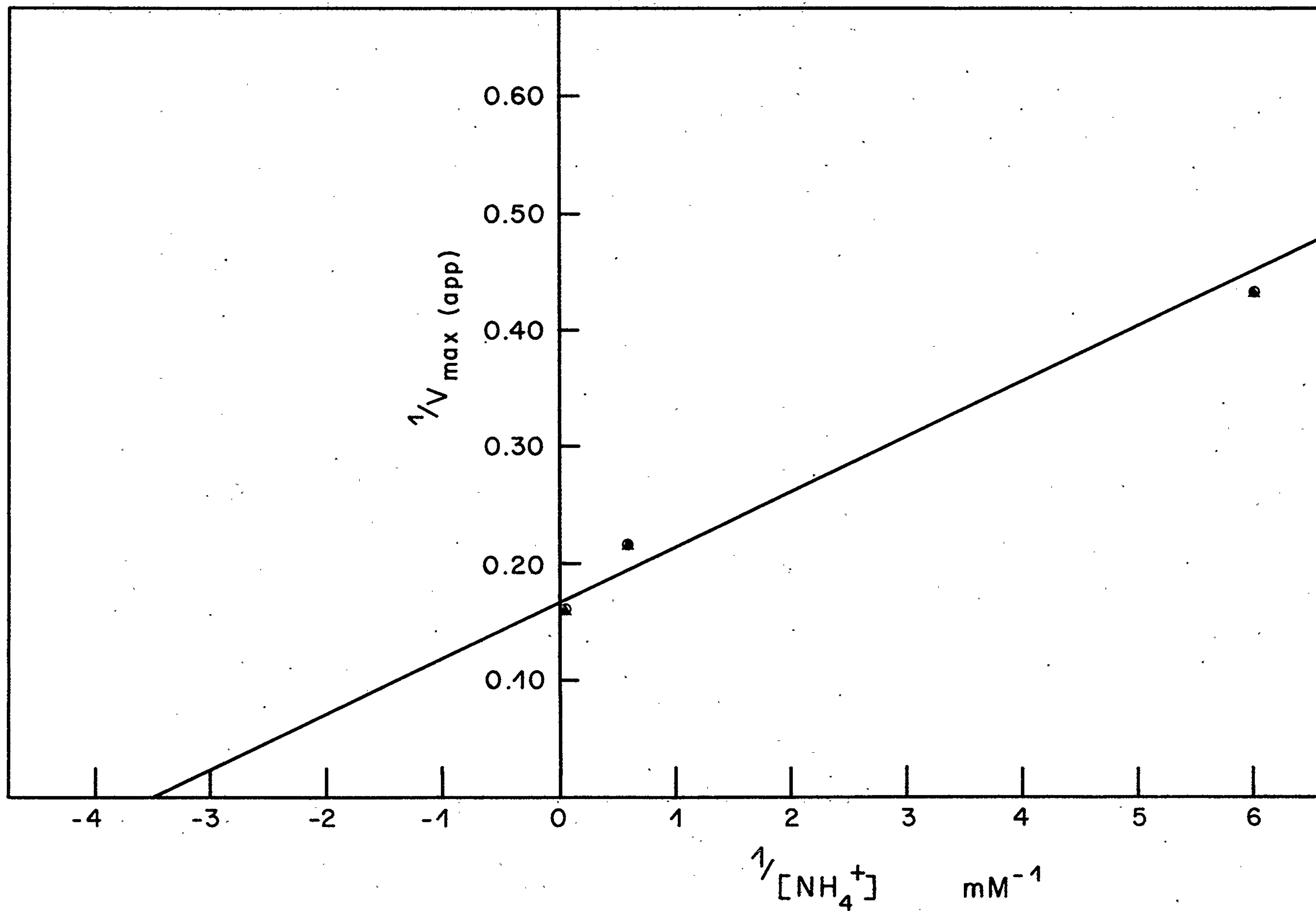
◑ 1.00 mM

● 5.00 mM



[α-KG]
0.20 mM
0.33 mM
0.50 mM
1.00 mM
5.00 mM
.540
.480
.420
.360
.300
.240
.180
.120
.060
1/vo [mμ MOLES NADPH / MIN.]-1
-4
-2
0
2
4
6
8
10
12
14
16
18
20
1/[NH4+] mM-1

Figure 17. Secondary plot derived from Figure 12. Intercepts of apparent $1/V_{max}$ versus the reciprocal of each of the changing fixed concentrations of ammonium ion gave a value for the $K_m$ of ammonium ion.


0.60
0.50
0.40
0.30
0.20
0.10
$1/V_{max}$ (app)
-4
-3
-2
-1
0
1
2
3
4
5
6
$1/[NH_4^+]$ $mM^{-1}$

c. α-ketoglutarate

Figure 18 shows initial velocity data obtained by varying α-ketoglutarate concentrations, in the presence of a constant level of NADPH (0.040 mM) and changing fixed ammonium chloride. From a family of straight lines intersecting on the negative abscissa, a Michaelis constant of 4.0 mM was calculated for α-ketoglutarate; this value was also found to be independent of ammonium ion concentrations. However, whether such a Michaelis constant is a dissociation constant would depend on whether an [enzyme-α-ketoglutarate] complex can be formed in the absence of either NADPH or $NH_4^+$. The kinetic analysis presented here does not provide any evidence for the existence of such a complex.

Again, the above-mentioned value of $K_m$ was verified by two secondary plots. From the double reciprocal plot of varying NADPH concentration versus changing fixed α-ketoglutarate, given in Figure 15, intercepts at the ordinate indicating apparent $1/V_{max}$ were plotted against the reciprocal α-ketoglutarate (Fig. 19). The extrapolated value for the Michaelis constant for α-ketoglutarate was 4.0 mM.

Similarly, from the data of Figure 16, a secondary plot was constructed, as represented in Figure 20, showing the values of apparent $1/V_{max}$ versus reciprocal α-ketoglutarate levels. A straight line was obtained which, upon extension to the abscissa, again gave a $K_m$ for α-ketoglutarate of 4.0 mM.

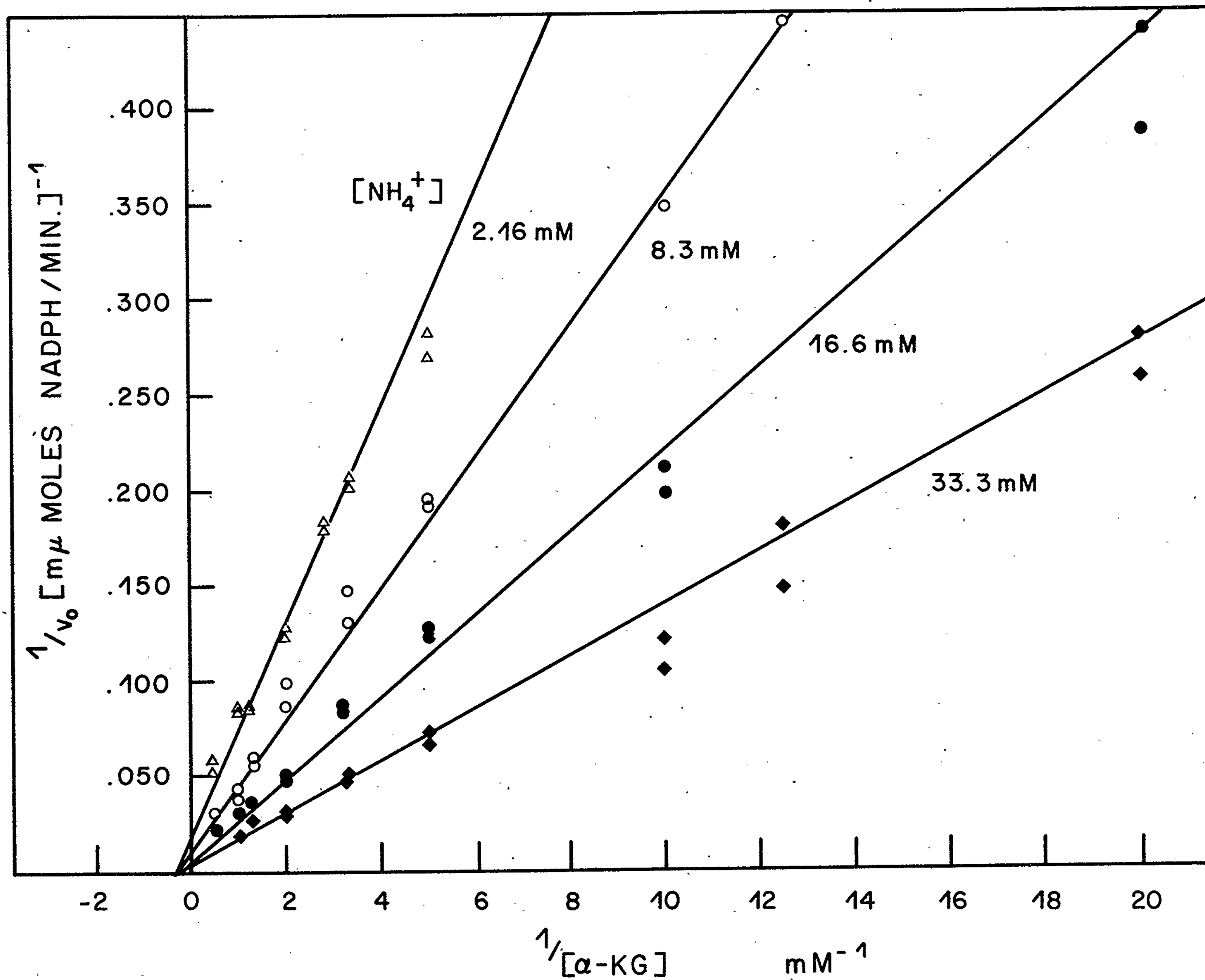
Figure 18. Double reciprocal plot of velocity against α-ketoglutarate concentration at a fixed level of NADPH (0.040 mM) and changing fixed levels of ammonium chloride. Assays were performed in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. Concentrations of ammonium chloride employed were

△ 2.16 mM

○ 8.3 mM

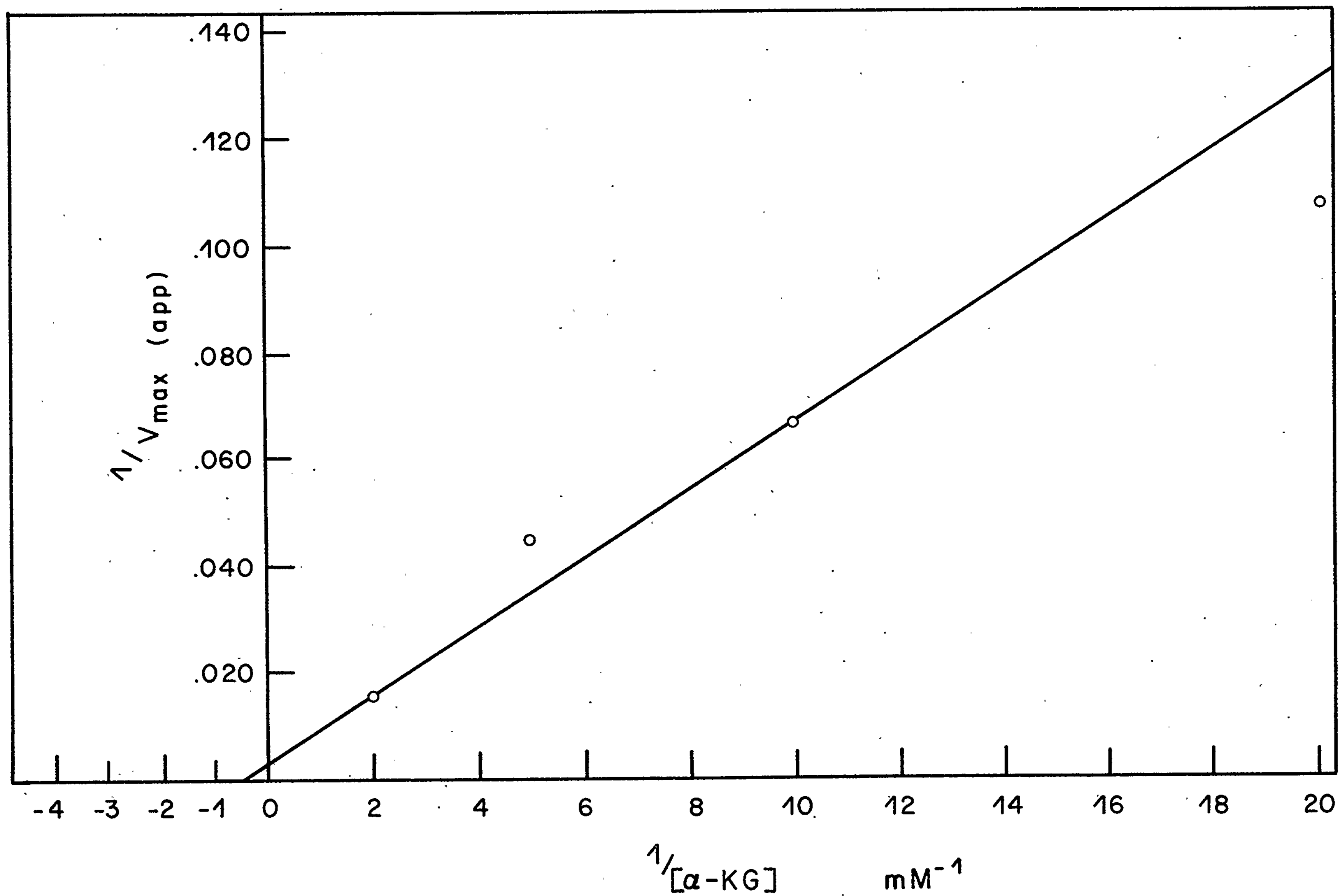
● 16.6 mM

◆ 33.3 mM



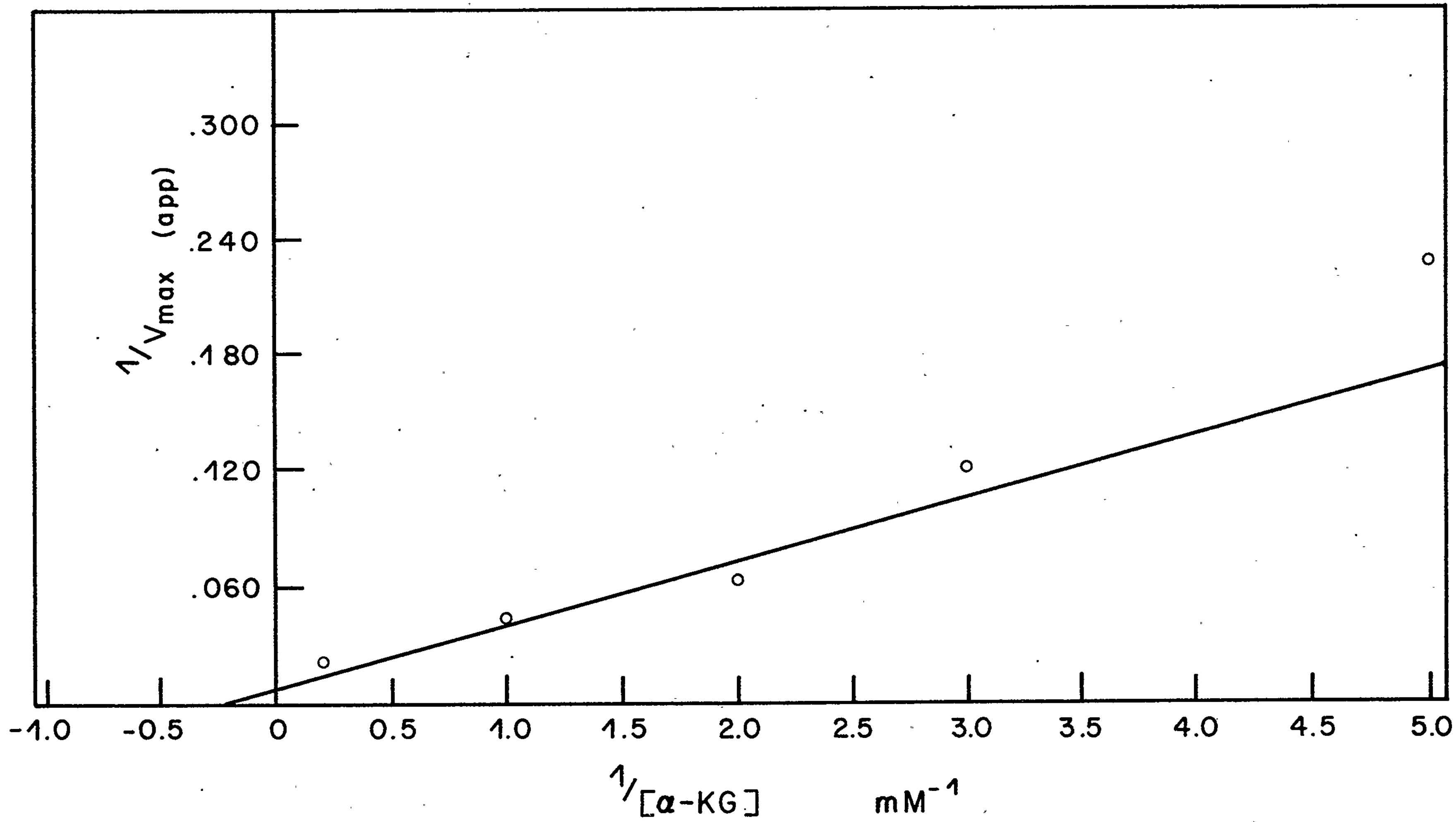
.400
.350
.300
.250
.200
.150
.100
.050
1/v₀ [mμ MOLES NADPH / MIN.]⁻¹
[NH4+]
2.16 mM
8.3 mM
16.6 mM
33.3 mM
-2
0
2
4
6
8
10
12
14
16
18
20
1/[α-KG] mM⁻¹

Figure 19. Secondary plot of intercepts of apparent $1/V_{max}$ derived from Figure 15 versus the reciprocal of changing fixed concentrations of α-ketoglutarate. The value of $K_m$ for α-ketoglutarate was determined as 4.0 mM.



.140
.120
.100
.080
.060
.040
.020
1/Vmax (app)
-4
-3
-2
-1
0
2
4
6
8
10
12
14
16
18
20
1/[α-KG] mM-1

Figure 20. Secondary plot of intercepts of apparent $1/V_{max}$ derived from Figure 16 versus the reciprocal of the changing fixed concentrations of α-ketoglutarate. From the best straight line through the points, a $K_m$ for α-ketoglutarate was determined as 4.0 mM.



.300
.240
.180
.120
.060
1/Vmax (app)
-1.0
-0.5
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
1/[α-KG] mM-1

3. Product Inhibition by $NADP^+$: In order to conclusively demonstrate that NADPH was the first substrate to bind to the enzyme in the amination reaction, product inhibition studies were undertaken. As suggested by Cleland (1963c), a product that reacts with only one enzyme form will give linear inhibition which may be competitive (slopes only vary), uncompetitive (intercepts only vary), or non-competitive (both slopes and intercepts vary). With $NADP^+$ as the constant varying inhibitor, and keeping α-ketoglutarate and ammonium ion at high levels, double reciprocal plots of reaction velocity against NADPH concentration showed clearly competitive inhibition (Fig. 21). The straight lines intersected at one point on the ordinate with the slopes varying; such a pattern is an expression of competitive inhibition. Since the product inhibitor is capable of interacting along a series of reversible steps with the variable substrates which may either precede or follow the inhibitor in binding to the enzyme, the competitive inhibition by $NADP^+$ with respect to NADPH is an indication that both these ligands bind to the same form of the enzyme.

By comparison, product inhibition by $NADP^+$ with α-ketoglutarate as the varying substrate produced a series of straight lines with a unique point of intersection on the horizontal axis (Fig. 22). Thus, $NADP^+$ would appear to be a non-competitive inhibitor of α-ketoglutarate. This result represents a deviation from an anticipated pattern of uncompetitive inhibition by $NADP^+$ with respect to α-ketoglutarate. Up to this point, the tentative order of binding of the substrates in the forward direction has been assigned as NADPH, $NH_4^+$, and α-ketoglutarate, with the products being released in the order glutamate

Figure 21. Lineweaver-Burk plot of product inhibition of the reductive amination reaction: reciprocal velocity versus reciprocal NADPH concentration at varying constant levels of $NADP^+$. High α-ketoglutarate (10.0 mM) and ammonium ion (16.6 mM) were maintained. Experiments were performed in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. Concentrations of $NADP^+$ used were

- ○ 0.308 mM
- ▲ 0.154 mM
- ◆ no $NADP^+$

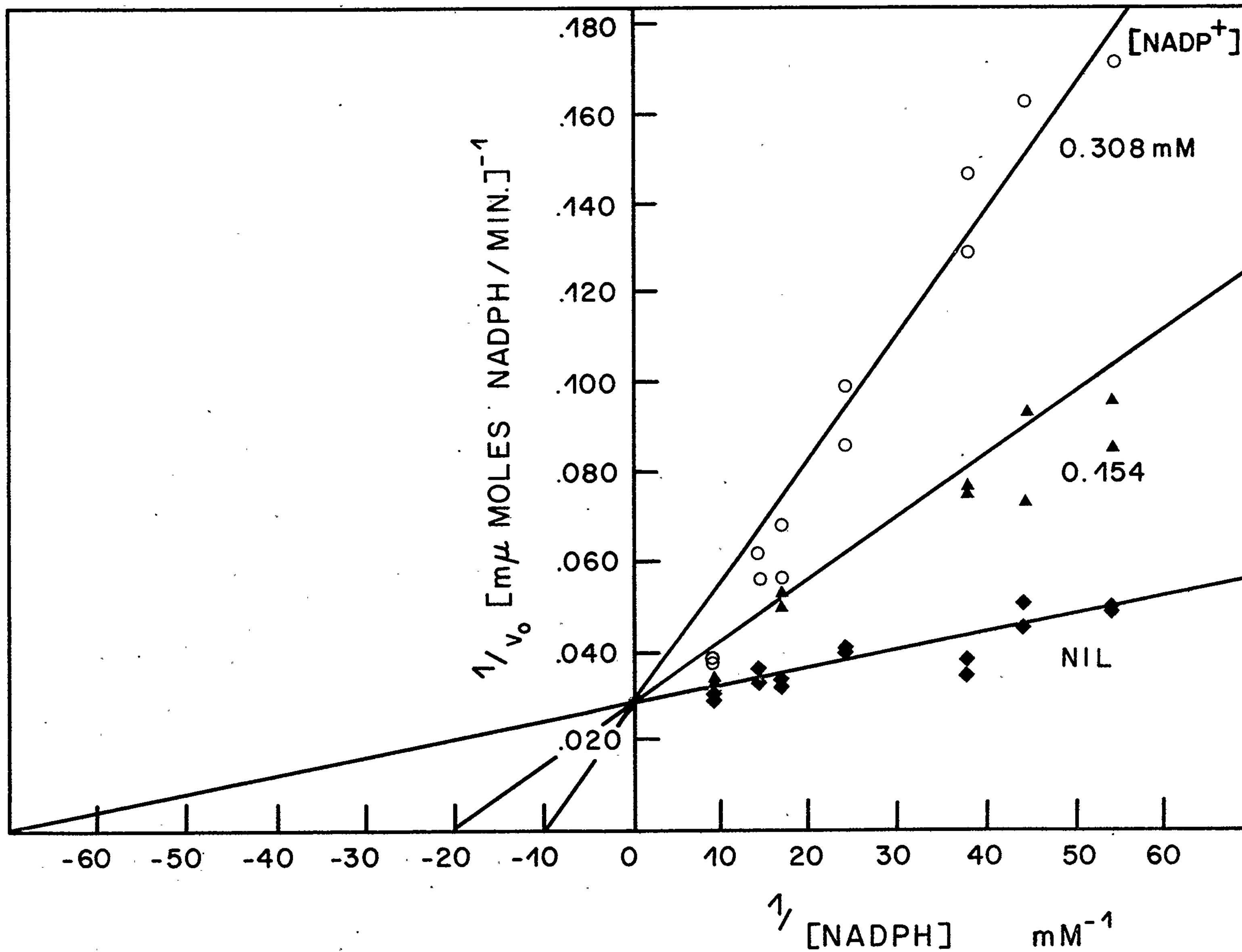

[NADP+]
0.308 mM
0.154
NIL
.180
.160
.140
.120
.100
.080
.060
.040
.020
1/vo [mμ MOLES NADPH / MIN.]-1
-60
-50
-40
-30
-20
-10
0
10
20
30
40
50
60
1/[NADPH] mM-1

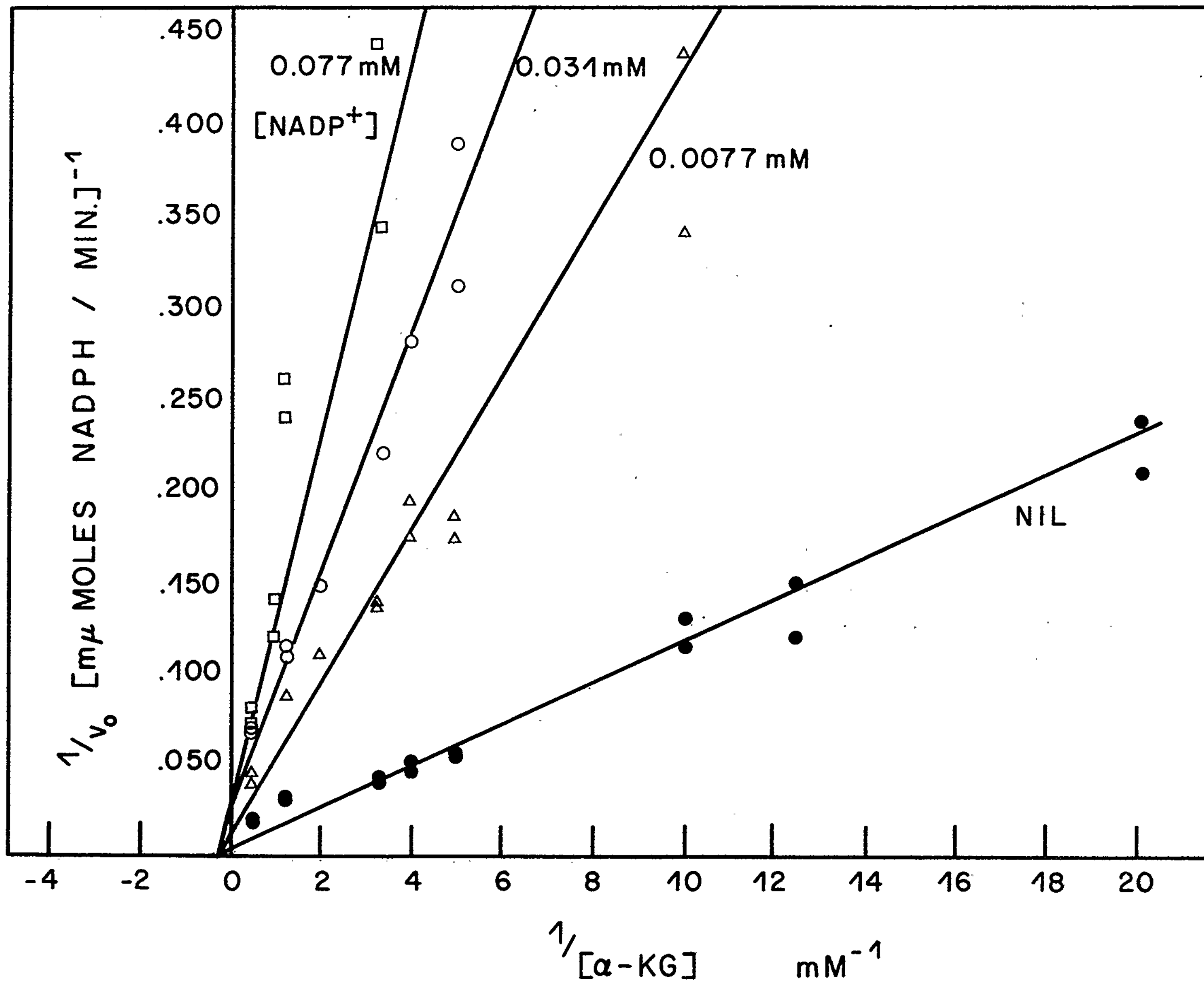
Figure 22. Lineweaver-Burk plot of product inhibition of the reductive amination reaction: reciprocal velocity versus reciprocal α-ketoglutarate concentration at varying fixed levels of $NADP^+$. Saturating ammonium ion (16.6 mM) and high NADPH (0.040 mM) were maintained. Assays were carried out in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. Concentrations of $NADP^+$ used were

□ 0.077 mM

○ 0.031 mM

△ 0.0077 mM

● no $NADP^+$



.450
.400
.350
.300
.250
.200
.150
.100
.050
0.077 mM
[NADP+]
0.031 mM
0.0077 mM
NIL
1/vo [mμ MOLES NADPH / MIN.]-1
-4
-2
0
2
4
6
8
10
12
14
16
18
20
1/[α-KG] mM-1

and $NADP^+$. Hence, $NADP^+$ and α-ketoglutarate bind to different forms of the enzyme, such that the double reciprocal plots of initial velocity versus α-ketoglutarate concentration in the presence of changing fixed levels of $NADP^+$ would show a group of straight lines with varying intercepts. In the absence of glutamate, however, a reversible connection between $NADP^+$ and α-ketoglutarate does not exist, so that one would predict $NADP^+$ to act as an uncompetitive inhibitor with respect to α-ketoglutarate. The possible significance of this result will be presented in the Discussion.

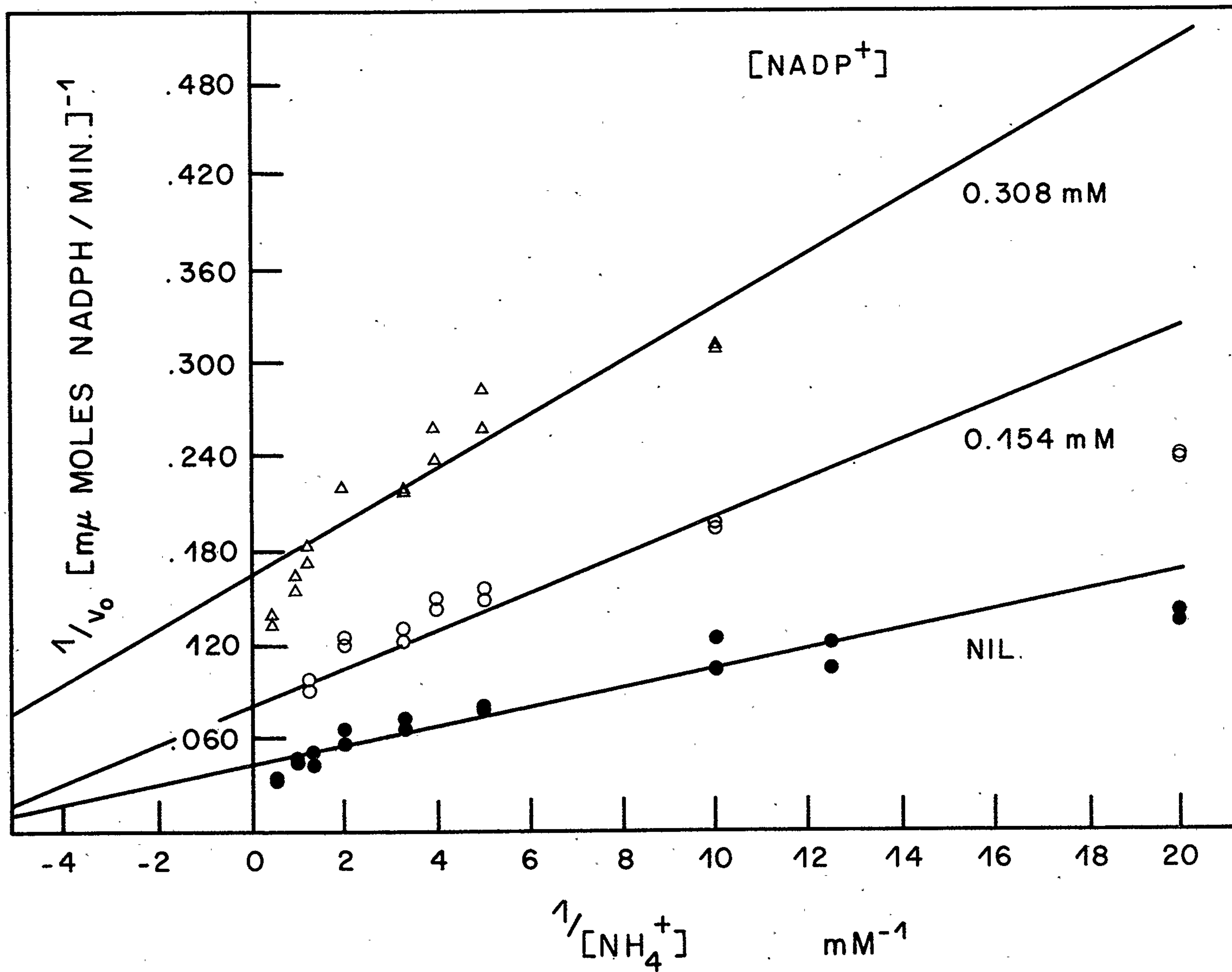
When product inhibition patterns were studied with varying ammonium chloride concentrations in the presence of high α-ketoglutarate and NADPH, double reciprocal plots of reaction velocity versus ammonia at fixed levels of $NADP^+$ showed a series of almost parallel lines (Fig. 23). Strictly parallel lines, according to Cleland, are indicative of uncompetitive inhibition by $NADP^+$ with respect to $NH_4^+$, and would be anticipated in this case, since $NADP^+$ and ammonia bind to different enzyme forms. The intercept, therefore, varies, and the sequence between them is interrupted by the release of glutamate.

Figure 23. Lineweaver-Burk plot of product inhibition of the reductive amination reaction: reciprocal velocity versus reciprocal ammonium chloride concentration at varying fixed levels of $NADP^+$. High α-ketoglutarate (10.0 mM) and NADPH (0.040 mM) were maintained. The experiments were carried out in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. Concentrations of $NADP^+$ employed were

△ 0.308 mM

○ 0.154 mM

● no $NADP^+$


[NADP+]
0.308 mM
0.154 mM
NIL.
1/v₀ [mμ MOLES NADPH / MIN.]⁻¹
.480
.420
.360
.300
.240
.180
.120
.060
-4
-2
0
2
4
6
8
10
12
14
16
18
20
1/[NH4+] mM⁻¹

I. Modulation of Glutamate Dehydrogenase by Effector Ligands

As glutamate dehydrogenase from diverse sources is known to be regulated by a variety of metabolites, it was of interest to study the interaction of regulatory ligands and Salmonella GDH. These compounds included glycolytic, TCA cycle, and related intermediates, such as PEP, pyruvate, acetyl CoA, citrate, OAA, L-malate, and L-glutamine. Stock solutions of the effector ligands were prepared, and appropriate quantities added to the standard assay system. Acetyl CoA was used at a concentration of 0.40 mM, and all of the other effectors were at 5.0 mM. The GDH reaction rate was measured in the presence and absence of these metabolites, and the effect of each expressed as per cent inhibition or activation. Details of the mechanism of inhibition were not investigated for any of the compounds included in this list. Of the intermediates thus tested, L-malate and L-glutamine showed a significant amount of inhibition — 29 and 28 per cent, respectively — at the concentration used (Table V). Citrate and OAA were inhibitory to the extent of 11 per cent and 18 per cent, pyruvate and PEP being even less effective.

The second class of cellular metabolites which were known to be active in modulation of GDH activity, the purine nucleotides — AMP, ADP, ATP, cAMP, GMP, GDP, GTP — were also tested for their possible effects on the enzyme. The results showed that when tested at 1.0 mM, adenine nucleotides consistently elicited an appreciable stimulatory response. ATP was found to be an activator to the extent of 68 per cent over the original activity, but the effect of AMP was much less

Table V.

Effect of Regulatory Ligands

| Ligand | concentration tested | per cent activation or inhibition |
|---|---|---|
| PEP | 5.0 mM | - 2 |
| pyruvate | 5.0 mM | - 9 |
| acetyl CoA | 0.4 mM | - 2 |
| citrate | 5.0 mM | - 11 |
| OAA | 5.0 mM | - 18 |
| L-malate | 5.0 mM | - 29 |
| L-glutamine | 5.0 mM | - 28 |
| AMP | 1.0 mM | + 6 |
| ADP | 1.0 mM | + 4 |
| ATP | 1.0 mM | + 68 |
| cAMP | 1.0 mM | - 17 |
| GMP | 1.0 mM | - 12 |
| GDP | 1.0 mM | + 15 |
| GTP | 1.0 mM | + 25 |

- indicates inhibition; + activation.

dramatic (Table V). The guanine nucleotides varied in their effects, with GTP showing an increase of 25 per cent over the original activity when tested at 1.0 mM. It is noteworthy that whereas GMP proved to be mildly inhibitory, GDP increased the activity of GDH by 15 per cent.

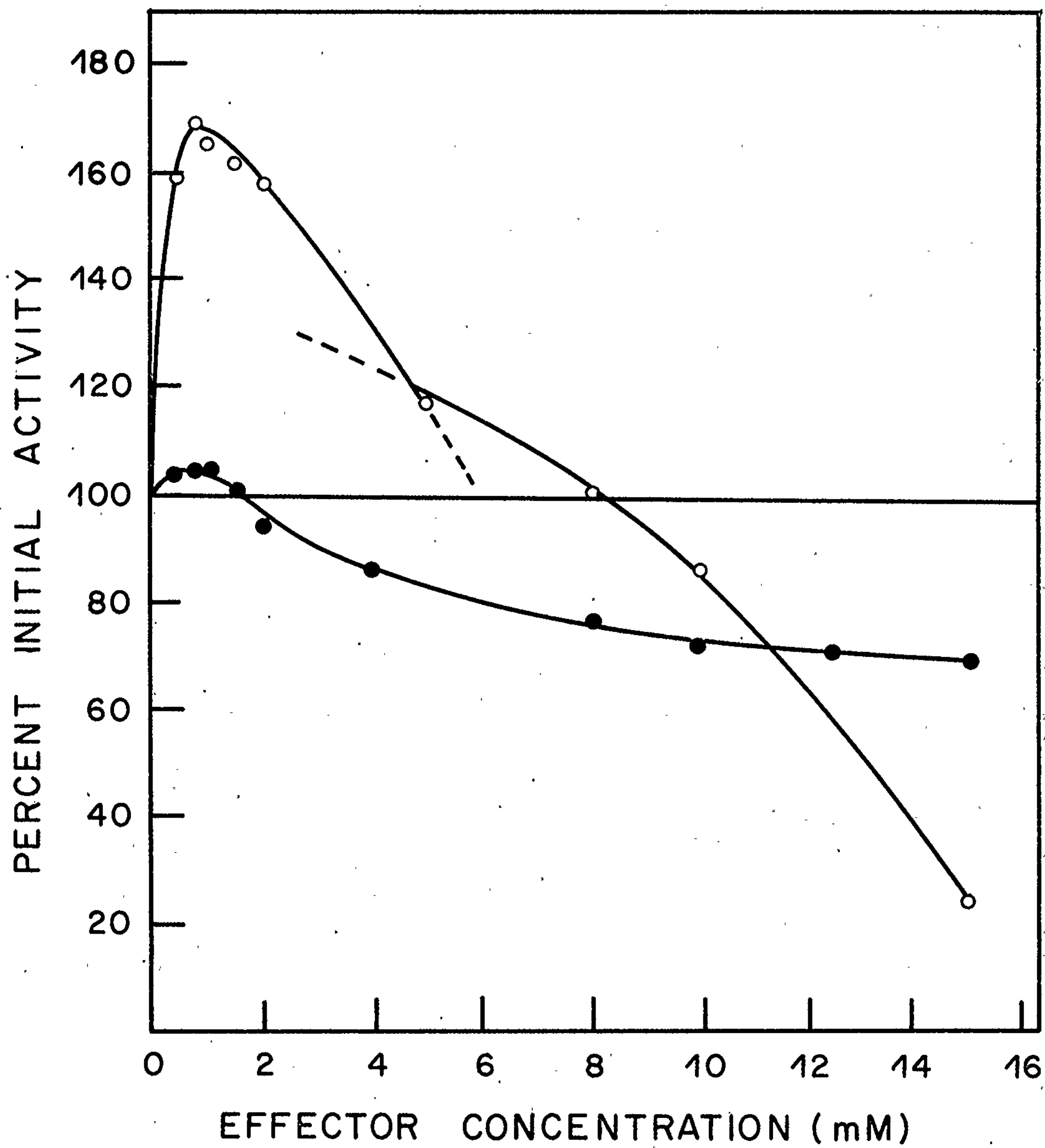
To elucidate the role of ATP and AMP in the regulation of activity of GDH, a range of concentrations, varying from 0 to 15.0 mM of each ligand, was included in the reaction system. As evidenced by the data of Figure 24, an enhancement of catalytic activity of up to 68 per cent was observed with 0.8 mM ATP, but at higher concentrations of this nucleotide, the level of stimulation progressively decreased, and at 8.0 mM no change in the initial velocity was recorded. At still higher concentrations, ATP exerted an inhibitory effect on GDH activity. It is apparent that the curve representing the ATP effect in Figure 24 can be resolved into two separate components, the first one encompassing concentrations up to 5.0 mM, and the second one including the response elicited by higher ATP levels. Similarly, AMP also behaved as an activator when employed at levels of up to 1.0 mM, but at concentrations ranging from 2.0 to 15.0 mM, it led to a moderate inhibition of the enzyme.

Since the range of concentrations utilized above was very wide, the effects of ATP and AMP were re-examined over a narrower range. An activatory response similar to that documented in the foregoing experiment was recorded when these two compounds were used in a 0 to 3.0 mM range (Fig. 25). The degree of activation was also comparable to that previously described.

Figure 24. Effect of regulatory ligands on the activity of GDH. AMP and ATP were tested separately over a range of concentrations from 0 to 15.0 mM; per cent initial activity was then determined. Assays were conducted in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C. The final protein concentration was 1.2 μg/ml.

○ ATP

● AMP

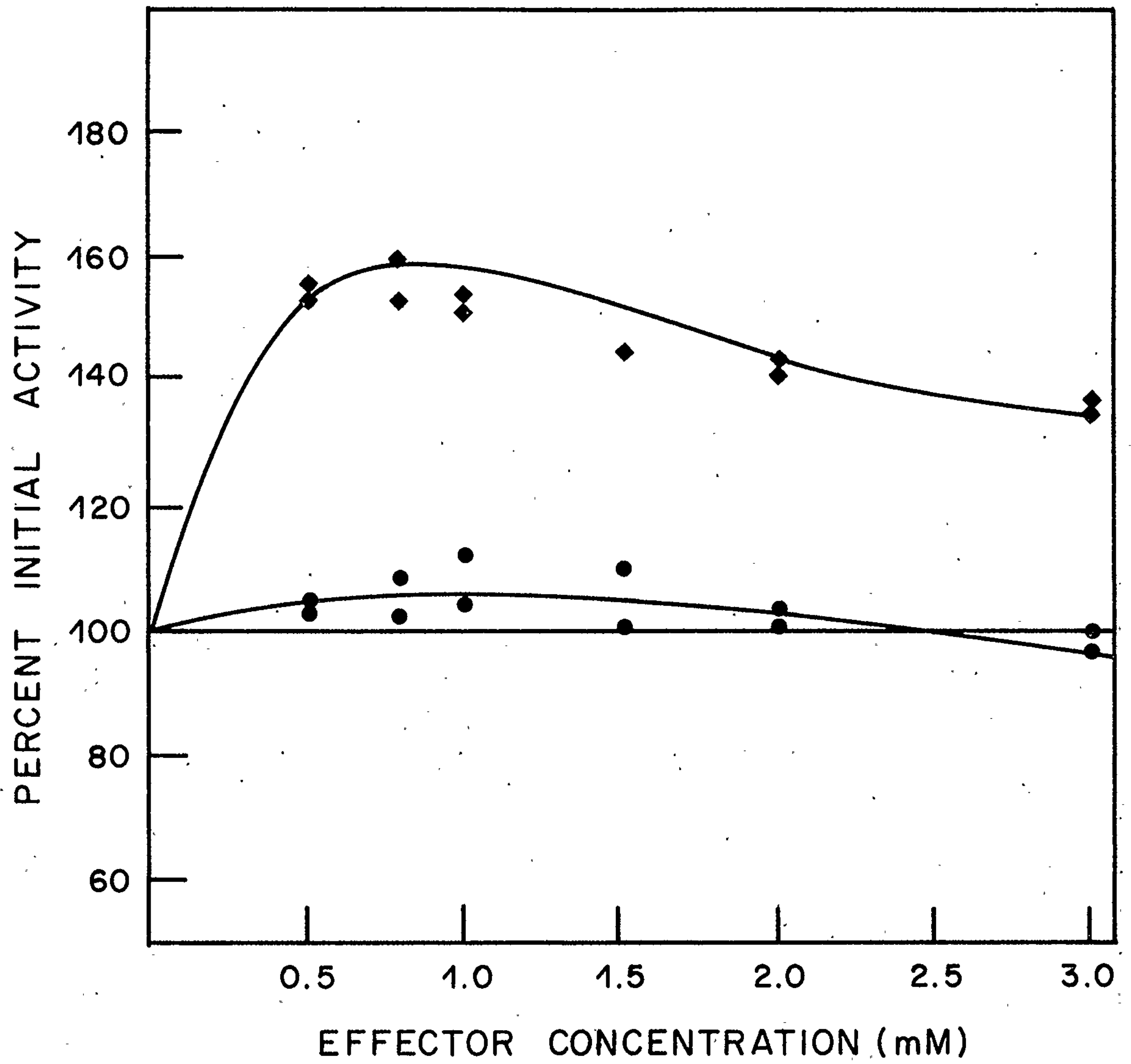


PERCENT INITIAL ACTIVITY
180
160
140
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
EFFECTOR CONCENTRATION (mM)

Figure 25. Effect of regulatory ligands on the activity of GDH. Identical conditions to those utilized in Figure 24, except that concentrations of AMP and ATP varied from 0 to 3.0 mM. Protein concentration was 1.2 μg/ml.

◆ ATP

● AMP



PERCENT INITIAL ACTIVITY
180
160
140
120
100
80
60
0.5
1.0
1.5
2.0
2.5
3.0
EFFECTOR CONCENTRATION (mM)

J. Inactivation by Urea, SDS, Guanidine Hydrochloride, and p-Hydroxymercuribenzoate

A kinetic investigation of the process of inactivation of glutamate dehydrogenase in the presence of protein denaturants, urea, guanidine hydrochloride, sodium dodecyl sulphate, and the sulphydryl reagent, p-hydroxymercuribenzoate, was carried out by determining the loss of enzymatic activity as a result of interaction with these agents. Freshly prepared solutions of reagents were used in all experiments. Stock solutions were prepared and diluted to the appropriate concentration just before use. Incubation mixtures for studies dealing with the first three denaturants were prepared by mixing 29 μg of the purified enzyme in a volume of 10 μl and 190 μl of a given concentration of the inactivating agent in 0.05 M Tris-HCl, pH 7.5. The test tubes containing the sample were allowed to stand in an ice bucket for a period of one hour, during which time enzyme activity was determined at regular intervals.

For pHMB experiments it was necessary to alter the composition of the mixture in order to observe the effect of ligand binding on the kinetics of inactivation. Thus, the control contained 10 μl enzyme, 10 μl 0.05 M Tris-HCl, pH 7.5, and 180 μl of a given concentration of pHMB. Preincubation in the presence of either NADPH or α-ketoglutarate was carried out by including appropriate concentrations of the ligand in the mixture. A constant amount of enzyme (29 μg protein) was used in the final mixture. The activity of the two ligands was evaluated by comparing the rate of enzyme inactivation caused by pHMB alone with

that observed in the presence of sulphydryl reagent plus the substrate. Aliquots were withdrawn at ten minute intervals and assayed for residual enzyme activity by means of the standard assay system.

1. Urea: When GDH was incubated in the presence of urea solutions, varying in concentration from 1.0 to 4.0 M, no inactivation was observed under the above conditions. Only when added at very high concentrations did urea bring about a loss of enzyme activity over a one hour period (Fig. 26). In 8.0 M urea, less than 10 per cent of the initial activity remained after 60 minutes, whereas interaction with 7.0 M urea left 50 per cent of the activity intact at the end of the same time interval. For 6.0 M urea, the rate of decrease in activity was considerably slower; a 20 per cent loss occurred after one hour. These observations attest to the extreme resistance of Salmonella glutamate dehydrogenase toward urea. On plotting log residual activity as a function of treatment time for the above-mentioned urea concentrations, straight lines resulted in all instances, indicating first order kinetics for the inactivation process.

2. Sodium dodecyl sulphate: Using the conditions described in the foregoing paragraphs, the effect of SDS was subsequently investigated. Since treatment in a 2.0 per cent (w/v) solution destroyed all activity within 10 minutes, considerably lower concentrations of SDS were employed in order to extend the inactivation time to 60 minutes. Thus, at 0.7, 0.5, and 0.3 per cent (w/v) SDS, the residual activity of GDH following incubations of one hour were 5 per cent, 12 per cent, and 47 per cent, respectively. Figure 27 shows that

Figure 26. Semilogarithmic plot of inactivation of GDH in various concentrations of urea. Final protein concentration was 29 μg in a volume of 200 μl.

● 6.0 M urea

△ 7.0 M urea

○ 8.0 M urea

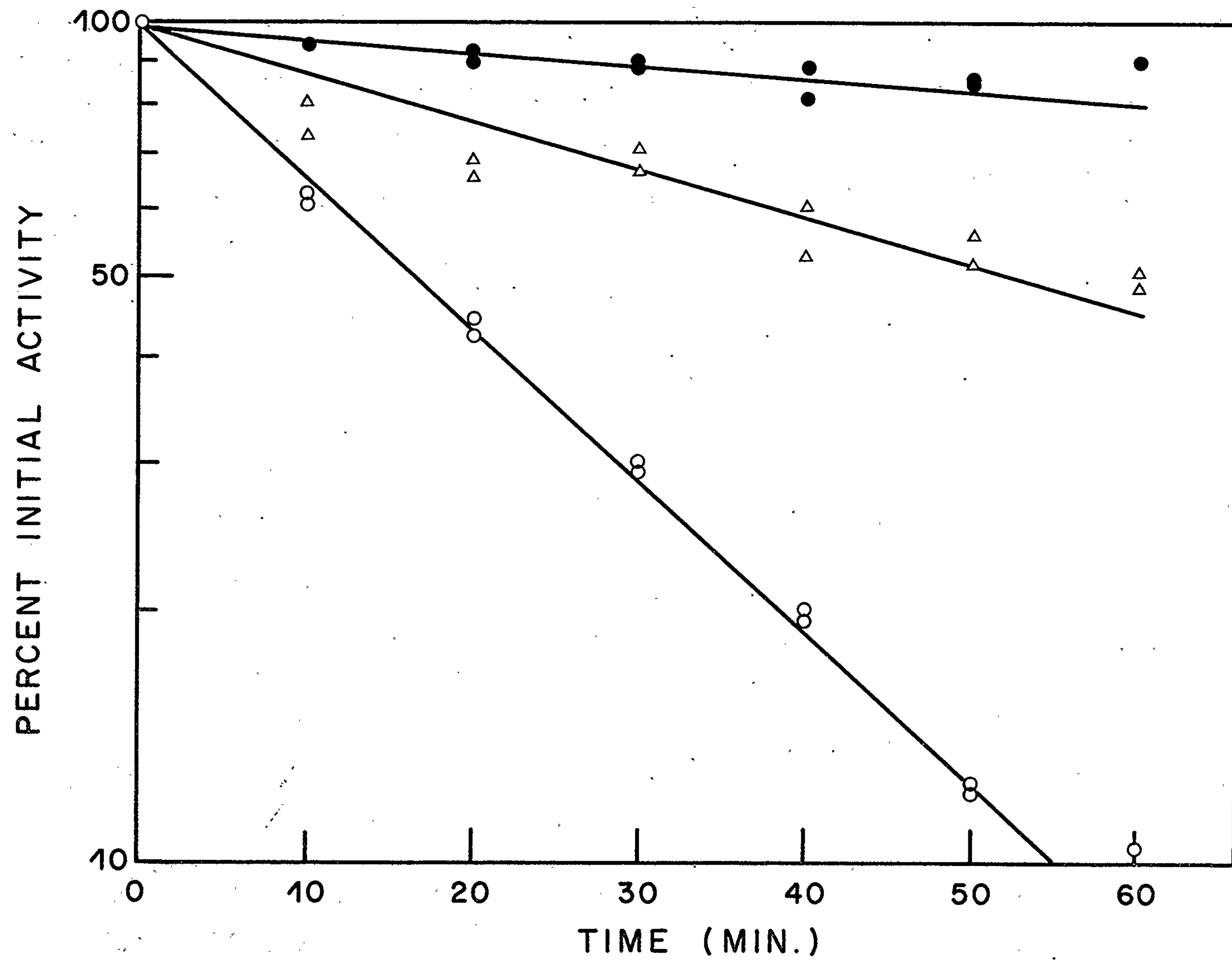
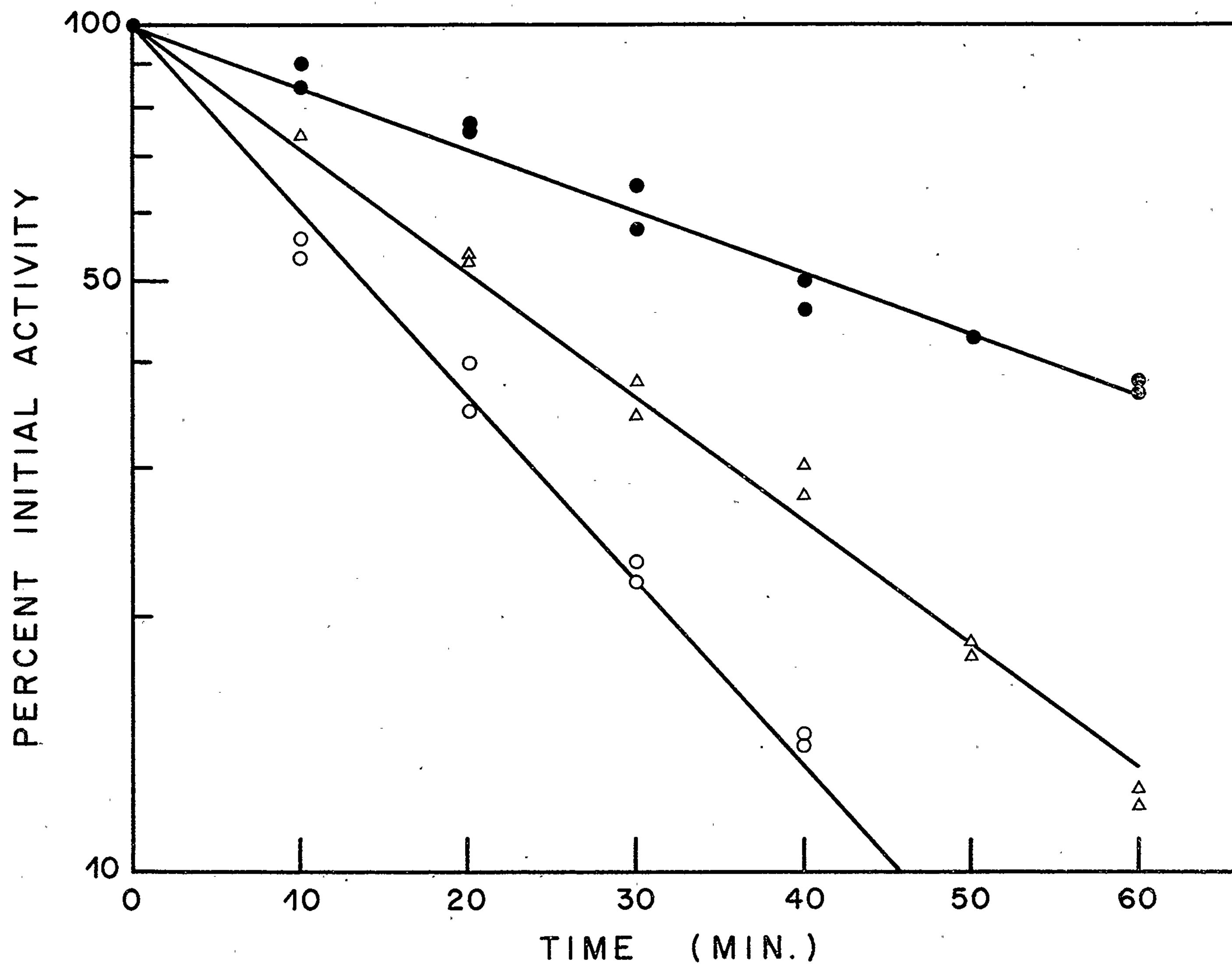

100
50
10
PERCENT INITIAL ACTIVITY
0
10
20
30
40
50
60
TIME (MIN.)

Figure 27. Semilogarithmic plot of inactivation of GDH in various concentrations of SDS over a period of 60 minutes. Final protein concentration was 29 μg in a volume of 200 μl.

● 0.3 per cent (w/v) SDS

△ 0.5 per cent (w/v) SDS

○ 0.7 per cent (w/v) SDS



100
50
10
0
10
20
30
40
50
60
TIME (MIN.)
PERCENT INITIAL ACTIVITY

semilogarithmic plots of residual activity versus time gave straight lines, indicating that SDS-induced inactivation of glutamate dehydrogenase follows first order kinetics.

3. Guanidine hydrochloride: The effect of guanidine hydrochloride differed from that observed with both urea and SDS in that the response of GDH towards this denaturant did not appear to be governed by a first order rate process. In the presence of 2.50 M, 2.75 M, and 3.00 M guanidine hydrochloride, plots of log residual activity versus sampling time revealed biphasic curves (Fig. 28). It is likely, therefore, that a complex mechanism is involved in the guanidine hydrochloride-catalyzed denaturation of this enzyme.

4. p-Hydroxymercuribenzoate: Determination of the rate of inactivation as a consequence of interaction with the sulphydryl reagent, pHMB, revealed that relatively high concentrations of the compound, ranging from 1.0 to 10.0 mM, were required to give a significant loss of catalytic activity over a one hour period. In the presence of 10.0 mM pHMB, less than 10 per cent of the initial activity remained after 40 minutes of incubation, whereas at 1.0 mM, only 30 per cent loss was recorded after one hour (Fig. 29).

Since it was of interest to determine the role, if any, of ligand binding at the active centre, two of the substrates of GDH were tested for their effects as possible protectants against inactivation by pHMB. At a level of 5.0 mM pHMB, a decrease of 80 per cent of the initial activity was observed in one hour. But if GDH were preincubated in the presence of 1.5 mM NADPH, and then transferred to the reaction

Figure 28. The rate of inactivation of GDH in various concentrations of guanidine hydrochloride in 60 minutes. Final protein concentration was 29 μg in 200 μl.

- ● 2.50 M GuHCl
- △ 2.75 M GuHCl
- ○ 3.00 M GuHCl

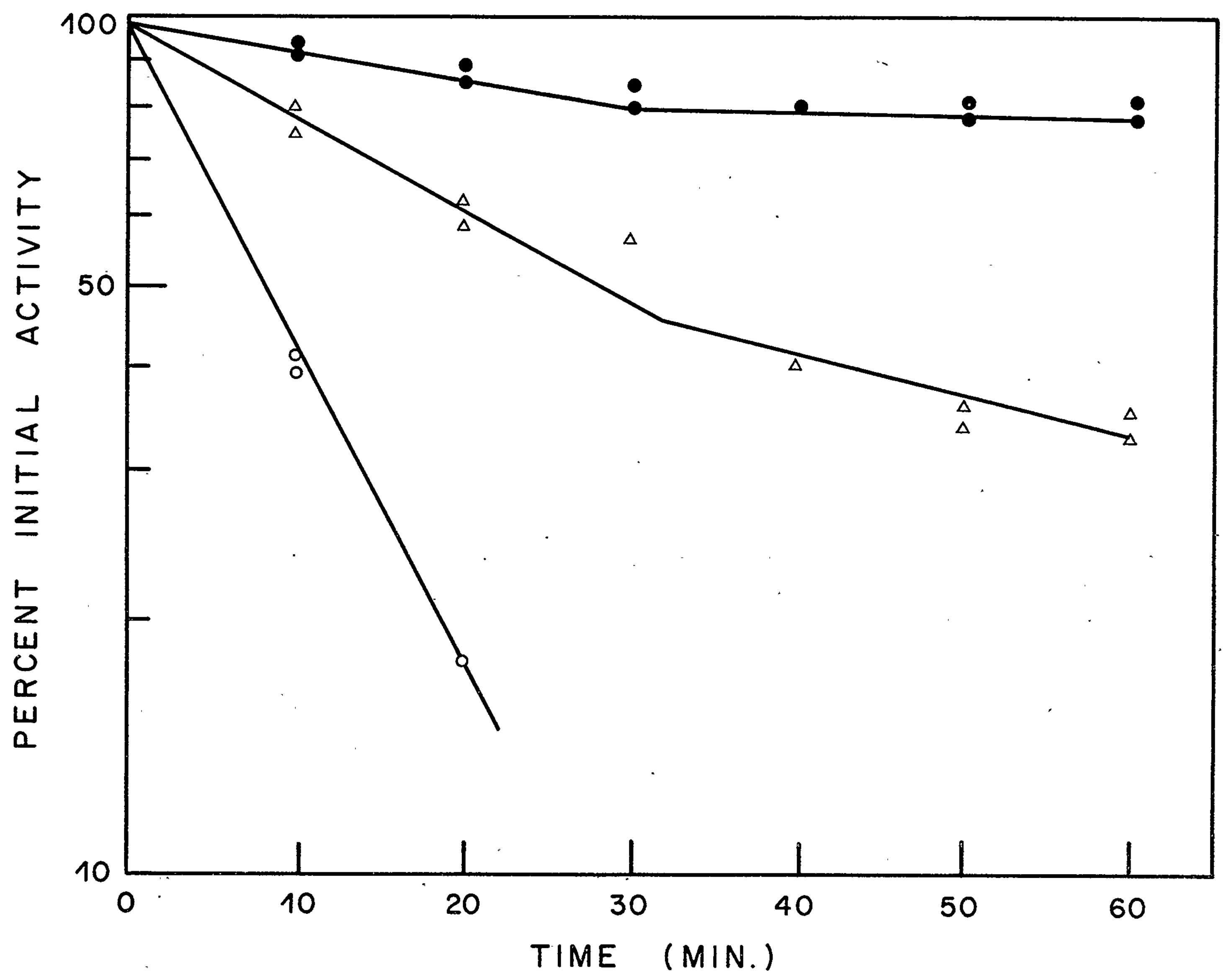
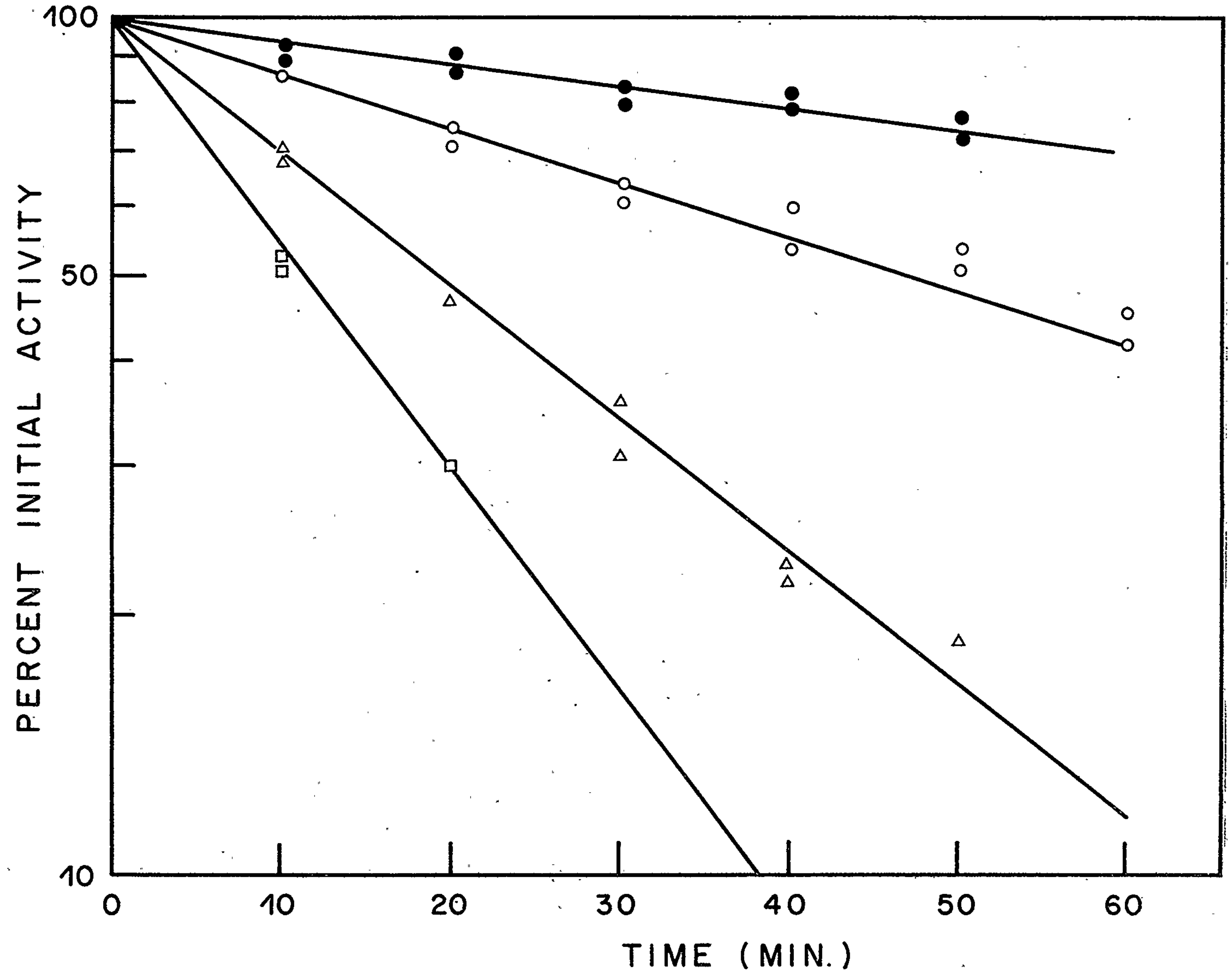

100
50
10
PERCENT INITIAL ACTIVITY
0
10
20
30
40
50
60
TIME (MIN.)

Figure 29. Inactivation of GDH in the presence of various concentrations of pHMB over a 60 minute period. Final protein concentration was 29 μg in 200 μl.

- ● 1.0 mM pHMB
- ○ 2.5 mM pHMB
- △ 5.0 mM pHMB
- □ 10.0 mM pHMB

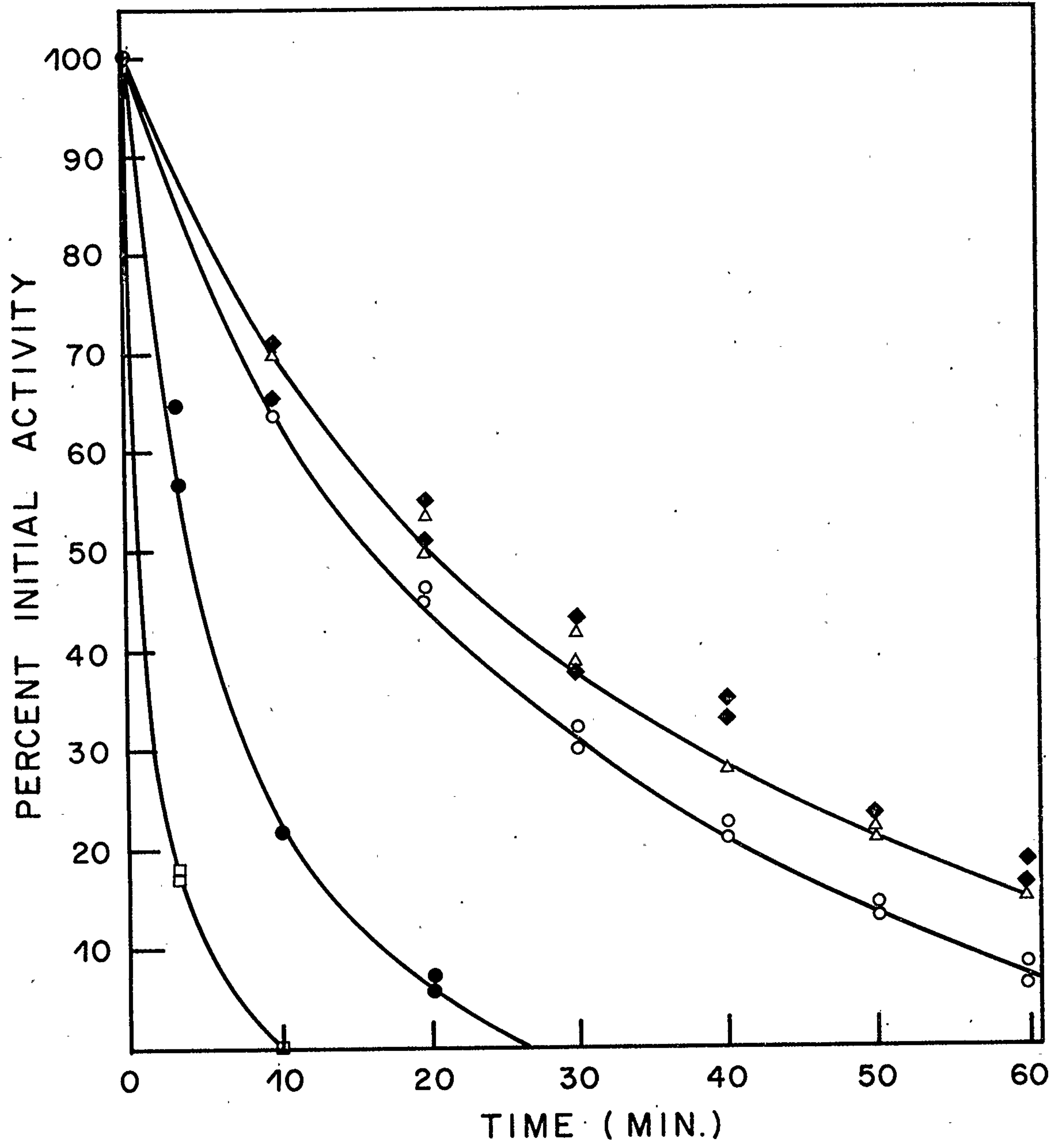

PERCENT INITIAL ACTIVITY
100
50
10
0
10
20
30
40
50
60
TIME (MIN.)

mixture containing the same level of pHMB, a rapid loss of activity occurred within 10 minutes (Fig. 30). Decreasing amounts of NADPH in the preincubation mixture gave corresponding increases in the times required for a comparable degree of inactivation. For instance, with 15.0 μM NADPH in the preincubation step, the loss of activity corresponded to that in the presence of pHMB alone. If a similar preincubation were carried out with α-ketoglutarate at concentrations of up to 5.0 mM, no difference in the rate of loss of activity was seen as compared to the control reaction mixture.

As the preparation used for the inactivation experiments was derived by ammonium sulphate precipitation of the Sephadex G-200 eluate, even after extensive dialysis of the enzyme it was not possible to eliminate all of the ammonium ions associated with the enzyme. For this reason, the effect of preincubation with $NH_4^+$ on pHMB-induced inactivation could not be investigated.

Figure 30. The effect of preincubation in the presence of substrates on pHMB-induced inactivation of GDH. Protein concentration was 29 μg in a total volume of 200 μl, and pHMB was 5.0 mM.

| Symbol | Addition |
| --- | --- |
| △ | no additions |
| □ | 1.5 mM NADPH |
| ● | 150. μM NADPH |
| ○ | 15.0 μM NADPH |
| ◆ | 5.0 mM α-ketoglutarate |


100
90
80
70
60
50
40
30
20
10
PERCENT INITIAL ACTIVITY
0
10
20
30
40
50
60
TIME (MIN.)

# CHAPTER IV

## DISCUSSION

Purification of glutamate dehydrogenase from Salmonella typhimurium LT-2 has shown that the enzyme in this organism comprises about 0.4 per cent of the total soluble protein. Judging by the results obtained from polyacrylamide disc gel electrophoresis, the 190 - fold purified preparation obtained following gel filtration was approximately 75 per cent of the staining material.

It is of interest that only an $NADP^+$-specific GDH was detected in this bacterium. In micro-organisms in general, $NAD^+$-dependent, $NADP^+$-dependent, or in some instances, two distinct enzymes, have been reported. The presence of only the $NADP^+$-GDH is not surprising, in the light of the possible role of this enzyme in the formation of glutamate. $NADP^+$ is considered to function chiefly in anabolic reactions, and it is logical, therefore, to expect that the $NADP^+$-dependent glutamate dehydrogenase performs a biosynthetic role in S. typhimurium.

In examining the kinetic parameters determined for the binding of substrates to Salmonella glutamate dehydrogenase, it is evident that the values derived in this study do not differ significantly from those reported for other organisms (Table VI). The $K_m$ for NADPH (0.019 mM) and for $NADP^+$ (0.013 mM) suggest that a very tightly bound [enzyme-cofactor] complex is the initial species formed in the reaction sequence in the forward and reverse directions, respectively. Similarly for Clostridium SB 4, Bacillus licheniformis, Brevibacterium flavum, Thiobacillus novellus, and Nitrosomonas europaea, Michaelis constants are close to the two values cited above. By comparison,

Table VI. Michaelis Constants* for GDH of Bacterial Origin

| Organism | α-KG | $NH_4^+$ | NADH | NADPH | Glutamate | $NAD^+$ | $NADP^+$ | assay conditions | reference |
|---|---|---|---|---|---|---|---|---|---|
| *Bacillus licheniformis* | 6.7 | 5.5 | | 0.12 | 39.0 | | N/R | 0.05 M Tris-HCl pH 8.0, 25°C | Phibbs and Bernlohr (1971) |
| *Brevibacterium flavum* | 5.72 | 3.08 | | 0.027 | 100 | | 0.033 | 0.1 M Tris-HCl pH 8.2, 25°C | Shiio and Ozaki (1970) |
| *Nitrosomonas europaea* | 4.3 | 16.1 | | 0.049 | 6.7 | | 0.0079 | 0.05 M $K_2HPO_4$ pH7.7, 25°C | Hooper *et al.* (1967) |
| *Clostridium* SB 4 | 0.65 | 0.32 | 0.01 | | 1.8 | 0.01 | | 0.1 M Tris-HAc pH 8.0, 25°C | Winnacker and Barker (1970) |
| *Rhodospirillum rubrum* | 2.3 | 12.0 | N/R | | N/R | N/R | | N/R | Bachofen and Neeracher (1967) |
| *Thiobacillus novellus* | 7.4 | 7.5 | | 0.077 | 35.5 | | 0.08 | 0.2 M Tris-HCl pH 7.5, 25°C | LéJohn *et al.* (1968) |
| *Thiobacillus novellus* (without AMP) | 0.67 | 0.50 | 0.004 | | 13.3 | 0.19 | | " | " |
| (with AMP) | 6.7 | 7.4 | 0.0363 | | 11.8 | 0.21 | | " | " |

*All $K_m$ values given in mM concentrations. N/R - not reported

considerable variation in the binding of $NH_4^+$ to bacterial glutamate dehydrogenases is apparent. A range from 0.32 mM for the Clostridium enzyme to 16.1 mM for Nitrosomonas indicates significant differences in binding efficiency, although such differences may be a reflection of the order of addition of this substrate to the enzyme. The third metabolite required in the amination reaction is α-ketoglutarate, where a ten-fold range of reported $K_m$ values is witnessed. Glutamate, on the other hand, has been shown to possess relatively high $K_m$'s in comparison to the value for α-ketoglutarate in the forward reaction; for GDH of Salmonella, the Michaelis constant for glutamate was determined as 50.0 mM. It is worthy of note at this point that the $K_m$ for glutamate in S. typhimurium is 12.5 times the $K_m$ for α-ketoglutarate; one may conclude from this that the reaction would appear to be essentially unidirectional, in favour of glutamate biosynthesis.

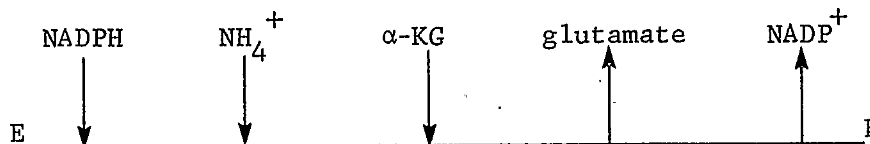
Detailed kinetic experiments were conducted following Cleland's recommendations (1963a,b,c) in order to arrive at a kinetic mechanism of addition of substrates to glutamate dehydrogenase. The plots of the data obtained by independently varying one substrate, at changing fixed concentrations of a second, and constant high levels of the third, were presented in Chapter III. The effect by $NADP^+$ on the binding of NADPH clearly demonstrated competitive product inhibition. Each of these cofactors, therefore, attaches to the same free enzyme form, and hence in the forward reaction, NADPH is the first of the three substrates to bind to GDH. Determination of the second substrate to bind to the enzyme required the evaluation of several kinetic plots. With NADPH as the variable substrate, at changing fixed levels of

α-ketoglutarate, and saturating $NH_4^+$ (16.6 mM), a series of near-parallel lines was obtained (Fig. 15). Such a pattern, according to Cleland, is exemplary of a ping pong type of mechanism. In the logical extension to the three substrate case however, such parallel lines indicate a block in the reaction sequence due to saturating concentrations of one substrate. By comparison, a double reciprocal plot of reaction velocity versus NADPH concentration at a level of ammonium chloride considerably lower than that employed above (2.76 mM) and again at changing fixed levels of α-ketoglutarate, revealed a set of straight lines which intersected at a unique point on the negative abscissa (Fig. 14). In such a case, the $K_m$ for NADPH became independent of ammonium ion, and a reversible connection was established between NADPH and α-ketoglutarate. Examination of the Lineweaver-Burk plots of reciprocal NADPH both at high and at low levels of α-ketoglutarate and changing fixed ammonium ion (Figs. 12, 13) revealed no evident differences in behaviour with respect to the enzyme form to which α-ketoglutarate was binding. No ping pong mechanism emerged from these data, in direct contrast to the situation with ammonium ion.

Although initial velocity studies discussed in the foregoing pages are consistent with ammonium ion and α-ketoglutarate binding second and third in the reaction sequence, an analysis of product inhibition patterns did not yield unambiguous results in this regard. For instance, in the product inhibition by $NADP^+$ with respect to α-ketoglutarate, the double reciprocal plot of initial reaction velocity versus α-ketoglutarate concentration at varying fixed levels

of the inhibitor (Fig. 22) showed a family of lines intersecting at a single point on the horizontal axis, indicating a non-competitive situation. However, a reversible connection between these ligands is not apparent in the complete absence of glutamate, and in such a case, the Lineweaver-Burk plot would be expected to show parallel lines. The implication of the results of product inhibition, considered in conjunction with the lack of absolutely parallel lines in Figure 15, is that the possibility of a random binding order of $NH_4^+$ and α-ketoglutarate cannot be ignored. On the other hand, the kinetic data earlier presented showed that the [enzyme-NADPH] complex did not respond in an identical manner towards $NH_4^+$ and α-ketoglutarate, supporting a sequential order of binding referred to earlier.

The results of these kinetic analyses of glutamate dehydrogenase of S. typhimurium may be tentatively summarized by postulating an ordered Ter-Bi mechanism for the addition of substrates: NADPH binds initially to the enzyme, $NH_4^+$ is the second substrate, and α-ketoglutarate binds third. Glutamate is the first product released, followed by $NADP^+$.




This proposed ordered mechanism is identical to that advanced by Frieden (1959) for the bovine liver GDH. Recently, with the use of difference spectroscopy, Cross (1972) was able to obtain support for

the occurrence of a [glutamate dehydrogenase-NADPH-α-ketoglutarate] complex as an active or transient intermediate in the reaction mechanism. This is consistent with a random addition of $NH_4^+$ and α-ketoglutarate, following the compulsory binding of NADPH as the first substrate.

In Clostridium SB 4, Winnacker and Barker (1970) postulated a sequential mechanism in which NADH binds first to the enzyme, and is followed in random order by α-ketoglutarate and ammonium ion. Shiio and Ozaki (1970) reported that the order of addition to the GDH of Brevibacterium flavum was NADPH, α-ketoglutarate, $NH_4^+$. LéJohn et al. (1968), on the other hand, proposed the order NADPH, $NH_4^+$, α-ketoglutarate for the $NADP^+$-specific GDH of Thiobacillus novellus. But for the $NAD^+$-dependent GDH from the latter organism, an ordered mechanism was evidenced only in the presence of AMP, the deduced order from kinetic data being NADH, α-ketoglutarate, $NH_4^+$. In the absence of AMP, the mechanism of the $NAD^+$-GDH was allosterically controlled, but the possibility of random addition was, again, not ruled out.

The proposed mechanisms for glutamate dehydrogenase from a variety of sources considered above do not lead to a consistent pattern. With the sole exception of bovine liver GDH, kinetic investigations on all enzymes were conducted by initial velocity and product inhibition studies only. It is appropriate to point out that these two methods alone are not sufficient to establish, unequivocally, the mechanism for complex reactions. It is often necessary to confirm the proposed mechanisms by the use of more refined techniques. The application of difference spectroscopy, isotope exchange, and relaxation methods is

imperative for this purpose. However, it is impossible to employ these refined techniques with enzyme preparations that are not homogeneous. A significant advance in this direction has been made with the bovine liver GDH because of its commercial availability in a highly purified state. Among the bacterial sources, GDH has been purified to homogeneity from Clostridium SB 4 and from Micrococcus aerogenes; however, the above-mentioned techniques have not been applied to determine their respective reaction mechanisms.

The most striking structural feature to emerge from the study of the Salmonella GDH was its remarkable resistance to high concentrations of urea, sodium dodecyl sulphate, and guanidine hydrochloride. For instance, upon exposure to 8.0 M urea, a period of about 40 minutes was required to destroy the integrity of the active site. Such a degree of resistance is probably a reflection of the paucity of hydrophobic residues around the active site of the protein. It was not possible to assess the contribution of subunit dissociation of the enzyme, as a result of the elevated concentrations of the denaturants, towards the inactivation process.

The use of the sulphydryl reagent, p-hydroxymercuribenzoate, clearly demonstrated that the molecular array of free thiol groups was necessary in order to maintain enzyme activity. Again, GDH proved to be remarkably resistant to this compound, since concentrations of 10.0 mM were required for inactivation over a one hour period. Experiments designed to test for binding of the substrates, NADPH and $\alpha$-ketoglutarate, to the native GDH revealed a dramatic enhancement of inactivation upon preincubation with NADPH. By comparison,

α-ketoglutarate did not appear to cause any alteration in the pattern of pHMB inactivation. An explanation that can be offered for this effect is that the binding of NADPH initiated a conformational change in the enzyme, leading to exposure of free -SH groups which were previously unavailable for pHMB attack. The inability of α-ketoglutarate to affect the course of pHMB-induced inactivation is probably because it binds only to the [enzyme-NADPH] complex, and not to the free enzyme. Information regarding the interaction of other bacterial glutamate dehydrogenases with protein denaturants is, unfortunately, not available; therefore the results of this study cannot be compared with any other.

A number of mechanisms for the regulation of GDH were outlined in the Introduction. Among these was the necessity of considering the role of glycolytic and TCA cycle intermediates in the metabolic circuitry. Investigation into the sensitivity of GDH to acetyl CoA, pyruvate, PEP, citrate, OAA, L-malate, and L-glutamine was undertaken. However, only the last two metabolites of this series were demonstrated to exert an appreciable inhibitory effect on Salmonella GDH, at 5.0 mM concentration. Whether, in fact, such inhibition was a regulatory effect or simply due to the structural similarity of these compounds to the substrate, α-ketoglutarate, remains undetermined.

ATP proved to be the most significant regulatory ligand in influencing the activity of GDH. The optimal effect of this adenylate was activation of the enzyme by 68 per cent above the initial reaction velocity at a concentration of 0.8 mM. Higher levels led to a reduced activation of the enzyme, and at concentrations greater than 8.0 mM,

an inhibitory effect was manifested. A similar result was found using the mononucleotide, AMP, although the enhancement of initial velocity was less pronounced. Again, an optimal concentration was revealed at 0.8 mM, with increasing concentrations giving rise to inhibition of catalytic activity. From these observations, one may therefore postulate the existence of one, or possibly two, regulatory sites for adenine nucleotides on the Salmonella GDH. A high intracellular energy level, represented by ATP, favours a decreased production of energy and a corresponding increase in the biosynthesis of the amino acid, glutamate. The action of ATP has amply demonstrated regulation at an essential link between carbohydrate metabolism and amino acid metabolism. On the other hand, the energy charge ratio, ATP/AMP, did not appear to be significant in modulating the activity of GDH in Salmonella, since an equal and opposite effect of these two nucleotides was not observed.

Rigorous control of glutamate dehydrogenase from several bacterial species appears to be lacking. In the glutamate-producing Brevibacterium flavum, an exhaustive investigation into the effect of twenty amino acids, ten metabolic intermediates, and the adenine and guanine nucleotides, revealed that only GMP, at 2 mM, inhibited the deamination reaction by 53 per cent. The physiological significance of this inhibition remains obscure. Similarly, in Nitrosomonas europaea, neither amino acids nor adenine and guanine nucleotides appeared to alter the behaviour of GDH. However, in this organism, NADPH oxidation was inhibited 50 per cent by the following additions to the assay mixture: 0.5 M NaCl or $NH_4Cl$, 20 mM D-glutamate,

$2 \times 10^{-4}$ M NADH, $10^{-3}$ M $NADP^+$, and $4.4 \times 10^{-4}$ M NADPH. By comparison, in the direction of glutamate breakdown, $NADP^+$ reduction was inhibited 50 per cent by 1.0 mM $NH_4Cl$, 20 mM D-glutamate, $4 \times 10^{-4}$ M NADH, and $3 \times 10^{-5}$ M NADPH. Phibbs and Bernlohr (1970) were unable to detect any changes in the kinetic behaviour of GDH from Bacillus licheniformis upon the addition of AMP, ADP, ATP, or cAMP. Nor was any regulatory influence witnessed by several effector ligands on a homogeneous preparation of glutamate dehydrogenase from the lysine-fermenting anaerobe, Clostridium SB 4 (Winnacker and Barker, 1970). And finally, in Thiobacillus novellus, LéJohn et al. (1968) found no effect of AMP on the activity of the $NADP^+$-specific enzyme. Inclusion of AMP in the assay for the $NAD^+$-dependent GDH, however, caused a dramatic alteration of the kinetic parameters.

In a review of amino acid metabolism in micro-organisms, Umbarger issued the rather dogmatic comment (1969), "In bacteria, there has been little evidence of a regulation of glutamate biosynthesis. The absence of regulation is not surprising since its formation, by either an NADPH-linked amination or amino group transfer to α-ketoglutarate, is reversible." By comparison, Shapiro and Stadtman (1970) felt justified in stating that "NADPH-dependent amination, catalyzed by glutamate dehydrogenase, has been shown to be subject to rather intensive control in micro-organisms". Their apparently conclusive observation was derived from experiments similar to those of Varrichio (1970) showing that in E. coli grown on selected nitrogen sources, there were low activities of glutamate dehydrogenase, but cells cultured on two alternate nitrogen sources yielded high GDH activities.

Nevertheless, in considering the regulation of glutamate dehydrogenase from a number of bacterial species, it is evident that the enzyme is not the target for complex metabolic control. Kinetic investigation into the relative affinities of glutamate versus α-ketoglutarate tends to substantiate the belief that GDH specific for the cofactor NADPH is primarily involved in the biosynthesis of glutamate. In Salmonella, therefore, the reaction significantly favours the formation of this amino acid and probably *in vivo* this reaction proceeds in a unidirectional manner. Similarly, GDH did not appear to be affected by energy charge ratios, or glycolytic intermediates, but showed a pronounced modulation in the presence of L-malate, L-glutamine, and ATP.

It is apparent from the foregoing discussion that glutamate dehydrogenase of a relatively few representative bacterial genera have been studied in a detailed manner. Admittedly, the nine or ten species investigated in this regard do not, by any means, warrant any generalizations about the behaviour of GDH of bacterial phyla as a whole. It is perhaps reasonable to state that so far, no novel regulatory features have been discovered in the bacterial glutamate dehydrogenases. This might be interpreted as an implication that in bacterial systems, the enzyme controlling the assimilation of ammonia is not GDH, but is located at some other control point.

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