GLUTAMATE DEHYDROGENASE:

KINETIC ANALYSIS AND ENZYME REGULATION

by

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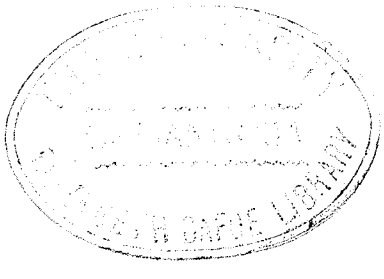

ABSTRACT

NAD-specific glutamate dehydrogenase from Neurospora crassa was purified 300 fold over non-induced levels by ammonium sulfate precipitation, alumina C γ adsorption and DEAE-cellulose chromatography. The purified enzyme was approximately 90 percent pure as judged by electrophoresis on polyacrylamide gel. The enzyme was characterized with respect to substrate specificity, optimum hydrogen ion concentration, for activity, energy of activation and effect of inhibitors. Molecular weight of NAD-specific glutamate dehydrogenase was estimated as 330,000 by sucrose density gradient method.

Detailed kinetic analyses of the enzyme revealed the mechanism to be ordered Bi-Ter. Order of substrate addition was found to be, NAD followed by L-glutamate. Products of the reaction were released in the following order: ammonia, α-ketoglutarate and NADH. Evidence obtained from spectrofluorometric analysis revealed approximately four NADH binding sites which were completely independent of one another.

Only NAD-specific glutamate dehydrogenase activity was inhibited by the purine nucleotides, GTP, GDP, GMP and IMP, at physiological concentrations. Inhibition of enzyme activity supports the contension that purine nucleotide 'pools' control NAD-specific GDH activity by an end-product inhibition mechanism.

Evidence is presented that in mutants of Neurospora which lack urease, NAD-specific glutamate dehydrogenase is not induced in the

presence of urea. Experiments with actinomycin-D support the contention that the induction of NAD-specific glutamate dehydrogenase and repression of NADP-GDH by L-glutamate and, possibly, ammonia is controlled at the genetic level.

The results indicate that glycine represses the formation of NAD-GDH at the genetic level. Glycine repression could be reversed by the addition of L-alanine, L-glutamine, L-asparagine, L-valine and L-leucine. The latter two amino acids acted by preventing entry of glycine into the cells.

A partial mutant of NAD-GDH (d^-) was obtained. Due to screening difficulties its precise genetic location could not be determined, however, results indicate that the d^- gene is not closely linked to am^-inos^- region.

Forced heterokaryon experiments showed that the effect of the *i* gene was in the *trans* position which suggests that it is a typical repressor gene.

In an attempt to explain 'inverse concurrent regulation' of glutamate dehydrogenases, a genetic model is proposed that appears to be compatible with the biochemical and genetic evidence.

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ABBREVIATIONS

AMP	-	adenosine 5'-monophosphate
ADP	-	adenosine 5'-diphosphate
ATP	-	adenosine 5'-triphosphate
CDP	-	cytidine 5'-diphosphate
CTP	-	cytidine 5'-triphosphate
GMP	-	guanosine 5'-monophosphate
GDP	-	guanosine 5'-diphosphate
GTP	-	guanosine 5'-triphosphate
IMP	-	inosine 5'-monophosphate
ITP	-	inosine 5'-triphosphate
UDP	-	uridine 5'-diphosphate
UTP	-	uridine 5'-triphosphate
NAD	-	Nicotinamide Adenine Dinucleotide
NADH	-	reduced nicotinamide adenine dinucleotide
NADP	-	nicotinamide adenine dinucleotide phosphate
NADPH	-	reduced nicotinamide adenine dinucleotide phosphate
ADH	-	alcohol dehydrogenase
GDH	-	glutamate dehydrogenase
PRPP-ATP-PPase	-	phosphoribosyl-ATP-pyrophosphorylase
GSH	-	reduced glutathione
DEAE	-	diethyl aminoethyl
DES	-	diethylstilbesterol
EDTA	-	ethylenediaminetetraacetic acid
S-RNA	-	soluble ribonucleic acid
M-RNA	-	messenger ribonucleic acid
DNA	-	deoxyribonucleic acid
CRM	-	cross reacting material
Hb	-	haemoglobin
PCMB	-	p-chloromercuribenzoate.

INTRODUCTION

The discovery of two separate glutamate dehydrogenases, one specific for NAD and another for NADP in Neurospora crassa (Sanwal and Lata, 1961) and some other organisms (Kaplan, 1963), posed two important questions which demanded an answer.

The first question arose regarding the necessity and physiological benefits derived by the organism possessing two enzymes which catalyze identical reactions which lead to the formation of the same product, glutamate. It will be recognized that this question has been posed for similar situations existing for other duplicate enzymes in diveral organisms (Aspartokinases in Escherichia coli: Stadtman et al., 1961; Threonine deaminases in E. coli: Umbarger and Brown, 1957; Carbamyl phosphate synthesizing enzymes in Neurospora: Davis, 1963). However, no unified concept has emerged from studies of such enzyme systems. The reason seems to be that the problem has been approached either from a purely genetic or purely biochemical angle. In the report that follows here, we have attempted to answer this question by using both genetic and enzymological techniques. This has, of necessity, led to an involved kinetic study of one of the glutamate dehydrogenases (NAD-specific).

The second question arose from the findings of Sanwal and Lata (1962) that when a wild-type strain of Neurospora

crassa was grown in the presence of exogenous glutamate, the specific activity of the NAD-dependent glutamate dehydrogenase increased approximately 20-fold and this increase was invariably accompanied by a corresponding decrease in the specific activity of the NADP-specific enzyme. This "inverse concurrent" regulation of the two dehydrogenases has since been shown to occur in yeast (Kaplan, 1963). In the case of Neurospora crassa it was considered likely (Sanwal and Lata, 1962) that the actual regulatory agent was urea which can be easily derived from glutamate in this organism. The mechanism of 'inverse concurrent' regulation is, however, obscure. On a 'molecular conversion' hypothesis, such as the one suggested by Monod and Jacob (1961) for beef-liver glutamate dehydrogenase, the function of the regulatory chemical could be considered as purely physical, resulting in the 'conversion' (by associative-dissociative changes of quarternary structure, for instance) of NADP-specific enzyme to the NAD-specific one in the presence of the inducer. Such molecular conversions seem to play a part in the formation of lactic dehydrogenase isozymes in various tissues of animals (Cahn et al., 1962), and such a mechanism has also been suggested to play a part in the regulation of beef-liver glutamate dehydrogenase in vivo (Yielding and Tomkins, 1960a) by certain steroid hormones.

In Neurospora crassa, however, loss of the NADP-specific glutamate dehydrogenase by mutation does not either affect the formation or the inducibility of the NAD-specific enzyme. This is true in all mutants which lack or possess a material antigenically related to the NADP-specific dehydrogenase (Sanwal and Lata, 1962). It thus seems that the regulation of glutamate dehydrogenases has a genetic basis, and it was to probe into the mechanism of this regulation that the present study was undertaken. It may be pointed out here that one of the least understood aspects of haemoglobin synthesis in humans is the 'concurrent' or 'compensatory' regulation of γ - and β - chains during foetal development (Baglioni, 1963), a situation which is strikingly similar to that obtained with the glutamate dehydrogenases of Neurospora. It was felt that work with the fungal system (with its attendant advantages of manipulation) could also yield clues to the solution of the haemoglobin problem.

For the ease of presentation, the thesis has been divided into two parts, one dealing with the kinetics of NAD-specific enzyme and its feed back inhibition by purine nucleotides, and another dealing with the genetic and biochemical basis of regulation of the two glutamate dehydrogenases.

SECTION I

NAD-SPECIFIC GLUTAMATE DEHYDROGENASE:

CHARACTERIZATION AND KINETIC ANALYSIS

I. HISTORICAL

I. HISTORICAL

In general, microorganisms possess two mechanisms for the incorporation of ammonia into amino acids, which serve as precursors of proteins. One such mechanism is the reversible incorporation of ammonia into fumarate forming aspartic acid. Quastel and Woolf (1926) showed that this reaction was catalyzed by the enzyme aspartase. Their work was confirmed and expanded upon by Cook and Woolf (1928), Haehn and Leopold (1937), and Gale (1938, 1940).

Another, more widely distributed and important mechanism is the reductive amination of α-ketoglutarate, catalyzed by the enzyme glutamate dehydrogenase (GDH). This enzyme plays an important role in living cells because the reaction catalyzed by it serves as a connecting link between carbohydrate and amino acid metabolism. Glutamate dehydrogenase presumably also controls the ammonium ion levels *in vivo* by either directly incorporating it into glutamic acid or indirectly by the formation of urea (Krebs-Henseleit cycle).

DISTRIBUTION AND COENZYME SPECIFICITY

In keeping with the importance of its function, glutamate dehydrogenase occurs in a variety of bacteria, fungi, plants and animal tissues. The coenzyme specificity of the enzyme has been found to depend upon its source. For instance, bacterial GDH is NADP-specific (Adler *et al*, 1938) or NAD-

specific (Barban, 1954, Nisman, 1954). The plant enzyme from pea seeds, cabbage and carrot roots, celery, radish, white cabbage (Adler et al 1938a), bean and pea seedlings (Damodaron, 1938; Davies, 1956; Bone,1959), oats (Berger, 1943 and 1944; Routanen, 1955) and corn leaves (Bulen, 1956) has been reported to be NAD-specific. In animal tissue (Von Euler et al, 1939; Von Euler 1938) and the green alga, Ulva lactuca (Jacobi, 1957), the enzyme utilizes either one of the coenzymes, as hydrogen acceptor. However, fungi like baker's and brewer's yeast (Holzer, 1957), Neurospora (Sanwal, 1961) and Fusarium (Sanwal, 1961a) possess two distinct enzymes, one NADP-specific and the other NAD-specific. Among the fungi investigated only Allomyces arbuscula (Klinkhammer, 1959) has been reported to possess one glutamate dehydrogenase (NAD-specific).

SUBSTRATE SPECIFICITY

Ever since the crystallization of glutamate dehydrogenase (Strecker, 1951 and Olson, 1951) from beef liver, the enzyme was believed to be completely specific for L-glutamate, α-ketoglutarate and ammonia. However, Bässler and Hammer (1958) demonstrated that the enzyme also utilized α-ketovaleric, α-ketobutyric and α-ketoisovaleric acids as substrates at a rate equal to 25%, 2% and 2% respectively, of α-ketoglutarate. The Michaelis constant (K_m) for α-ketovaleric acid was calculated to be ten times that of

α-ketoglutarate (Bassler, 1958) under similar assay conditions. Struck and Sizer (1960) reported the following amino acids to be active at pH 8.0 in relation to glutamate oxidation to the extent of: norvaline (17%), L-α- aminobutyric (2.3%), L-leucine (1.7%), L-valine (1.6%), DL-norleucine (1.6%), L-isoleucine (0.95%), L-methionine (0.82%) and L-alanine (0.3%). Purified GDH-NADP from Neurospora appears to be similar to the liver glutamate dehydrogenase with respect to α-keto acid specificity (Barratt, 1963).

Although Fisher and McGregor (1961) showed that the glutamate binding site was the same for the amino acids tested by Struck and Sizer (1960), the rather low reaction rates reported leaves their metabolic significance in doubt.

PHYSICAL ASPECTS

Many of the physical properties of the crystalline liver glutamate dehydrogenase were first reported by Olson and Anfinsen (1952). By sedimentation coefficient measurements, they arrived at a molecular weight value of 1×10^6 for this enzyme. More recent determinations using bovine (Kubo, 1959) and ox liver (Kubo et al., 1957) glutamate dehydrogenase range between $1 - 1.3 \times 10^6$. Employing light scattering techniques Kubo (1957, 1959) showed that liver GDH underwent a reversible dissociation into three or four subunits of molecular weight 300,000. Sedimentation (Frieden,

1957), and electron microscopic studies (Horne, 1963) suggested four subunits of MW 250,000. The latter estimate is most widely accepted. Crystalline bovine liver GDH may undergo further dissociation (irreversible) into low molecular weight (30,000 to 60,000) fragments which display no enzymatic activity (Jergensons, 1961).

Earlier it was assumed that the 'dissociated' form (subunits) of MW 250,000 was less active in catalysis than the 'associated' form (tetramer). Subsequent experiments (Fisher et al., 1962, Frieden, 1963) demonstrated that the 'association-dissociation' phenomenon is concentration dependant. Under normal enzyme assay conditions the enzyme (GDH) exists as subunits and association into the tetramer form occurs at enzyme concentrations greater than 2.5 - 3.0 mg./ml.

Recently, Barratt and Strickland (1963) reported a molecular weight of 267,400 $\pm$ 3,000 for the Neurospora GDH-NADP. Their experiments were performed with a high concentration of enzyme ($>$ 2.3 mg./ml.), thus minimizing dissociation (if indeed the enzyme was subject to it).

The inhibitory effects of several hormones (diethylstilbesterol, estradiol, progesterone, Δ^4-androsterone-3, 17-dione and testosterone) on liver GDH activity in the presence of NADH, has been extensively studied by Yielding et al, (1960). No inhibition occured in the absence of coenzyme. Yielding and Tomkins (1960a) also observed that

in the presence of NADH, diethylstilbesterol, ostradiol and progesterone cause dissociation of the enzyme which can be completely reversed by ADP. Therefore, there is agreement between the results of Yielding (1960a) and those of Frieden (1959a) who reported reversal of NADH-induced dissociation by ADP.

Examining the effect of nucleotides on the velocity of the glutamate dehydrogenase catalyzed reaction, Frieden (1959a) observed that at high NADH concentrations, ADP increased and ATP decreased the reaction rate. Sedimentation studies revealed that ATP enhanced NADH-induced dissociation, whereas ADP prevented it. Other purine nucleotides shown to be potent inhibitors of coenzyme oxidation and reduction were GTP, GDP, and ITP (Wolff, 1962 and Frieden, 1962).

Consideration of results obtained from kinetic, fluorescence quenching and light scattering experiments led Frieden (1963a) to propose the existence of three different, highly specific, binding sites:

(1) an active site binding oxidized and reduced di- or triphosphopyridine nucleotides,

(2) a highly specific binding site for purine nucleotides, and,

(3) a non-active site which binds only NADH.

It was also suggested by Frieden (1963a) that inhibition by purine nucleotides was due to a configurational change in the

enzyme structure which prevented coenzyme binding.

KINETIC ANALYSIS

Kinetic analysis has proven to be a useful tool for establishing the mechanism of enzyme reactions. Frieden (1959b) utilized kinetic techniques to distinguish between two of the simplest mechanisms possible with respect to addition of substrate molecules to the enzyme glutamate dehydrogenase. In one mechanism binding of one substrate is independant of the other substrates (Random substrate addition). In the other mechanism, substrates must bind to the enzyme in a compulsory sequence (Ordered substrate addition). The random mechanism predicts the same type of kinetic relationship between all substrates, whereas the ordered mechanism does not. Frieden (1959b) showed by the usual initial velocity studies (double reciprocal plots of velocity versus NADPH concentration and velocity versus α-ketoglutarate concentration at various ammonium chloride levels), that the same kinetic relationships held for both substrates, NADPH and α-ketoglutarate. That is, the plotted lines intersected on the abscissa. Therefore, the Michaelis constants (K_m's) for NADPH and α-ketoglutarate were independent of ammonium ion concentrations. Similar studies of velocity versus NADPH at several α-ketoglutarate concentrations resulted in plots which were almost parallel and

intersected far below the abscissa. These results demonstrated that the enzyme did not act in a similar manner toward each substrate. Therefore, the concept of a random substrate addition was discarded in favour of the ordered mechanism.

The order of addition of substrates by liver GDH was postulated to occur in the following sequence: NADPH, ammonium ion and α-ketoglutarate (Frieden, 1959b; Fisher, 1960). That NADPH binds to the free enzyme form was shown by several methods:

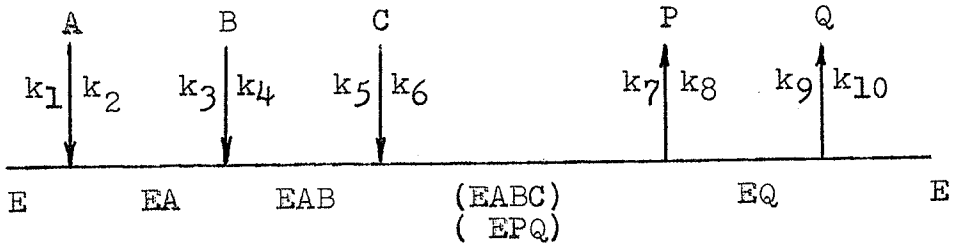
(1) NADPH effect on sedimentation coefficient (Frieden, 1959).

(2) Quenching of enzyme fluorescence in the absence of other substrates.

Ammonium and α-ketoglutarate had little or no effect on (1) and (2). Evidence that α-ketoglutarate did not bind to free enzyme was obtained from isotope exchange experiments (Fisher, 1958) and equilibrium dialysis of enzyme with tritiated α-ketoglutarate (Fisher, 1960).

In a series of papers on kinetics of enzyme catalyzed reactions, Cleland (1963, 1963a, 1963b) proposed a nomenclature, short hand notations and a general method for describing the steady state rate equations in measurable kinetic constants (concentration and concentration/unit time). He also emphasized the use of product inhibition data for determining enzyme mechanism and sequence. According to Cleland's nomenclature the liver glutamate dehydrogenase would be an

ordered ter-bi reaction mechanism, graphically expressed as:



(where A is NADPH, B is ammonium ion, C is ∝ -ketoglutarate and P and Q either glutamate or NADP).

Although the binding sequence for NADPH oxidation has been substantiated for liver GDH, such is not the case for NADP or NAD reduction, nor has the proposed mechanism been tested on GDH from other sources.

II. METHODS AND MATERIALS

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CHEMICALS

Reduced and oxidized NAD, α-ketoglutaric acid, α-ketovaleric acid, α-ketoisovaleric acid, α-ketobutyric acid, α-ketoisocaproic acid, ATP and ADP were obtained from Sigma Chemical Company. The nucleotides UTP, UDP, GTP, GDP and NAD analogues were purchased from the Pabst Company. Crystalline enzymes were obtained from Sigma Chemical Company.

GENETIC STRAINS

Glutamate dehydrogenase was isolated from the following strains of Neurospora crassa: STA-4 (wild type, am^+i^+); am^3 (am^-i^+); am^4 (am^-i^+) and 1499-12 (am^-i^-). The first three strains were obtained from the culture collection of the Fungal Genetics Stock Centre located at Dartmouth College, New Hampshire. Strain 1499-12 was kindly donated by Dr. J. R. S. Fincham of the John Innes Institute, England. The amination-deficient strains (am^-), lack the NADP-specific glutamate dehydrogenase and require amino-nitrogen for growth (Fincham, 1950, 1951). Strain 1499-12 lacks the NADP-GDH and its growth is inhibited by the presence of ammonium salts in medium-N (see later) supplemented with a source of amino-nitrogen (Fincham, 1957). All strains described above possessed an inducible NAD-specific glutamate

dehydrogenase. Stock cultures of these strains of Neurospora crassa were maintained on agar slants of Neurospora Culture Agar (Baltimore Biological Laboratories, Inc.).

GROWTH CONDITIONS

The basic growth medium used for culturing Neurospora crassa was Vogel's medium-N (Vogel, 1956). Stock solution of medium-N contained: sodium citrate, 120 gm., KH_2PO_4, (anhydrous) 250 gm., NH_4NO_3, 100 gm., $MgSO_4 \cdot 7H_2O$, 10 gm., $CaCl_2 \cdot 2H_2O$, 5 gm., trace element solution, 5 ml. and 0.01% biotin, 2.5 ml. dissolved in glass-distilled water to yield a final volume of 1000 ml. Chloroform (5.0 ml./l.) was added as a preservative. The trace element solution consisted of: citric acid, 5 gm., $ZnSO_4 \cdot 7H_2O$, 5 gm., $FeSO_4 \cdot 7H_2O$, 1 gm., $CuSO_4 \cdot 5H_2O$, 0.2 gm., $MnSO_4 \cdot H_2O$, 0.05 gm., anhydrous H_3BO_3, 0.05 gm. and $Na_2MoO_4 \cdot 2H_2O$, 0.05 gm. dissolved in 100 ml. of water. The stock solution was diluted fifty fold with distilled water and supplemented with 2% sucrose before use.

Conidia from stock cultures of am^3, am^4, and wild type (STA-4) were grown on medium-N agar at 28°C in the dark until abundant hyphal formation was evident. The cultures were then exposed to light for conidiation. Conidia were washed off the agar with sterile deionized water, passed asceptically through four layers of gauze to remove

hyphal fragments and washed three times by alternate centrifugation and suspension in water.

Erlenmeyer flasks of 500 ml. capacity, containing 100 ml. diluted medium-N, were seeded with approximately 5 x 10^8 conidia (washed) and grown at 28°C for the required length of time. Vigorous aeration was accomplished on a New Brunswick rotary shaker. The resulting cells were harvested by filtration through two layers of cheese cloth and repeatedly washed with distilled water. The cells were pressed dry between paper towels and used for the preparation of cell-free enzyme extracts.

Large quantities of mycelia for enzyme purification were obtained by inoculating conidia into fifteen liter carboys containing ten liters of medium-N supplemented with inducer (0.05 M L-glutamate). Aeration was achieved by forcing sterile air through the medium. Carboys were incubated at 28°C for 24 to 60 hours. The mycelial mass was harvested in the usual manner, washed, and excess water removed by wrapping the mycelia in cheese cloth and passing it through a washing machine ringer. The squeeze-dried mat was then lyophylized, ground into a fine powder by means of a Waring Blender and stored at -20°C. The lyophylized powder could be stored for at least four months without loss of NAD-specific glutamic dehydrogenase activity.

EXTRACTION PROCEEDURE

When working with small quantities of mycelia (1-10 gm. wet weight) as in preliminary purification studies, the cells were disrupted and the enzyme extracted by grinding according to the method of Sanwal (1961). For the extraction of large quantities of NAD-specific glutamate dehydrogenase the lyophilized powder was suspended in ten times its weight of 0.05 M Tris buffer, pH 7.5, containing 1×10^{-3}M reduced glutathione (GSH). This suspension was mechanically stirred at 4^{o}C for 30 minutes. The extract was filtered through cheese cloth and centrifuged at 12,000 xg for 15 minutes. The supernatant solution constituted the crude enzyme extract.

ASSAY PROCEEDURE

NAD-specific glutamate dehydrogenase was assayed in a mixture containing 20 µmoles α-ketoglutarate, 120 µmoles ammonium sulphate, 0.50 µmoles reduced NAD, enzyme preparation and 273 µmoles Tris buffer at pH 9.0. The final volume in silica cuvettes of 1 cm. light path was 3.0 ml. The reaction was started by the addition of enzyme and the reaction rate determined by calculating the decrease in optical density at 340 mµ per unit time. Assays were carried out on a Gilford model 2000, recording spectrophotometer. The reference cuvette contained all components of the

reaction mixture except α-ketoglutarate. A unit of enzyme activity is defined as a change in optical density at 340 mμ of 0.10 per minute. Specific activity is defined as units per mg. protein. Protein concentrations were determined according to the method of Lowry et al., (1957) using twice crystallized serum albumin as standard.

QUANTITATIVE AND QUALITATIVE DETERMINATION OF KETO ACIDS

The keto acids, when individually present, were assayed according to the method of Friedmann and Haugen (1943). Chromatographically pure α-ketoglutarate was used for the preparation of the standard curve. Qualitatively, keto acids were detected by the method of Cavallini (1949). Assay mixtures (3.0 ml.) were deproteinized by the addition of one volume of metaphosphoric acid (24%). The deproteinized mixture (4.0 ml.) was then treated with 2.0 ml. of 0.2% 2,4-dinitrophenylhydrazine in 2N hydrochloric acid at 37°C for 20 minutes. The resulting hydrazones were repeatedly extracted with equal volumes of ethyl acetate. The combined ethyl acetate fractions were then extracted with one half their volume of 10% (W/V) sodium carbonate solution in portions. Free 2,4-dinitrophenylhydrazone was removed by further ethyl acetate extractions. Cooled carbonate fractions were then acidified with concentrated hydrochloric acid and again extracted with ethyl acetate.

The extract was evaporated to dryness by negative pressure and the residue dissolved in 0.2 ml. ethanol which was neutralized with 0.1M phosphate buffer, pH.7.5. Aliquots of the extracted material were then applied to Whatman #1 chromatographic paper and chromatographed in n-butanol-ethanol (20:80) as solvent. The keto acid spots were intensified by spraying the paper with a 1.0% sodium carbonate solution.

STARCH GEL ELECTROPHORESIS

Vertical starch gel electrophoresis was performed on an apparatus as described by Smithies (1959), with some slight modifications of his earlier proceedure (1955). Hydrolyzed starch used as the supporting medium was obtained from Connaught Laboratories, Toronto. The gel was prepared by suspending 48.5 gm. of starch in 500 ml. of a 0.026 M boric acid 0.01 M sodium hydroxide buffer (pH 8.3), and immediately mixed. The starch suspension was then heated over a naked flame with continuous swirling until the starch became a viscous gel. This proceedure was not interupted until the gel was of the proper consistency. The viscous gel was then immediately degassed by applying negative pressure with a vacuum pump. The gel was then poured on a template and allowed to cool to room temperature. Liquid protein samples were directly pipetted into the slots. These were than sealed with cover slips and coated with paraffin

jelly. To prevent drying of the gel during a run, it was wrapped in Saran Wrap.

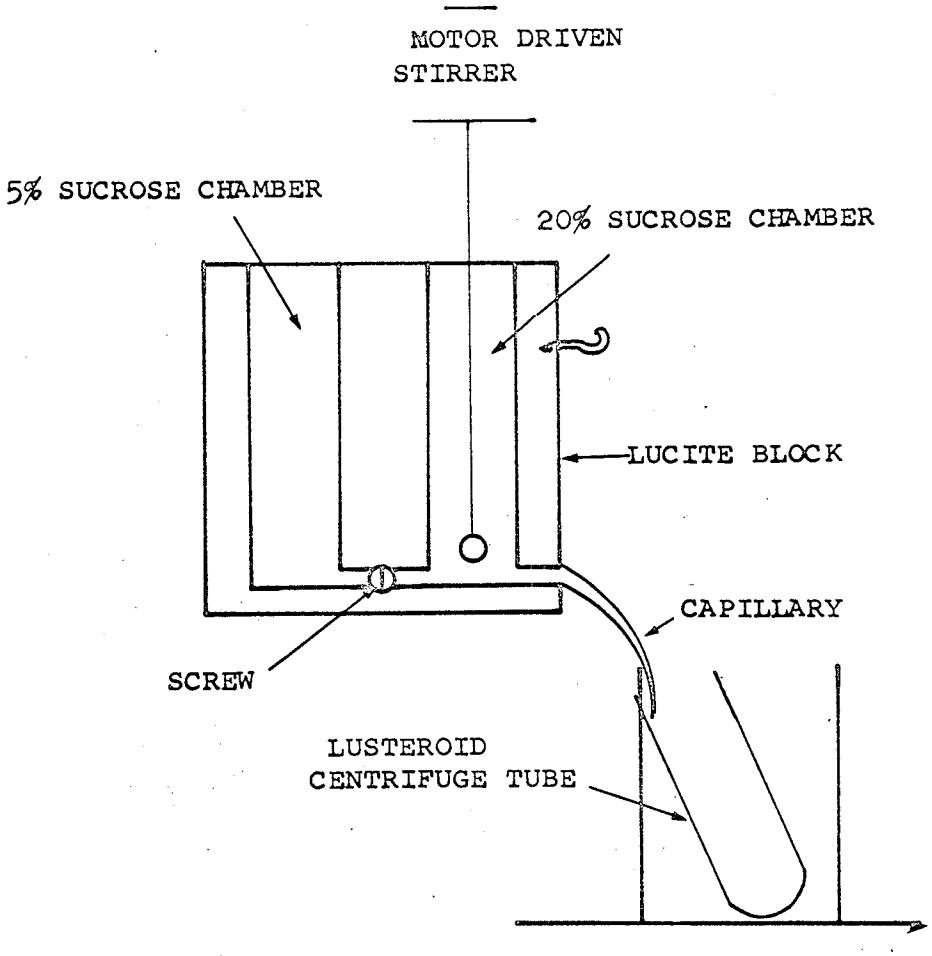
The buffer used in the electrode compartment was 0.15 M boric acid-sodium hydroxide, pH 8.0. Electrophoresis was carried out at 150 volts, 20 milliamperes, at 4°C for 12 hours. Protein bands were stained with Amido Black 10B and the gel destained by repeated methanol-water-acetic acid (50:50:10) washings. Individual dehydrogenases were localized by the specific neo-tetrazolium method of Markert (1959).

DISC ELECTROPHORESIS

Disc electrophoresis was performed as described by Ornstein and Davis (preprint available from Distillation Products Industry. Eastman Kodak, Rochester, N.Y.), using Tris-glycine buffer, pH 8.3. Dilute protein solutions were first concentrated before applying onto the polyacrylamide gel. Concentration of protein solutions was achieved by placing the solution in a tightly sealed dialysis bag and burying it in crystalline sucrose (fine grained). The volume was reduced ten fold in 3 to 6 hours at 4°C. The sucrose which entered the bag by diffusion was removed by dialysis against buffer.

MOLECULAR WEIGHT DETERMINATIONS

The molecular weight of glutamate dehydrogenase was estimated by ultracentrifugation in a linear sucrose



SUCROSE GRADIENT APPARATUS

gradient according to the method of Martin and Ames (1961). The apparatus used (see diagram) to produce a linear sucrose gradient consisted of a Lucite block containing two chambers of equal size connected by a screw pin at the bottom. The outflow tube, connected to one of the chambers was a short length of fine bore rubber tubing with a glass capillary at one end. The glass tube was drawn out in a flame so that the emptying time of the sucrose in both chambers (total volume of 4.8 ml.) was approximately 20 minutes. A platinum bacteriological inoculating loop mounted on a rheostated motor was used to stir the sucrose in the chamber adjacent to the outflow tube. The stirrer speed was adjusted so that the meniscus was not disturbed. Before the chambers were filled with 2.4 ml. of sucrose-buffer solution, the passage between the chambers was closed and the outflow tube bent up so that the tip was above the top of the chambers. The "mixing" chamber was then filled with 20% (W/V) cold sucrose in 0.10 M Tris-HCl buffer at pH 7.5. The adjacent chamber was filled with 5% (W/V) cold sucrose in 0.10 M Tris-HCl buffer at pH 7.5.

The screw was then withdrawn just enough to allow free flow of solutions between chambers. The tip of the outflow tube was placed at the top of a Lusteroid centrifuge tube. Initially the linearity of the gradient was established by mixing dichlorophenolindolphenol with the

20% sucrose solution. A linear relationship was obtained by plotting absorbancy at 600 mu against fraction number. Gradients prepared in this manner could be stored up to 12 hours at 3°C before use.

A mixture (total volume 0.2 ml.) of glutamate dehydrogenase (0.2 mg.), and reference enzyme, alcohol dehydrogenase (0.3 mg.), was layered on the gradient by means of a lambda pipette, avoiding any bubble formation. It was essential that the protein solution float on 5% sucrose and its concentration was less than 2% in order to obtain convection-free sedimentation. The gradient tubes were centrifuged in a model-L Spinco centrifuge using an SW39 swinging bucket rotor. Initially, the rotor was accelerated slowly to prevent back-lash. Then the speed control was immediately set at 39,000 r.p.m. Samples were centrifuged at this speed for 6 hours at 5°C. At the end of the run, the rotor was allowed to decelerate with the brakes off. Two-drop fractions were collected by puncturing the bottom of the tube with a fine needle, and these were assayed for enzymatic activity.

KINETIC MEASUREMENTS

All kinetic studies were performed with a Gilford Model 2000 optical density converter connected to a Beckman-DU monochromator and a 10 inch self-balancing servo-recorder with a multiple chart drive. Absorbancy changes in the

presence of NADH or α-ketoglutarate as inhibitor of the enzymatic reaction were recorded after "blanking-out" the original absorbance. Reduced and oxidized NAD were prepared just before use and kept at 0°C throughout the experiment. The reaction mixture, lacking enzyme, was preincubated to 25-26°C before the addition of properly diluted enzyme. The purified enzyme preparation was freed of ammonia by dialysis against frequent changes of 0.1 M Tris-HCl buffer, pH 7.5, containing 2×10^{-3} M reduced glutathione at 4°C for 5 hours. The recorder curves were extrapolated to zero time and the slopes were taken as initial velocities. The reaction was linear for at least three minutes.

SPECTROFLUOROMETRIC ANALYSIS

Purified NAD-specific glutamate dehydrogenase (DEAE-cellulose fraction) was concentrated and dialyzed at 4°C for six hours against 0.2 M Tris-HCl buffer, pH 7.5 plus 1×10^{-3} M reduced glutathione. The dialyzed enzyme, as well as all other reagents (NAD, NADH, GTP, glutamate and buffer), was clarified by filtration through a millipore filter (pore size 0.45 μ). These experiments were performed with NAD-GDH at a concentration of 16×10^{-6} M (assuming 330,000 as its molecular weight). Total volume of the reaction mixture in 1 cm cuvettes was 1.0 ml. All spectrofluorometric analyses were performed with an Aminco-Bowman Spectrofluorometer equipped with a 416-992 Xenon lamp and

an Electro Instruments flat-bed X-Y Recorder. The protein emission hand recorded at a wave length of 340 mμ when excited at a wave length of 290 mμ. Reduced NAD emitted fluorescent light at 460 mμ when excited at a wave length of 350 mμ.

III. RESULTS

III RESULTS

NAD-SPECIFIC GLUTAMATE DEHYDROGENASE

SPECIFIC ACTIVITY OF THE ENZYME DURING GROWTH

Before attempting to purify the NAD-specific glutamate dehydrogenase, it was necessary to determine the stage of growth at which maximum amount of the enzyme was present. To accomplish this, conidia from strain $\underline{am}^3$ (total number, 5×10^8) were inoculated into 100 ml. of Vogel's medium-N in Erlenmeyer flasks of 500 ml. capacity. The flasks were incubated at 28°C with vigorous shaking and samples were assayed for NAD-specific glutamate dehydrogenase at 12 hour intervals. The enzyme attained a peak level after 36 hours (Figure 1). Subsequent experiments revealed that the time of appearance of maximum enzyme activity depended on inoculum size, aeration and temperature. Therefore, these conditions were kept constant throughout the course of this investigation.

PURIFICATION OF ENZYME

All purification proceedures described here were performed at 4°C. A crude enzyme extract (100 ml.) was prepared from lyophilized mycelia of Neurospora crassa ($\underline{am}^3$) according to the proceedure described in Methods.

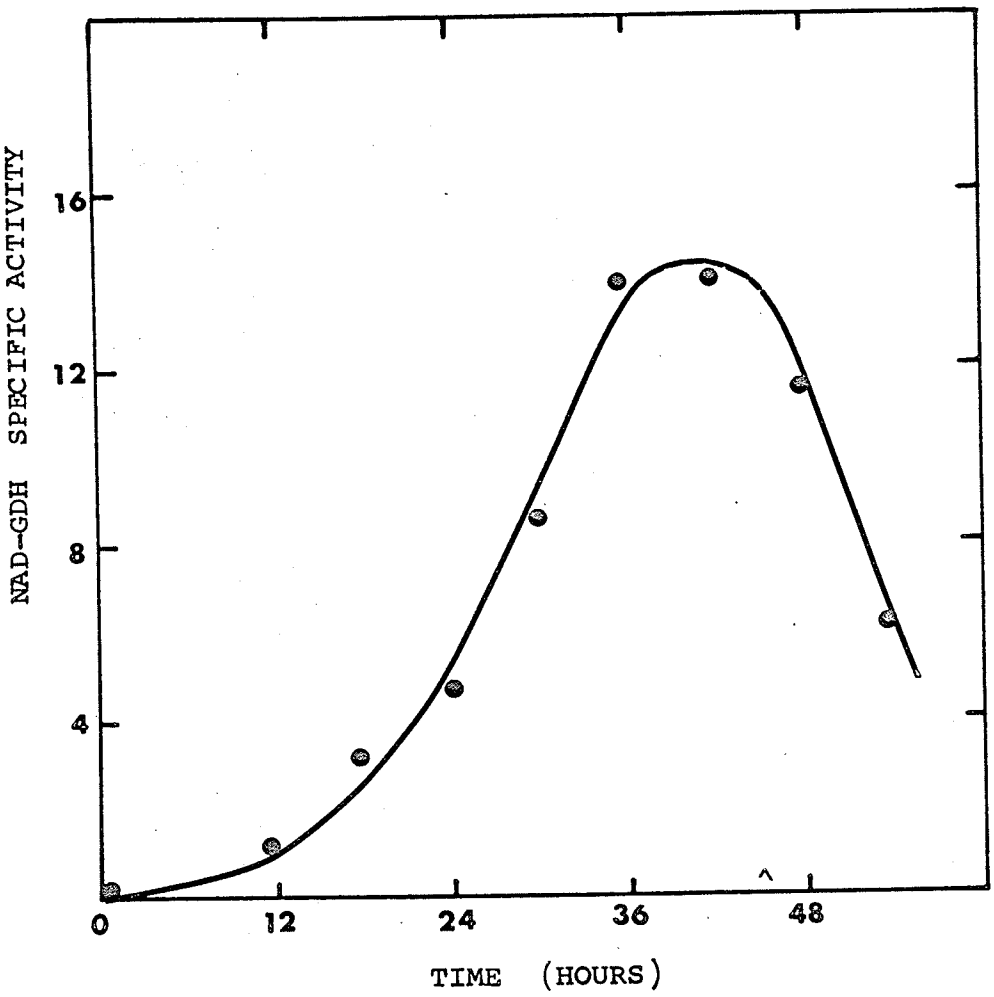


Figure 1. Specific activity of NAD-GDH during growth. Strain 523 (am⁻) grown in Vogel's minimal N medium.

Solid ammonium sulphate was added to the supernatant solution to 0.30 saturation. The precipitate which resulted after 30 minutes of stirring was removed by centrifugation at 12,000 x g for 15 minutes. To the resulting supernatant fluid, solid $(NH_4)_2SO_4$ was added to 0.40 saturation. After the precipitate was stirred for 30 minutes, it was separated by centrifugation at 20,000 x g for 20 minutes and dissolved in 1/10 the original volume of 0.10M Tris-HCl buffer, pH 7.5, containing 1 x 10^{-3}M reduced glutathione. Further purification was achieved by C_{γ} aluminum hydroxide gel fractionation according to the method of Ochoa *et al.*, (1948). C_{γ} aluminum hydroxide gel was added at a concentration of 5 mg. gel/mg. protein, and the suspension was gently stirred over a period of 20 minutes. The gel was recovered by centrifugation at 10,000 x g for 10 minutes, and washed twice with 0.1 M KH_2PO_4-NaOH buffer, pH 7.5, containing 1 x 10^{-3} M GSH. The adsorbed enzyme was eluted from the gel with 5.0 ml. of 0.5 M KH_2PO_4-NaOH buffer, pH 6.5, containing 1 x 10^{-3} M GSH. Gel adsorption and elution was repeated five times and the eluates having the highest specific activities were pooled. The enzyme preparation obtained in this way was dialyzed against 0.10 M Tris-HCl buffer, pH 7.2, for at least 6 hours. The dialyzed enzyme was then subjected to column chromatography on DEAE-cellulose (diethylaminoethyl-cellulose). The column was prepared by a proceedure similar

to that reported by Sober et al., (1956). DEAE-cellulose (obtained from Sigma Chemical Company) was sifted through a fine wire mesh (#50, Endecotts (Filters)Ltd., London, England), suspended in 1.0 N sodium hydroxide and decanted after 2-4 hours to remove the fine particles. The ion exchanger was suspended and decanted alternately with 0.01 M KH_2PO_4-NaOH buffer, pH 6.5, until the hydrogen ion concentration was equal to that of the buffer (pH 6.5). The cellulose was transferred to a chromatographic column (Pharmacia, Uppsala, Sweden) of dimensions 2.5 x 43 cm., packed to a height of 30 cm. and equilibriated with 0.01 M KH_2PO_4-NaOH buffer, pH 6.5, containing 10^{-3}M GSH. Dialyzed $C_{\frac{1}{2}}$ fraction containing from 20-50 mg. of protein was layered on the column. The NAD-specific glutamate dehydrogenase was eluted by applying a continuous linear gradient from 0.01 M to 0.5 M KH_2PO_4-GSH (10^{-3} M) buffer, pH 6.5. Fractions of approximately 3.5 ml. were collected with the aid of a Research Specialties Fraction Collector fitted with a drop counter. Enzyme elution pattern was determined by spectrophotometric assay (Figure 2). Fractions showing highest NAD-glutamate dehydrogenase activity were pooled (usually fractions 50 to 55) and used for further study. This proceedure yielded an enzyme preparation approximately 330-fold purified compared with basal levels normally found in am^3 strain under uninduced conditions.

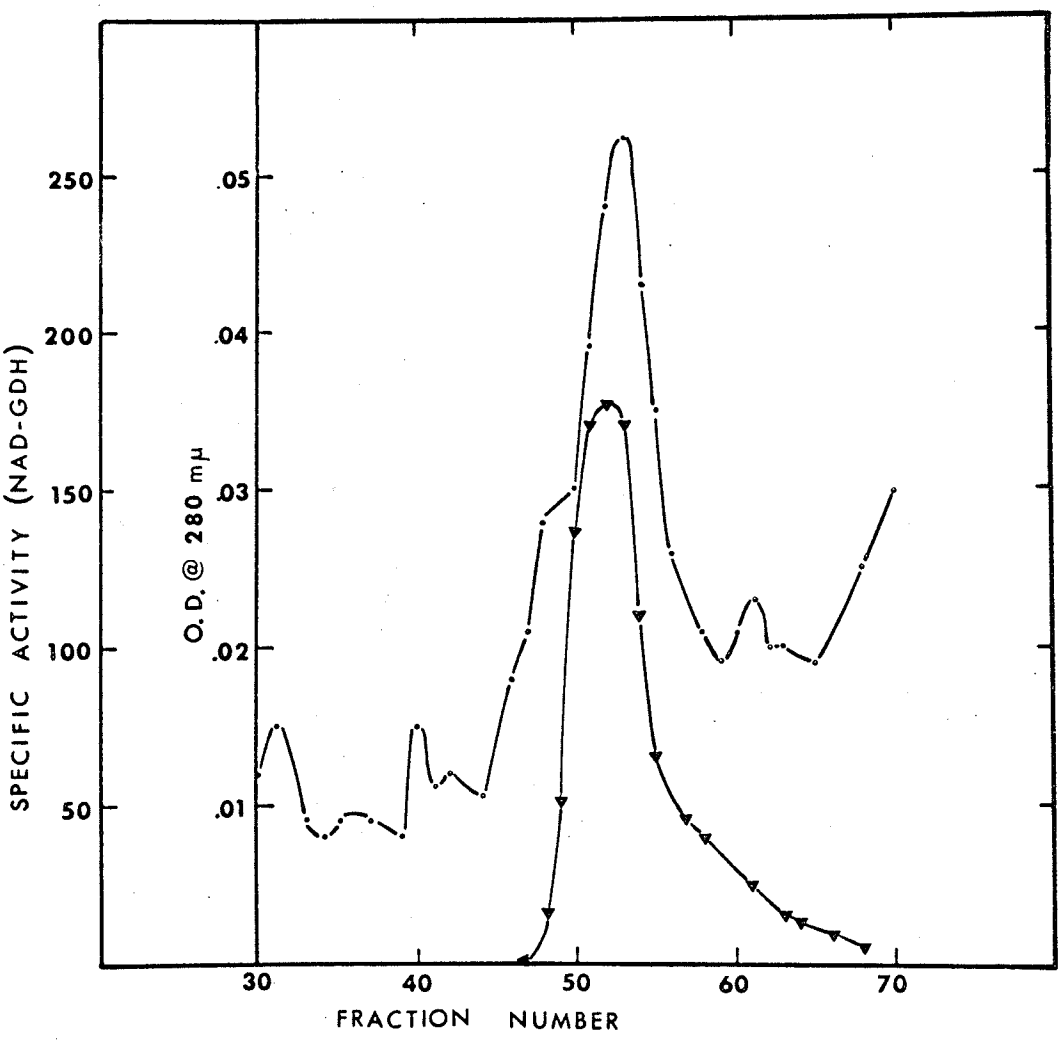


Figure 2. Elution pattern of NAD-specific glutamate dehydrogenase from DEAE cellulose column. ● - ● - ●, extinction coefficient at 280 mu; ▼-▼-▼, specific activity with ∝- ketoglutarate.

Summary of a typical purification proceedure for NAD-specific glutamate dehydrogenase from Neurospora crassa is presented in table (1).

STORAGE OF ENZYME

Experiments showed that enzyme stability was dependent upon the presence of reduced glutathione. When GSH was omitted, the NAD-specific enzyme lost approximately 50% of its activity in 6 hours at 0°C and 75% in 12 hours at -20°C. However, the purified enzyme suspended in buffer containing GSH could be stored frozen at -20°C. or lyophilized at room temperature for at least 4 months without significant loss of enzymatic activity.

HOMOGENEITY OF PURIFIED ENZYME

The purity of the enzyme was determined by disc electrophoresis (Ornstein and Davies (preprint)). The purified enzyme was mixed with equal amounts of Upper-gel and 0.20 ml. of the mixture (Sample-gel) was layered on the Spacer-gel. Sample-gel usually contained 0.1 to 0.2 mg. protein. Figure (3) shows that the purified enzyme exhibits only one protein band. However, electrophoresis of concentrated (up to .5 mg. protein) enzyme solution revealed additional one or two faint protein bands associated with the glutamate dehydrogenase band. These results suggest that the enzyme preparation was approximately 90% pure.

TABLE 1. Summary of purification procedure for NAD-specific glutamate dehydrogenase from Neurospora crassa.

Step	Volume ml.	Protein mg.	Total units/ml.	Specific Activity	Purification
1. Crude extract	60	882	600	40	
2. Ammonium sulfate 0-0.30 saturation (supernatant)	60	714	1120	94	2.3
3. Ammonium sulfate 0.30-0.40 saturation (precipitate)	20	204	1720	193	4.8
4. Pooled C γ eluates	20	57	1360	544	13.6
5. DEAE-cellulose column	5	0.75	180	1200	30

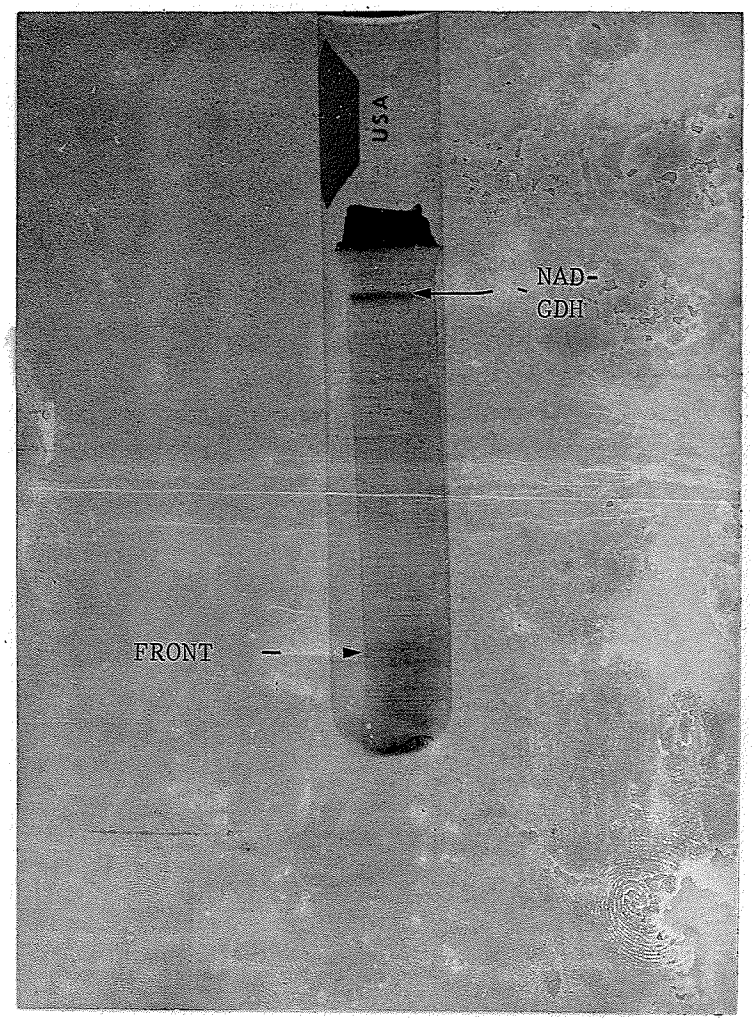


Figure 3. Polyacrylamide electrophoresis of purified NAD-specific glutamate dehydrogenase from Neurospora.

MOLECULAR WEIGHT DETERMINATION OF ENZYME

The molecular weight of purified glutamate dehydrogenase was determined by the sucrose density gradient method of Marten and Ames (1961). The reference enzyme used in these experiments was crystalline alcohol dehydrogenase which has a molecular weight of 150,000 (Hayes and Velick, 1954). Knowing the molecular weight of the reference enzyme (ADH) and the distance which both ADH and NAD-specific glutamate dehydrogenase travel in the sucrose gradient (Figure 4), the molecular weight of NAD-glutamate dehydrogenase was calculated by substituting into equation (1).

$$\frac{S_1}{S_2} = \left(\frac{MW_1}{MW_2}\right)^{\frac{2}{3}} \qquad (1)$$

(where S_1 and S_2 equal the distance travelled from the meniscus by enzymes of unknown and known molecular weights respectively, and MW_1 and MW_2 are equal to the molecular weights of unknown and known proteins). From equation (1), NAD-GDH was estimated to have an average molecular weight of about 330,000 (assuming a partial specific volume for the protein of 0.725 cm^3/gm.).

OPTIMUM HYDROGEN ION CONCENTRATION FOR ACTIVITY

The optimum pH for the oxidative deamination of L-glutamate was found to be 9.5 (Figure 5), whereas the

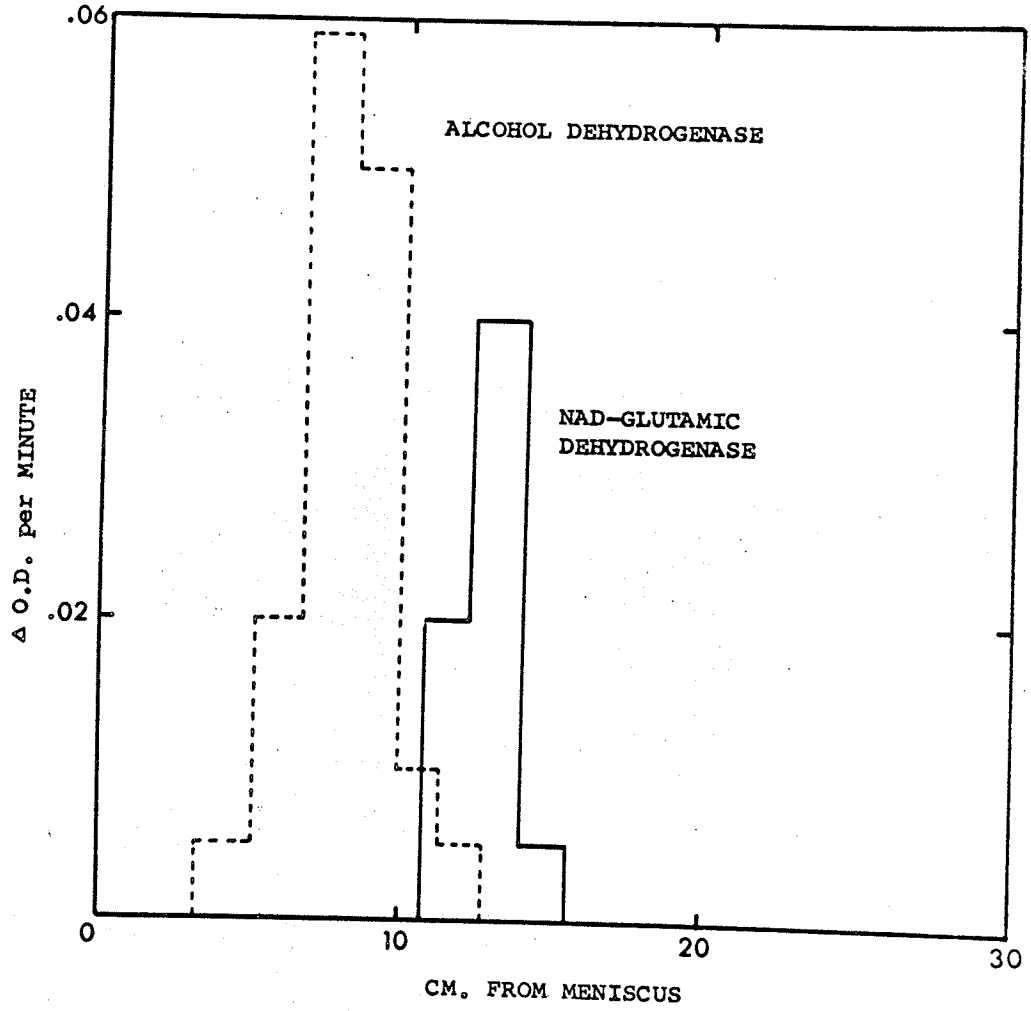


Figure 4. Molecular weight determination of NAD-specific glutamate dehydrogenase by ultracentrifugation in sucrose density gradient. Crystalline alcohol dehydrogenase as reference protein.

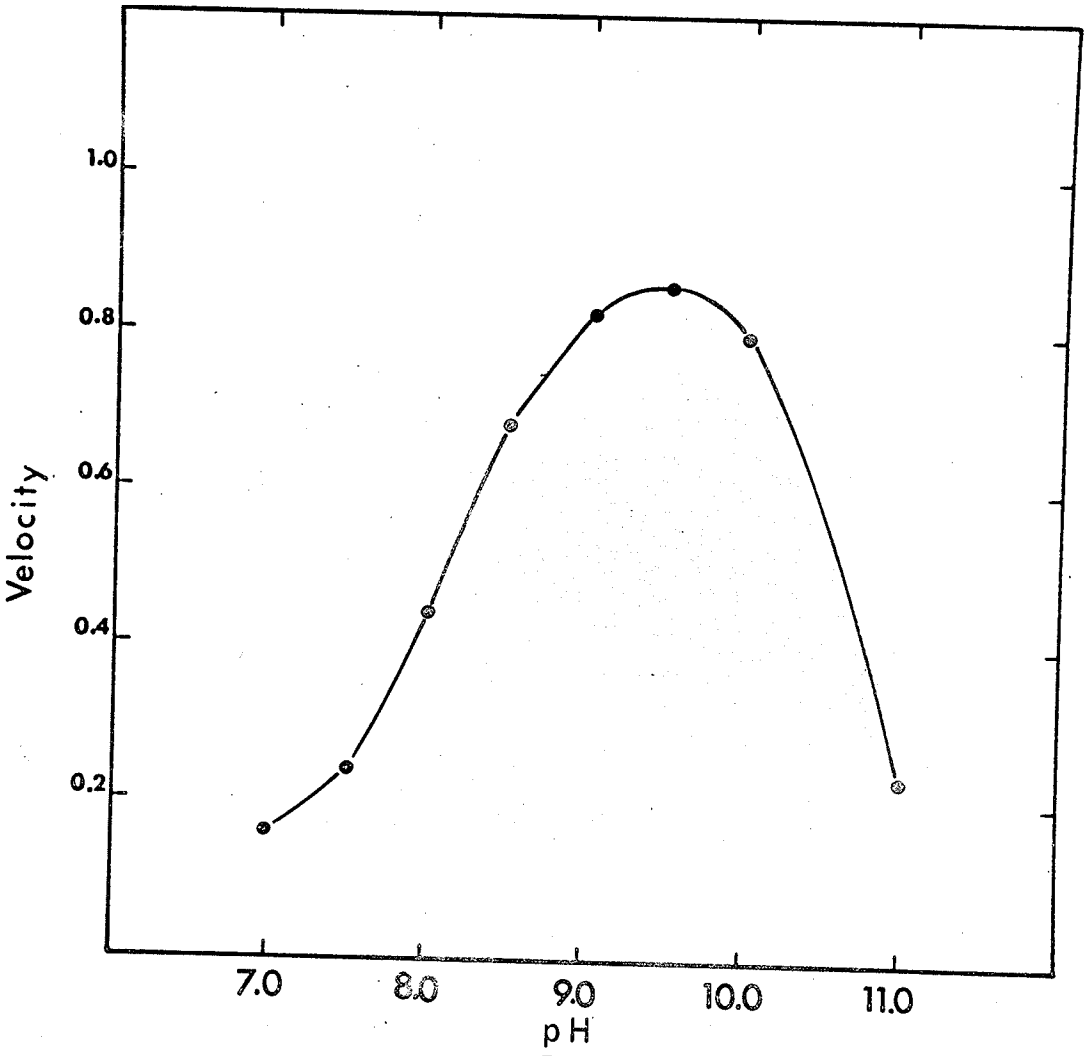


Figure 5. Effect of hydrogen ion concentration on the oxidative deamination of L-glutamate by NAD-specific glutamate dehydrogenase. All measurements were carried out in Tris-HCl buffer.

reductive amination of α-ketoglutarate showed an optimum activity at pH 8.0 (Figure 6). The oxidative deamination curve shows a much broader optimum pH range than the curve for reductive amination of α-ketoglutarate.

PRODUCTS OF THE REACTION

Qualitative identification of α-ketoglutarate as an end product of oxidative deamination of L-glutamate was made by the chromatographic method of Cavallini et al., (1949). Only one spot corresponding to the hydrazone derivative of an authentic sample of α-ketoglutarate was obtained from a reaction mixture containing 20 µmoles L-glutamate, 1.0 µmole NAD and 40 µg enzyme (total volume, 3.0 ml.). To determine the product of the reductive amination of α-ketoglutarate, the usual reaction mixture (see Methods) containing 35 µg. protein was incubated at 28°C for 30 minutes. The reaction was stopped by the addition of 1.0 ml. of trichloroacetic acid (20% W/V) and the supernatant chromatographed on paper with phenol:water (80:20) as solvent. Only the complete reaction mixture gave a spot which corresponded to authentic α-ketoglutarate when developed with ninhydrin spray.

COENZYME SPECIFICITY OF ENZYME

The reduced form of NAD in the reductive amination assay

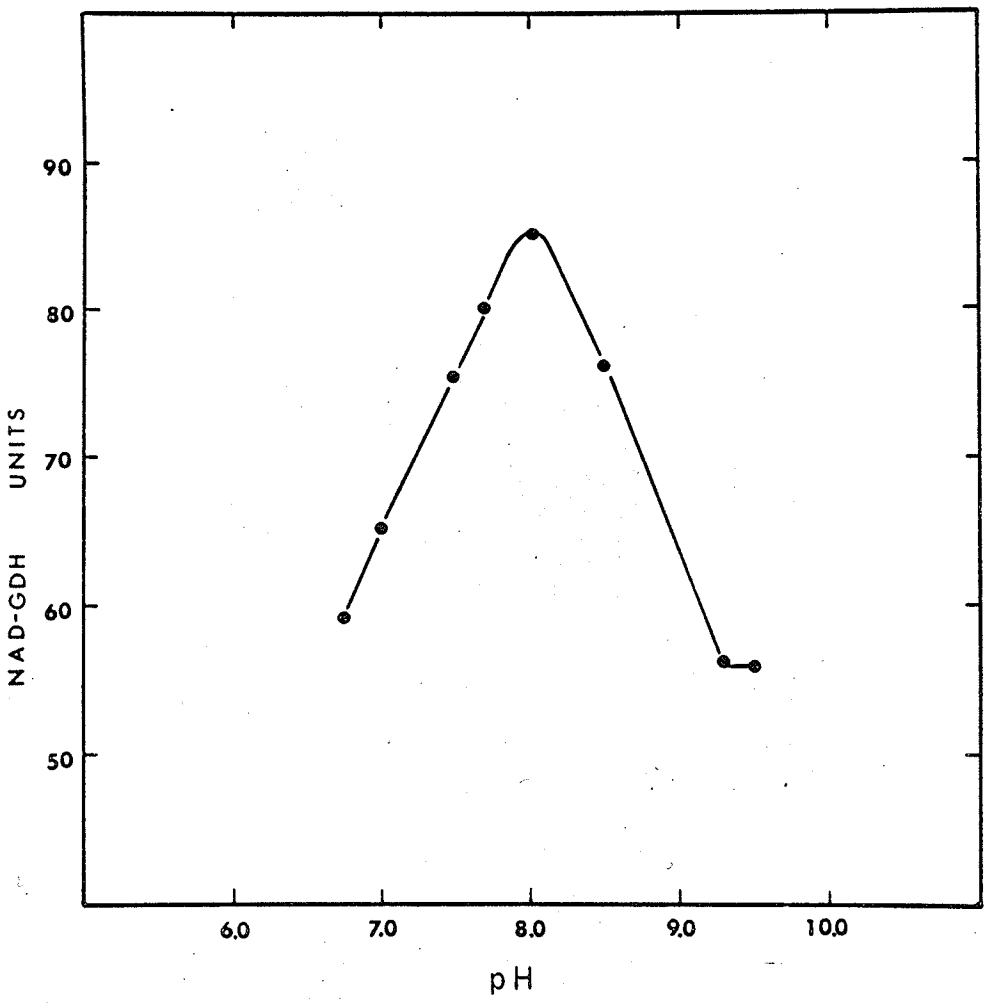


Figure 6. Effect of hydrogen ion concentration on the reductive amination of α-ketoglutarate by NAD-specific glutamate dehydrogenase.

and oxidized form of NAD in oxidative deamination assay, could not be replaced by NADPH or NADP respectively in the reaction mixture. The enzyme, when assayed with NADP at concentrations fifteen times that of NAD used normally in assays (see Methods), at various hydrogen ion concentrations still failed to catalyze the oxidative deamination of L-glutamate. The glutamate dehydrogenase isolated from Neurospora crassa, therefore, appears to be completely specific for the oxidized and reduced forms of NAD. In this respect it differs from the beef-liver glutamate dehydrogenase (Olson and Anfinsen, 1951) and is similar to one of the glutamate dehydrogenases of Fusarium (Sanwal, 1961a).

COENZYME ANALOGUES

The NAD analogues, deamino-NAD, 3-pyridinealdehyde-NAD, 3-pyridinealdehyde-deamino-NAD and 3-acetylpyridine were tested as coenzymes of the NAD-specific glutamate dehydrogenase. Only deamino-NAD functioned as a coenzyme, but activity with the analogue was only 1/20 of that obtained with NAD (Figure 7). 3-Pyridinealdehyde-NAD (at a concentration of (1×10^{-4}M) caused a 65% inhibition of activity in either the oxidative (NAD concentration, 5×10^{-3}M) or reductive (NADH concentration, 1×10^{-3}M) deamination assay. Again these results are unlike those obtained with beef liver glutamate dehydrogenase which shows as great or greater activity in the presence of NAD analogues than it

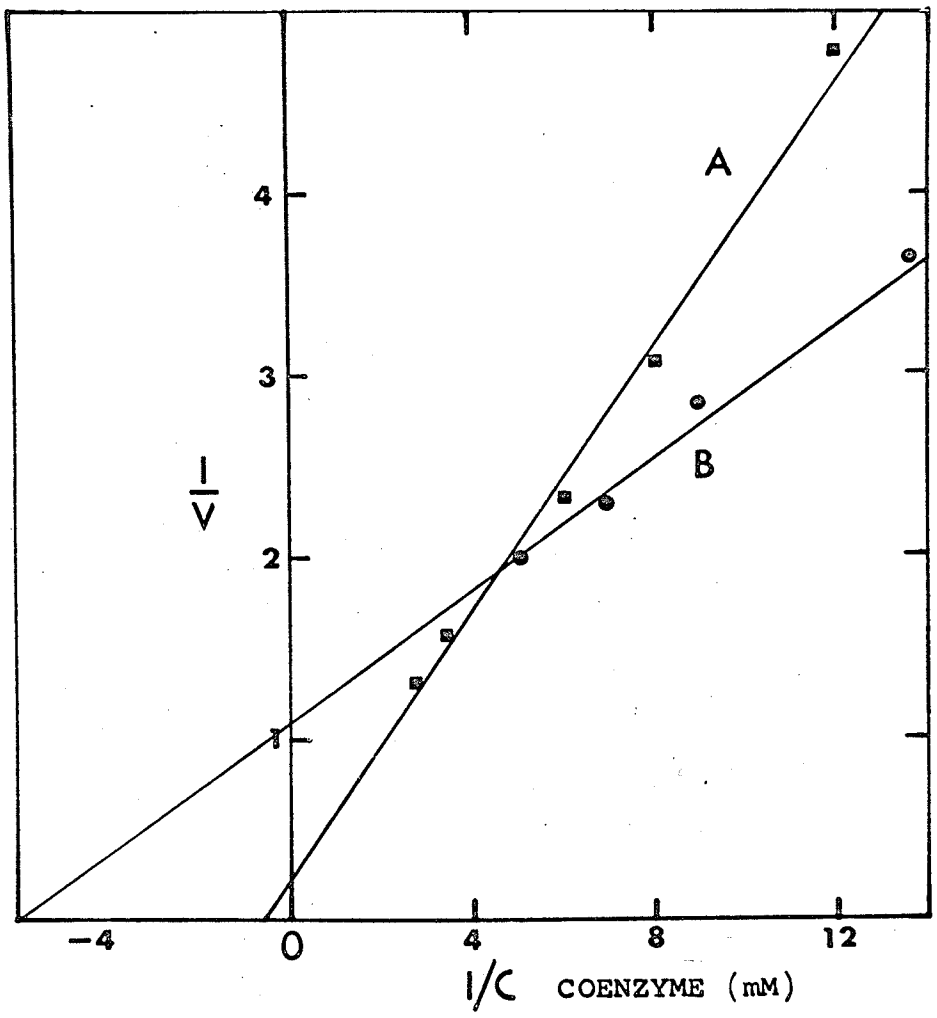


Figure 7. Double reciprocal plot of the rate of oxidative deamination of L-glutamate as a function of (A) deamino-NAD and (B) NAD by NAD-specific glutamate dehydrogenase.

does with NAD (Olson and Anfinsen, 1953; Kaplan et al., 1956).

AMMONIA ANALOGUES

None of the following compounds; methylamine (0.1M), dimethylamine (0.1M), ethylamine (0.1M) and urea (2×10^{-3}M) could be substituted for ammonium sulfate in the reductive amination assay. Olson and Anfinsen (1953) obtained similar results with beef-liver GDH.

SUBSTRATE SPECIFICITY

All amino and α-keto acids tested as substrates of the purified glutamate dehydrogenase were assayed at pH 7.0, 8.0, 9.0 and 10.0. At a concentration of 6.6×10^{-3}M, L-alanine, L-leucine, L-valine, L-isoleucine and L-aspartate were completely inactive as substrates, even when assayed at enzyme concentration twice that used in normal assays. The α-keto acids, pyruvate, α-ketobutyric acid, α-ketovoleric acid, α-ketoisovaleric acid, α-ketocaproic acid and α-ketoisocaproic acid were also inactive as substrates in the reductive amination assay.

EFFECT OF INHIBITORS

The effect of various inhibitors on oxidative deamination of L-glutamate is given in Table 2. Metal-chelating reagents such as,1,10-phenathroline, sodium sulfate, sodium

TABLE 2. Effect of inhibitors on NAD-glutamate dehydrogenase activity.

Inhibitor	Concentration M	Percent Inhibition
EDTA	1×10^{-3}	43
pCMB	1×10^{-3}	100
pCMB	1×10^{-4}	86
KCN	1×10^{-3}	-
NaN_3	1×10^{-2}	-
glutaric acid	3×10^{-2}	41
O-phenanthroline	2×10^{-3}	-
Diethylstilbesterol	1×10^{-3}	-
NaF	1×10^{-2}	-
$\alpha\alpha$ - dipyridyl	1×10^{-3}	-
8 - hydroxyquinoline	2×10^{-4}	-
Thiourea	1×10^{-3}	-
Glycine amide	2×10^{-2}	-
L-glycine	16×10^{-2}	-
L-Serine	2×10^{-2}	-
GTP, GMP, IMP	1×10^{-3}	100
3-pyridinealdehyde-NAD	1×10^{-4}	65

axide, 8-hydroxyquinoline, thiourea, potassium cyanide, sodium fluoride and $\alpha\alpha$-dipyridyl were ineffective as inhibitors of the reaction. Ethylenediamine tetra-acetic acid exhibited only 43% of the enzymatic activity at a concentration of 10^{-3} M.

The NAD-GDH was found to be sensitive to the sulf-hydryl reagent, p-hydroxymercuribenzoate (PCMB) and the inhibition could be reversed by the addition of 2×10^{-3} M reduced glutathione. The sensitivity to PCMB suggests that -SH groups are involved in the catalytic activity of the enzyme.

The Neurospora NAD-GDH was not inhibited by diethyl-stilbesterol (DES), unlike the beef-liver glutamate dehydro-genase for which the hormone serves as a potent inhibitor. Yielding et al., (1960, 1960a) reported that the inhibition of beef-liver GDH by DES was due to dissociation of the enzyme polymer into enzymatically inactive subunits.

EQUILIBRIUM CONSTANT

A reaction mixture containing 20 µmoles L-glutamate, 0.5 µmoles NAD, 273 µmoles Tris-HCl buffer, pH 9.5 and 40 µg. enzyme in a total volume of 3.0 ml. was incubated at 26.5°C for 30 minutes. The NADH concentration was determined by measuring the extinction coefficient at 340 mµ and using the molar absorbancy index of 6.22×10^6 sq.cm./mole (Horecker and Kernberg, 1948). α-Ketoglutarate was

quantitatively determined by the colorimetric proceedure of Friedmann and Haugen (1943). The equilibrium constant of the reaction (K_{eq}) is defined as:

$$K_{eq} = \frac{(NADH)(NH_4^+)(\quad\text{-ketoglutarate})(H^+)}{(\text{L-glutamate})(NAD^+)}$$

and the value, uncorrected for ionization of the reactants, was found to be, 5.2×10^{-12} at pH 9.5.

ENERGY OF ACTIVATION

The effect of temperature on the velocity of the oxidative deamination of L-glutamate is given in Figure 8 in the form of an Arrhenius plot. The reaction mixture containing 16 m moles L-glutamate, 0.22 m moles NAD, 0.10 M Tris-HCl buffer, pH 9.5, in a final volume of 3.0 ml. was equilibrated at various temperatures for 15 minutes, after which 30 µg. enzyme was added. The reaction was stopped by the addition of 1.0 ml. Trichloroacetic acid (10% W/V) after a further 15 minute incubation period, and the α-ketoglutarate produced was determined according to Friedmann and Haugen (1943). The Arrhenius plot (Figure 8) of logarithm of µgm. α-ketoglutarate as a function of the reciprocal of the absolute temperature showed linearity between 0°C to 27°C as predicted by the Arrhenius equation. An integrated form of the Arrhenius equation is expressed as:

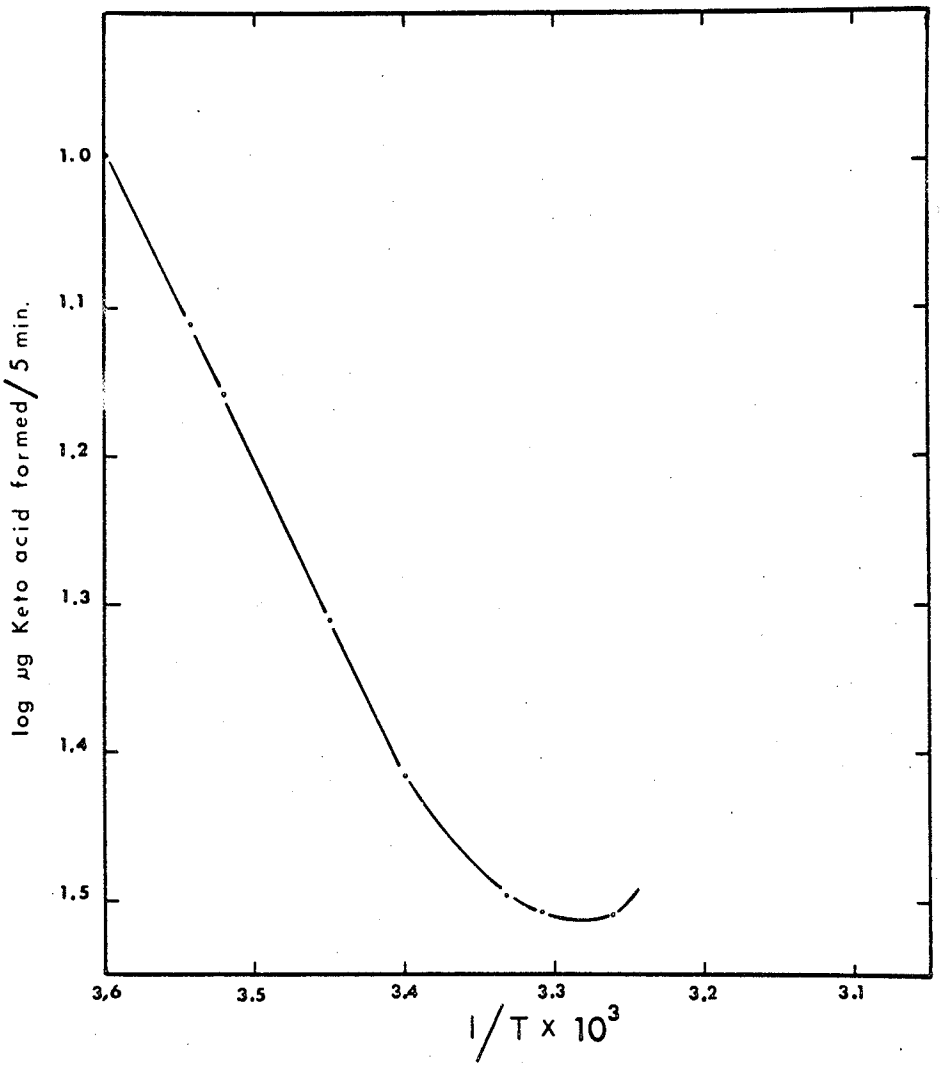


Figure 8. Arrhenius' plot of NAD-specific glutamate dehydrogenase activity of Neurospora crassa.

$$\log \frac{k_2}{k_1} = \frac{E(T_2 - T_1)}{RT_1T_2} \qquad (2)$$

where k_1 and k_2 are the reaction rates at two temperatures, T_2 and T_1 are the absolute temperatures, R is the gas constant and E is the activation energy. By direct substitution in equation (2), the energy of activation for NAD-specific glutamate dehydrogenase was calculated to be 11,835 calories/mole. Activation energies of 4,185 calories/mole for L-leucine dehydrogenase and 11,112 calories/mole for NADP-specific glutamate dehydrogenase have been reported by Zink (1963), whereas Olson and Anfinsen (1953), reported a value of 14,000 calories/mole for ox-liver glutamate dehydrogenase.

THERMAL INACTIVATION

The effect of temperature on the NAD-specific glutamate dehydrogenase enzyme was determined by heating the enzyme at the indicated temperatures for various time intervals. The enzyme was suspended in 0.1 M Tris-GSH buffer, pH 8.0. Portions of the enzyme (0.5 mg. protein/ml.) solution were dispensed into tubes, incubated in a water-bath set at the desired temperature and samples removed at 1 to 5 minute intervals. Samples were immediately cooled to 5°C by placing in an ice-salt bath. The results are shown in Figure (9) where velocity is plotted

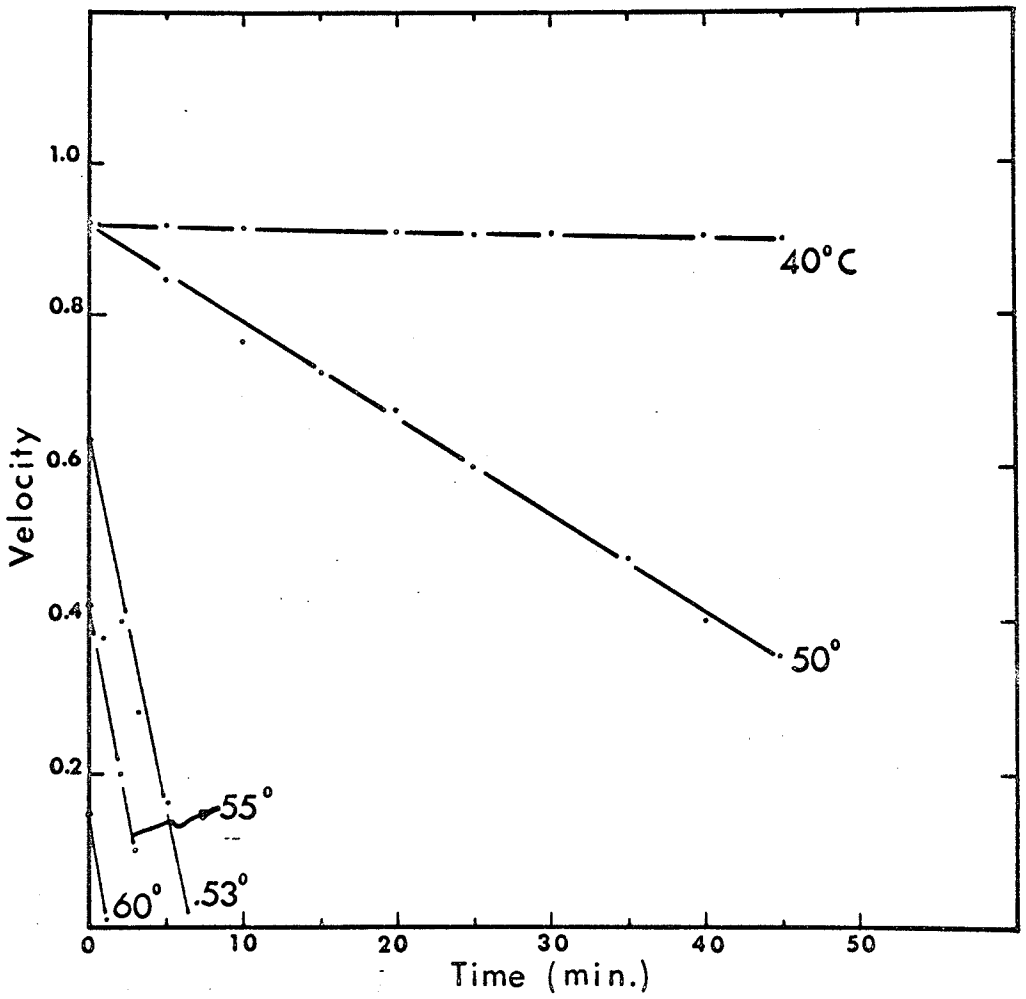


Figure 9. Thermal inactivation of NAD-specific glutamate dehydrogenase.

as a function of time. From these plots the half life of glutamate dehydrogenase can be determined as 2 minutes at 53°C.

EFFECT OF PURINE NUCLEOTIDES

Following the report that purine nucleotides act as inhibitors or activators of beef-liver GDH (Wolff, 1962; Frieden, 1963), the effect of these compounds was tested on the activity of NADP and NAD-specific glutamate dehydrogenases of Neurospora crassa. The NAD-GDH was purified according to the proceedure outlined in this section and the NADP-specific GDH by the method of Sanwal and Lata (1962). Figure 10 represents the results of experiments with NADP-specific glutamate dehydrogenase in which NADPH was varied in the absence and presence of 1.1×10^{-3} M GTP. NADPH shows substrate inhibition which is also the case for NADH as substrate of the NAD-specific glutamate dehydrogenase (Figure 11). However, GTP had no effect on the velocity of the NADP-GDH reaction (Figure 10). Similar results were obtained when ATP, ADP, AMP, GTP, CDP, UTP and GDP (all tested at 10^{-3}M) were used in the place of GTP. With the NAD-specific enzyme, however, GTP caused a marked inhibition of the reaction when measured either in the oxidative deamination (Figure 12) or reductive amination assay. The inhibition was competitive with the oxidized

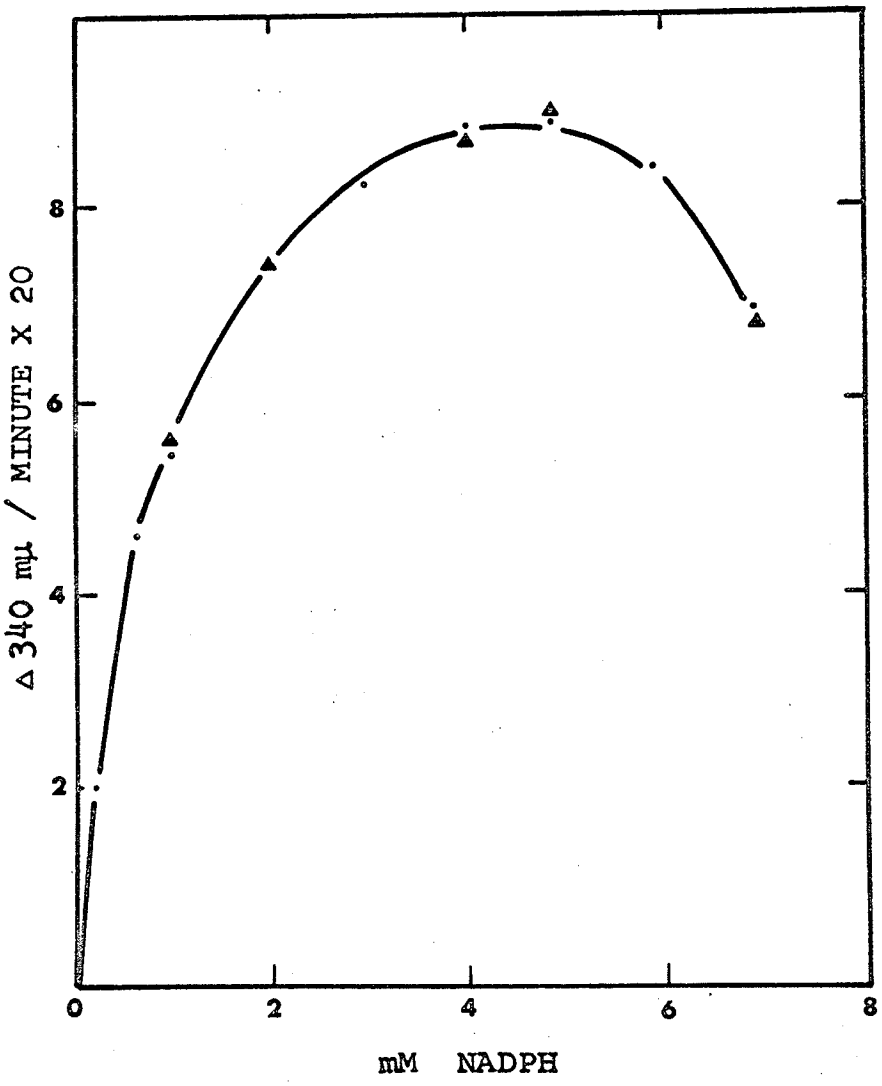


Figure 10. NADPH inhibition of NADP-specific glutamate dehydrogenase catalyzed reductive amination of ∝-ketoglutarate. Assay mixture contained: 13 mM ∝-ketoglutarate; 80 mM $(NH_4)_2SO_4$; 0.02 M Tris-HCl buffer (pH 7.5) and 1.2 μg./ml. enzyme. (●-●-●) minus GTP; (▲-▲-▲), 1.1×10^{-3} MGTP.

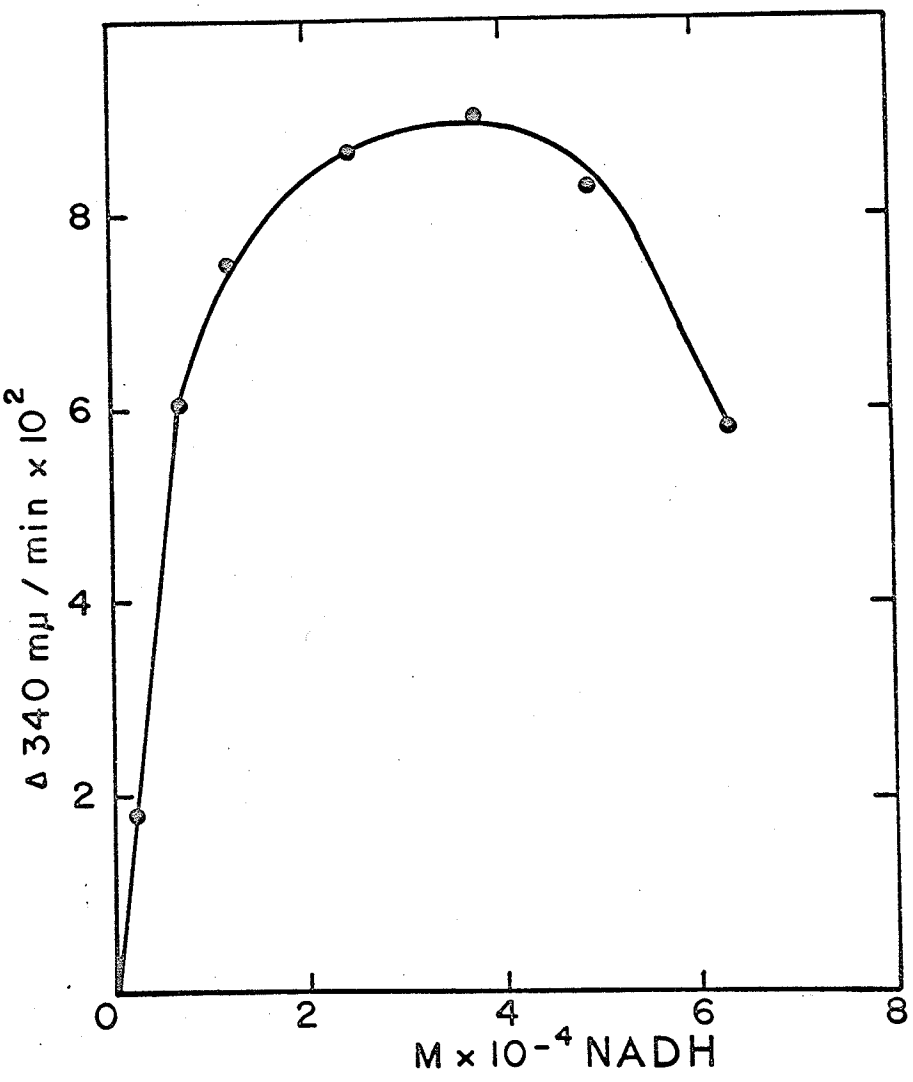


Figure 11. Substrate inhibition of NAD-specific glutamate dehydrogenase by high NADH concentrations.

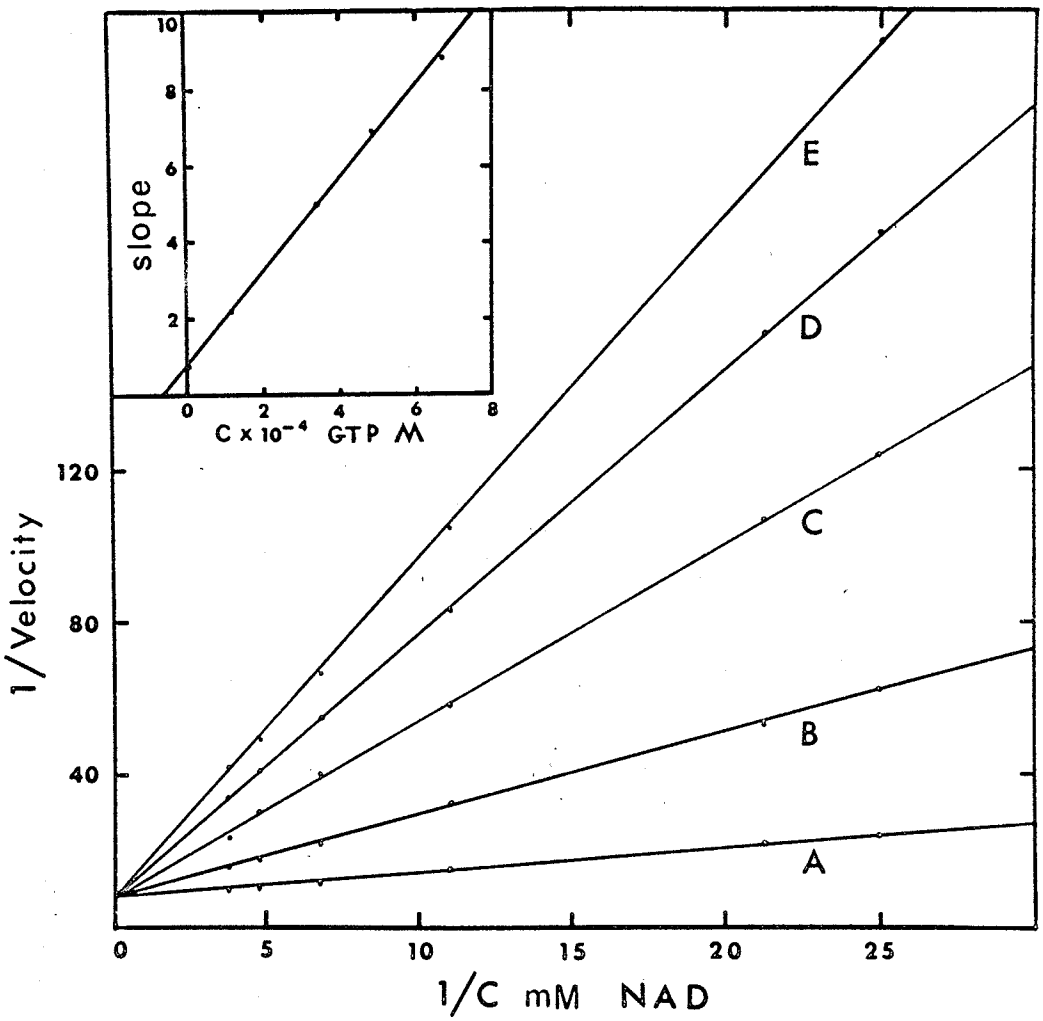


Figure 12. Double reciprocal plots of velocity versus NAD concentrations at several fixed concert rations of GTP L-glutamate concentration 25 mM. GTP concentrations: (A) none; (B) 0.13 mM; (C) 0.33 mM; (D) 0.49 mM; (E) 0.66 mM. Insert is replot of slopes versus GTP concentration.

(Figure 12) or reduced coenzyme and the K_i calculated according to Cleland (1963) was 6×10^{-5} M. ATP, ADP, CTP, CDP, UTP and UDP, when tested separately at a concentration of 1×10^{-3} M, had no effect on the enzyme activity. The nucleotides GDP, GMP and IMP inhibited the NAD-specific enzyme competitively (Figures 13, 14 and 15). The K_i values given in Table 3 show that GDP and GMP values are nearly similar to GTP, but inhibition by IMP is considerably smaller. ATP by itself does not alter enzyme activity, yet it must complex with the NAD-specific glutamate dehydrogenase because, in its presence the inhibition caused by GTP is alleviated considerably (Figure 16).

KINETIC ANALYSES

The kinetic data presented here were all processed according to Cleland (1963c) using an IBM-1620 digital computer. The nomenclature of the reaction mechanism and description of kinetic constants used here is also that proposed by Cleland (1963). Iterative least square fits of the data were made to equation (3) when preliminary plotting of the data in the reciprocal form ($\frac{1}{V}$ vs. $\frac{1}{C}$) showed that they were straight lines.

$$v = \frac{V_S}{K+S} \qquad (3)$$

Replots of slopes or intercepts obtained from this

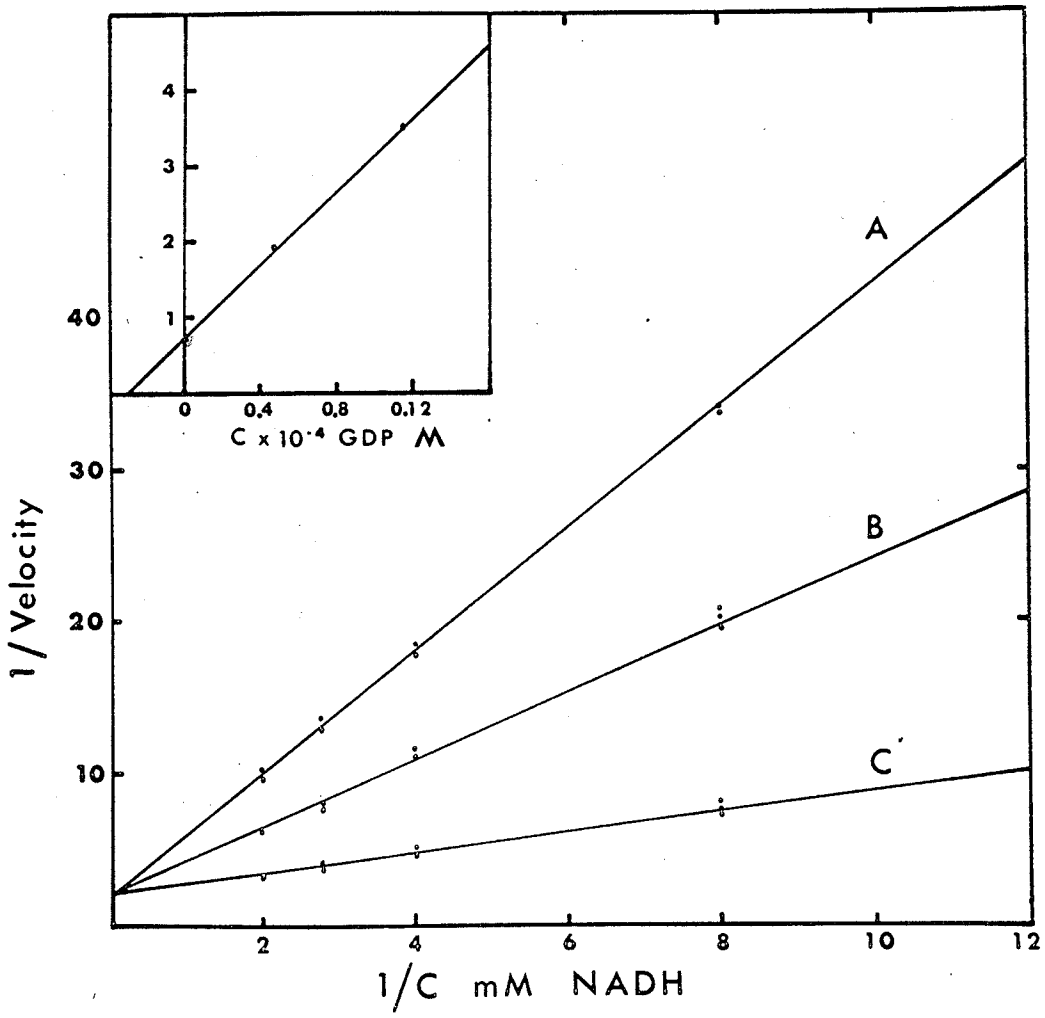


Figure 13. Double reciprocal plots of velocity versus NADH concentrations at several fixed concentrations of GDP. α-ketoglutarate concentration; 30 mM. GDP concentrations; (A) 0.115 mM; (B) 0.046 mM; (C) none. Enzyme concentration: 1.5 µg./ml. The insert is replot of slopes versus GDP concentrations.

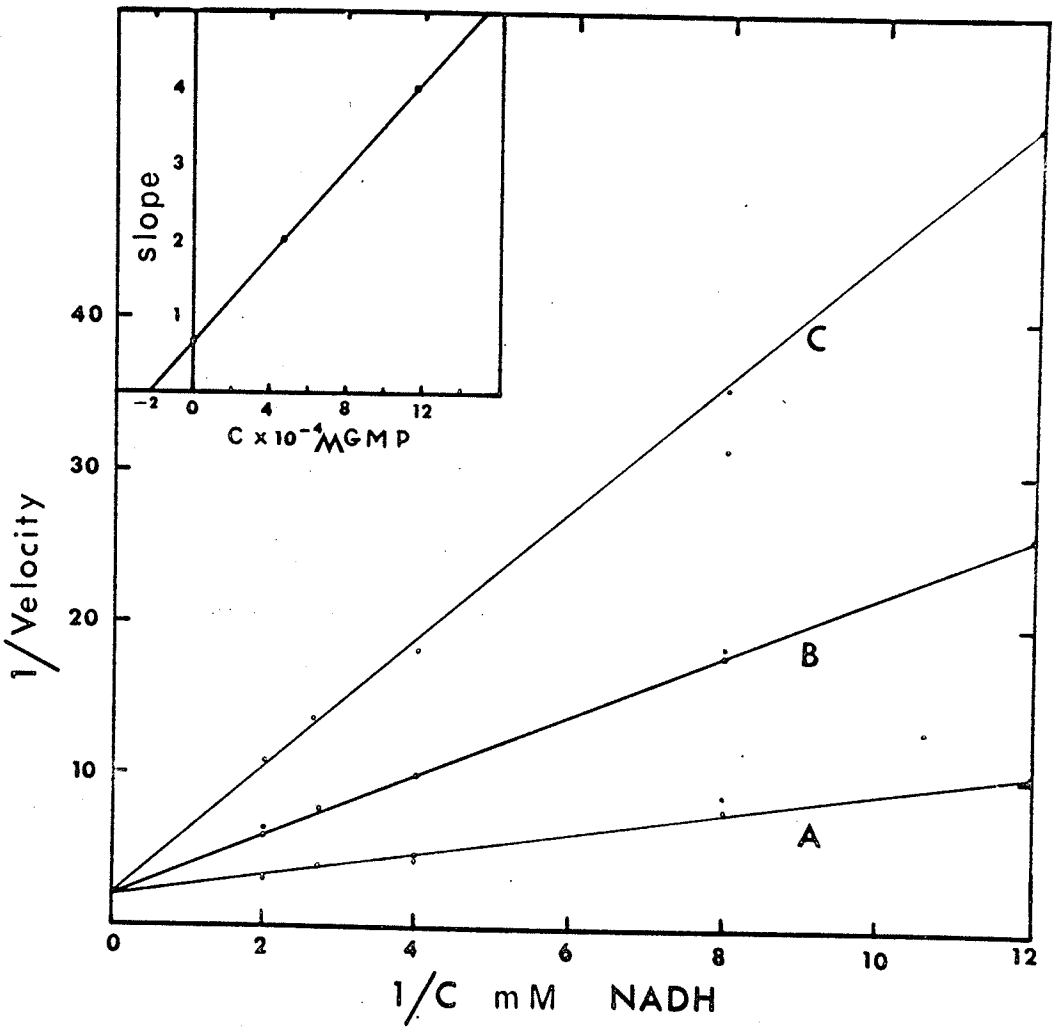


Figure 14. Double reciprocal plots of velocity versus NADH concentration at several fixed concentrations of GMP. GMP concentrations; (A) none; (B) 0.046 mM; (C) 0.115 mM; α-ketoglutarate concentration: 25 mM. Insert is replot of slopes versus GMP concentrations.

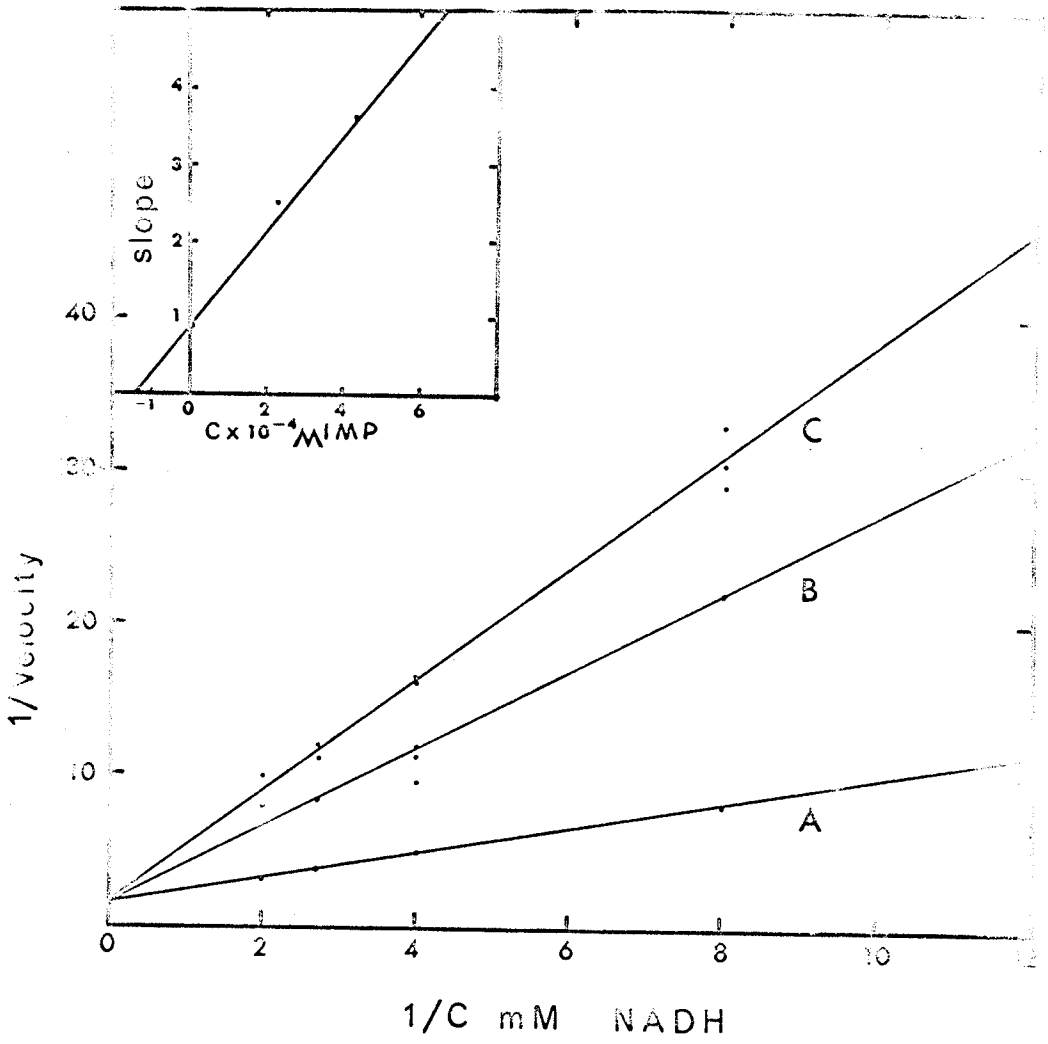


Figure 15. Double reciprocal plots of velocity versus NADH concentration at several fixed concentrations of IMP. IMP concentrations: (A) none; (B) 0.215 mM; (C) 0.43 mM; α-ketoglutarate concentration: 25 mM. Insert is replot of slopes versus IMP concentration.

TABLE 3. Some purine nucleotide inhibition constants of NAD-specific glutamate dehydrogenase.

Purine Nucleotide	Inhibition Constant
Guanosine-5'-triphosphate (GTP)	6×10^{-5}M
Guanosine-5'-diphosphate (GDP)	3×10^{-5}M
Guanosine-5'-phosphate (GMP)	2.5×10^{-5}M
Inosine-5'-phosphate (IMP)	1.5×10^{-4}M

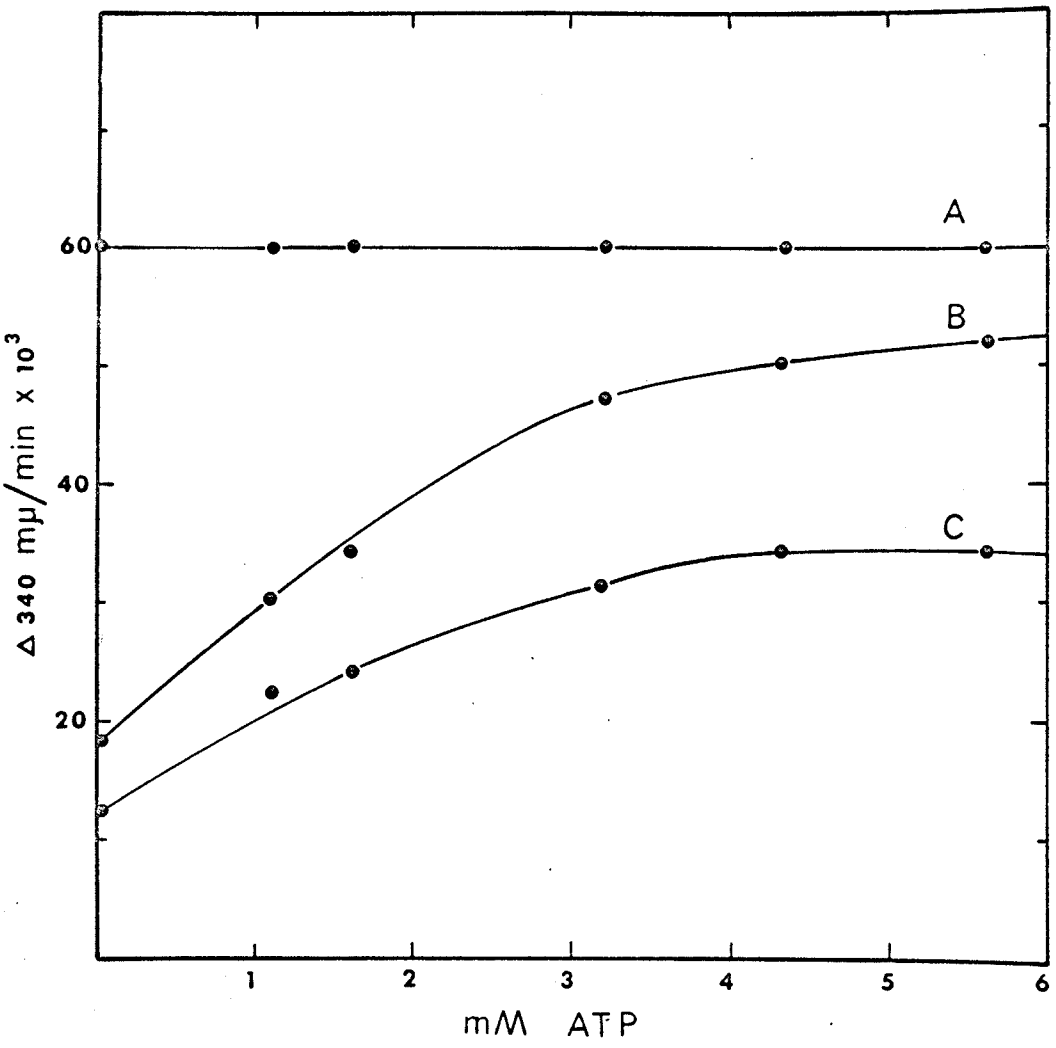


Figure 16. Reversal of GTP inhibition of the NAD-specific glutamate dehydrogenase by ATP. α-ketoglutarate concentration; 40 mM. Enzyme concentration; 0.66 μg./ml. GTP concentrations: (A) none; (B) .03 mM; (C) .05 mM.

analysis were made against inhibitor concentration to determine the nature of the inhibition, or against the reciprocal of the concentration of the non-varied substrate in initial velocity studies. Replot data was fitted to a straight line, $y = ax + b$, or a parabola, $y = a + bx + cx^2$ using weighting factors supplied by the first fits to equation (3). Whenever possible the data was fitted to an overall rate equation describing the type of inhibition. Data was fitted to equation (4) for linear competitive inhibition,

$$v = \frac{VS}{K(1 + I/K_i) + S} \qquad (4)$$

to equation (5) for linear uncompetitive inhibition,

$$v = \frac{VS}{K + S(1 + I/K_i)} \qquad (5)$$

and to equation (6) for linear non-competitive inhibition,

$$v = \frac{VS}{K(1 + I/K_{is}) + S(1 + I/K_{ii}} \qquad (6)$$

and equation (7) for I-parabolic uncompetitive inhibition.

$$v = \frac{VS}{K + S\,(1 + I^1/K_{i_1} + I^2/K_{i_2})} \qquad (7)$$

INITIAL VELOCITY STUDIES

All initial velocity studies were performed at pH 9.0 in 0.2 M Tris-HCl buffer. With NAD as a variable substrate at several fixed concentrations of L-glutamate, the double

reciprocal plots were linear (Figure 17). The values of slopes (K/V) and intercepts (1/V) obtained from fits to equation (3) when plotted against reciprocal of L-glutamate concentrations were also linear (Figure 18). The double reciprocal plots and replots of K/V and 1/V, with L-glutamate as variable and NAD as the changing fixed substrate, were also linear.

In the reductive amination direction with ∝-ketolglutarate as the variable substrate at several fixed concentrations of NADH, the double reciprocal plots were linear (Figure 21). The slope and intercept replots when plotted against reciprocal of NADH concentrations were also linear (Figure 22). The initial velocity analysis of both substrates, NAD and L-glutamate, correspond to equation (8)

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_{ia}K_b}{AB} + \frac{K_a}{A} + \frac{K_b}{B}\right) \qquad (8)$$

where A and B are substrate concentrations, K_a and K_b are Michaelis constants of A and B and K_{ia} is the dissociation constant for A. A distinction between A and B is not possible in this case, unless product inhibition or binding studies show one of them to be binding to the free enzyme form (see later).

KINETIC CONSTANTS

Michaelis constants: The K_m values are defined here

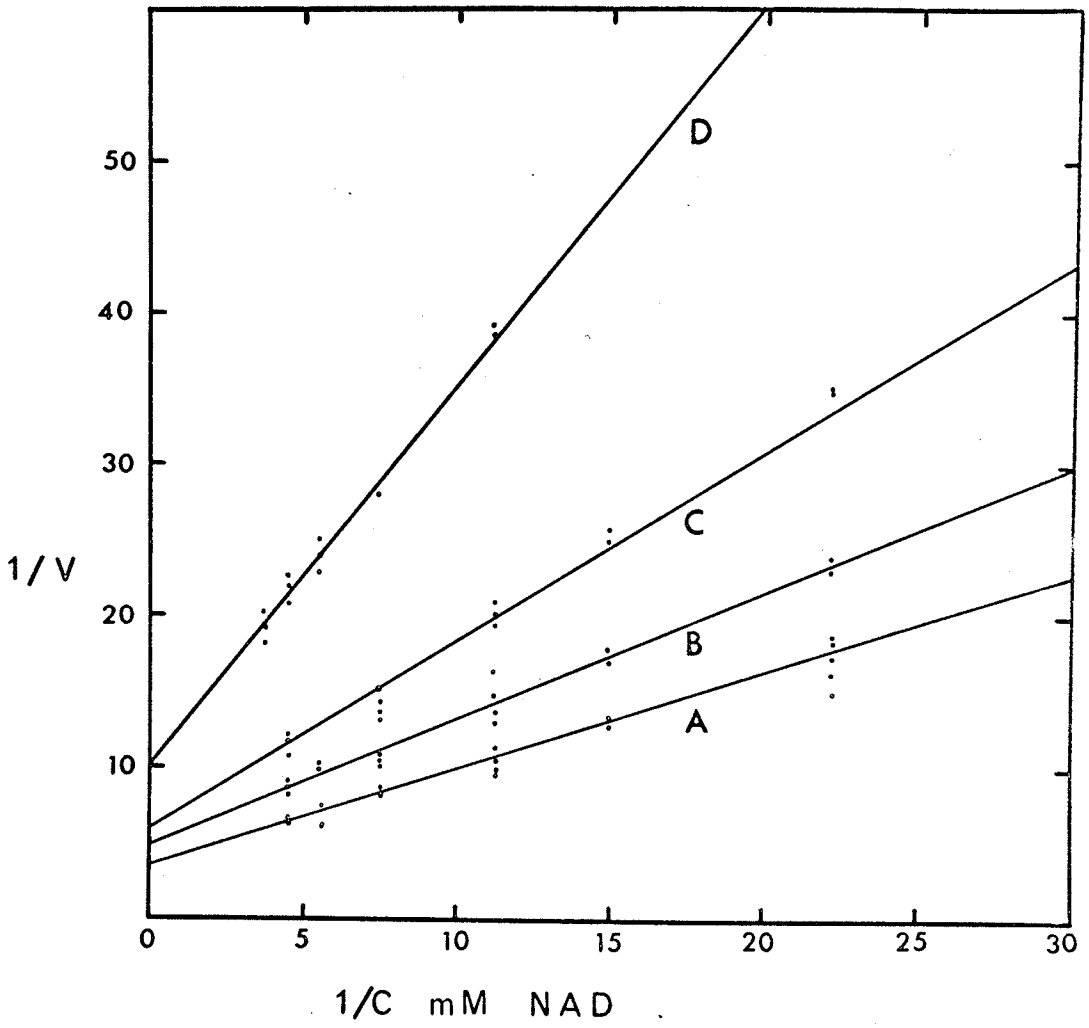


Figure 17. Double reciprocal plots of velocity versus NAD concentration at several fixed concentrations of L-glutamate. L-glutamate concentrations: (A) 6.66 mM; (B) 3.33 mM; (C) 1.99 mM; (D) 0.66 mM. Enzyme concentration: 0.7 μg./ml.

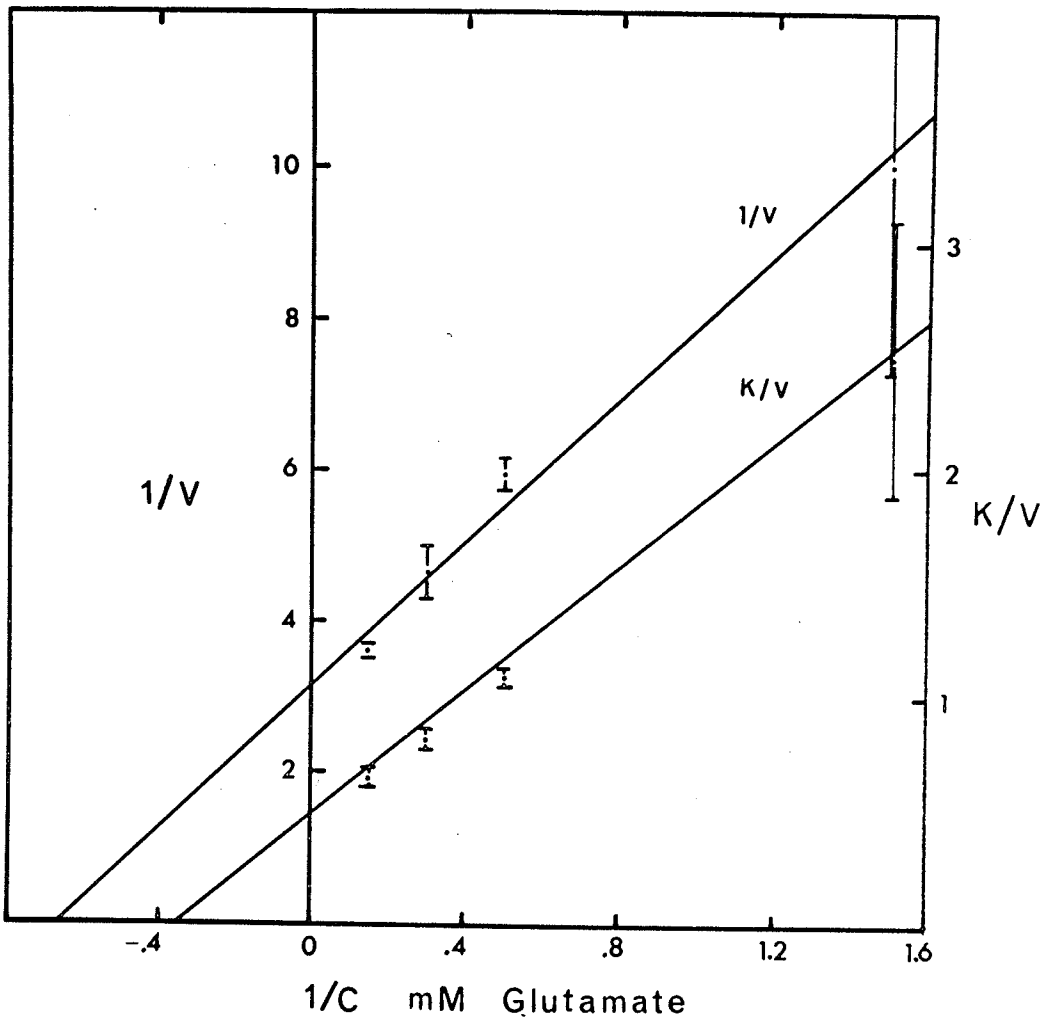


Figure 18. Replots of slopes and intercepts from Figure 17 versus reciprocal of L-glutamate concentration.

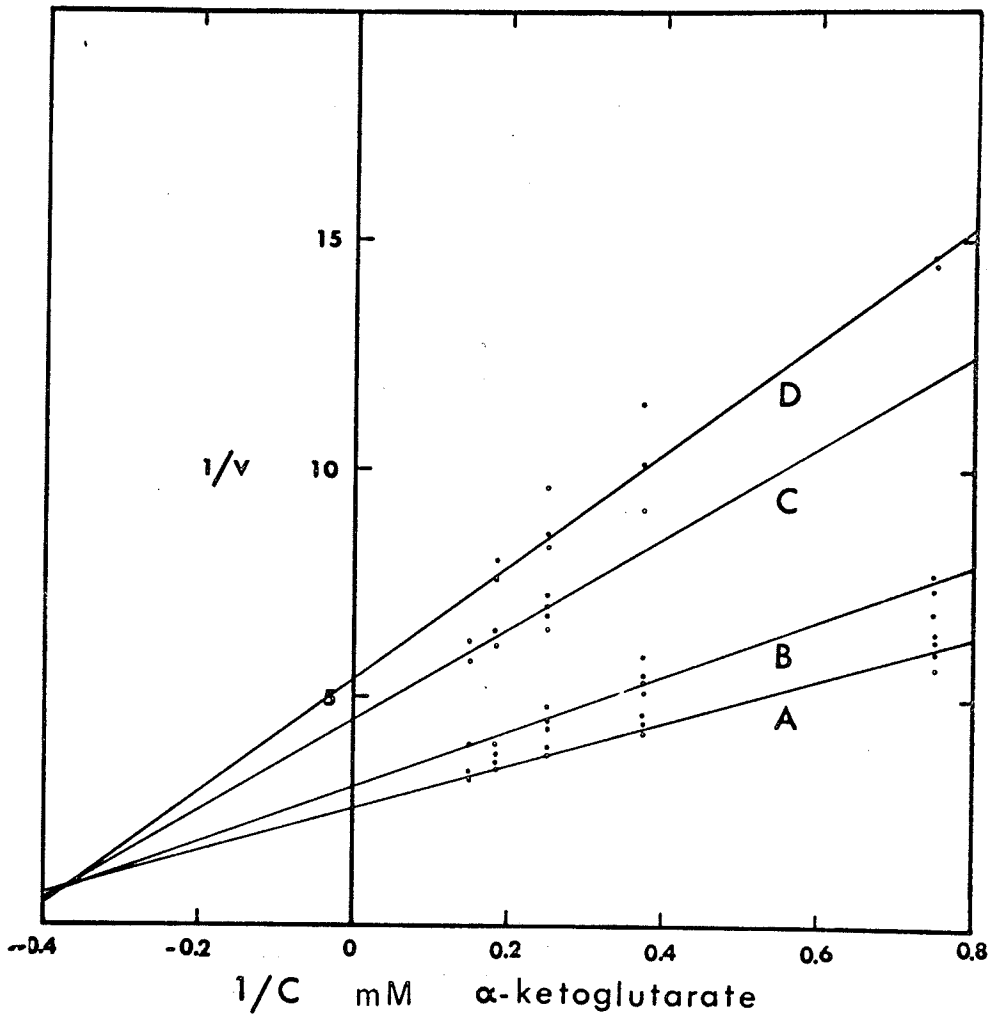


Figure 21. Double reciprocal plots of velocity versus α-ketoglutarate at several fixed concentrations of NADH. Ammonia sulfate concentration constant at 1.7 mM. NADH concentrations: (A) 0.174 mM; (B) 0.087 mM; (C) 0.065 mM; (D) 0.043 mM.

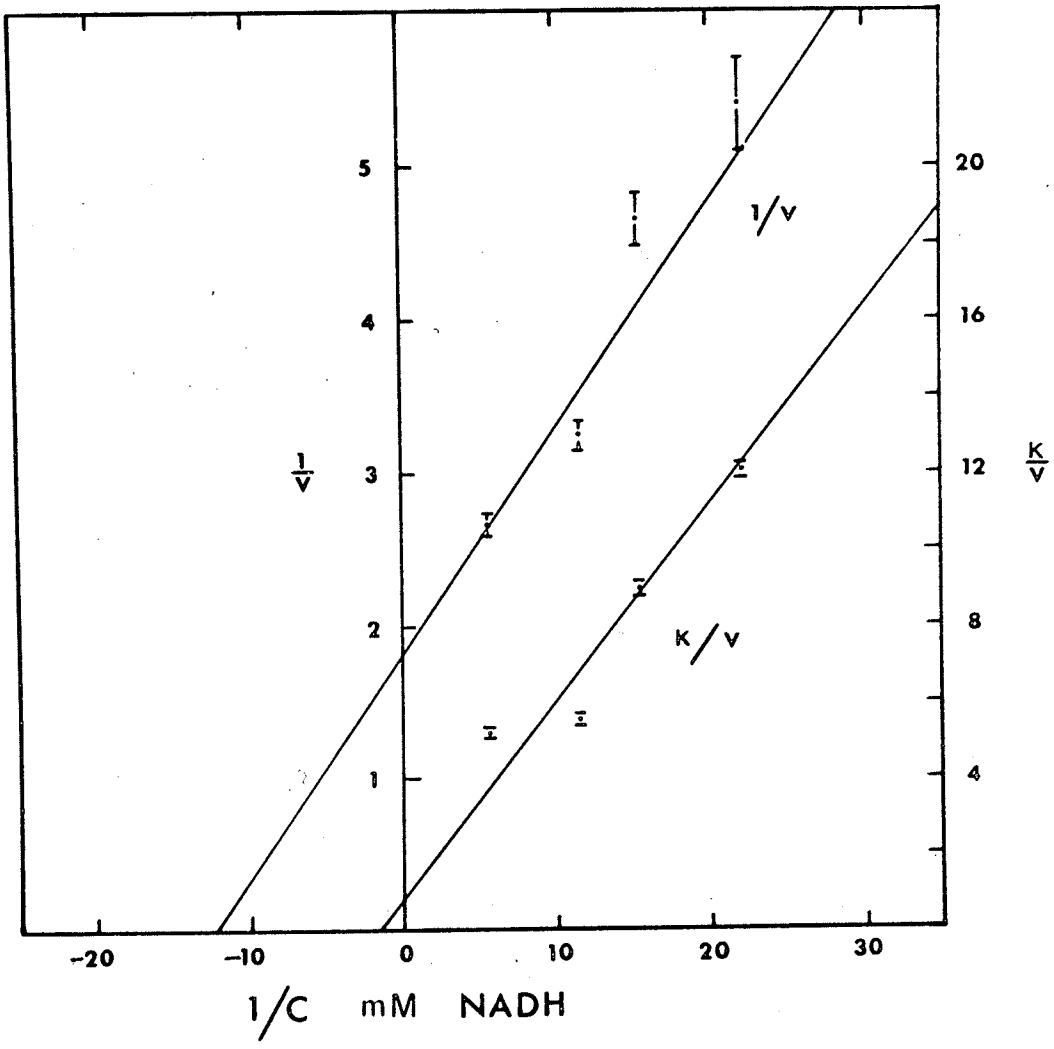


Figure 22. Replots of slopes and intercepts from Figure 21 versus reciprocal of NADH concentration.

as the concentration of substrate giving 1/2 value of V_{max} in initial velocity studies, when the enzyme is saturated with the second reactant. The K_m for fixed changing substrate is obtained from the intersection point on the horizontal axis of a replot of intercepts against reciprocal of fixed changing substrate. The K_m for the varied substrate is also obtained from the ratio of the intersection points on the vertical axis of the 1/V and K/V replot (Florini and Vestling, 1957).

INHIBITION CONSTANTS

The inhibition constant for the first substrate (A) to combine with the enzyme (K_{ia}, which is also the dissociation constant of A) is determined from the common intersection point of the reciprocal plots (above, on, or below the horizontal axis) where A is the variable substrate (Frieden, 1957a).

Product inhibition constants are determined in the case of competitive inhibition from the intersection point on the horizontal axis of a slope versus inhibitor replot (e.g. Figure 24). In cases of non-competitive inhibition, replot of slopes versus inhibitor gives a horizontal intersection point of

$$- K_i \left(1 + \frac{X}{K_{ix}}\right)$$

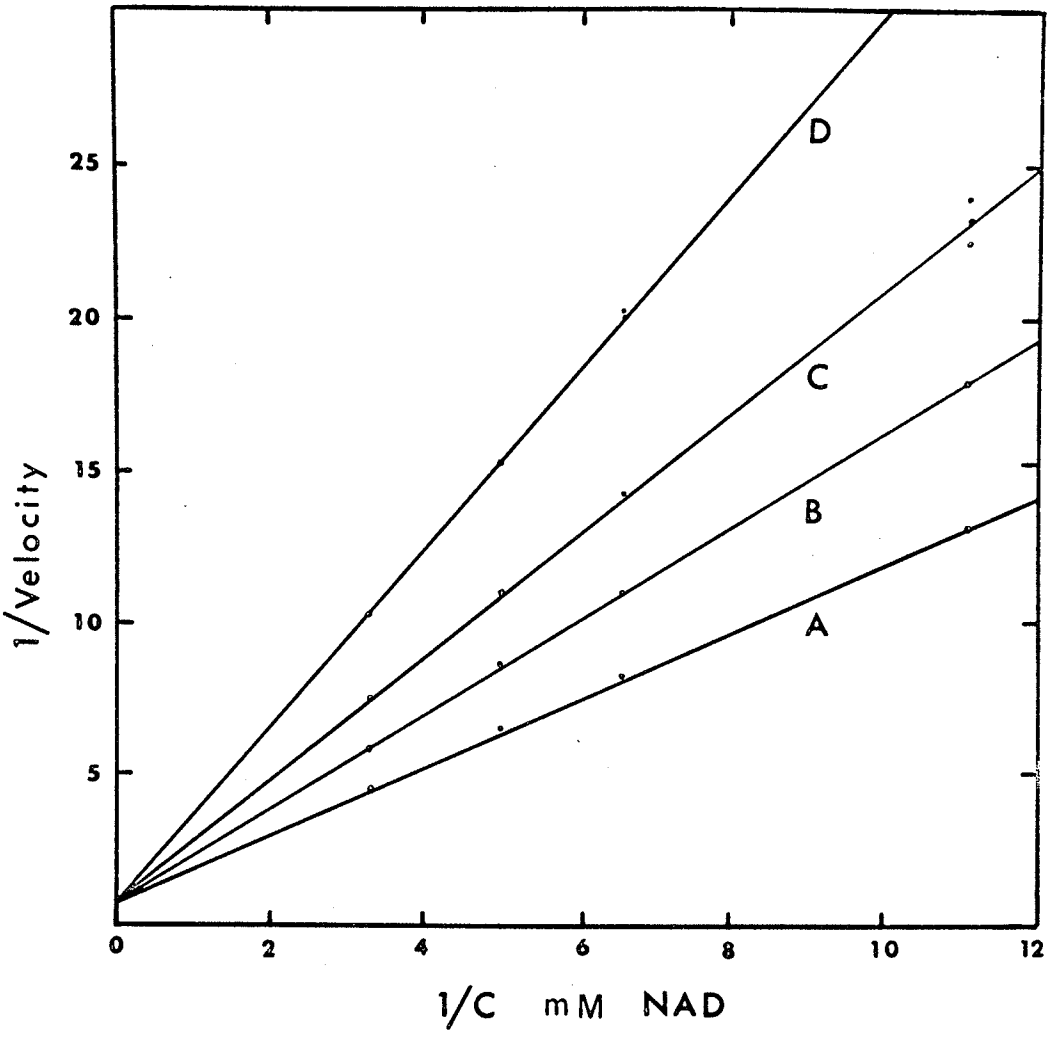


Figure 23. Product inhibition of glutamate dehydrogenase by NADH with NAD as varied substrate and a constant high concentration of L-glutamate (5.3 mM). NADH concentrations: (A) 0.15 mM; (B) 0.21 mM; (C) 0.32 mM; (D) 0.43 mM.

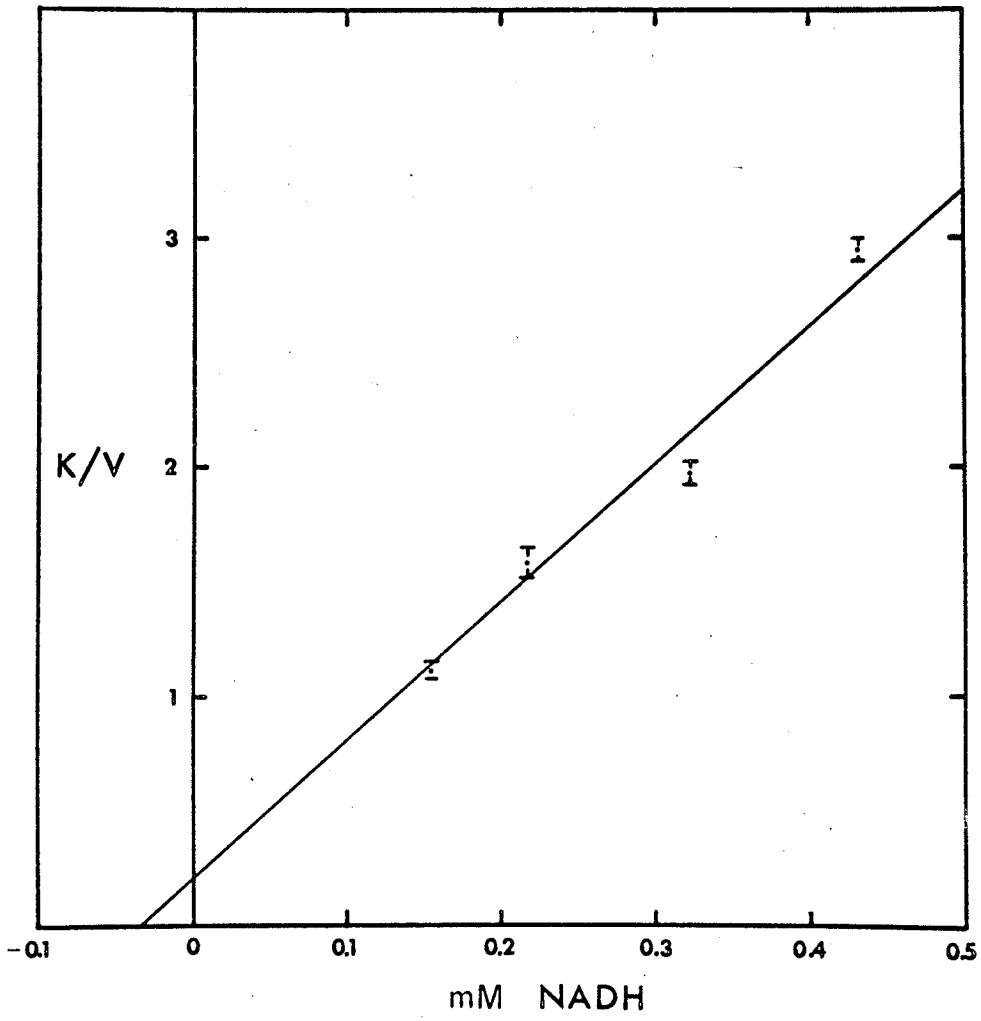


Figure 24. Replots of slopes from Figure 24 versus NADH concentrations.

TABLE 4. Some kinetic constants of NAD-specific glutamate dehydrogenase.

Constant	mM
Michaelis constants	
K_a	0.154 ± 0.010
K_b	1.48 ± 0.223
K_q	0.51 ± 0.200
K_r	0.081 ± 0.037
Inhibition constants	
K_{ip}	11.70 ± 0.94
K_{iq}	1.26 ± 0.30
K_{ir}	0.035 ± 0.31

and a replot of 1/V gives

$$- K_i \left(1 + \frac{X}{K_X} \right)$$

where X is the concentration of non-varied substrate and K_{ix} and K_X are the inhibition and Michaelis constant of X, and K_i is the inhibition constant of product. In the case of uncompetitive inhibition, the inhibition constant is obtained from the horizontal intercept of a 1/V versus inhibitor replot.

All constants which could be evaluated from data presented in Figures 17 to 34 are given in Table 4.

PRODUCT INHIBITION STUDIES

Product inhibition studies were made to determine the order of substrate addition and the release of products. Double reciprocal plots of NAD as varied substrate at different fixed NADH concentrations gave competitive inhibition (Figure 23), and replots of slopes against NADH concentration were linear (Figure 24). Linear non-competitive inhibition was obtained when L-glutamate was used as the variable substrate at several fixed levels of NADH (Figure 25). A linear relation also existed when the slopes or intercepts were reploted against NADH concentrations (Figure 26). The fact that NADH shows competitive inhibition with NAD and non-competitive with L-glutamate

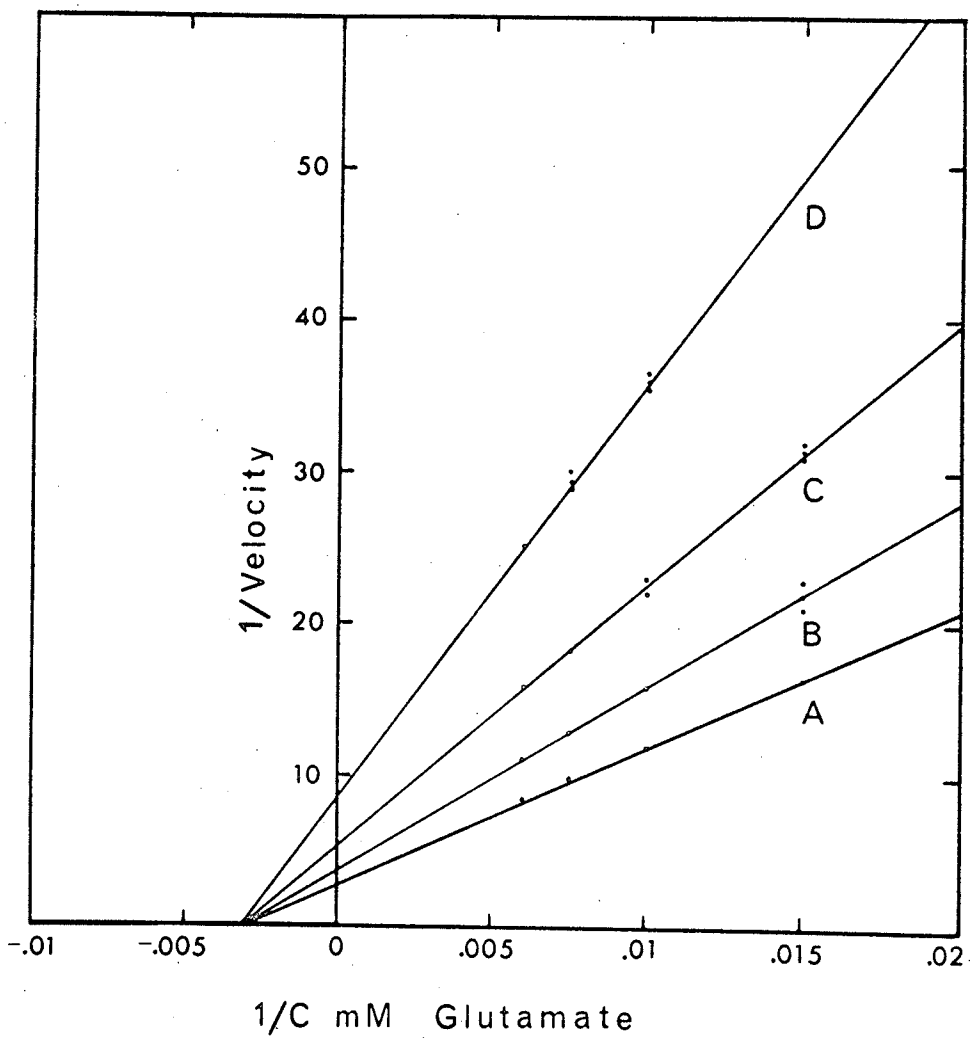


Figure 25. Production inhibition of glutamate dehydrogenase by several fixed levels of NADH with L-glutamate as variable substrate. Constant NAD concentration (9.2×10^{-4}M). NADH concentrations: (A) none; (B) 0.04 mM; (C) 0.10 mM; (D) 0.21 mM.

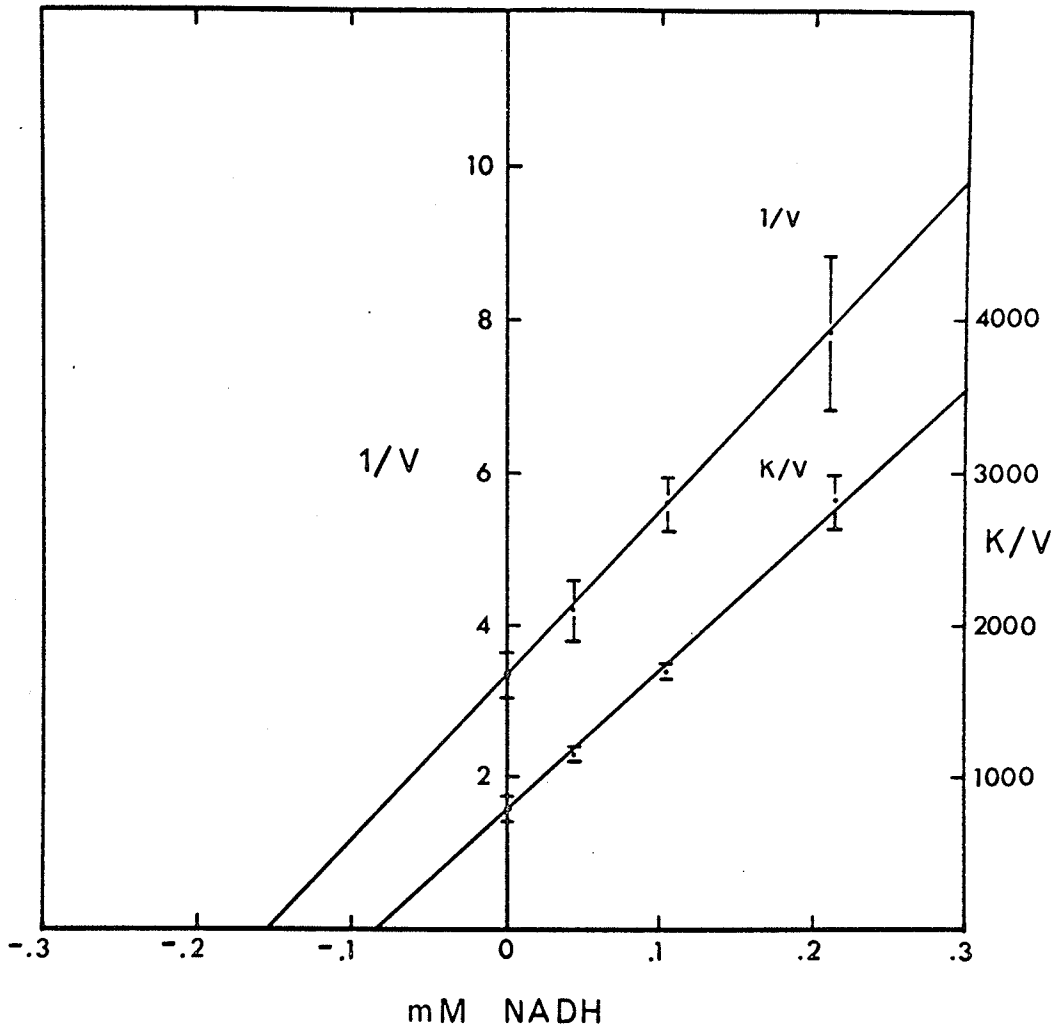


Figure 26: Replots of slopes and intercepts from Figure 25 versus NADH concentrations.

strongly suggests that NAD and NADH are able to bind to the free enzyme form.

Using ammonium sulphate as inhibitor, NAD was varied at constant L-glutamate concentration (13 times K_m). The inhibition was non-competitive (Figure 27) and replots of slopes and intercepts against inhibitor concentrations were linear (Figure 28). Again, non-competitive inhibition (Figure 29) and linear slope or intercept replots (Figure 30) resulted when ammonium sulphate was used as inhibitor and L-glutamate was the varied substrate.

With α-ketoglutarate as inhibitor and L-glutamate as variable substrate in the presence of high NAD concentrations (approximately 10 times K_m), the inhibition was uncompetitive (Figure 31) and replots of intercepts against varying α-ketoglutarate concentrations was linear (Figure 32). Although the inhibition was also uncompetitive with α-ketoglutarate as inhibitor and NAD as variable substrate (Figure 33) the replot of the intercepts versus inhibitor concentrations was parabolic (Figure 34). Slope replot did not vary. Therefore the inhibition by α-ketoglutarate when NAD was varied is actually I-parabolic S-linear uncompetitive and the data corresponds to equation (7). This suggests that α-ketoglutarate may combine with the enzyme-NAD complex in a dead-end manner as well as combining as a product inhibitor.

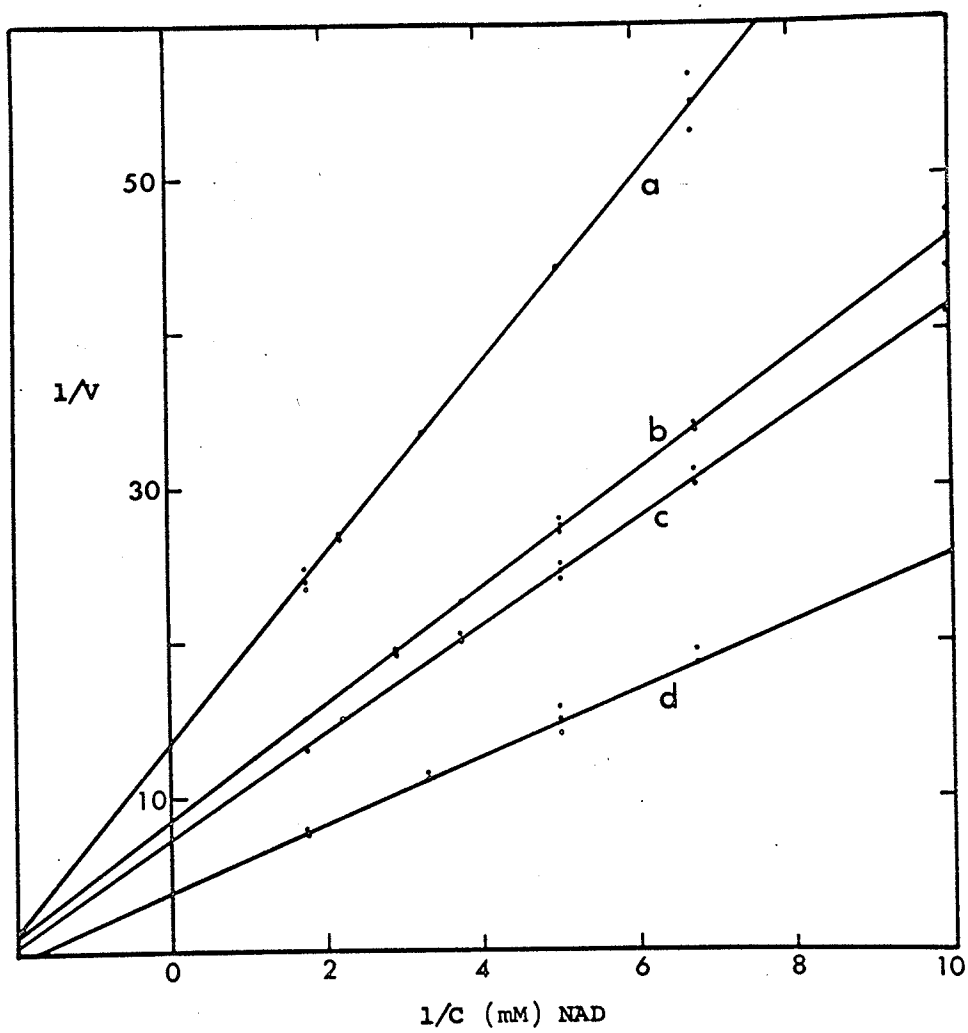


Figure 27. Product inhibition of glutamate dehydrogenase by several concentrations of ammonium sulfate with NAD as variable substrate at constant L-glutamate concentrations (13 times K_m). Ammonium sulfate concentrations: (a) none; (b) 12 mM $(NH_4)_2SO_4$; (c) 15 mM $(NH_4)_2SO_4$; (d) 28 mM $(NH_4)_2SO_4$.

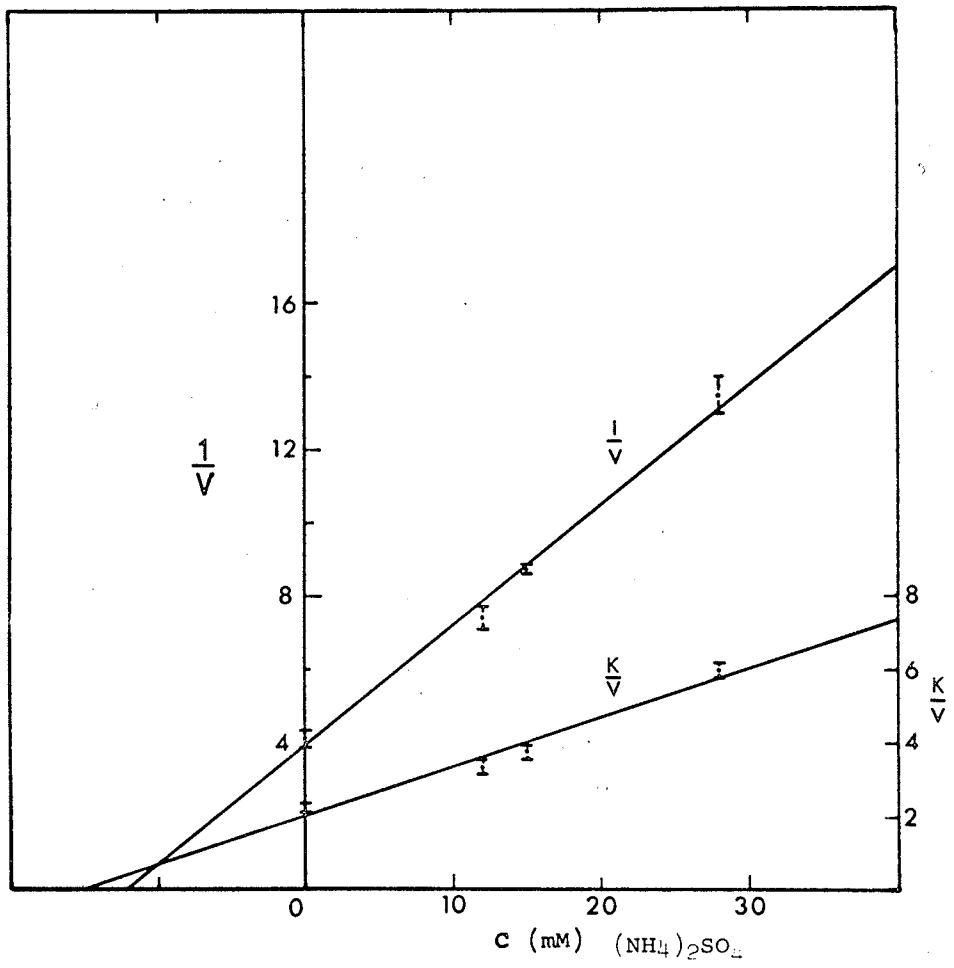


Figure 28. Replots of slopes and intercepts from Figure 27 versus $(NH_4)_2SO_4$ concentrations.

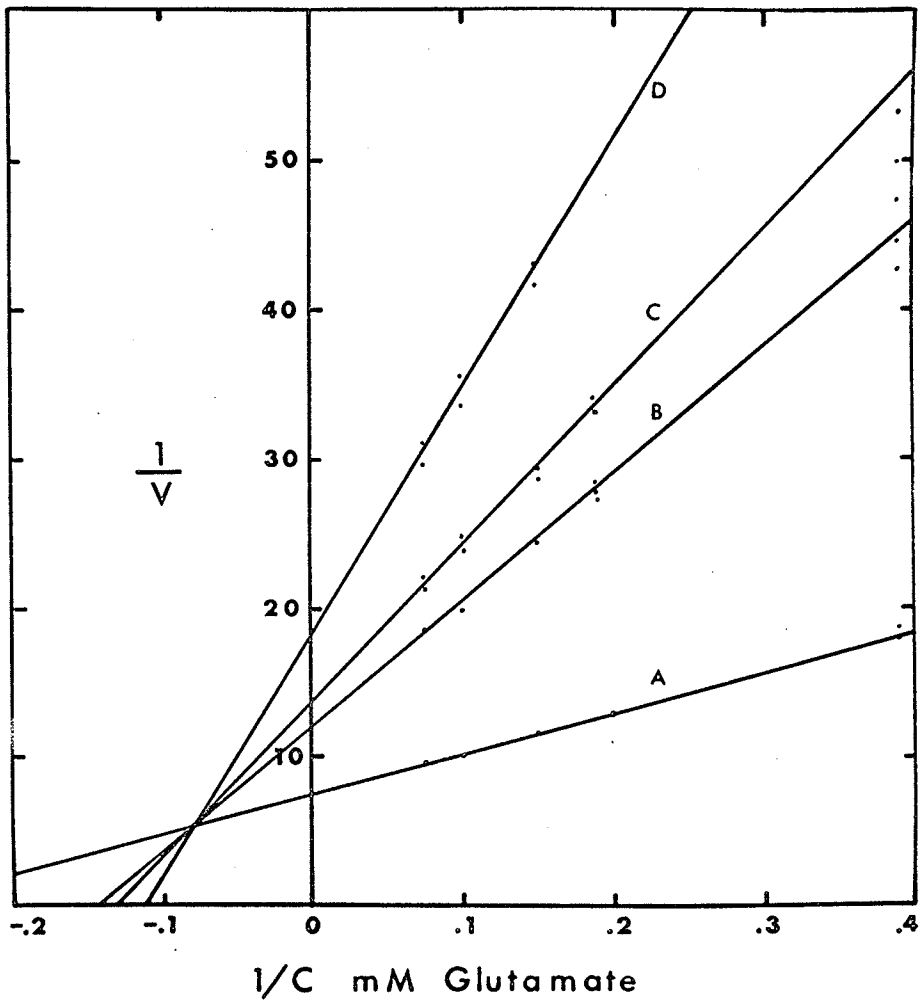


Figure 29. Product inhibition of glutamate dehydrogenase with ammonium sulfate as inhibitor and L-glutamate as varied substrate. Constant NAD concentration (4 mM). Ammonium sulfate concentrations: (A) none; (B) 10 mM $(NH_4)_2SO_4$; (C) 20 mM $(NH_4)_2SO_4$; (D) 40 mM $(NH_4)_2SO_4$.

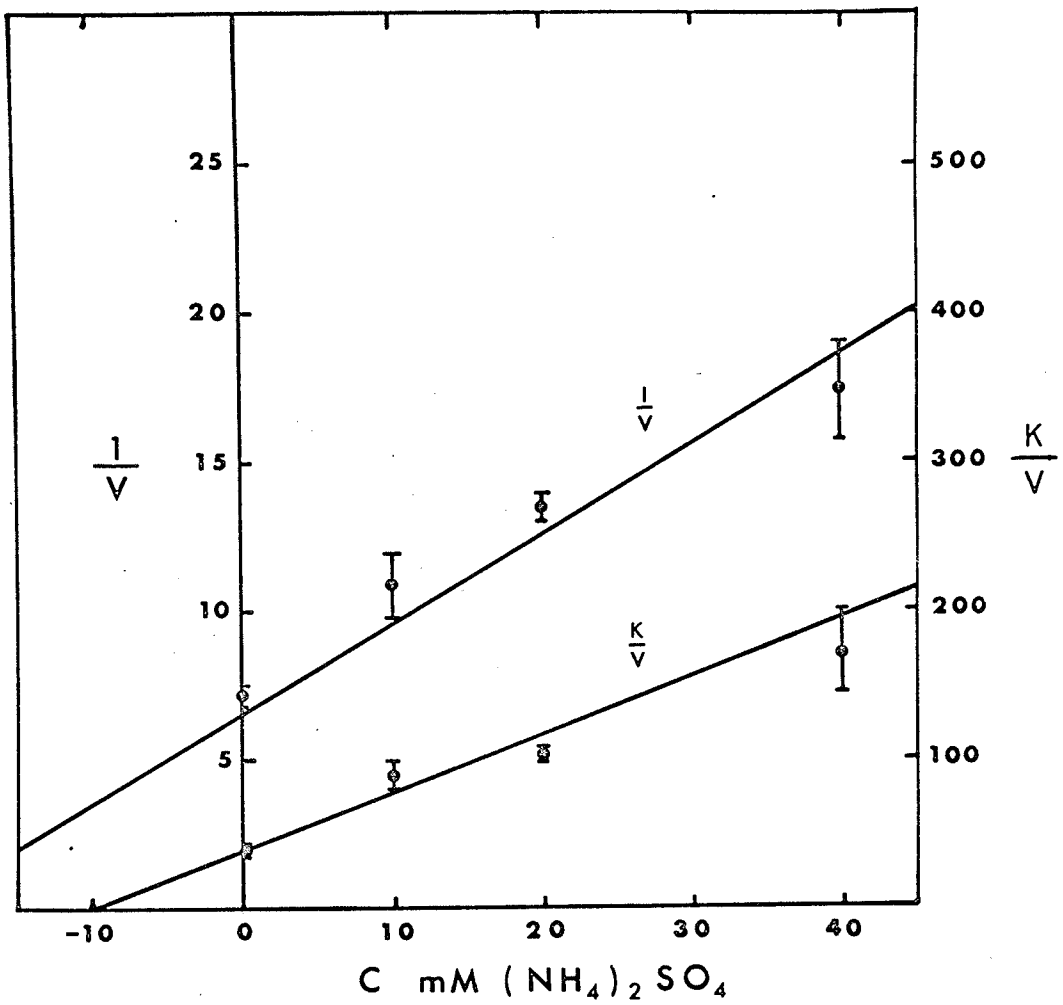


Figure 30. Replots of slopes and intercepts from Figure 29 versus ammonium sulfate concentrations.

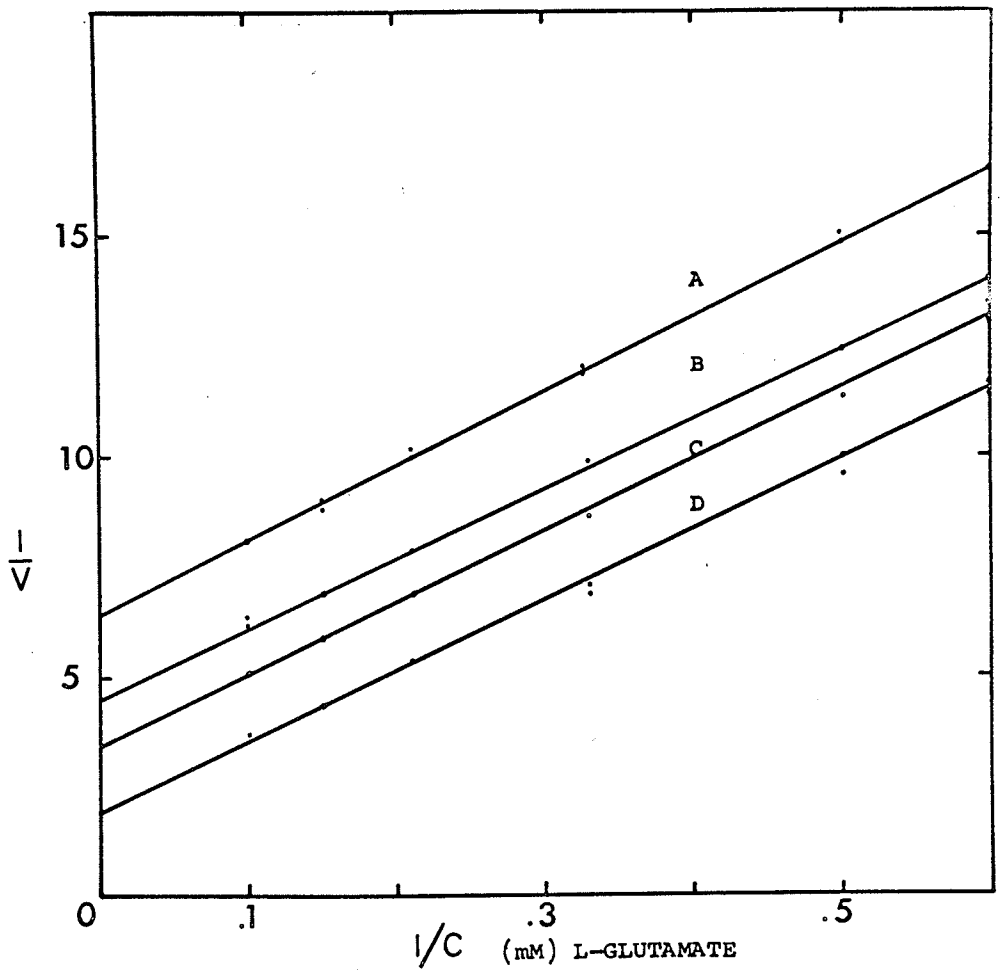


Figure 31. Product inhibition of glutamate dehydrogenase by several concentrations of α-ketoglutarate with L-glutamate as variable substrate in the presence of constant high concentrations of NAD (approximately 10 times K_m). α-ketoglutarate concentrations: (A) 20 mM; (B) 13.3 mM; (C) 10 mM; (D) 4.6 mM.

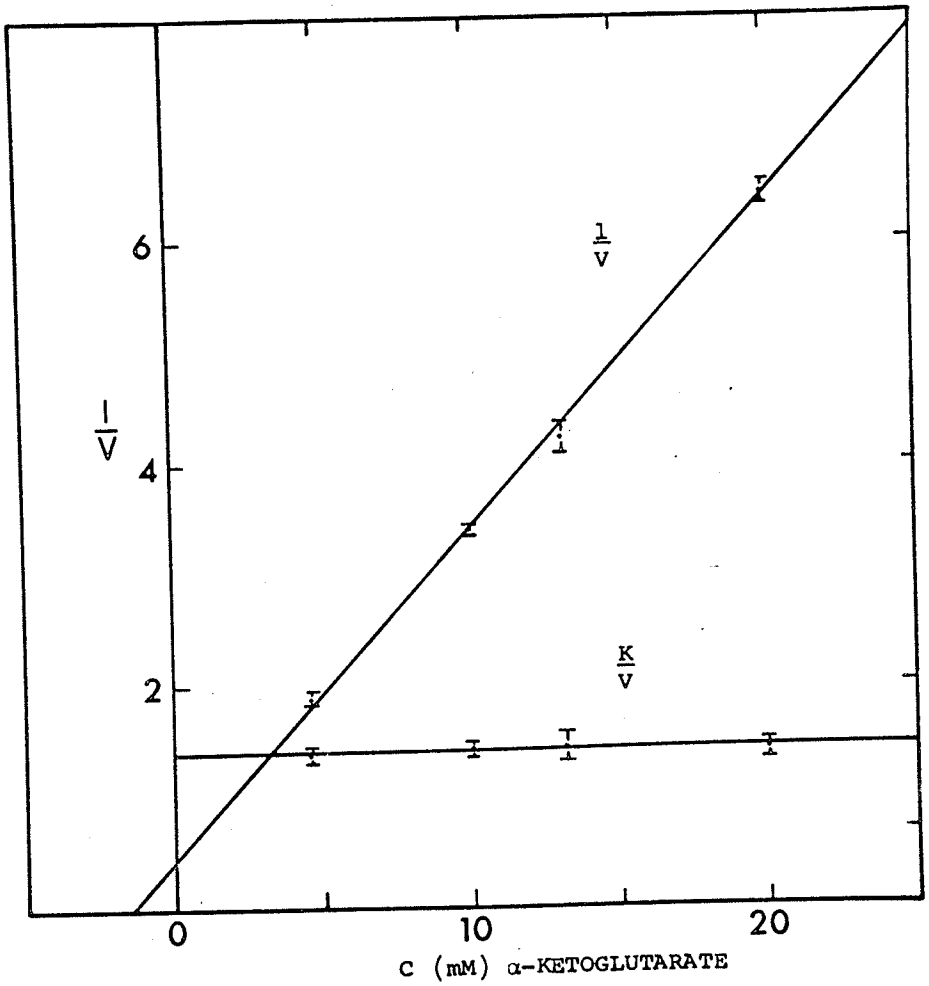


Figure 32. Replots of slopes and intercepts from Figure 31 versus ∝-ketoglutarate concentrations.

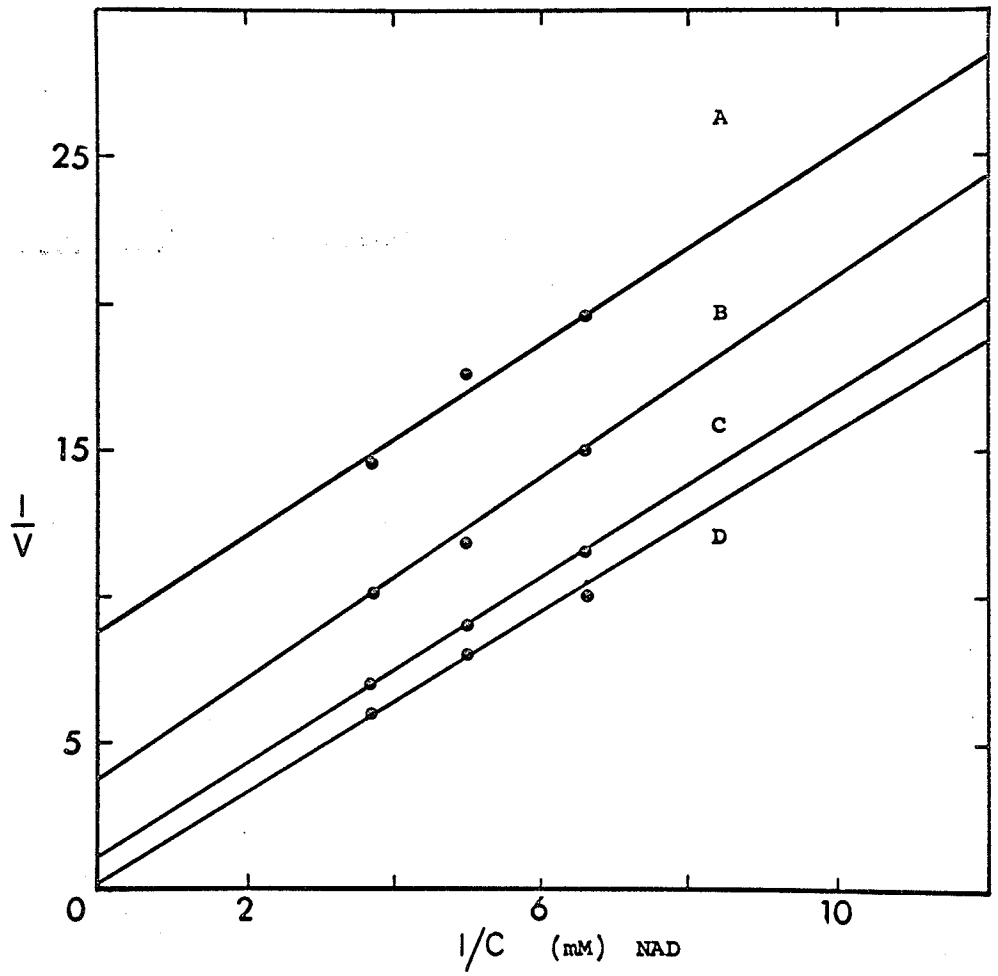


Figure 33. Product inhibition of glutamate dehydrogenase by ∝-ketoglutarate at various fixed concentrations with NAD as variable substrate in the presence of saturing L-glutamate concentration (20 mM). ∝-ketoglutarate concentrations. (A) 26.66 mM; (B) 20 mM; (C) 13.33 mM; (D) 6.66 mM.

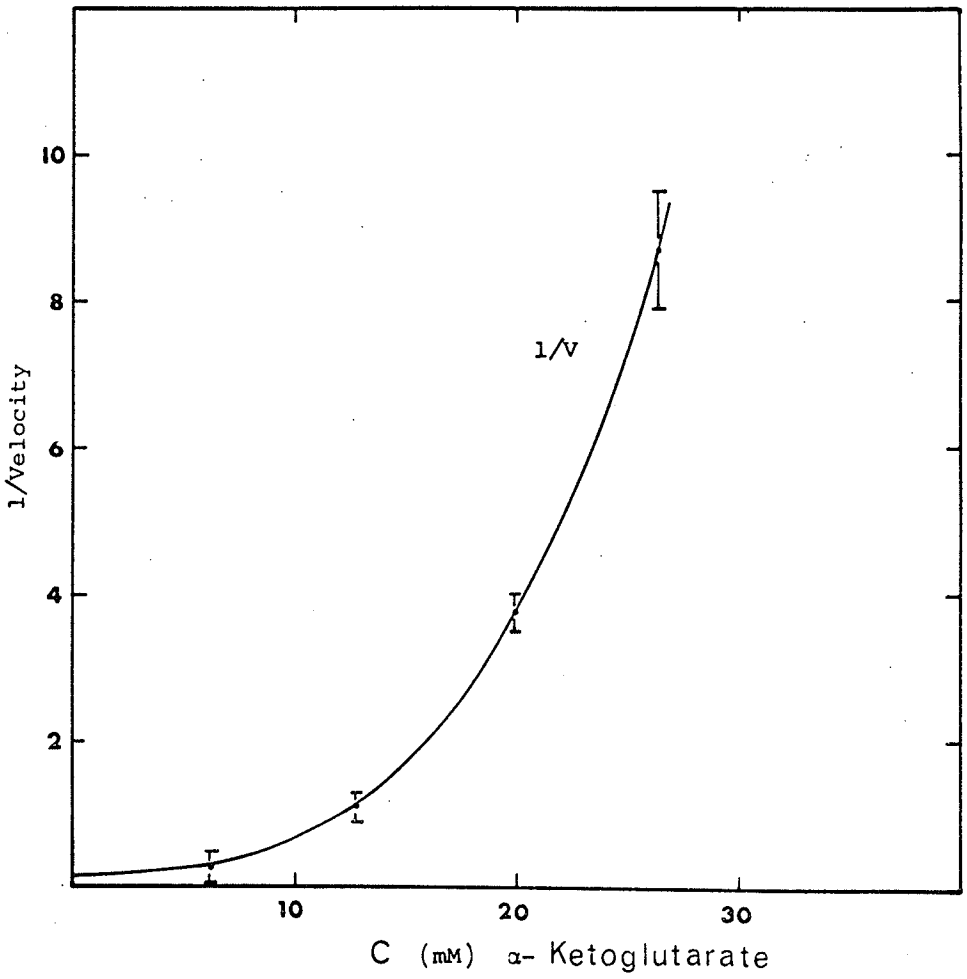


Figure 34. Intercept replots from Figure 33 versus ∝-ketoglutarate concentrations. Slopes were unchanged.

SPECTROFLUOROMETRIC STUDIES OF THE ENZYME

Fluorescence quenching of enzyme by coenzymes, or fluorescence enhancement of reduced coenzymes by enzymes, has been extensively utilized by Velick (1958), Fisher (1960), McKay and Kaplan (1964) and others to show that the coenzymes of most dehydrogenases bind to free enzyme form. Spectrofluorometric techniques have been also used to determine the number of binding sites on the enzyme surface.

Glutamate dehydrogenase, when excited at a wavelength of 290 mu, yields a fluorescence emmission band at 350 mu and this emmission is quenched by NAD (Figure 35) and NADH in the absence of L-glutamate. In a similar experiment in which free enzyme is titrated with L-glutamate in the absence of NAD, no quenching of enzyme fluorescence occurs (Figure 36). Figure 37 shows that GTP can also bind to the free enzyme form. These results show that both reduced and oxidized coenzymes bind to free enzyme, whereas L-glutamate does not.

ESTIMATE OF THE NUMBER OF BINDING SITES AND DISSOCIATION CONSTANT

In Figure 38 is shown the fluorescence titration curve of reduced NAD in the presence of increasing amounts of NAD-specific glutamate dehydrogenase. When the change in

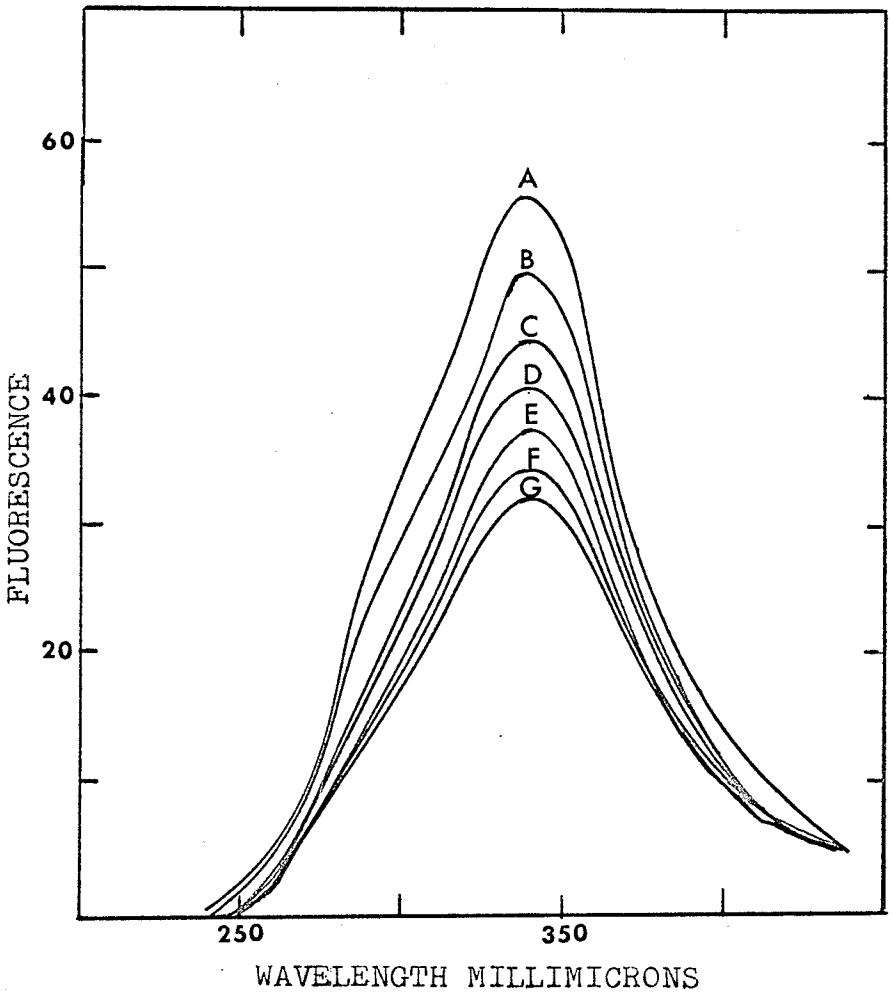


Figure 35. Quenching of NAD-glutamate dehydrogenase fluorescence by varying concentrations of NAD. Enzyme (80 µg.) was excited at 290 mµ. NAD concentrations: (A) none; (B) 33 µM; (C) 67 µM; (D) 100 µM; (E) 134 µM; (F) 168 µM. Plots represented here were not corrected for dilution.

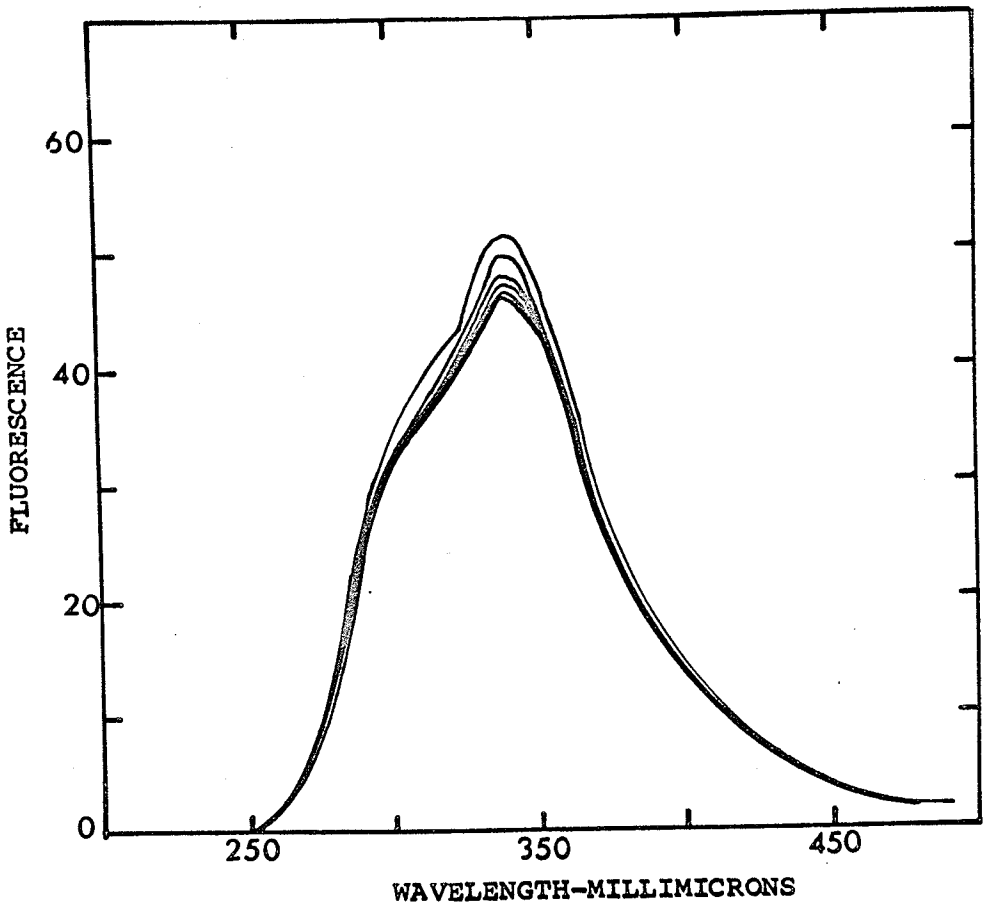


Figure 36. Fluorescence titration of NAD-specific glutamate dehydrogenase by various L-glutamate concentrations. NAD-GDH concentration was 85 µg. Protein excitation at 290 mµ. Plots not corrected for dilution.

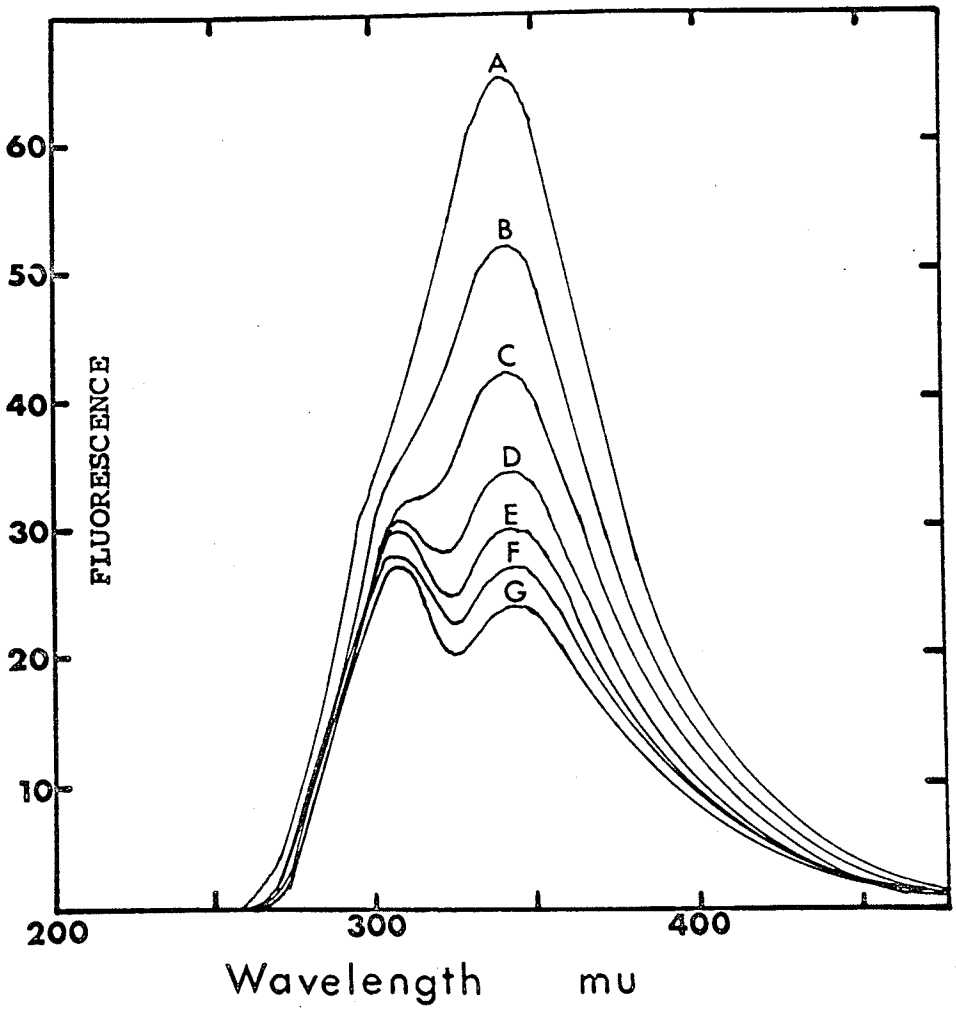


Figure 37. Quenching of NAD glutamate dehydrogenase (105 μg.) fluorescence by various GTP concentrations when excited at 290 mu. GTP concentrations: (A) none; (B) 45 μM; (C) 90 μM; (D) 135 μM; (E) 180 μM; (F) 225 μM; (G) 270 μM.

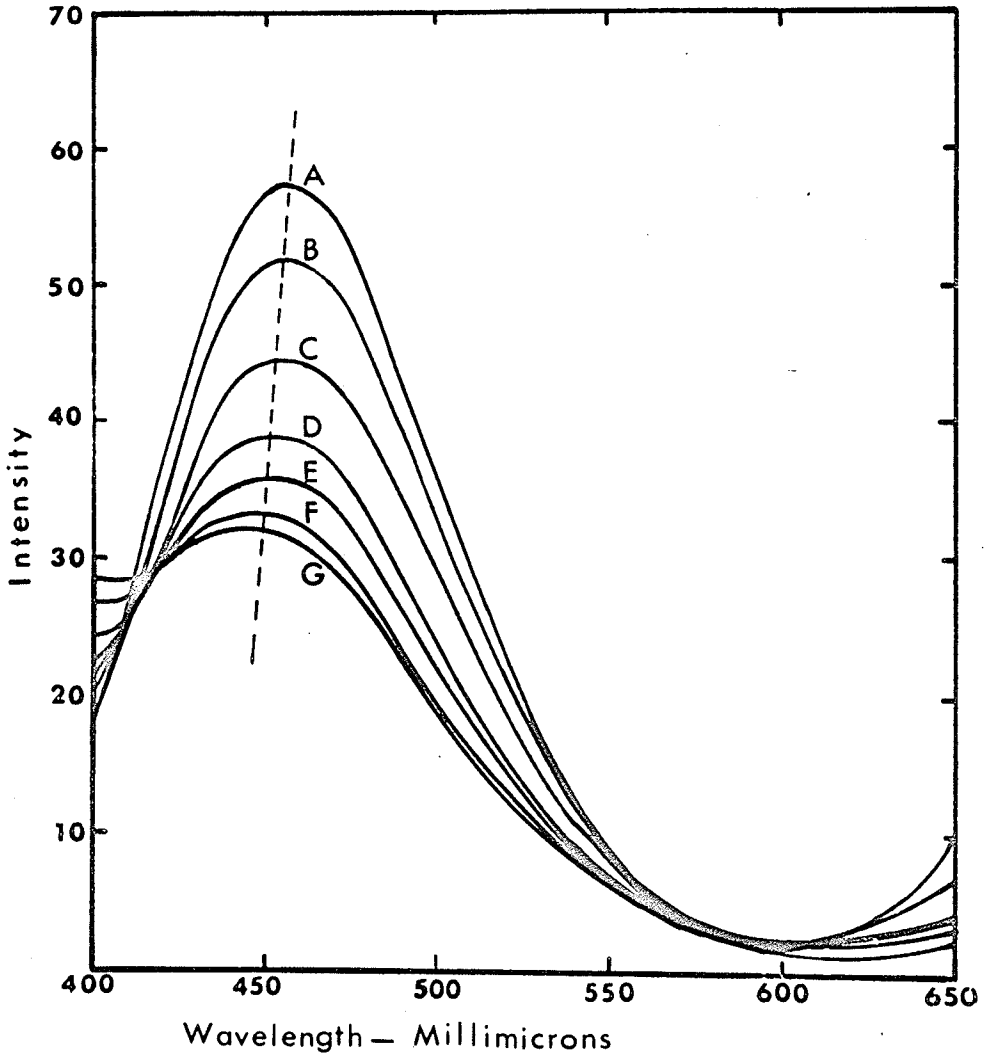


Figure 38. Fluorescence titration of NADH in the presence of increasing amounts of NAD-specific glutamate dehydrogenase. Reduced nucleotide was excited at 350 mμ, (emission at 460 mμ). Purified NAD-specific glutamate dehydrogenase concentrations were: (A) none; (B) 0.61×10^{-6}M; (C) 1.18×10^{-6}M; (D) 1.72×10^{-6}M; (E) 2.22×10^{-6}M; (F) 2.66×10^{-6}M; (G) 3.13×10^{-6}M. Vertical dashed-line illustrates characteristic shift in emission peak.

fluorescence intensity (Δ F) was plotted against enzyme concentration (Figure 39), a characteristic adsorption isotherm was obtained showing good fits to equation (9) (Klotz, 1953),

$$r = \frac{nK_A [A]}{1 + K_A[A]} \qquad (9)$$

where r is the mole ratio of bound enzyme to enzyme, n is the number of binding sites, [A]is the free coenzyme concentration and K_A the association constant. As predicted from equation (9) the double reciprocal plot of $1/\Delta F$ and 1/ [enzyme] were linear (Figure 39). The horizontal intercept of this plot gave the $1/F_{max}$ value which was used to calculate the fraction of bound coenzyme from equation (10)

$$X = \frac{F - 1}{F_{max} - 1} \qquad (10)$$

where X is the fraction of coenzyme bound, F is the fluorescence intensity of the enzyme-coenzyme complex relative to that of the coenzyme alone at the same concentration and F_{max} is the limiting value of F, obtained by titrating the coenzyme with an excess of enzyme. It may be mentioned that equation (10) does not distinguish between a change in X and a redistribution of already bound molecules among adsorption sites.

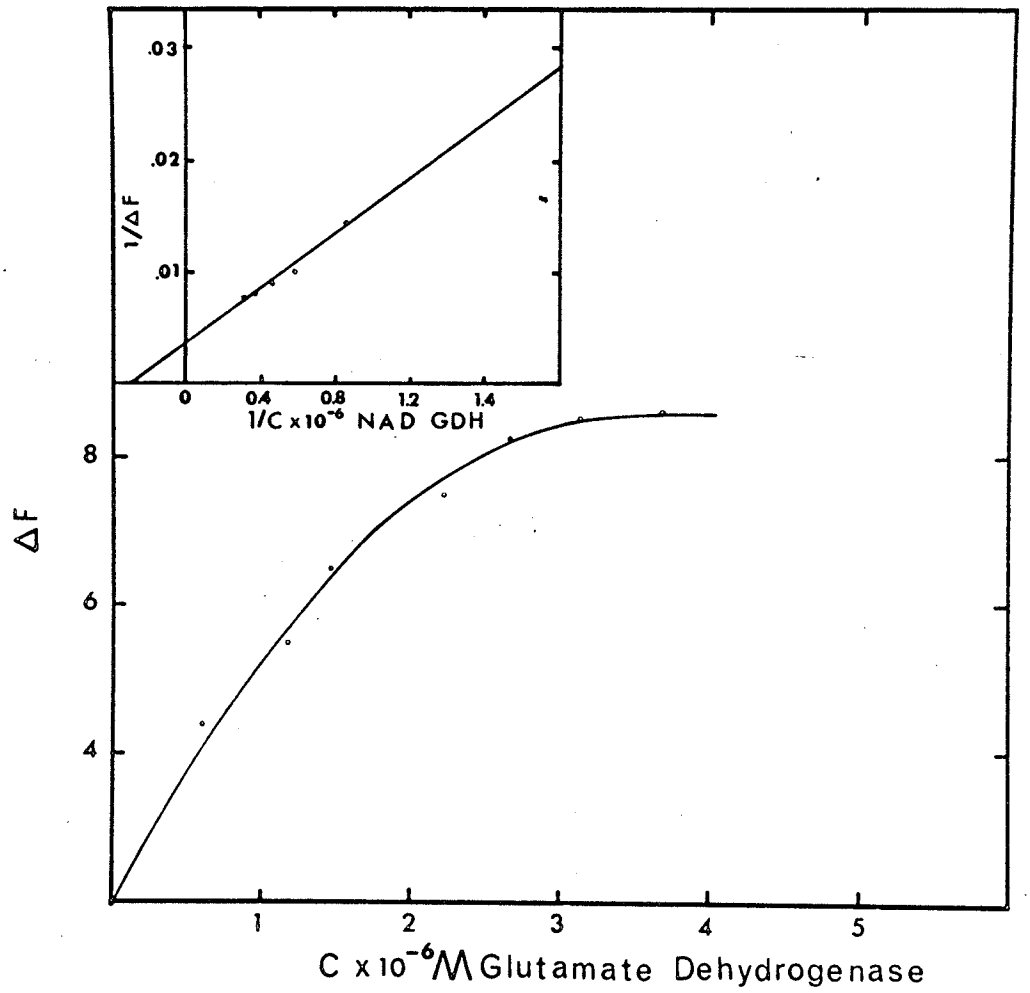


Figure 39. Adsorption isotherm of NAD-specific glutamate dehydrogenase concentrations versus change in fluorescence (ΔF). Insert is double reciprocal plot of change in fluorescence against enzyme concentration.

The number of NADH binding sites was estimated to be 3.6 per mole of NAD-GDH by plotting r against r/ [A] (Figure 40) according to Klotz (1953). This estimate is similar to that reported for lactic dehydrogenase. However, 20 binding sites have been reported for beef-liver glutamate dehydrogenase (Kubo, 1957) which has a molecular weight of 1×10^6.

The dissociation constant for NAD was calculated as 0.167 mM from the double reciprocal plots of NAD concentrations versus change in fluoresence intensity (Figure 41). A comparison of the value of the dissociation constant obtained from kinetic studies (Table 4) and fluorometric studies shows that the value obtained by the latter technique is fully 3 times higher than that obtained by the former method. The causes of this discrepancy are not known. Frieden (1963) has also reported that beef-liver glutamate dehydrogenase gives different dissociation constants depending upon the technique used.

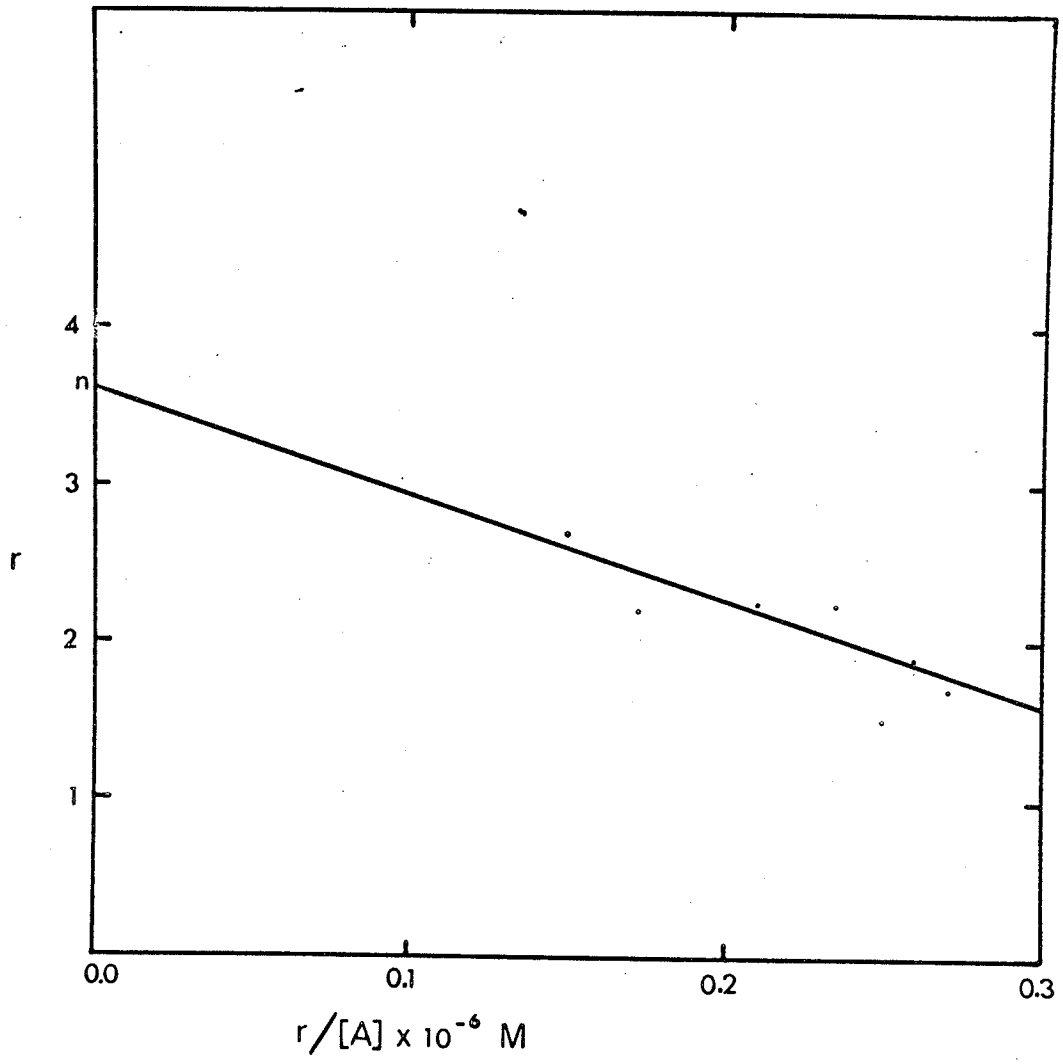


Figure 40. Estimate of number of NADH binding sites for NAD-glutamate dehydrogenase. r vs. r/ A plot for the titration of NADH with enzyme.
r = mole ratio of bound coenzyme to enzyme;
A = free coenzyme concentration;
n = number of NADH binding sites. Line was fitted statistically.

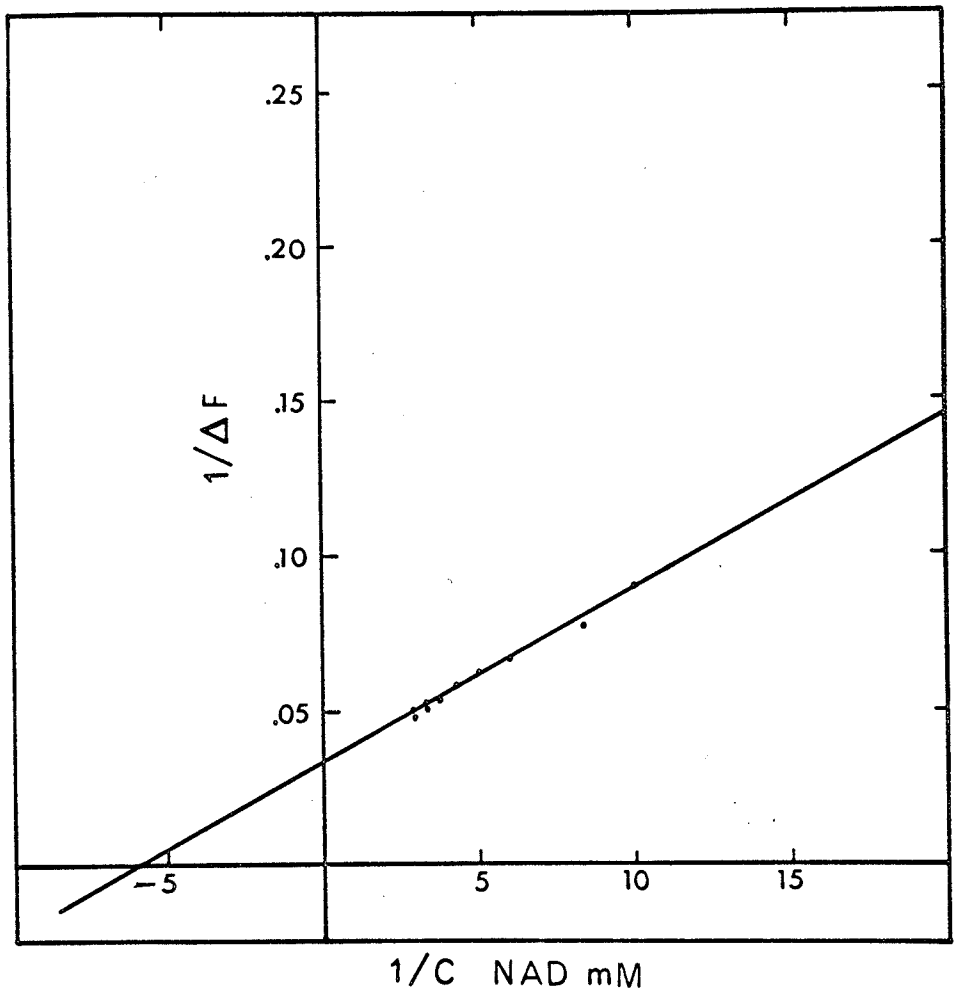


Figure 41. Spectrofluorometric determination of dissociation constant for NAD of glutamate dehydrogenase. Double reciprocal plots of NAD concentrations versus change in fluorescence intensity (ΔF) when various concentrations of NAD were titrated with NAD-glutamate dehydrogenase.

IV. DISCUSSION

evidence that zinc is involved in the catalytic process brought about by the enzyme. It has been estimated that the beef-liver enzyme possesses one atom of zinc per monomeric subunit (molecular weight 250,000), and more than one binding site per subunit for coenzyme (Frieden, 1959). Consequently, these results suggest that zinc is related to enzyme activity in some way other than its involvement at a site which the coenzyme binds. In contrast, the results obtained with metal chelating agents in Neurospora glutamate dehydrogenase seem to exclude the possibility that a metal is necessary for enzyme activity. However, this does not imply that the glutamate dehydrogenase enzyme has no metal moiety. It simply may not be involved in the catalytic process.

The results obtained by Yielding et al., (1960) show that diethylstilbesterol (DES), in the presence of NADH, dissociates beef-liver glutamate dehydrogenase into inactive subunits. There also appears to be a correlation between the extent of dissociation by DES, and the inhibition of catalytic activity. Other workers (Frieden, 1963; Yielding and Tomkins, 1960a) suggest that hormonal inhibition of animal glutamate dehydrogenase may play an important role in the regulation of glycogen content in cells. Since Neurospora crassa has not been reported to synthesize hormones, and both its glutamate dehydrogenases are unaffected by DES, it seems most unlikely that its glycogen content

could be regulated by the same mechanism.

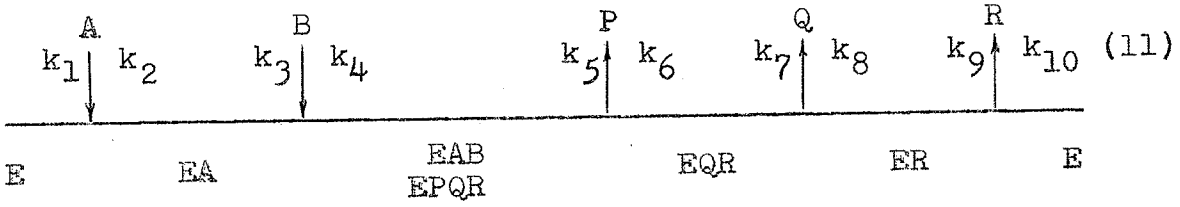
The finding that GTP was a competitive inhibitor of NAD-specific glutamate dehydrogenase led to experiments designed to study its effect on the NADP-specific enzyme. The results obtained with the latter showed that there was no inhibition of activity by GTP. This finding is in contrast to the results obtained by Wolff (1962) and Frieden (1962) for the beef-liver enzyme. They showed GTP to be an uncompetitive inhibitor of both NADH and NADPH oxidation as well as NAD and NADP reduction.

Of all the nucleotides tested, only guanine nucleotides and to a much lesser degree, inosine nucleotides inhibited the NAD-specific glutamate dehydrogenase. None of the nucleotides examined acted as inhibitors of the NADP-specific enzyme. Frieden (1963) has reached the conclusion that the beef-liver enzyme, in addition to an active coenzyme site, also has a highly specific purine nucleotide binding site and the inhibition observed at the high NADH concentrations (Yielding et al., 1964) may be due to its binding at the purine nucleotide site. Also, some purine nucleotides (ATP,ADP), by binding at the latter site, cause activation while others (GTP,ITP, etc.,) cause inhibition. It is tempting to suggest that the NAD-specific glutamate dehydrogenase of Neurospora may also have two sites, one of which readily binds GTP and ATP, and the other binds the coenzyme. More sophisticated approaches would have to

be developed to prove the conjecture. If GTP caused any conformational changes by combining at a site different from coenzyme binding site on the protein molecule, a competitive inhibitor would result. It is clear that kinetic analysis could not be used to detect such changes. The physiological significance of the differential purine nucleotide effect on the activity of the two glutamate dehydrogenases of Neurospora will be discussed in the next section (see Discussion II).

REACTION MECHANISMS

The initial velocity studies (Figure 17) showed that the reaction corresponds to equation (8). The presence of the $\frac{K_{ia}K_b}{AB}$ term in equation (8) indicates that both NAD and L-glutamate must bind to the enzyme before the products are released. This rules out a Ping-Pong type of mechanism, where the $\frac{K_{ia}K_b}{AB}$ term would be missing in the initial velocity equation (8) (Cleland, 1963; 1963a). The simplest mechanism which fits the product inhibition data reported here is the Ordered Bi-Ter mechanism (Mechanism 11) where



(11)

A and B are substrates and P, Q and R represent products.

The steady-state rate equation for the ordered Bi-Ter mechanism (mechanism 11) can be written in the form of equation (12), which was derived according to the method of King and Altman (1956) using kinetic constants described by Cleland (1963).

$$V = \frac{V_1 \left(AB - \frac{PQR}{K_{eq}} \right)}{K_{ia}K_b + K_bA + K_aB + AB + \frac{K_{ia}K_bK_qP}{K_pK_{iq}} + \frac{K_{ia}K_bR}{K_{ir}} + \frac{K_bAP}{K_{ip}} + \frac{K_{ia}K_bK_rPQ}{K_pK_{iq}K_{ir}} + \frac{K_aBR}{K_{ir}} + \frac{K_{ia}K_bQR}{K_{iq}K_{ir}} + \frac{K_{ia}K_bK_qPR}{K_pK_{iq}K_{ir}} + \frac{ABP}{K_{ip}} + \frac{K_{ia}K_bPQR}{K_pK_{iq}K_{ir}} + \frac{K_rK_bAPQ}{K_pK_{iq}K_{ir}} + \frac{ABQ}{K_{iq}} + \frac{K_{ia}K_bBQR}{K_{ib}K_{iq}K_{ir}} + \frac{ABPQ}{K_{ip}K_{iq}} + \frac{K_{ia}K_bBPQR}{K_p K_{ib}K_{iq}K_{ir}}} \qquad (12)$$

Equation (12) was used to distinguish between an ordered mechanism and a Theorell-Chance or a Rapid Equilibrium Random mechanism (in which both A and B can bind to the free enzyme, but the rate limiting step is the interconversion of the central complexes). The presence of ABPQ and BPQR terms in the denominator of equation (12) and the fact that Wratten and Cleland (1963) have shown that mechanisms without central complexes are unlikely, rules out the Theorell-Chance type of mechanism. A Rapid Equilibrium

Random mechanism with some dead-end complexes would give a steady state rate equation similar to equation (12) but this can be eliminated from the product inhibition studies with α-ketoglutarate presented earlier. Further, a Random mechanism can also be discarded on the basis of product inhibition data which show that slopes and intercepts of double reciprocal plots are in general linear functions of inhibitor concentrations. The one case which did not give linear replots (Figure 34), could be explained on the grounds that α-ketoglutarate acts as a product as well as a dead-end inhibitor. Therefore, it seems reasonable to propose an Ordered Bi-Ter mechanism for the reaction catalyzed by glutamate dehydrogenase.

Equation (12) was also used to predict product inhibition patterns. Equations for the three types of product inhibition experiments reported here were obtained by setting either P, Q or R equal to zero in equation (12), and rearranging into the form of equations (13) to (17). Thus when P is inhibitor and A the variable substrate;

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{KB}\right)\left(1+ \frac{P}{\frac{K_pK_{ia}}{K_q}\left(1+ \frac{K_aB}{K_{ia}K_b}\right)}\right)\frac{1}{A} + \frac{1}{V}\left(1+ \frac{K_b}{B}\right)\left(1 + \frac{P}{\left(\frac{1 + \frac{K_b}{B}}{\frac{1}{K_{ip}} + \frac{K_b}{K_{ip}B}}\right)}\right) \qquad (13)$$

When Q is inhibitor and A the variable substrate;

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{K_b}{B}\right)\left(1 + \frac{Q}{K_{iq}\left(1 + \frac{K_b}{B}\right)}\right) \qquad (14)$$

and with B the variable substrate;

$$\frac{1}{v} = \frac{K_b}{V}\left(1 + \frac{K_{ia}}{A}\right)\frac{1}{B} + \frac{1}{V}\left(1 + \frac{K_a}{A}\right)\left(1 + \frac{Q}{K_{iq}\left(1 + \frac{K_a}{A}\right)}\right) \qquad (15)$$

With R as inhibitor and A the variable substrate;

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left(1 + \frac{R}{K_{ir}}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{K_b}{B}\right) \qquad (16)$$

and with B the varying substrate;

$$\frac{1}{v} = \frac{K_b}{V}\left(1 + \frac{K_{ia}}{A}\right)\left(1 + \frac{R}{K_{ir}\left(1 + \frac{A}{K_{ia}}\right)}\right)\frac{1}{B} + \frac{1}{V}\left(1 + \frac{K_a}{A}\right)\left(1 + \frac{R}{K_{ir}\left(1 + \frac{A}{K_a}\right)}\right) \qquad (17)$$

Since NADH shows competitive inhibition with NAD (equation (16)) and non-competitive with L-glutamate (equation (17)), it is reasonable to assume that NAD and NADH rather than L-glutamate, bind to free enzyme form. The quenching of enzyme fluorescence by either NAD or NADH (Figure 35), but not by L-glutamate (Figure 36) gives further support to this contention. Therefore, the order of substrate addition would be NAD, first, followed by L-glutamate and since NAD and NADH bind to the same enzyme form NADH would be the last product released. By examining the product inhibition patterns of the remaining two products, their order of

release can be determined. Equation (13) predicts that the first product to be released would display non-competitive inhibition when either substrate was varying. The results presented (Figure 27 and Figure 29) show ammonium sulphate to be a non-competitive inhibitor for both NAD and L-glutamate. Therefore ammonium sulphate appears to be the product which is released first. With α-ketoglutarate as inhibitor, uncompetitive inhibition (equations (14) and (15)) results in the presence of either substrate. The parallel pattern (Figure 33) shows that there is no reversible connection between $(^{EAB}_{EPQR})$-EQR-enzyme forms. The sequence E-EA-$(^{EAB}_{EPQR})$-EQR-ER is interrupted by the absence of P. (Some P is indeed produced, but since only initial velocities are considered here, the concentration for purposes of analysis is zero). Therefore α-ketoglutarate is released after ammonium sulphate.

The model that emerges as a result of the data presented, suggests that the mechanism of the glutamate dehydrogenase reaction is Ordered Bi-Ter, with A(NAD) adding first, followed by B(L-glutamate). The release of products then occurs in the following order; P(NH_4^+), Q(α-ketoglutarate) and R(NADH).

It may be mentioned in passing that most pyridine nucleotide dependent dehydrogenases studied so far have been shown to bind the coenzymes (NAD or NADP) first

obligatorily or preferentially (Schwart and Winer, 1963; Sund et al., 1964) before binding of the second substrate. It is little realized, however, that physiologically this observation may have great importance in the control of overall rates of dehydrogenase reactions depending upon the availability of free coenzymes in the cell.

SECTION II

REGULATION OF GLUTAMATE DEHYDROGENASES

I. HISTORICAL

I. HISTORICAL

The ability of microorganisms to modify their metabolism in response to environmental changes and utilize available metabolites more efficiently is due to three well documented control mechanisms: (1) Feed-back inhibition, (2) induction and (3) repression.

"FEED-BACK" INHIBITION

The terms "feed-back" and "end-product" inhibition have been used interchangeably and refer to the inhibition of the first enzyme of a biosynthetic pathway by its terminal metabolite. This control mechanism prevents the over-function of an enzyme rather than its over-synthesis.

The earliest report of a regulatory feed-back mechanism was by Dische (1941), who showed inhibition of glucose phosphorylation by phosphoglyceric acid in erythrocytes. However, the physiological significance of this phenomenon was not appreciated until Novick and Szilard (1954) discovered inhibition of tryptophan biosynthetic pathway by its end-product. One year later, Roberts et al., (1955) showed that when a wild type strain of E. coli was grown in a medium containing C^{14}-labelled glucose but unlabelled isoleucine 95% of the radioactivity from glucose was excluded from incorporation into cellular isoleucine. It became evident that the exogenous source of isoleucine was utilized

preferentially and its presence inhibited the endogenous synthesis although all enzymes of the pathway were present.

Working on the early steps in the biosynthetic pathway leading to isoleucine in E. coli, Umbarger (1956) demonstrated that isoleucine was a potent inhibitor of threonine deaminase, which catalyzes the irreversible deamination of threonine to α-ketobutyrate. The general pattern of this type of feed-back control was further revealed by the studies of Yates and Pardee (1956) on the effect of excess uracil on orotic acid accumulation in pyrimidine auxotrophs of A. aerogenes. These workers demonstrated that the end-product (actually the entire pool of pyrimidine nucleotides) controlled its synthesis by inhibiting the enzyme aspartate-transcarbamylase. Numerous enzymes are now known to be controlled by the end product of their respective pathways. A partial list of such enzymes include: aspartate-transcarbamylase (Yates and Pardee, 1956; Gerhart and Pardee, 1962), threonine deaminase (Umbarger, 1956; Changeux, 1961, 1962), phosphoribosyl-ATP-pyrophosphorylase (Moyed, 1958; Martin,1962), aspartokinase I and II (Stadtman, et al., 1961) homoserine dehydrogenase (Patte et al., 1962), inosine-5'-phosphate dehydrogenase and guanosine-5'-phosphate dehydrogenase (Magasanik, 1958) and acetohydroxy acid synthetase (Bauerel, 1964).

Monod (1963) has stated two rules which all feed-

back systems seem to obey. They are: (1) the regulatory enzymes (each of them acting immediately after a metabolic branching point) are all strongly and specifically inhibited by the terminal metabolite of the pathway; intermediary metabolites in each pathway do not inhibit the regulatory enzyme.

(2) the enzymes which intervene after the regulatory one in each pathway are not significantly sensitive to inhibition by the terminal metabolite. Another general property of the control mechanism may be added to the list and this is:

(3) the inhibition exerted by the terminal metabolite is reversible. In other words, as the concentration of end-product decreases below a certain level, the enzyme is able to resume its physiological function.

With feed-back inhibition, difficulties, however, arise in the control of early enzymes of pathways which feed intermediary metabolites into branched pathways. Thus β-aspartyl phosphate and aspartate semialdehyde are common intermediates for the reaction sequences leading to the formation of the end products lysine, methionine, threonine and isoleucine. If threonine, for instance were to inhibit the activity of the first enzyme, aspartokinase it would simultaneously lead to the cessation of lysine and methionine synthesis. Two strategies seem to be employed in nature

to control the activity of such enzymes. One of these is to produce two or more isoenzymes, each specifically under the control of one end product. Thus, in E. coli, aspartokinase I is inhibited by lysine and aspartokinase II by threonine (Stadtman et al., 1961). The second strategy (concerned with feed-back inhibition) is to have only one enzyme which is not inhibited by either end product separately, but powerfully inhibited in the simultaneous presence of two or more end products. Such seems to be the mechanism of control of the lone aspartokinase found in Rhodospirillium capsulatus which is only inhibited by the combined presence of threonine and lysine (Datta and Gest, 1964).

The sensitivity of regulatory enzymes to end-product inhibition is a labile property which can be removed by mutation or alterations of these proteins by heat, urea and mercuric compounds without appreciable loss of catalytic activity. Such "desensitization" has been accomplished with homoserine dehydrogenase (Patte et al, 1962), PRPP-ATP-PPase (Martin, 1962), threonine deaminase (Changeux, 1961), aspartate transcarbamylase (Gerhart and Pardee,1962) and acetohydroxy acid synthetase (Bauerle et al., 1964). The sensitivity of the last enzyme to valine can be restored by treating the desensitized enzyme with reduced glutathione (Bauerle et al., 1964).

Monod (1963) suggests that the desensitizing agents do

not destroy the inhibitor binding site (allosteric site), but rather, cause a conformational change in the enzyme molecule. This view obtains support from the observations of Martin (1962) that "desensitized" PRPP-ATP-PPase retains its capacity for the binding of the end-product inhibitor, histidine.

The currently accepted hypothesis on the mechanism of feed-back inhibition is that:

(a) the end-product of a pathway binds to a site on the regulatory enzyme which is distinct from the substrate binding site (active site). The inhibitor binding site has been termed the "allosteric site" (Monod, 1963).

(b) the binding site of the inhibitor ("effector") to the allosteric site causes a reversible conformational alteration in the active site thus affecting the substrate binding. Koshland (1958) has provided ample evidence that the binding of any substrate involves an induced alteration of the shape of the active site.

REGULATION BY ENZYME INDUCTION AND REPRESSION

As early as 1900 Dienert described a phenomenon that resembled enzyme induction. He also reported on antagonism of enzyme formation. The latter phenomenon is now called enzyme repression and is defined by Vogel (1956) as:

"A relative decrease, resulting from the exposure of cells to a given substance (repressor), in the rate of synthesis of a particular apoenzyme" or metabolically related enzymes. Enzyme induction, as defined by Cohn, Monod, Pollock, Spiegelman and Stanier (1953) can be stated as "a relative increase, resulting from the exposure of cells to a given substance (inducer), in the rate of synthesis of a particular apoenzyme."

Both these phenomena are extremely widespread in bacteria, (Vogel, 1956; Pardee, 1955; Gorini, 1957; Ames, 1959; Monod, 1953, etc.), mammalian cells (De Mars, 1958; Knox, 1958; Walker, 1959; 1960) and in fungi (Horowitz et al., 1960; Sanwal, 1961).

The early work of Vogel (1953) showed that when a culture of E. coli was grown in a medium supplemented with L-arginine, the level of acetylornithinase, an enzyme of the arginine pathway, decreased below the level of that obtained in cells grown in the absence of L-arginine. Later evidence revealed that though none of the intermediate metabolites of the pathway acted as repressors, the entire arginine pathway was repressed by L-arginine (not only acetylornithinase, but also ornithine transcarbamylase (Gorini and Maas, 1957), acetylornithine transaminase (Albrecht and Vogel, 1960) and acetylornithine permease (Vogel, 1960). Tryptophan has been shown to be a regressor of a series of enzymes in the pathway involved in

the synthesis of tryptophan (Cohen and Jacob, 1959). In these cases the repressor is also the end-product of the pathway.

Only trace amounts of the enzyme β-galactosidase are present in E. coli cells when they are grown in the absence of lactose. However, the addition of lactose or some of its analogues to the same cells increases the rate of synthesis of the synthesis of the enzyme about 10,000 fold. As in the case of repression only the lactose utilization system is effected and the inducer has to be lactose or a 'gratuitous' analogue (Jacob and Monod, 1961).

The extreme specificity of the inducer or repressor underlines the physiological importance of this type of control mechanism. It is unlike feed-back inhibition in that induction and repression control enzyme synthesis. This specific influence of metabolites on the production of enzymes ensures that the levels of cells' enzymes are adjusted continually to the needs of its metabolism.

MECHANISM OF INDUCTION AND REPRESSION

Vogel (1957) was first to suggest that induction and repression of enzyme synthesis had a common basis. According to Vogel's regulator hypothesis, the interaction of inducer or repressor with the enzyme synthesizing system (secondary template-ribosomes) was responsible for induction

and repression of enzymes. Vogel proposed that inducers and repressors acted by increasing or decreasing the rate of enzyme dissociated from its ribosomal template. Under repressed conditions the newly formed protein is bound to its site of synthesis by the repressing agent and the presence of inducer exerts a neutralizing effect on binding. It follows that rapid removal of the template product from the template would accelerate enzyme formation (induction), whereas occupation of the template by its product would prevent the template from functioning (repression).

Genetic analysis and biochemical characterization of mutations which affected inducibility and repressibility led Jacob and Monod (1961) to propose a genetic model for the control of protein synthesis in bacteria. Jacob and Monod's model is largely based on the discovery and involvement of three distinct types of genes: structural gene (SG), regulator gene (RG) and operator gene (O), in the control of β-galactosidase synthesis in E. coli. A wide variety of pathways which are regulated coordinately (Ames and Garry, 1959) have been shown to have a genetic make-up similar to the lactose pathway. These are; enzymes concerned with histidine synthesis (Ames and Garry, 1959; Ames et al., 1960; Ames, Martin and Garry, 1961), pyrimidine synthesis (Beckwith and Pardee, 1961), arginine biosynthetic enzymes (Gorini, 1961), enzymes involved in isoleucine-

valine synthesis (Wagner and Berquist, 1960) and many others. The β-galactosidase-permease system has received the most intensive genetic and biochemical analysis and for these reasons it will be used to explain Monod's model.

STRUCTURAL GENES:

Mutation of the structural gene has long been recognized to lead to a loss of the gene-specific enzyme or an alteration of the enzyme molecule in such a way that enzymatic activity is undetectable. Yanofsky (1961) showed that all mutations which changed the molecular properties of an enzyme accurred in a small segment of the genetic map and belonged to the same "cistron" (Benzer, 1957). Mutation of the structural gene did not in any way effect the inducibility or repressibility (Sanwal and Lota, 1962b; Jacob et al., 1960). Using immunological techniques, Perrin, Jacob and Monod (1960) demonstrated that on SG mutant of β-galactosidase (Z) synthesized an enzymatically inactive, antigenically related protein (CRM) which responded to induction in the same manner as the prototroph (wild type). Detailed genetic maps of structural genes controlling a metabolic sequence of enzymes such as the histidine and arginine pathways revealed a clustering of these genes.

REGULATOR GENES

Mutation of a regulator gene (RG) leads to an uncontrolled synthesis of protein even in the presence of a

repressing agent (end-product). In the case of enzymes controlled 'coordinately', such a mutation simultaneously affects several proteins of the pathway to the same degree. Jacob and Monod (1961) demonstrated that mutation of a regulator gene (i) resulted in the uncontrolled synthesis of both β-galactosidase and permease proteins. These mutations mapped in a region distinct from the structural genes.

Genetic recombination studies of Jacob and Monod (1961) show the regulator gene mutation to be "recessive", which suggests that the factor produced by this gene is released in the cytoplasm. Earlier the nature of the compound formed by the repressor gene (apo-repressor) was thought to be ribonucleic acid. However, the recent work of Garen (1963; 1964) strongly indicates that the repressor may actually be a protein.

Regulator gene mutants have been reported for a series of inducible systems such as: amylomaltase (Cohen-Bazure and Jolet, 1953), glycuronidase (Stober, 1961) and penicillinase (Kogut, Pollock and Tridgell, 1956), and for repressible systems such as; tryptophan pathway (Cohen and Jacob 1959), arginine pathway (Gorini, 1961) and alkaline phosphatase (Echols, Garen, Garen and Torriani, 1961). The latter enzyme appears to have two regulator genes controlling their activity.

OPERATOR GENES

These are proposed to be loci, lying adjacent to the structural genes, which have the capacity to switch transcription of m-RNA off and on for a coordinately controlled system. Operator gene mutants were shown to map at the extremity of the structural gene for β-galactosidase (Z) (Jacob, Perrin, Sanchez and Monod, 1960). Jacob's group also showed that a single operator gene controls the expression of the two adjacent structural genes (Z) and (Y), located on the same chromosome.

Operator mutants have been isolated for the galactose system (Lederberg, 1960) and enzymes of the histidine pathway (Ames, Garry and Herzenberg, 1960). There are many regulatory systems however, for which no operator mutants have been obtained. Indeed, after the demonstration of the operator gene, Jacob, Ullmann and Monod (1964) have recently revised their earlier concept of operator. According to present day ideas, the extremity of a structural gene may be considered to be a 'promoter' where transcription initiates (Jacob et al., 1964). Their first model (Jacob and Monod, 1961) can be presented as follows:

RG is the regulator gene, O is operator gene, SG and SG_2 are structural genes and m-RNA is messenger ribonucleic acid. In this model (Figure 56) R represents the product of RG and is termed "repressor". The repressor is transformed into R' by an effector (F), which may be either the repressing or inducing metabolite.

According to the model (Figure 56) for a repressible system, the repressing metabolite (F) combines with R which results in a modification of R into R'. The modified repressor substance (R') is thought to act on the sensitive site, genetically specified as the promoter region. Once this occurs, transcription of the linked structural genes is switched off and the ultimate product of these genes (enzymes) is not synthesized.

In inducible systems, the effector (inducer) associates with R in such a manner that the repressor (R) is rendered inactive and no longer has the capacity to exert any effect on the promotor region. Therefore, transcription of the operon occurs and the structural genes are expressed. The operon has been defined as, "the genetic unit whose phenotypic function is regulated by the action of a specific repressor", (Riley and Pardee, 1962). This model (Figure 56) has been shown by Jacob and Monod (1961) to explain most of their genetic and biochemical data pertaining to the β-galactosidase system.

However, the model can not be considered as universal

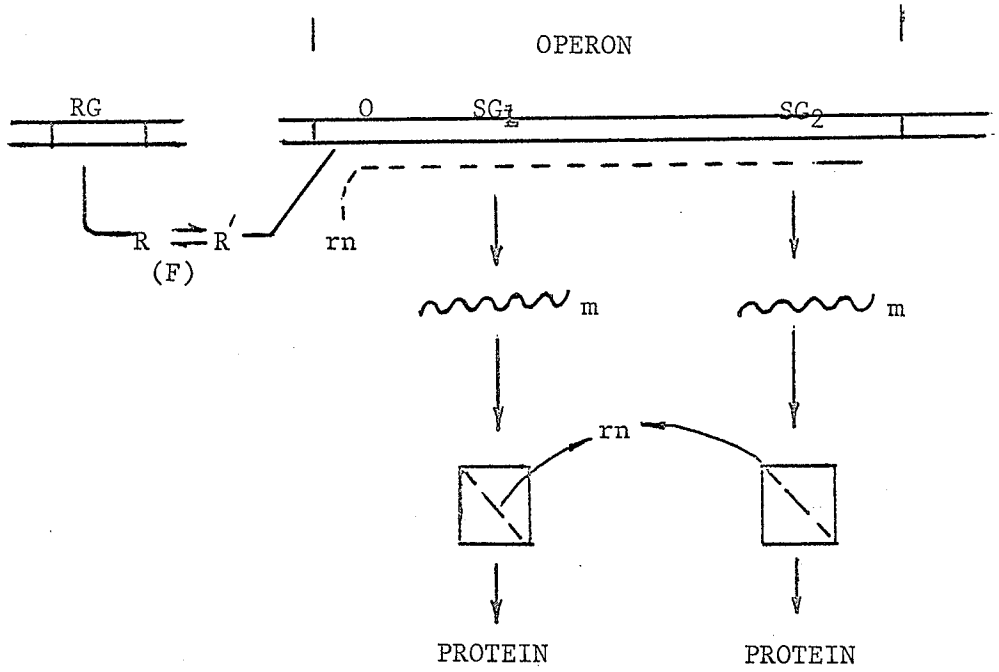


Figure 56. Genetic model for protein regulation proposed by Jacob and Monod (1961).
RG = regulator gene; R = repressor converted to R' in the presence of F (inducing or repressing metabolite); O = operator; SG_1, SG_2 = structural genes; rn = ribonucleotides; M = messengers.

for induction and repression since certain characteristics have been found in other inducible systems which are not observed in the lactose system. For example, the permease in the arabinase system is not linked to the arabinase operon (Isaacson and Englesburg, 1964) and Helling and Weinberg, 1963) have obtained evidence for the presence of a gene which appears to govern the formation of an "active inducer". Further, as in the case of the induction of D-serine dehydrogenase in E. coli (McFall, 1964) and repression of alkaline phosphatase of E. coli, more than one regulatory gene may be involved in regulation. The semisequential induction of enzymes of madelate catabolism in Pseudomonas (Stanier, Hegeman and Ornston, 1963) seems to involve further complications. The pathway of mandelate catabolism is:

mandelate $\xrightarrow{a}$ benzoylformate $\xrightarrow{b}$ benzaldehyde $\xrightarrow{c\ c1}$ benzoate $\xrightarrow{d}$ catechol $\xrightarrow{e}$ β-oxoadipate $\longrightarrow$ succinyl-CoA + acetyl CoA. In this pathway, mandelate coordinately induces enzymes a to c1, benzoate induces only enzyme d and catechol coordinately induces the remaining sequence of enzymes. Unlike the histidine pathway (Ames et al., 1960) the product of each sequence of the mandelate pathway coordinately induces the following sequence of enzymes. Stanier et al., (1963) suggested that the three enzyme groups constitute an operon and Mandelstam (1964) has showed

further complications by demonstrating that the enzymes of each group are coordinately repressed by their end-product.

Within the last year many comprehensive reviews and symposia have been published on the subject of cellular regulation. Among these are: Ames and Martin, Ann. Rev., Biochem. (1964); Synthesis and Structure of Macromolecules (Cold Spring Harbor Symp., Quant. Biol.,1963); Mechanisms of Regulation of Cellular Activities in Microorganisms (Centre Natl. Rech. Sci., Symp., Marseilles, 1963); Informational Macromolecules (Vogel, Bryson and Lampen, Eds., 1963) and Multiple Forms of Enzymes and Control Mechanisms (Kaplan, 1963).

II. METHODS AND MATERIALS

II. METHODS AND MATERIALS

CHEMICALS

The 1-C^{14}-DL-glutamatic acid and 1-C^{14}-glycine were obtained from the California Corporation for Biochemical Research, 3625 Medford Street, Los Angeles 63, California, U.S.A. Freund's adjuvant was supplied by Difco Laboratories, Detroit 1, Mich., U.S.A. Merck, Sharp and Dohme Research Laboratories of Canada, Ltd., P.O. Box 899, Montreal 3, Quebec were suppliers of Actinomycin-D ('Lyo' Meractinomycin). All other chemicals were obtained from Sigma Chemical Co., 3500 DeKalb Street, St.Louis 18, Mo., U.S.A.

GENETIC STRAINS

The phenotypes and genotypes of the cultures used are presented in Table 5a. All the am strains were periodically tested for reversion to the wild type by the glycine inhibition method of Pateman (1956). The method is based on the observation that glycine, at a concentration of 20 mM, strongly inhibits growth of am strains but not the wild type.

All mutant strains were maintained on minimal medium (Vogel and Bonner, 1956) with suitable supplementation where necessary.

MEASUREMENT OF GROWTH

The amount of growth was determined in two ways. (a) For data reported as mg. dryweight, Neurospora mycelia were col-

TABLE 5a. Description of Genetic Strains

Strain Designation	Locus	Linkage Group	Other Phenotypic Characters	Growth requirement	Source
STA 4	Wild-type		-	none	FGSC*
#am^{3}	am	V	leaky	amino-N	Fincham
#am^{4}, am^{5}, #am^{6}, am^{8}, #am^{9}	am	V	"	"	FGSC
am^{2}i^{-}	am,i	V	inhibited by ammonia	amino-N	Fincham
Pyr^{3}	pyr	IV	-	uridine	FGSC
Td^{2}	td	VI	-	trypto-phan	FGSC
inos^{-}	inos	V	-	inositol	FGSC
Suc^{-}	suc^{-}	I	very leaky	succi-nate	FGSC

* Fungal Genetics Stock Cultures.

\# These become non-leaky when supplemented with .02M glycine and lack the NADP-specific glutamate dehydrogenase.

lected on filter paper discs and excess moisture removed by suction. The mycelial pad was then dried over night at 80°C and weighed. (b) Growth tube method: the ends of a length of glass tubing (1 x 25 cm.) were bent upwards, at a 45° angle approximately 5 cm. from each end. Solid medium, sufficient to cover the bottom of the horizontal portion of the tube, was added, the ends plugged with cotton and autoclaved in an upright position. Conidia were powdered at one end of the agar and mycelial growth along the tube measured at time intervals.

SELECTION OF UREASE-MUTANTS

The "inositol-less death" method was used to select mutants of Neurospora crassa lacking urease (Lester and Gross, 1959). This method is based on the observation that a reduced rate of death of germinating conidia of an inositol-requiring strain occurs when a second mutation is imposed (Strauss, 1958) on top of inositol requirement.

Conidia from the inositol-requiring strain were harvested from three day old cultures grown at 28°C in Vogels N synthetic minimal medium with 2% sucrose and 10 µg of inositol/ml. The mycelial fragments were removed by filtration through four layers of cheesecloth. Conidia were repeatedly washed with sterile distilled water by alternate centrifugation and resuspension in conical glass capped

centrifuge tubes. The conidia were then counted on a Coulter Counter equipped with a 100 μ orifice, and adjusted to a concentration of 1 x 10^8 conidia/ml. Five ml. (5 x 10^8 conidia) of the suspension was placed in a petri plate and irradiated with ultraviolet light (Westinghouse Sterilamp G15T8) at a distance of 15 cms. until a survival rate of 0.1% was obtained. The time of irradiation necessary to obtain 0.1% survival at 15 cm. was 2.25 minutes. The irradiated conidia were stored in the dark for one hour to prevent photoreactivation. After this time the conidia were inoculated into 100 ml. of Vogel's medium (lacking ammonium nitrate, subsequently referred to a Vogel's- N medium) supplemented with 0.5% urea as the sole source of nitrogen and 0.25 ug. of inositol/ml. This was vigorously shaken for 6 hours at 28°C. Vigorous shaking prevented heterokaryosis. Germinated conidia were removed by filtration through six layers of gauze and the remaining conidia were washed three times by alternate centrifugation and resuspension in sterile distilled water. The final conidial pellet was suspended in enough sterile water to yield 200 viable conidia per ml. Portions (0.10 ml.) of the suspension were plated with a glass spreader on the following medium: 50 times diluted Vogel's medium-N (lacking a nitrogen source); 1.0% sorbose (autoclaved separately); 0.2% glycerol; 0.2% glucose; 0.5% urea (sterilized by milli-

pore filtration, pore size 0.45 μ) and 2% Noble agar. The plates were incubated at 34°C for two days. Colonies which appeared during the incubation period were removed. Following incubation, the agar plates were supplemented with 2.0 ml. of 0.5 mg./ml. inositol plus 2% ammonium nitrate and again incubated for two days. The resulting Neurospora colonies were transferred to agar plates containing Vogel's medium-N lacking ammonium nitrate, 2% sucrose, 1.0% sorbose, 0.5% urea, 5 mg./100 ml. inositol and 2% agar. These plates were then incubated at 30°C for 24 hours. Those colonies which displayed no growth on medium with urea as sole nitrogen source were selected. These were transferred to a complete medium (Vogel and Bonner, 1956) supplemented with inositol (5 mg./10 ml.) and assayed for urease activity.

UREASE ASSAY

The mycelial mat of Neurospora crassa was collected and washed in the usual manner (Methods I). The enzyme was extracted by grinding with levigated alumina powder and 5 volumes of 0.1 M phosphate buffer, pH 7.2, containing 1×10^{-3} M EDTA, at 4°C. The debris was removed by centrifugation at 10,000 x g for 10 minutes. Supernatant constituted the source of urease. The assay mixture, together with the enzyme, was incubated in the peripheral well of a Conway diffusion dish, the central well of which con-

tained 0.1M HCl. The reaction mixture (total volume 2.0 ml.) contained 25 mM urea, 0.2 ml. enzyme and 0.1M phosphate buffer, pH 7.0, containing 1 x 10^{-3}M EDTA. The reaction was stopped by the addition of 1.0 ml. of staturated K_2CO_3 after 10 minutes incubation. The ammonia was allowed to diffuse into the centre well for 1.5 hours after which time 0.5 ml. aliquots were removed and added to 1.0 ml. of water. The amount of ammonia was determined according to the method of Crowther and Large (1956). One unit of urease activity is defined as : μg. ammonia produced per 10 minutes.

PREPARATION OF ANTI-GLUTAMATE DEHYDROGENASE

Antiserum against NAD-specific glutamate dehydrogenase was prepared by injecting adult albino rabbits subcutaneously with a highly purified glutamate dehydrogenase emulsified with Freund's adjuvant (Cohn, 1952). The rabbits were injected weekly with 4 mg of protein each time for a period of five weeks. One week after the last injection, when titres were high enough, the rabbits were exsanguinated and the serum obtained was clarified by centrifugation. The resulting supernatant solution was dispensed in portions in small tubes and stored frozen at -20°C. All precipitation reactions were performed according to the standard serological technique described by Cohn (1952).

PROCEDURES FOR OBTAINING C^{14}-LABELLED ENZYMES

Wild type (STA 4) conidia of Neurospora crassa were inoculated into one litre of Vogel's medium-N containing 0.005 M L-leucine-1-C^{14} (1 x 10^6 CPM) in a two litre flask. They were vigorously aerated by means of a rotary shaker at 28°C for 18 hours. The resulting mycelial mat was asceptically collected by filtration with a Buchner funnel and washed with two litres of sterile water. The washed mycelial mat was then sucked dry by vacuum, and maintained for six hours at 28°C under aerated conditions in Vogel's medium-N minus a nitrogen source. After the starvation period, 2% NH_4NO_3(W/V) and 0.05 M L-alanine (inducer) were added to the medium and the cells were allowed to grow for an additional 18 hours. The second 18 hour incubation was necessary for the induction of the NAD-specific glutamate dehydrogenase. The cells were then collected by filtration through gauze and washed with copious amounts of water. The pressed dry mycelial mat was then divided into two equal lots and used for purification of both the NADP-specific and the NAD-specific glutamate dehydrogenases.

The NADP-specific enzyme was purified according to the method of Sanwal and Lata (1962b) and the NAD-specific enzyme as discribed earlier (Methods I). In both purification proceedures the DEAE-cellulose chromatography step

was omitted. Instead the enzymes were precipitated with $(NH_4)_2SO_4$ at 0.80 saturation. After 20 minutes of stirring at 4°C the precipitates which resulted were recovered by centrifugation at 20,000 x g for 15 minutes, and resuspended in 2 ml. of 0.1 M Tris-HCl buffer at pH 7.7. These fractions were then further purified by starch gel electrophoresis (220 V, 25 mA). The electrophoresis was carried out for 5 hours at 4°C after which the enzymes were located on the starch gel by the specific dehydrogenase staining method of Markert (1959). The NADP- and NAD-specific glutamate dehydrogenases and malic dehydrogenase were eluted from the gel with 1.0 ml of 0.1 M phosphate buffer, pH 7.5.

The enzyme, malic dehydrogenase, which is not subject to increase or decrease during the various growth stages, served as a control. The radioactivity of all three enzymes was measured with a Nuclear Chicago Scintillation Counter. Samples were counted for a period of 100 minutes.

ESTIMATION OF AMMONIA

Ammonia was extracted from thoroughly washed cells with 0.1 M HCl in a Ten Brock homogenizer and its concentration determined in aliquots by the Conway diffusion method. The sensitive sodium phenoxide reagent (Crowther

and Large, 1956) was used for the estimation of ammonia.

ESTIMATION OF AMINO ACIDS

For the determination of amino acids, thoroughly washed cells were extracted with cold perchloric acid (6%) in a Ten Brock homogenizer. The acid was removed as potassium perchlorate, by adjusting the pH to neutrality with 2N potassium hydroxide. After 30 minutes at 4°C, the precipitated potassium perchlorate was separated by centrifugation at 10,000 x g for 5 minutes. The amino acid fraction was separated from carbohydrates and nucleic acids by ion exchange chromatography with Dowex 50W-8X ion exchange resin. The Dowex resin was washed in distilled water, allowed to settle and the small particles removed by decantation. The Dowex resin slurry was poured into a chromatography column (1.5 x 30 cm) and packed to a height of 2.5 cm. Charging of the resin was accomplished by cycling 3 times with 6N HCl and 2N NaOH. Following the final 6N HCl cycle, distilled water was passed through the column until the eluent was neutral.

The extract containing amino acids was applied to the column, washed with water and the amino acids eluted with 4N NH_4OH. The eluent was concentrated to 2.0 ml with a Buchler Instruments flash-evaporator under negative pressure. Aliquots of the concentrated eluent were applied

on Whatman #1 chromatography paper (40 x 52 cm) and chromatographed with n-butanol-acetic acid-water (40:10:50) as solvent for 24 hours.

The dried paper was sprayed with 0.4% solution of ninhydrin in water saturated n-butanol. L-alanine spots were cut out and eluted with 5.0 ml. ethanol (70%) containing 0.005% copper sulphate (Giri et al., 1952). The color intensity was measured at 540 mu with a Klett-Summerson colorimeter and the concentration of L-alanine was calculated from an L-alanine standard curve.

MEASUREMENT OF RADIOACTIVE AMINO ACID UPTAKE

Conidia from am^5 mutant of Neurospora crassa were inoculated into 25 ml. of Vogel's medium-N in 125 ml. Erlenmeyer flasks and incubated at 30°C for 3 days as a standing culture. A drop of Tween 80 was added to the medium to prevent conidiation (Zalokar, 1954). The mycelial mat was filtered in a Buchner funnel with gentle suction and washed thoroughly with one litre of fresh medium. The mycelial mat was transferred onto filter paper and discs were cut from it with a cork borer 10 mm in diameter. The mycelial discs were placed on filter paper (Whatman #1) moistened with Vogel's medium-N in a petri plate. These were incubated for one hour at 28°C to allow healing of mycelia which were damaged by the cutting.

After this recovery period the discs were gently sucked dry in a Buchner funnel. Drops of Vogel's medium-N containing radioactive amino acids (.05 ml. each) were spotted on a non-wetting petri plate. The dry discs were transferred onto the drops and exposed to the label for various time intervals (usually 5, 10, 15 and 20 minutes). After the appropriate time interval, the discs were transferred to a Buchner funnel and washed with a continuous stream of ice-cold medium containing non-radioactive amino acid for 60-80 seconds. The discs were directly mounted onto planchettes, covered with filter paper discs and pressed flat with a rubber stopper. The whole assembly was dried in a heating oven at 80°C for 30 minutes. This proceedure caused the mycelia discs to adhere firmly to the planchette but not the filter paper. The radioactivity was measured with a windowless Nuclear Chicago micromil gas-flow counter for 15 minutes. According to Zalokar (1960) discs prepared in this manner gave 25% self-absorption. Therefore, the data presented here were corrected accordingly for 25% self-absorption.

SELECTION OF NAD-SPECIFIC GLUTAMATE DEHYDROGENASE MUTANT

In order to facilitate selection of a mutant lacking NAD-specific glutamate dehydrogenase (d^- mutant) by the "inositol-less death" method (Lester and Gross, 1959), it was necessary to obtain a double mutant lacking the NADP-specific enzyme and inositol requiring. The only method available for selecting d^- mutants would be

its requirement for L-glutamate. Therefore, the starting culture would have to be deficient in the NADP-specific glutamate dehydrogenase since it is capable of synthesizing glutamate from α-ketoglutarate and ammonia.

CROSSING MEDIUM

All crossing experiments reported in this work were preformed on corn meal agar (Difco) supplemented with 0.2% inositol; 0.5% L-glutamate; 0.2% glucose and 2% Vogel's medium-N (stock solution). Filter paper (Whatman #1) strips, 1 x 8 cm, were placed in test tubes (1.5 x 15 cm) prior to the dispensing of the crossing medium. The presence of filter paper in the medium increased the fertility of the crosses.

GENETIC CROSSES

Slants of crossing medium were inoculated with conidia from $\underline{am}^6$ (mating type, $\underline{a}$) and allowed to form mycelia in the dark at 22°C. After incubation for 2 days, conidia harvested from an inositol requiring mutant ($inos^-$, mating type, A) were powdered onto the $\underline{am}^6$ mycelia and again incubated at 22°C in the dark. The immature perenthecia which were formed after 3 days of incubation required an additional 5 day incubation period to attain maturity. The mature perenthecia were transferred to a small 4% agar block (1 cm^2) and asci were liberated by breaking the

perithecia with dissecting needles. Single ascospores were picked at random with the aid of a binocular stereomicroscope. The spores were treated with 0.01% sodium hypochlorite and transferred to agar slants containing Vogel's medium-N supplemented with 0.1% inositol. Dormancy of the individual ascospores was broken by heat-activation at 60°C for 35 minutes. The activated spores were incubated at 30°C for 5 days and the resulting conidia were used to determine the phenotype.

Cultures which displayed a nutritional requirement for inositol and were inhibited in the presence of 0.02 M L-glycine were selected. These cultures were then assayed for the presence of NADP-specific glutamate dehydrogenase. The double mutant *am*^{6} inos^{-} (hereafter S_1(A)) served as the parent strain for further mutation experiments.

SELECTION OF NAD-SPECIFIC GLUTAMATE DEHYDROGENASE MUTANTS

Conidia from S_1(A) were irradiated according to the method previously described for the urease mutant, until 0.1% survival (2.5 minutes) was attained. Irradiated conidia were inoculated into minimal medium plus inositol (0.25 µg./ml.) and aerated by shaking for 6 hours at 30°C. The germinated conidia were removed by filtration through 6 layers of gauze. The ungerminated conidia which escaped

filtration were plated on minimal sorbase medium containing inositol (5 µg.) and L-glutamate (.05 M). Following 2 days of incubation at 28°C, the colonies were transferred to minimal sorbase medium lacking L-glutamate. Those colonies which showed no growth in the absence of L-glutamate and growth in its presence were selected and assayed for NAD-specific glutamate dehydrogenase.

FORMATION OF HETEROKARYONS

The strains $pyr^3\underline{a}$ was crossed with $\underline{am}^8i^+$ and $td^2\underline{a}$ was crossed with the $\underline{am}^1i^-$ strain of *Neurospora crassa*. am^8i^+ pyr^3a and $am^1i^-td^2\underline{a}$ mutants were selected each according to its specific auxotrophic requirements. The conidia from these two multiple mutants were harvested, washed twice with sterile distilled water by alternate centrifugation and resuspension, and mycelial fragments removed by filtration through gauze. Following the final centrifugation, the conidial concentration of both strains was adjusted to 5×10^8 per ml. One millilitre of each conidial suspension was inoculated into a 125 ml. Erlenmeyer flask containing 25 ml. of Vogel's minimal medium lacking nitrogen source (V-N) and supplemented with L-glutamate (0.1%). The flasks were incubated at 30°C until a mycelial mat was formed under still culture conditions (without shaking). The mat was collected in a Buchner funnel, thoroughly washed with sterile water, blended

lightly (30 seconds) and inoculated into test medium.

III. RESULTS

III. RESULTS

INDUCTION OF NAD-SPECIFIC GLUTAMATE DEHYDROGENASE

STUDIES OF UREASE$^-$ STRAINS

It has been previously shown that urea is capable of induction of NAD-specific glutamate dehydrogenase which simultaneously results in a corresponding decrease of NADP-specific glutamate dehydrogenase (Sanwal and Lata, 1962). To establish whether the induction was actually due to urea or some break-down product, a mutant lacking urease was isolated from an inositol requiring mutant by irradiation. The double mutant, urease$^-$inositol$^-$ ($am^+ u_3^- inos^-$) was then back-crossed to $am^{6-} inos^+ u_3^+$, and a $am^{6-} u^{3-}$ mutant isolated. Since urease is a non-essential enzyme for growth, the mutant ($am^{6-} u_3^-$) displayed a phenotype identical to the parent strain (am^{6-}).

The strains $am^- u^+_3$ and $am^+ u^-_3$ were grown in minimal medium N supplemented with 0.062M urea and grown for 36 hours. While NAD-specific glutamate dehydrogenase was not induced in the urease$^-$ mutant, the urease$^+$ type displayed induced levels of this enzyme (Table 5). However, the NAD-specific glutamate dehydrogenase of the urease$^-$ mutant could be induced by all amino acids which are capable of inducing the enzyme in am$^-$ mutants. Therefore, a prerequisite for NAD-GDH induction by urea appears to be

TABLE 5. Comparison of NAD-glutamate dehydrogenase induction by urea (0.062M) in am^- and $urease^-$ strains of Neurospora crassa.

Supplement	NAD-GDH Specific Activity	
	Strain	
	$Urease^-$	am^-
None	6.0	7.3
Urea (0.062M)	6.8	72.0
Methyl urea (0.05M)	5.3	6.3
L-alanine (0.05M)	41.0	52.0
Ammonium succinate (0.05M)	35.0	43.4

the presence of the enzyme urease.

To distinguish which of the two break-down products of urea (CO_2 and NH_3^+) act as inducer, it became necessary to study the effect of urea on a mutant with a defective carbon dioxide fixation mechanism. A mutant of this kind described by Barratt et al., (1954) requiring succinate in the growth medium was used for this purpose. The results presented in Table 6 show that in this mutant (suc^-), NAD-specific glutamate dehydrogenase was induced by urea (0.062M). These results suggest that ammonia, but probably not CO_2, may in some way induce or accelerate induction of the enzyme.

Results obtained when an am^- mutant, was grown in the presence of increasing amounts of ammonia, clearly indicated induction of NAD-specific glutamate dehydrogenase and a concurrent repression of the NADP-specific enzyme (Figure 42). Similar experiments demonstrated that the internal glutamate levels reached saturation just prior to induction (Figure 42).

EFFECT OF ACTINOMYCIN D ON THE INDUCTION OF NAD-SPECIFIC GLUTAMATE DEHYDROGENASE

Since glutamate and ammonia both act as inducers as well as substrates for the enzymes, the possibility exists that these compounds complex with the 'nascent' enzymes and detach them from their sites of synthesis in the cytoplasm. This possibility has, indeed, been suggested for

TABLE 6. Induction of NAD-specific glutamate dehydrogenase in succinate mutant (suc $^-$) of Neurospora crassa by urea (0.06M).

Supplement	NAD-glutamate dehydrogenase Specific Activity
None	5.1
succinate (0.02M)	4.2
succinate plus urea (.06M)	69.0

Conidia harvested from suc$^-$ mutant (377) were inoculated into 100 ml Vogels' minimal medium, containing supplements in 500 ml. Erlenmeyer flasks and vigorously aerated at 28^{o}C for 36 hours.

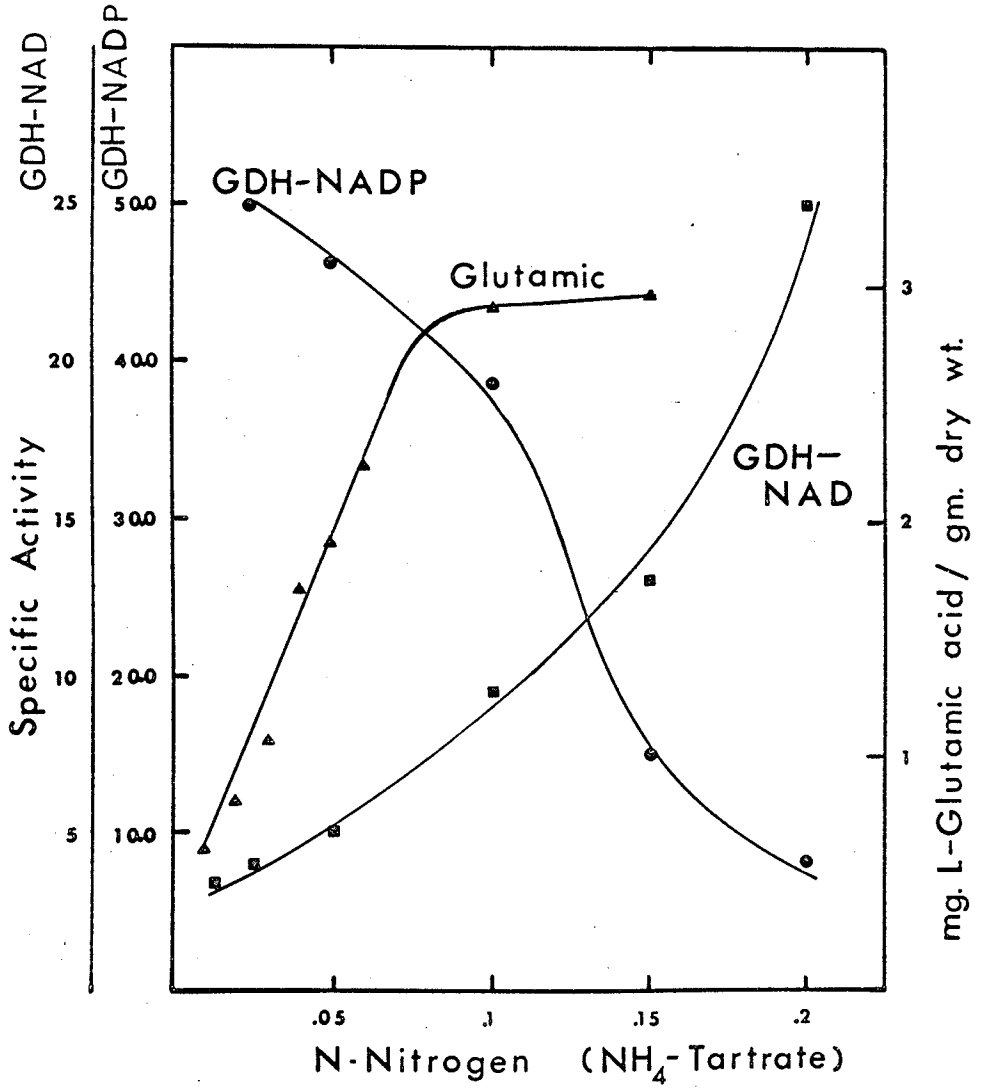


Figure 42. Effect of increasing ammonia concentrations on the induction and repression of NAD-GDH and NADP-GDH (respectively) from Neurospora crassa (W.T.). Endogenous L-glutamate concentrations were determined manometrically with commercial glutamate decarboxylase (Sigma Chemical Co., U.S.A.). L- glutamate was extracted from mycelia in boiling water and concentrated *in vaccu* prior to determination.

the induction of alanine dehydrogenase of Bacillus subtilis by Freese's group (Freese and Oosterwyk, 1963). Thus the increase or decrease in enzyme levels could be due to a simple interaction of enzymes and inducers in the cytoplasm and not due to interactions at the nuclear level.

It has been shown that actinomycin D selectively inhibits synthesis of m-RNA from the DNA template (Hurwitz, 1962). If this induction process occurs at the genetic level, the antibiotic should inhibit the induction of NAD-specific glutamate dehydrogenase. To test this possibility, conidia from a five-day old culture of wild-type (STA 4) Neurospora crassa were inoculated into 25 ml. of minimal medium supplemented with urea (0.05M) and varying concentrations of actinomycin D. The 125 ml. capacity Erlenmeyer flasks were aerated at 28°C. for 36 hours. A portion of the cell mass was saved for dry weight determinations and the remainder was assayed for NAD-specific glutamate dehydrogenase activity. It can be seen from Figure 43 that 0.25 µg/ml. of actinomycin D retarded enzyme induction by 55% whereas growth only decreased by 15%

GRATUITOUS INDUCERS

Four analogues of glutamic acid were tested as gratuitous inducers of glutamate dehydrogenase. Jacob and Monod (1961) and Müller-Hill, Rickenberg and Wallenfels (1964) showed that induction of β-galactosidase occurs in the

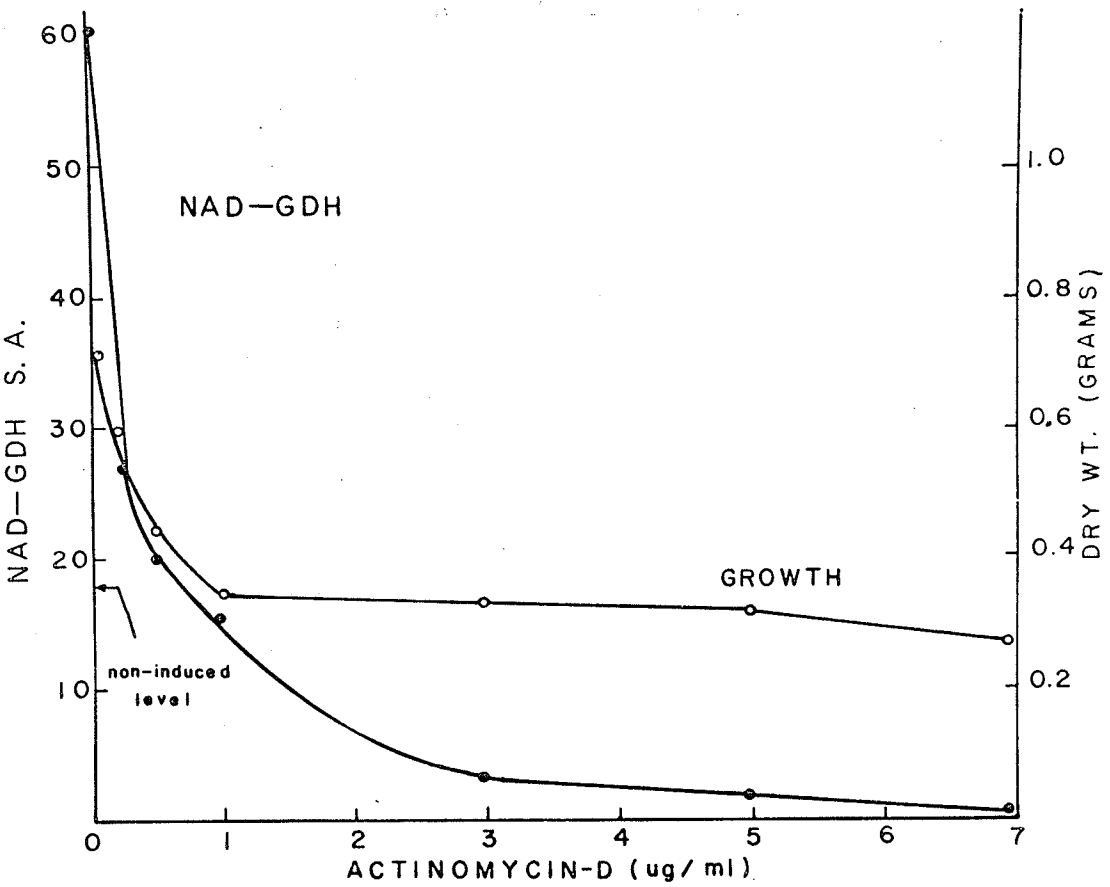


Figure 43. Effect of various actinomycin-D concentrations on the induction of NAD-glutamate dehydrogenase of Neurospora crassa (W.T.). Actinomycin-D was dissolved in 70% ethyl alcohol and stored frozen in the dark.

the presence of non-metabolizable thio derivatives of lactose as efficiently as in the presence of lactose itself. Table 7 shows that the analogues, p-aminobenzoyl-glutamate, N-acetyl-DL-glutamate, L-pyroglutamic acid and ∝-methyl-DL-glutamate were ineffective as inducers.

Also, several compounds of the tricarboxylic acid acid cycle were tested as possible inducers of NAD-specific glutamate dehydrogenase of amination deficient mutants. None of the following compounds acted as inducers; malic acid, citric acid, fumaric acid, succinic acid and ∝-keto-glutarate, tested at a concentration of 0.05 M. Compounds structurally similar to urea such as, methylurea, ethyl urea and thio urea were also found to be ineffective as inducers, possibly because they are not attacked by Neurospora urease.

CONVERISON OF NAD-SPECIFIC GLUTAMATE DEHYDROGENASE

The results presented in Figure 44 show that in the presence of an inducer (urea) the effect upon the two glutamate dehydrogenases differs. In one case the exogenously supplied metabolite causes a derepression (NAD-GDH), while in the other (NADP-GDH) it brings about repression. This peculiar phenomenon has been labelled 'concurrent regulation' (Sanwal and Lata, 1962). The possibility arises that, in the presence of inducer, the NADP-specific glutamate dehydrogenase is converted or modified to NAD-specific glutamate

TABLE 7. Effect of L-glutamate analogues on the induction of NAD-specific glutamate dehydrogenase of Neurospora crassa.

L-glutamate analogues*	NAD - GDH Specific Activity
Control (no analogue)	4.6
p-aminobenzoyl-glutamate	3.8
N-acetyl-DL-glutamate	4.8
L-pyro-glutamic acid	3.0
∝-methyl-glutamate	3.2

*Minimal medium was supplemented with 0.05M of L-glutamate analogues. Conidia from wild-type Neurospora were incubated at 30°C for 48 hours.

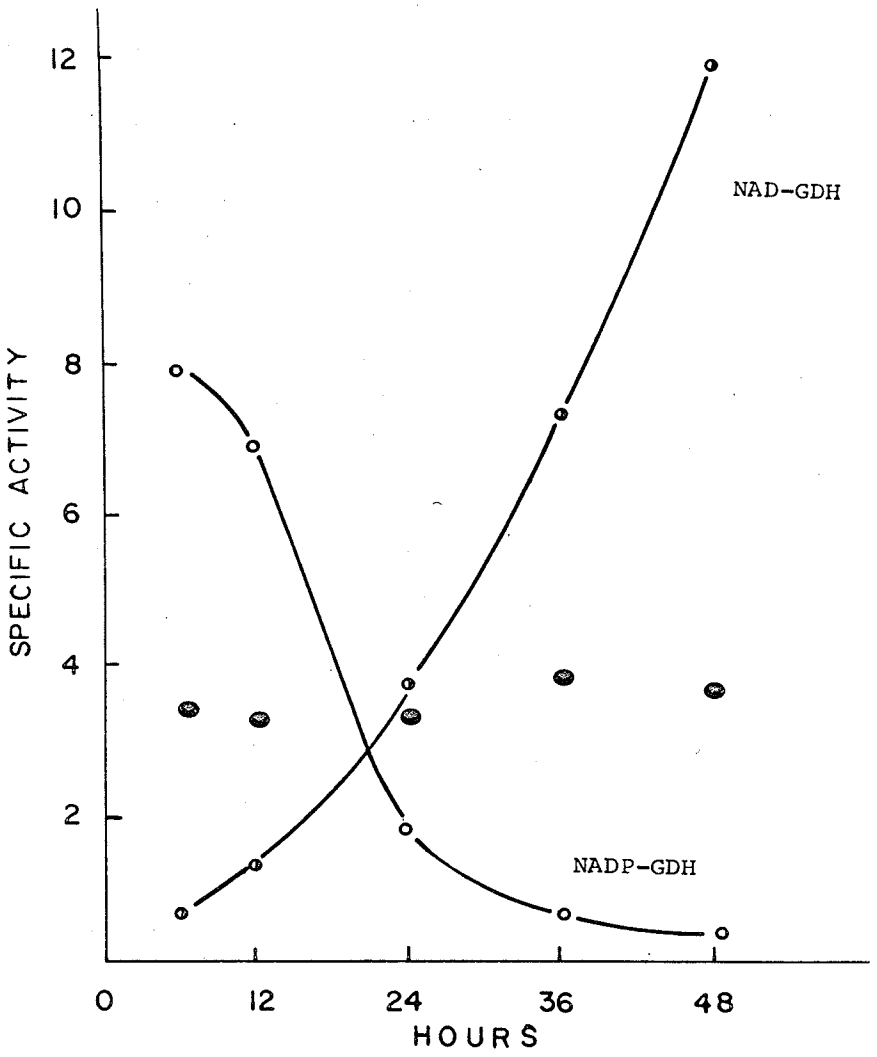


Figure 44. Effect of urea on the induction and repression of both glutamate dehydrogenases of Neurospora crassa. Wild-type strain (262) was grown in Vogel's minimal medium N supplemented with 0.062 M urea (sterilized by Millipore filtration). Solid circles represent urease specific activity.

dehydrogenase during growth. To test this hypothesis an experiment was designed in which the NADP-specific enzyme was labelled with 1-C^{14}-leucine and then transferred to a label-free medium containing inducer. If conversion of one enzyme to another occurred, the label incorporated in the NAD-specific enzyme should be equal to or greater than that in the NADP-specific enzyme. The incorporation of radioactivity into NAD-specific glutamate dehydrogenase was less than 50% of that of the NADP-specific enzyme (Table 8). This indicates that interconversion is unlikely, although the experiment does not completely exclude the possibility.

GLUTAMATE DEHYDROGENASE ISOZYMES

Crude extracts prepared from the wild-type (STA 4) strain of Neurospora crassa grown under induced conditions (0.05 M L-alanine) were subjected to starch gel electrophoresis. A portion of the gel strips were stained specifically for the NAD-specific glutamate dehydrogenase and the others for NADP-specific glutamate dehydrogenase (Markert and Moller, 1959). Figure 45 reveals the presence of three and possibly four NAD-specific enzyme bands. The second gel strip, specifically stained for NADP-specific glutamate dehydrogenase shows only one band. The occurence of isozymes of dehydrogenases is not uncommon. Markert and

TABLE 8. Conversion of NADP-specific glutamate dehydrogenase during induction.

Enzyme	Incorporation of 1-C^{14}-leucine into enzyme dpm per mg. protein*
Control-Malic dehydrogenase	834
NADP-glutamate dehydrogenase	595
NAD-glutamate dehydrogenase	293

*Samples were counted for 100 minutes with a Nuclear Chicago Sintilation counter. Counts reported are corrected for background. For details of procedure see text.

Figure 45. Starch gel electrophoresis of a crude extract of Neurospora crassa (W.T.) grown under induced conditions (see text). Gel strip:

(A) stained specifically for NAD-GDH whereas

(B) was stained specifically for NADP-GDH.

(according to Markert and Moller, 1959).

Moller (1959) were able to separate lactic dehydrogenase into several molecular forms which they called 'isozymes'. Multiple forms of serum esterases and cholinesterases have also been reported (Harris et al., 1962; Augustinsson,1959), as well as animal glutamate dehydrogenase (Van der Helm, 1962).

COMPARISON OF PURIFIED CONSTITUTIVE AND INDUCED ENZYME

Results of the interconversion experiments as well as those with actinomycin D make it likely that regulation of the dehydrogenases occurs at the primary (DNA) template level. However, it is possible that the NAD-specific dehydrogenase induced in the cells is not the same as that present in basal (or constitutive) levels under uninduced conditions. In other words, there might be two types of NAD-specific glutamate dehydrogenases, one inducible and the other constitutive. Such a situation has been found in the case of repressible β-glucosidases in yeast by Duerksen and Fleming (1963). It is at once clear that if such a situation were found to be the case in Neurospora it would have a tremendous bearing on the hypothesis that can be suggested as a basis of 'concurrent regulation' of the dehydrogenases. A comparison of the NAD-specific glutamate dehydrogenases produced under uninduced and induced conditions was, therefore, undertaken.

(a) Immunological Comparison

Prior to comparing purified 'constitutive' and induced NAD-GDH by serological means, it was necessary to determine the equivalence point. This was determined by titrating a fixed amount of anti-GDH (0.2 ml.) with purified glutamate dehydrogenase. The usual precipitin reaction was carried out in physiological saline (0.15 M), containing 5×10^{-3} M reduced glutathione (final volume of 1.5 ml.). Tubes were incubated at 6°C for 18 hours. Non-immune rabbit serum was used as control. The reduced glutathione was incorporated into the saline in an attempt to stabilize the enzyme during the incubation period. However, the enzyme still lost more than 80% of its catalytic activity. After incubation, the precipitate was recovered by centrifugation at 5,000 x g for 10 minutes and washed three times with saline-GSH mixture. The washed precipitate was resuspended in 0.5 ml. of 0.1 M Tris-HCl buffer, pH 7.5.

The results presented in Figure 46 demonstrate that the precipitation reaction reaches saturation when 14 units of NAD-specific glutamate dehydrogenase are added. The enzyme-antibody complexes were found to be completely active. Non-immune rabbit sera controls showed no neutralization of glutamate dehydrogenase activity.

Subsequent experiments, in which enzyme was titrated with anti-enzyme, revealed that the overall precipitation patterns of both constitutive and induced glutamate dehydro-

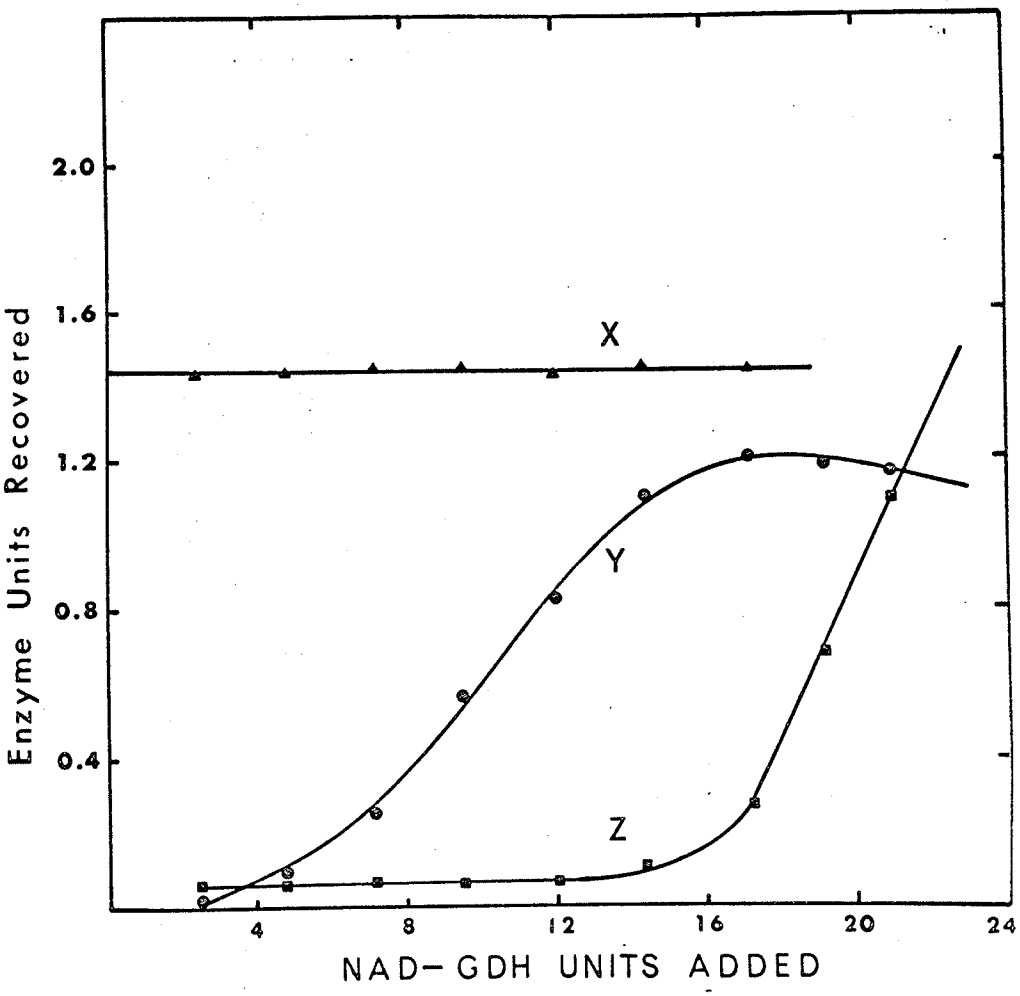


Figure 46. Precipitin tests with rabbit antibody and NAD-specific glutamate dehydrogenase. Anti-NAD-GDH serum was constant at 0.2 ml. Line (X) represents control (non-immune rabbit serum; (Y) NAD-GDH units recovered from pellet; (Z) NAD-GDH enzyme units remaining in supernatant.

genases were similar (Figure 47). Such results are expected in cases in which two protein molecules are antigenically related.

(b) Kinetic Comparison

In this work the induced and constitutive glutamate dehydrogenase from *am*^{5} and *am*^{1} i$^-$ (1499-12) were compared kinetically. The Michaelis-Menton constants were calculated graphically from double reciprocal plots (Lineweaver and Burk, 1934) by varying one substrate in the presence of constant amounts of the other substrates. The difference between K_m values presented in Table 9 and those previously reported (Results I), was due to assay differences. The values given in Table 9 were obtained under conditions in which the non-variable substrates were not completely saturating. Nevertheless, the K_m values (Table 9) indicate that the kinetic parameters of the induced and constitutive enzymes are the same, within limits of the experimental error.

(c) Heat Inactivation

Both constitutive and induced enzymes were extracted from *am*^{6} mycelia and purified (C_γ fraction) according to the method previously outlined. The protein concentration of each enzyme preparation was adjusted to 0.1 mg./ml. Each sample of enzyme was suspended in 0.1 M KH_2PO_4-KOH buffer, pH 7.5, containing 10^{-3} M reduced glutathione. Figure 48

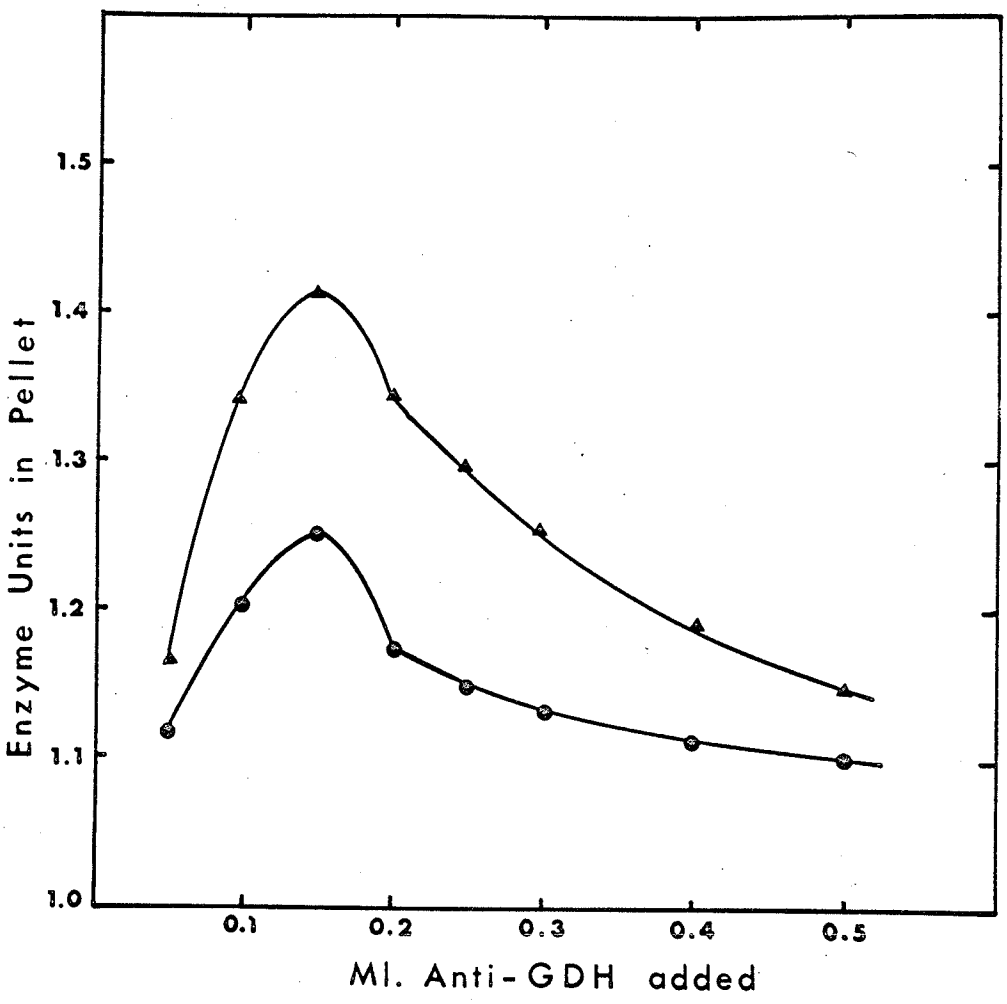


Figure 47. Immunological comparison of precipitation pattern of induced and constitutive NAD-specific glutamate dehydrogenase (see text for procedure). Curve (▲-▲) induced NAD-GDH; (●-●) constitutive NAD-GDH.

TABLE 9. Comparison of some kinetic constants of induced and constitutive NAD-glutamate dehydrogenase of Neurospora crassa.

Substrate	K_m values in moles/liter.*		
	Wild-type	am⁻ (525)	am⁻ i⁻ (1499-12)
	Non-induced	induced	induced
L-glutamate	5.5×10^{-3}	5.0×10^{-3}	3.8×10^{-3}
NAD	3.3×10^{-4}	3.5×10^{-4}	2.3×10^{-4}
∝-ketoglutarate	4.6×10^{-3}	3.0×10^{-3}	4.8×10^{-3}
$(NH_4)_2SO_4$	17.0×10^{-3}	8.3×10^{-3}	5.2×10^{-3}
NADH	5.5×10^{-4}	2.6×10^{-4}	6.6×10^{-4}

*The values presented here are an average of three separate determinations.

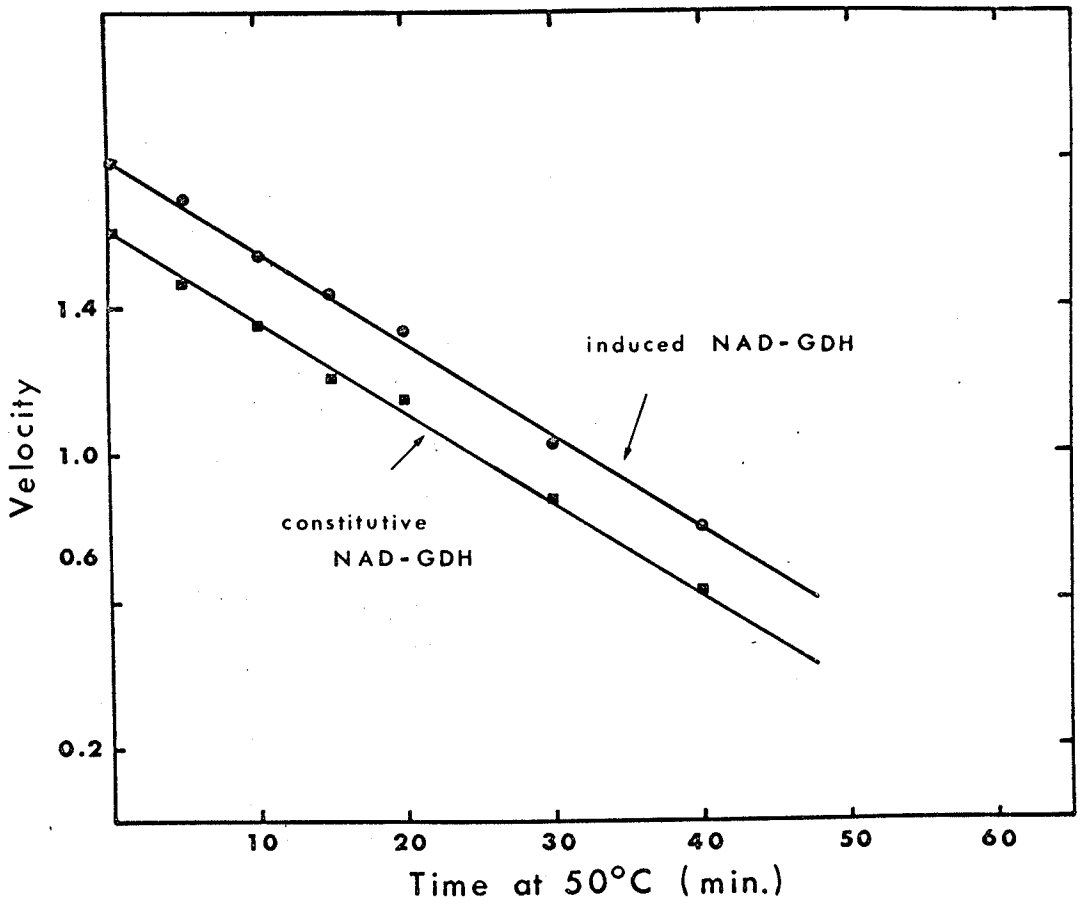


Figure 48. Heat inactivation of induced and constitutive NAD-specific glutamate dehydrogenase at 50°C. Velocity was plotted as a function of time.

shows that both enzymes are heat inactivated at the same rate at 50°C.

INHIBITION OF GROWTH OF am MUTANTS BY GLYCINE

SPECIFICITY OF GLYCINE INHIBITION

The effect of glycine on the growth of both *am* and prototrophic strains is shown in Figure 49. It may be noted that glycine inhibits the growth of *am* strain completely without interfering with that of the wild type. In order to understand the mechanism of this inhibition and its bearing on regulation, it was necessary to determine whether the inhibition was restricted to glycine alone or whether related compounds could bring about the same effect. A number of compounds related to glycine were tested in growth assays. Table 10 represents the results of this investigation where compounds are listed in the order of their potency in bringing about growth inhibition. None of these compounds show any effect on the wild-type strain. Specially interesting among these compounds is methylamine which is not utilized by the cell at all. The inhibition displayed by this compound suggested the possibility that all these substances may inhibit growth by interfering with the uptake of ammonia. However, when such experiments were performed, the results (Table 11) showed that in the presence of glycine (.02 M), ammonia was

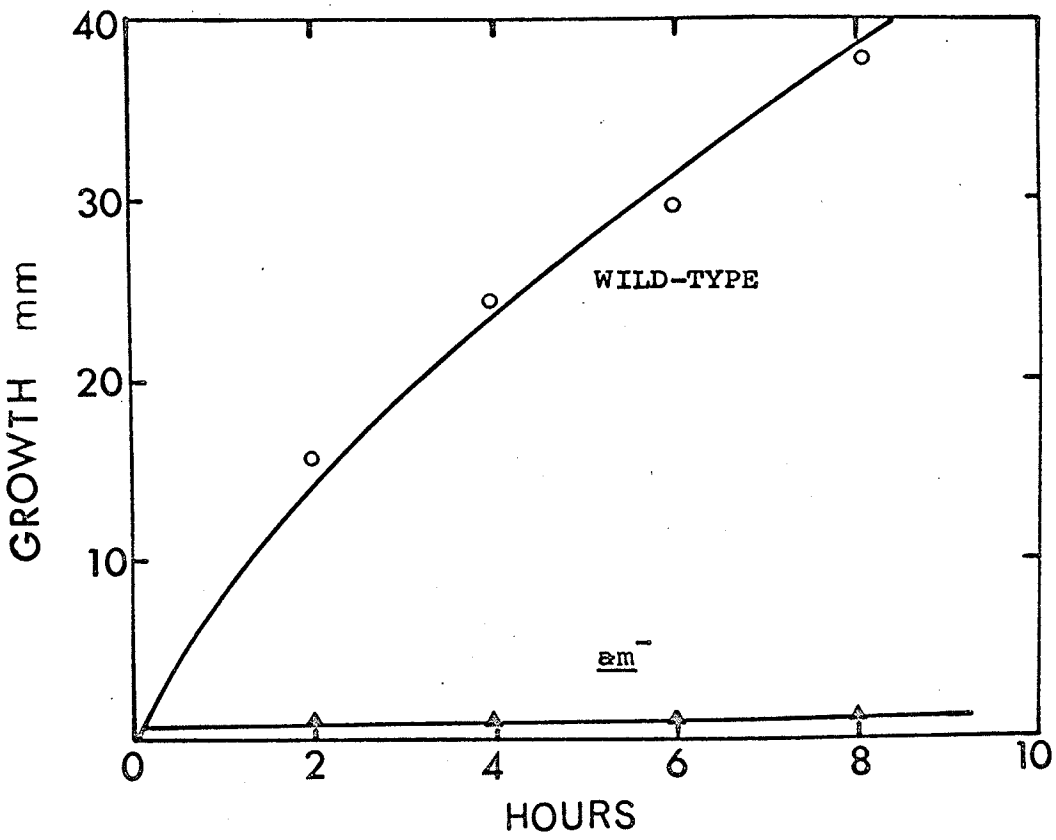


Figure 49. Effect of glycine on the growth of both am⁻ and prototrophic (W.T.) strains. Conidia from each strain were inoculated onto minimal medium N containing glycine (0.02M) in growth tubes. Distance of mycelial growth was measured at various time intervals. Cultures were incubated at 28°C.

TABLE 10. Growth inhibition of am^- strain by compounds related to L-glycine.

Growth Inhibitors*	Growth (am^-) Time	
	24 Hours	48 Hours
L-glycine	-	-
L-serine	-	-
Sarcosine	-	-
glycine-methyester	-	-
methylamine	-	-
ethylamine	-	sl.
glycine-amide	+	++
N-acetylglycine	++	+++
glyoxylic acid	+	++

*All inhibitors were tested at a concentration of 0.02M. Conidia harvested from am^-(525) were inoculated into tubes containing Vogel's minimal medium N plus inhibitor. Tubes were incubated at 30°C. Growth: (-) no visible hyphae; (sl) hyphae visible over surface of media; (+) degrees of growth.

TABLE 11. Effect of L-glycine on ammonia uptake in Neurospora crassa mycelium.

Minimal medium lacking carbon source plus inhibitor*	Ammonia μg NH_3-N per ml.
Control no inhibitor	3.4 5.7
L-glycine (0.02M)	9.0 8.4
L-serine (0.02M)	7.4 9.2

*Mycelium from am^- growth in Vogel's minimal medium N for 24 hours, were collected, washed and starved for 6 hours in minimal medium lacking carbon and nitrogen source. Cells were then resuspended in minimal medium containing L-glycine (.02M). Ammonia was extracted by grinding with boiling water in a Ten-Brock homogenizer.

taken up by the cells. This was also the case in experiments with other growth inhibitors listed in Table 10.

ABSENCE OF NADP-SPECIFIC GLUTAMATE DEHYDROGENASE AS A PREREQUISITE FOR GLYCINE INHIBITION

Since glycine inhibition is shown only by mutants lacking NADP-glutamate dehydrogenase, it was desirable to know whether the absence of this enzyme was necessary for the inhibition. Attempts were made to obtain mutants from strain $\underline{am}^6$ which would grow in the presence of glycine or serine. Conidia from a 5 day culture of $\underline{am}^6$ were irradiated as previously described. The irradiated conidia were diluted and spread on plates (200-300 per plate) containing minimal medium N, sorbose (1.0%) and glycine (0.02 M) or serine (0.02 M). After incubation at 30°C the few colonies appearing were selected at random and analyzed for the presence of NADP-specific glutamate dehydrogenase. Table 12 presents the results of this investigation. All the strains capable of growth in the presence of glycine (0.02M) or serine (0.02M) possessed the NADP-specific glutamate dehydrogenase, although the specific activity of the enzyme differed in different strains.

Therefore, all the colonies tested were revertants for the am locus. It is clear that glycine or serine resistence of wild-type or revertant strains is connected with the

TABLE 12. Absence of NADP-specific glutamate dehydrogenase as a prerequisite of glycine inhibition.

Revertants of am6*	NADP-specific glutamate dehydrogenase (units)
G-5	800
G-6	170
G-7	2,000
G-8	600
G-10	3,000
G-11	3,000
G-13	4,000
G-14	1,800
G-15	2,000

*See text for details

presence of NADP-specific glutamate dehydrogenase.

REVERSAL OF GLYCINE INHIBITION BY L-GLUTAMATE

Since NADP-glutamate dehydrogenase synthesized glutamate, and only in the absence of this enzyme are cells inhibited by glycine, it was desirable to determine whether an exogenous supply of L-glutamate would alleviate glycine inhibition in *am* mutants. Increasing concentrations of L-glutamate (0.02M - 0.10M) were supplied in the minimal growth for *am*^{5}, as well as 0.02M L-glycine. Maximum growth of *am*^{5} occurred in flasks containing minimal medium or minimal medium plus 0.02 M glutamate within 48 hours of incubation. Very little growth occurred in flasks supplemented with 0.02 M L-glycine in combination with various concentrations of L-glutamate (Table 13).

INCORPORATION OF 1-C^{14}-GLUTAMATE

To determine whether glycine was interfering with the entry of glutamate into the internal 'pool', experiments were conducted with 1-C^{14}-glutamate. Results of these experiments are given in Table 14. It was shown that glutamate was incorporated into the internal pools at a much lower rate than labelled glycine. Furthermore, glutamate uptake was almost completely retarded in the presence of glycine (.02M).

TABLE 13. Effect of L-glutamate on glycine inhibition of am⁻ mutant growing in minimal medium.

Supplement	Growth (dry weight) mg.*
No supplement	95
L-glycine (.02M)	none
L-glutamate (.01M)	137
L-glycine (.02M) plus L-glutamate (.005M)	22
L-glycine (.02M) plus L-glutamate (.01M)	29
L-glycine (.02M) plus L-glutamate (.02M)	36
L-glycine (.02M) plus L-glutamate (.05M)	35

*Values presented are the average of duplicate flasks.

TABLE 14. Incorporation of 1-C^{14}-glutamate into am$^-$ mutant of Neurospora crassa in the presence of glycine.

Radioactive Compound	Time (Hours)	Incorporation (CPM)*
1-C^{14}-glycine (20 mM)	0	4568
	1	378
	3	959
	4	1821
1-C^{14}-glutamate (20 mM)	0	2100
	1	51
	3	145
	4	204
Cold L-glycine (20 mM) + 1-C^{14}-glutamate (20 mM)	0	2131
	1	12
	3	27
	4	43

*Samples were counted for 30 minutes and corrected for background, as was self adsorption.

REVERSAL OF GLYCINE INHIBITION BY AMINOACIDS

If glycine was repressing or inhibiting the activity of some enzyme essential to the *am* cells, the nature of this enzyme should be revealed by establishing which amino acid on addition to the growth medium would alleviate the "glycine effect". Of the amino acids tested for the reversal of glycine inhibition, it was found that L-alanine, L-leucine, L-valine, L-glutamine and asparagine reversed inhibition to various degrees (Table 15).

COMPETITION FOR SITE OF ENTRY BETWEEN L-GLYCINE AND OTHER AMINO ACIDS

Among other alternatives, it seemed possible that the amino acids which relieved the "glycine effect", did so by competing for the site of entry to the exclusion of glycine from the cells. Figure 50 shows that L-alanine, asparagine and L-glutamine did not reduce glycine uptake appreciably. Competition of amino acids for entry into *Neurospora* is not unusual. Using labelled arginine and lysine, Bauerle and Garner (1964) demonstrated that arginine, lysine and canavanine are competitive for assimilation into the mycelia of *Neurospora crassa*.

When various concentrations of glycine were tested with a range of alanine concentrations (Figure 51) it was found that at all concentrations of the inhibitor, growth of *am*5 was proportional, to a point, to the amount of alanine supplied exogenously.

TABLE 15. Reversal of glycine inhibition by amino acids in the am⁻ mutant of Neurospora crassa.

Supplement*	Growth (mg.dry weight) Inhibitor: L-glycine (.02M)	Growth (mg.dry weight) Inhibitor: L-serine (.02M)
Control - no inhibitor added	95	98
- no supplement	-	-
Supplements:		
L-alanine	234	239
L-valine	212	220
L-asparagine	167	170
L-leucine	165	173
L-glutamine	120	132
L-ornithine	74	72
L-arginine	26	15
L-proline	21	24
L-glutamate	20	23
L-threonine	-	-
L-glutaric acid	-	-
L-aspartic acid	-	-
L-cysteine	-	-
glycerine (.02M)	-	-
pyruvate (.08M)	-	-

*Strain 525 (am⁻) was cultured in Vogel's minimal medium N at 28°C for 36 hours. All amino acid supplements were present at a concentration of 0.02M. Dry weights reported are average of duplicate experiments.

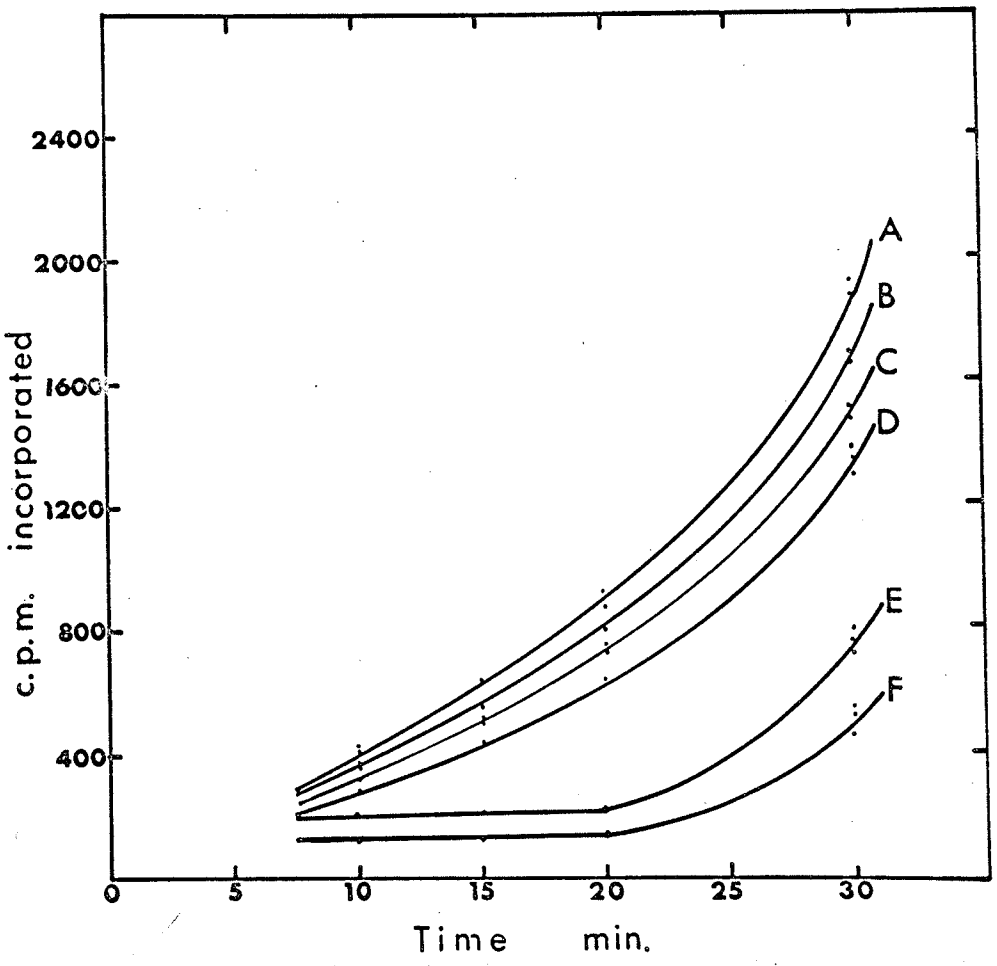


Figure 50. Competition for site of entry between 1-C^{14}-L-glycine and other amino acids. Incorporation of 20 mM of 1-C^{14} glycine (sample contained 4000 C.P.M.) was plotted against time in the presence of 20 mM of various amino acids. Curve (A) no added amino acid; (B) L-alanine; (C) L-asparagine; (D) L-glutamine; (E) L-valine; (F) L-leucine.

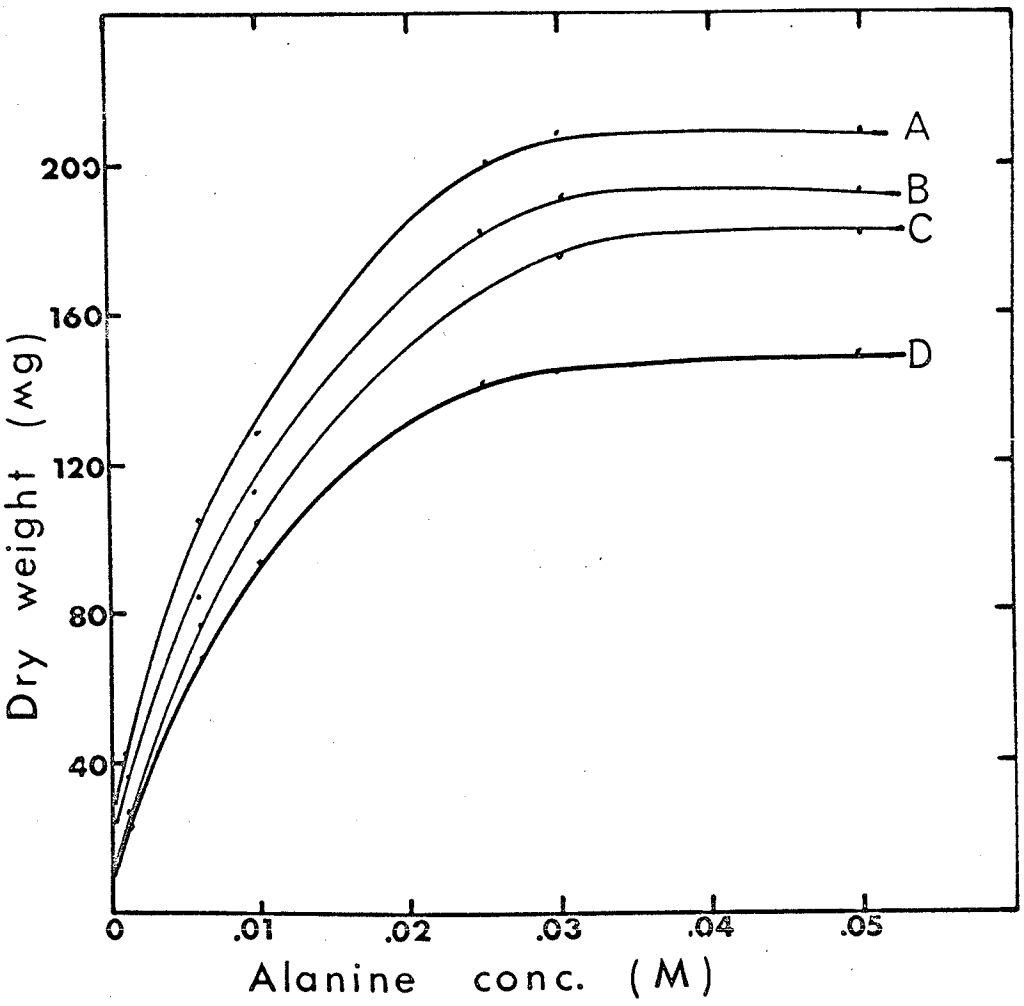


Figure 51. Effect of increasing L-alanine concentrations on growth (dry weight) of am in the presence of varying L-glycine concentrations. Glycine concentrations: (A) 5 mM; (B) 10 mM; (C) 20 mM; (D) 30 mM.

TABLE 17. Effect of L-glycine on glutamate-alanine transaminase of wild-type Neurospora.

Growth Medium	Glutamate-alanine Transaminase Specific Activity*
Minus L-glycine	3.2×10^{-6}
	4.0×10^{-6}
	4.2×10^{-6}
L-glycine (0.02M)	4.0×10^{-6}
	3.8×10^{-6}
	4.4×10^{-6}

*Specific activity of glutamate-alanine transaminase is defined as; moles pyruvate formed per hour per mg. protein. See text for assay procedure.

EFFECT OF GLYCINE ON GLUTAMATE-ALANINE TRANSAMINASE

The relief of inhibition of *am* mutants in the presence of glycine by alanine, asparagine, glutamine, leucine and valine is perhaps explainable by assuming that these substances give rise to glutamate through transamination. To study the effect of glycine on glutamate-alanine transaminase, wild-type conidia were grown in minimal medium N supplied with 0.02 M L-glycine for 24 hours and the enzyme extracted in the usual manner (see Methods). The reaction mixture contained; 50 mM L-alanine, 20 mM α-ketoglutarate, 0.1 ml. enzyme preparation and enough 0.1 M Tris-HCl buffer, pH 9.0,

containing 1 x 10^{-3}M pyridoxal phosphate, to make a final volume of 1.0 ml. The reaction was linear for 20 minutes (Figure 52). After incubation at 28°C for 15 minutes, the reaction was stopped by the addition of 1.0 ml. of 20% (W/V) TCA. The precipitated protein was removed by centrifugation at 27,000 x g for 10 minutes and the resulting supernatant neutralized by the addition of 0.5 ml. of 2N sodium hydroxide. Pyruvate was estimated by the oxidation of NADH at 340 mμ in a reaction mixture containing neutralized samples, lactic dehydrogenase and NADH (Molar extinction coefficient of NADH equal to 6.72 x 10^6). The controls were lacking in L-alanine, α-ketoglutarate and boiled enzyme, respectively.

The results presented in Table 17 show that the presence of glycine in the growth medium had no effect on the glutamate-alanine transaminase. Addition of glycine to the assay mixture also showed no effect on the transaminase activity.

POSSIBILITY OF INVOLVEMENT OF A REGULATORY GENE IN THE SYNTHESIS OF GLUTAMATE DEHYDROGENASES

HETEROKARYON EXPERIMENTS WITH THE DOUBLE MUTANT am^1i^- (1499-12)

Fincham (1950) first reported a spontaneously occurring mutant called i^- which when recombined in the same genome as *am* (double mutant, am^1i^-) produced a non-leaky *am* phenotype.

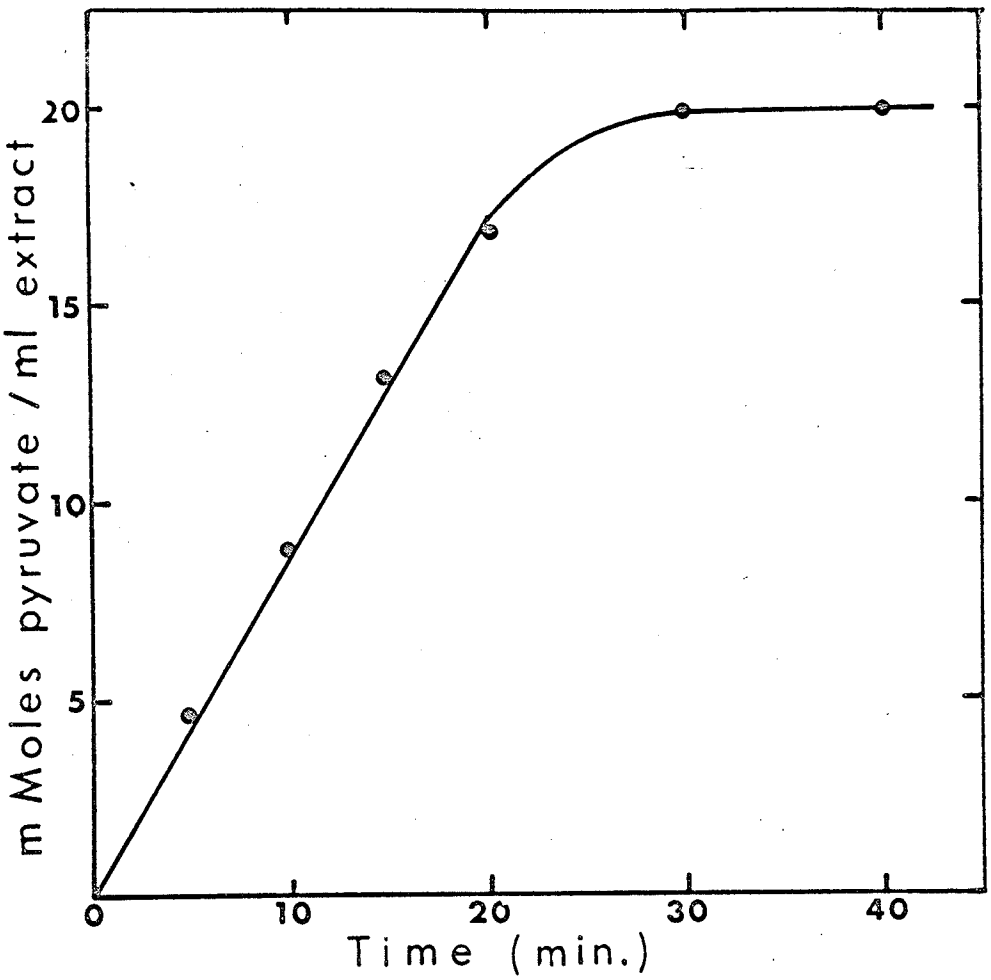


Figure 52. Linearity of glutamate-alanine transaminase with time.

That is, such double mutants failed to grow on minimal medium, although single am mutants are capable of slow growth on such a medium. This is clearly seen in Figure 53. Single mutant, i^-, behaves phenotypically as a wild-type.

In order to determine whether the i locus behaved as a repressor gene, forced heterokaryons of i^+/i^- type were produced (Table 18). Confirmation of the resulting strain as a true heterokaryon was achieved by selecting 100 single conidia on a complete medium (Vogel's medium supplemented with glutamate, uridine and tryptophane). Out of these, 73 colonies could grow on minimal medium plus glutamate which shows that these were heterokaryons. Fourteen of the remaining colonies displayed $am^-i^-td_2^-$ phenotypes and the other 13 colonies were $am^-i^+pyr^-$. Therefore the strain produced by mixing $am^-i^+pyr^-$ with $am^-i^-td_2^-$ was indeed a true heterokaryon.

When the forced heterokaryons of i^+/i^- were produced and tested, the resulting combination behaved as am (Table 18). Thus, like a proper repressor gene, the effect of i^+ gene is in the 'trans' position and is dominant. These experiments suggest that the i^- strain produces an altered repressor of low affinity for ammonia, and under such conditions ammonia is not able to induce the NAD-specific glutamate dehydrogenase by release of repression.

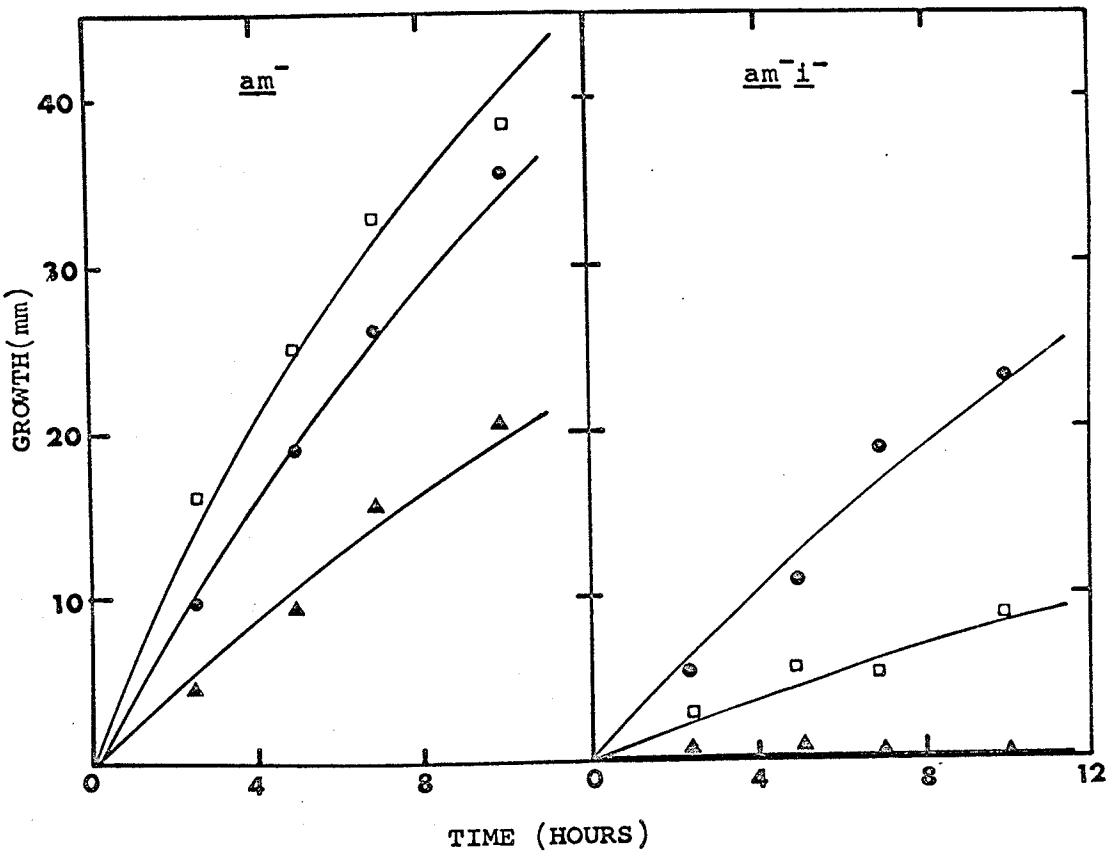


Figure 53. Comparison of am^- and am^-i^- strains growth on various media. Curve (▲-▲) minimal medium; (□-□) minimal medium plus L-glutamate (20 mM); (●-●) minimal medium-nitrogen source, supplemented with L-glutamate (20 mM).

TABLE 18. Phenotype of homo- and heterokaryons carrying genes i^- or i^+ or i^-/i^+.

Genotype	Nuclear Type	Growth in Medium (mg.) after 48 Hours								
		V+U*	V + G+U	V-N +G+U	V+Gl	V+U +Gl	V+T	V-N+G +T	V+G +T	V+T +Gl
$am^8, i^+, pyr^{3-}, td^{2+}, a$	homokaryon	100	690	380	-	-	-	-	-	-
$am^9, i^+, pyr^{3-}, td^{2+}$ a	homokaryon	75	502	212	-	-	-	-	-	-
$am^1, i^-, pyr^{3+}, td^{2-}$ a	homokaryon	-	-	-	-	-	trace	160	50	-
$\left(\frac{am^8, i^+ pyr^{3-} td^{2+} a}{am^1, i^- pyr^{3+} td^{2-} a}\right)$	heterokaryon	50	470	220	-	-				
$\left(\frac{am^9 i^+ pyr^{3-} td^{2+} a}{am^1 i^- pyr^{3+} td^2 a}\right)$	heterokaryon	40	470	240	-	-				

* Symbols:

V = Vogel's medium N (with NH_3)
V-N = Vogel's medium N (without NH_3)
U = uridine (.005%)
G = glutamate (0.1%)
Gl = glycine (0.05%)
T = tryptophan (.001%)

CHARACTERIZATION OF A POSSIBLE NAD-GDH STRUCTURAL GENE MUTANT

From the irradiated $\underline{am^{6}inos}^{-}$ conidia, a strain, denoted as d^{-}, was isolated, which grew slowly in the presence of L-glutamate, (0.1%), but not in its absence. The nutritional requirements of d^{-} was established by placing a drop of conidial suspension in the centre of a petri plate containing minimal medium supplemented with inositol (.05%) and various amino acids (0.1%). The results presented in Table 19 demonstrate that the nutritional requirements of d^{-} was fulfilled by L-alanine, L-glutamate, L-aspartate, L-glutamine and L-ornithine. No growth occurred in medium supplemented with cytidine, adenine, guanosine, thymine, or uridine. The nutritional requirements of d^{-} were not satisfied by a mixture of cofactors which included riboflavin, thiamine, niacin, pyridoxal, biotin, vitamin B_{12}, calcium pantothanate and p-aminobenzoic acid.

The d^{-}-mutant differs from the $\underline{am^{-}i^{-}}$ double mutant in its ability to grow equally well in the presence or absence of ammonia when the medium was supplemented with L-glutamate.

The mutant strain (d^{-}) was assayed for the presence of NAD-specific glutamate dehydrogenase. Conidia obtained from d^{-} grown on corn meal agar supplemented with inositol (.05%) and L-glutamate (.05%) were harvested and washed twice with sterile water. The conidia were grown under

TABLE 19. Nutritional requirements of am⁻inos⁻d⁻ mutant of Neurospora grown in minimal medium containing inositol (.05%).

Supplements*	Growth after 24 Hours (mM) at 28°C
None	-
L-alanine	55
L-glutamate	22
L-aspartate	23
L-glutamine	33
L-ornithine	43
DL-histidine	-
DL-aminobutyrate	7
L-proline	-
L-serine	-
L-glycine	-
L-threonine	-
isoleucine	2
L-leucine	5
methionine	-
tyrosine	-
L-valine	18
L-arginine	5
phenylalanine	2
tryptophan	-
citrulline	4 (after 36 hrs.)

* All supplements were at a concentration of 0.1%.

urea-induced and non-induced conditions at 28°C for 36 hours, and the NAD-GDH activity was measured. Results of these experiments, summarized in table 20, reveal that the d^- mutant possesses NAD-specific glutamate dehydrogenase. However, its level is considerably lower than that in the parent strain.

These results suggest that the d^- mutant may possess an altered NAD-GDH enzyme. However, experimentation revealed that its Michaelis constants, pH optimum and heat inactivation properties were similar to the wild-type enzyme. Therefore, not until a mutant strain is isolated which completely lacks NAD-specific glutamate dehydrogenase activity, can the d^- mutation be considered a structural gene mutation.

MAPPING OF THE d^- MUTANT

To determine if the d locus was linked to am^-inos^-, a cross of $am^-inos^-d^-$(A) X am^+ $inos^+d^+$ (a) was made on corn meal crossing medium. Ascospores were isolated and tested on various media to check for phenotypic differences. Table 21 lists the possible recombinants of such a cross and their expected phenotypic expression. From this table it is evident that the selection proceedure is inadequate for differentiating between all 8 possible types. Certain recombinants, which possess the am^+ allele, behave as wild-types. For example $am^+inos^+d^-$ and $am^+inos^+d^+$ express identical phenotypes. Such is also the case for $am^+inos^-d^-$

TABLE 20. Presence of NAD-glutamate dehydrogenase in am⁻inos⁻d⁻ mutant of Neurospora.

Growth Conditions	NAD-GDH Specific Activity	
	Parent am⁻inos⁻	Mutant am⁻inos⁻d⁻
VI*	1.08	-
VIG	6.83	0.71
VIGU	12.91	2.28

* V = Vogel's minimal medium N
I = inositol (.05%)
G = L-glutamate (.05%)
U = urea (.06 M)

TABLE 21. Possible phenotypes from $am^{+}inos^{+}d^{+}$ x $am^{-}inos^{-}d^{-}$ cross of Neurospora.

Genotype	V*	VI	VG	VIGLI	VIG
Parental					
$am^{-}inos^{-}d^{-}$	-	-	-	-	+
$am^{+}inos^{+}d^{+}$	+	+	+	+	+
Recombinants					
$am^{-}inos^{+}d^{-}$	-	-	+	-	+
$am^{-}inos^{+}d^{+}$	+	+	+	-	+
$am^{+}inos^{-}d+$	-	+	-	+	+
$am^{+}inos^{-}d^{-}$	-	+	-	+	+
$am^{-}inos^{-}d+$	-	+	-	-	+
$am^{+}inos^{+}d^{-}$	+	+	+	+	+

*V = Vogel's minimal medium
I = inositol (.05%)
G = L-glutamate (.07%)
GLY = L-glycine (.02M)

and $am^{+}inos^{-}d^{+}$. For this reason a precise genetic location of the d^{-} mutant could not be achieved.

With the knowledge that the am and inositol loci are closely linked on chromosome V (12 recombination units apart, Strickland, et al., (1959), it is possible to determine whether the d locus is or is not linked to the am locus. Assuming that the d locus is not linked to the am inos region, the theoretically expected numbers of progeny of the classes $am^{+}inos^{+}d^{+}$, $am^{+}inos^{+}d^{-}$, $am^{-}inos^{-}d^{+}$ and $am^{-}inos^{-}d^{-}$ would be 88% of the total population. The remaining 12% would be recombinants; $am^{+}inos^{-}d^{+}$, $am^{+}inos^{-}d^{-}$, $am^{-}inos^{+}d^{+}$ and $am^{-}inos^{+}d^{-}$ in which crossing-over has occurred between the am and inos loci.

Results of the $am^{-}inos^{-}d^{-}$ x $am^{+}inos^{+}d^{+}$ cross (Table 22) showed that 87% of the progeny were of the parental and single recombinant classes with no crossing-over between the am and inos loci. Eleven percent of the progeny tested were of the class which did show crossing-over between these loci.

If the d locus was linked to the am inos loci, the ratios would change proportional to the proximity of its linkage and the two recombinants classes would be greater than 88% and less than 12% respectively.

TABLE 22. Results of $am^{+}inos^{+}d^{+}$ x $am^{-}inos^{-}d^{-}$ cross of Neurospora.

Genotype	Number	Frequency percent
$am^{+}inos^{+}d^{+}$ $am^{+}inos^{+}d^{-}$	81	42
$am^{-}inos^{-}d^{-}$	58	29.7
$am^{-}inos^{+}d^{-}$	6	3.7
$am^{-}inos^{+}d^{+}$	6	3.7
$am^{-}inos^{-}d^{+}$	33	17.1
$am^{+}inos^{-}d^{+}$ $am^{+}inos^{-}d^{-}$	11	5.6
Total	195	

IV. DISCUSSION

It has been proposed and widely accepted that in situations where two enzymes catalyzing identical reactions are present in an organism, they may have two different physiological functions to fulfill. According to Umbarger (1957, 1964), if a reaction provides metabolites for both catabolic and anabolic pathways, possession of two enzymes at this juncture is a physiological necessity in order to avoid competition between the two pathways. In such a case, one of the enzymes serves a biosynthetic function and another a degradative function. Support is lent to this suggestion by the finding that in diverse organisms two enzymes catalyzing the same reaction are generally found at a point in the biochemical pathway which furnishes the same intermediates for synthesis into different end-products by different enzymatic routes. In accord with this view point are the demonstrations of two threonine deaminases in Escherichia coli (Umbarger, et al., 1957), and two acetolactate synthetases in Aerobacter aerogenes (Halpern, 1959). However, in recent years multiple enzyme forms of purely biosynthetic enzymes have been uncovered. Such, for example, is the situation with regard to two (or, possibly three) aspartokinases found in E. coli and Saccharomyces cervisae (Stadtman et al., 1961; Stadtman, 1963).

IV. DISCUSSION

In the case of multiple forms of dehydrogenases distinguished on the basis of their coenzyme requirements, such as the NADP- and NAD-specific isocitrate dehydrogenases of yeast, mammalian tissues etc., another physiological basis of their presence has been suggested. According to Holzer and Schneider (1957), NADP- and NAD-linked glutamate dehydrogenases of yeast could act as a coenzyme transhydrogenase system in the following manner:

(1) L-glutamate + $NAD^+ \rightleftharpoons$ α-ketoglutarate + NH_4^+ + NADH + H

(2) α-ketoglutarate + NH_4^+ + NADP + $H^+ \rightleftharpoons$ L-glutamate + $NADP^+$

Sum of (1) and (2) = (3).

(3) NAD^+ + NADPH $\rightleftharpoons$ NADH + $NADP^+$

However it has been shown that in Fusarium (Sanwal 1961a) and Neurospora crassa (Sanwal, 1961), the levels of NADP-specific glutamate dehydrogenase are high during early growth stages when the levels of NAD-specific glutamate dehydrogenase are extremely low, making it unlikely for them to act as a coupled transhydrogenase system. Furthermore, mutational loss of NADP-linked glutamate dehydrogenase leads to a partial L-glutamate requirement in Neurospora (as in am mutants) inspite of the presence of

NAD-specific enzyme, suggesting that the NADP-specific enzyme has a biosynthetic function. In view of these findings, it seems most unlikely that the glutamate dehydrogenases of Neurospora function as a transhydrogenase system. In keeping with Umbarger's suggestion (1957), Sanwal and Lata (1961) indicated that NADP-GDH has a biosynthetic function whereas the NAD-GDH serves a catabolic role.

However, the observation (presented in Section I) that only the NAD-specific glutamate dehydrogenase is inhibited by various purine nucleotides such as GTP, GDP, GMP, and IMP, suggests a significant biosynthetic role for this enzyme also. This role may be the control of the purine nucleotide 'pools'. The NAD-linked enzyme produces glutamic acid, which may serve as the precursor of glutamine to the exclusion of glutamate produced by the NADP-specific enzyme. NAD-specific glutamate dehydrogenase would thus be the first enzyme in a sequence of reactions described by Buchanan and Hartman (1959), which result in the synthesis of the end products of purine nucleotides. On this assumption, inhibition of the activity of NAD-specific glutamate dehydrogenase by purine nucleotides would be an end-product control mechanism. The specificity of purine nucleotides, coupled to the very low concentrations required to inhibit to enzyme activity are evidences in favour of the hypothesis. The concentration necessary

to inhibit NAD-specific glutamate dehydrogenase *in vitro* is well within the expected physiological concentration. This concept of enzyme regulation, not by individual metabolite but 'pools' of related metabolites, is not without precedent. For example, Gerhart and Pardee (1962) reported a system in *Escherichia coli* in which aspartate transcarbamylase is inhibited by the pyrimidine nucleotide 'pool'.

On the basis of our experiments, a hypothesis for the simultaneous presence of two glutamate dehydrogenases can be advanced. It can be suggested that in the cell 'protein specific' glutamate and 'purine nucleotide specific' glutamate (produced by NADP- and NAD-specific glutamate dehydrogenases, respectively) are distinguished. Exclusive compartmentation perhaps does not exist, rather, glutamate produced by one enzyme is *predominantly* used for one purpose. Were this not so, the *am* mutants, where glutamate can only be produced by the NAD-specific enzyme, would not be able to grow without a supplement of purines. A situation analogous to that discussed here has also been discovered by Davis (1963) in *Neurospora* in the partition of carbamyl phosphate for uridine and arginine synthesis. Genetic experiments have shown that two carbamyl phosphate synthesizing enzymes must exist, one specific for arginine and another for uridine pathway (Davis, 1963).

INDUCTION

In order to explain the induction of NAD-specific glutamate dehydrogenase (which simultaneously and invariably results in the corresponding repression of NADP-dependent GDH) in the presence of urea (Sanwal and Lata 1962a), experiments were designed to establish whether urea was the actual inducer. Mutant studies revealed that urea could only act as inducer when the cells possessed the enzyme urease which is capable of hydrolyzing this compound into ammonia and carbon dioxide. The possibility that carbon dioxide induced the NAD-specific enzyme was ruled out by studies performed with a mutant unable to fix carbon dioxide (suc^-).

Barratt (1963) reported that low concentrations of ammonia derepress NADP-specific GDH in nitrogen-starved mycelia and that high concentrations tended to repress the enzyme. The results reported in this work confirmed these findings. However, the data obtained here (Figure 42) indicated that as the exogenous ammonia concentration was increased, a corresponding increase in the endogenous glutamate level also occurred. An ambiguity is thus introduced in the interpretation of these results. For reasons given later, it seems that both ammonia and glutamate can repress the NADP-specific enzyme. It has also

pointed out by Barratt (1963) that glutamate (supplied exogenously) was not as effective a repressor of NADP-dependent GDH as ammonia. From this work could be concluded that ammonia rather than glutamate repressed NADP-specific glutamate dehydrogenase. This conclusion may not be valid in view of the results presented in Figure 42 and Table 14.

Table 14 indicates that the rate of 1-C^{14}-glutamate uptake by the cells was low in comparison to the monocarboxylic amino acid, glycine. Low potency of enzyme induction by glutamate may, therefore, be related to its low permeability in the cells. This experimental evidence suggests the possibility that *in vivo* L-glutamate as well as ammonia are able to induce NAD-specific glutamate dehydrogenase and repress the NADP-linked enzyme.

The effect of actinomycin D on the induction of NAD-specific GDH (Figure 43) and the evidence that serological cross-reacting material related to NADP-dependent GDH in *am* mutants is regulated in the same manner as the active enzyme (Sanwal and Lata, 1962b) indicates that regulation of these enzymes is independent of cytoplasmic events, and must occur at the primary template level.

It has already been mentioned elsewhere that the two glutamate dehydrogenases show a pattern of regulation which is unusual. Under *all* environmental conditions,

induction of the NAD-specific glutamate dehydrogenase leads invariably to a corresponding repression of the NADP-specific enzyme and vice-versa (Figure 42). This type of regulation can be, for want of a better term, labelled 'inverse concurrent regulation'. This label distinguishes it from other systems of regulation like 'coordinate regulation' (where two or more enzymes of a sequence are simultaneously induced or repressed to the same extent, Ames, (1959). Two other cases of inverse concurrent regulation have come to light in recent years. Jacobs et al., (1964) demonstrated that under the influence of some hormones, the two isocitric dehydrogenases (NAD- and NADP-specific) of mammalian cells show this kind of regulation. A better known case (although never interpreted in this light until very recently: ZuckerKandl, 1964) is the regulation of haemoglobin chains in humans during foetal development. It is well established that haemoglobin F is composed of equal numbers of α and γ polypeptide chains. During development this is gradually replaced by HbA which is constituted of α and β chains (the minor component of HbA, viz. $\alpha - \delta$ haemoglobin is disregarded here). As in Neurospora system, the related peptides, β and γ, show a 'compensatory' increase in one type of haemoglobin (HbA) when the rate of synthesis of the other type falls

(HbF). One explanation that can be given for this inverse concurrent regulation is that perhaps one chain is being converted into another, or in the case of Neurospora, one enzyme is being converted into another.

This possibility can be ruled out for both haemoglobin and glutamate dehydrogenases. In haemoglobin the amino acid composition of β and γ chains is nearly similar, yet the amino acid sequence of the chain is sufficiently different to rule out this presumed interconversion. Also, in Neurospora crassa, results of tracer experiments although admittedly not conclusive, (Table 8) lead to the inference that there is no interconversion of glutamate dehydrogenases. Since the results (Figure 45) indicate that under induced conditions Neurospora contains isozymes of NAD-specific GDH, a comparison of the uninduced and induced enzymes was undertaken to see whether both enzymes were similar. It is clear that, if found different, it would have a bearing on the hypothesis that can be produced for the genetic basis of inverse concurrent regulation.

In a few cases where attempts have been made to find differences between induced and uninduced ('constitutive') enzymes, only conflicting reports are available. Thus, from a physio-chemical comparison of constitutive and inducible β-glucosidase isolated from the yeast hybrid S. dobzhanskii x S. fragilis, Hu et al., (1960) reached the

conclusion that both enzymes were identical. However, in the same organism, using extremely sensitive immunological techniques, Duerksen and Fleming (1963) demonstrated that two distinct β-glucosidases were present. Comparison of kinetic parameters, heat sensitivity, pH optima and antigenic relationships of Neurospora NAD-dependent glutamate dehydrogenases show that the uninduced and induced enzymes are identical.

GLYCINE REPRESSION

Results indicated that glycine inhibits growth of am mutants without any effect on the prototrophic strain. The possibility is thus suggested that glycine either repressed or inhibited an enzyme responsible for synthesis of essential metabolites. Evidence has been presented which establishes the absence of NADP-specific glutamate dehydrogenase as an absolute prerequisite for glycine 'inhibition'. Compounds related to glycine, such as serine, glycine methylester and sarcosine, behaved in the same manner as glycine, i.e., they inhibited the am mutants but not the prototrophic strains. However, inhibition by these compounds was of a magnitude lower than that by glycine, and could perhaps be due to a reduced affinity for the inhibited system.

It is pertinent to point out here that am strains grow

slowly in the minimal medium in the absence of NADP-linked GDH. Under such conditions, their requirement for glutamate is fulfilled by the NAD-specific enzyme (Sanwal and Lata, 1961b). If it is assumed that in the presence of glycine the NAD-GDH is either repressed or inhibited, cessation of growth would occur because of an effective glutamate starvation. Even if glycine repressed the NAD-specific enzyme in prototrophic cells, it would not result in growth retardation because such cells would have a continuous supply of glutamate due to the activity of the NADP-specific GDH. A series of experiments, designed to test the hypothesis that glycine inhibition led to effective glutamate starvation, disclosed that L-glutamate supplied exogenously was relatively ineffective in the alleviation of inhibition.

The interpretation of these experiments is, however, not straight forward. It is possible that glycine interfered with the uptake of glutamate. Such competition for entry of one amino acid in the presence of others has been reported to occur in many microorganisms. Growth of lysineless mutants of Neurospora is inhibited by arginine (Doermann, 1944) and that of arginineless mutants by lysine (Srb, 1953). Similarly, histidineless mutants are inhibited by branched-chain monocarboxylic amino acids, methionine, glycine, tyrosine and many others (Haas, et al.,

1952). The amino acid inhibition of histidine utilization (Mathieson et al., 1955) and tyrosine and phenylalanine utilization (Brockman, et al., 1959) has been explained on the basis of a competition for entry into the cells. Recently Bauerle and Garner (1964) demonstrated that canavanine is competitive for arganine, and lysine, and that all three compounds are assimilated by a common mechanism in Neurospora. It can be seen from the radioactive incorporation experiments (Table 14) that L-glutamate uptake is reduced in the presence of glycine. However, in its absence, the rate of glutamate uptake into the cell was far below that of glycine (Table 14).

Owing to the sluggish permeation of glutamate in Neurospora cells and the inconclusive results obtained with this approach, it was decided to test the effect of other amino acids which can easily give rise to glutamate in vivo. L-alanine, L-glutamine, L-asparagine, DL-valine and L-leucine were found to relieve effectively, the inhibition of am strains by glycine. The relief of inhibition, however, need not necessarily have been due to the quick interconversion of these amino acids to glutamate in vivo. As outlined earlier, these amino acids could have stopped the entry of glycine into the cells. Further, experiments, however, demonstrated that three of the amino acids (alanine, glutamine and asparagine) reversing inhibition

did not significantly hinder glycine uptake into the am cells (Figure 50). The converse was true for valine and leucine (Figure 50). Therefore, valine and leucine acted only by preventing entry of glycine.

Insight into the reversal mechanism of glycine inhibition by alanine was obtained from the experiment in which the concentration of glycine was varied with a range of alanine concentrations (Figure 51). Similar results were obtained with asparagine and glutamine. The results of this experiment suggested that we were not dealing with a situation where glycine and alanine were competing for a site (or a reaction) but rather with 'end-product' substitution, i.e., alanine seemed not to effect the site of glycine inhibition but rather supplied the cell with end-product of the glycine inhibited reaction.

The experiments mentioned above narrowed the range of glycine-inhibited or repressed reactions to those leading to the formation of glutamate, alanine, glutamine and asparagine. It is difficult to visualize glycine as inhibitor of all these reactions simultaneously. However, the field is narrowed further when we connect the inhibition reversal experiments with the observation that glycine only inhibits when a product formed by NADP-specific dehydrogenase is lacking. Since glycine inhibition is not seen in wild-type strains, it is clear that the NADP-

linked glutamate dehydrogenase must be capable of synthesizing compounds *in vivo* which reinstate growth of *am* mutants in the presence of glycine.

Burk and Pateman (1962) demonstrated that the NADP-GDH of *Neurospora* is capable of forming L-alanine as well as glutamate. It seems reasonable to assume therefore, that glycine represses or inhibits either the NAD-specific glutamate dehydrogenase or some alanine forming enzyme such as glutamate-alanine transaminase. Highly purified NAD- and NADP-specific glutamate dehydrogenases, however, were not inhibited by glycine *in vitro* up to a concentration of 0.5 M, and preliminary experiments on glutamate-alanine transaminase revealed that glycine did not inhibit this enzyme also in cell-free extracts.

This leaves us with the only likely possibility that glycine represses the formation of NAD-specific glutamate dehydrogenase at the genetic level. This perhaps could be brought about by its binding with a weak affinity to a glutamate specific aporepressor (see discussion of genetic model later) without deactivating it. It is clear that if enough glutamate is present endogenously (as in prototrophic strains, where the presence of the NADP-specific glutamate dehydrogenase ensures a plentiful supply, or when alanine, asparagine etc. are present in the medium for growth of *am* strains), glycine will be unable to bind

to the aporepressor in competition with glutamate, which, because it can induce the NAD-specific enzyme so well, must have a high affinity for the hypothetical aporepressor. The relief of inhibition of *am* mutants in the presence of glycine by alanine, glutamine and asparagine is perhaps explainable by assuming that these substances give rise to glutamate *in vivo* through non-specific channels, possibly transaminases (Fincham and Boulter, 1956).

PROPOSED GENETIC MODEL

In an attempt to explain the 'inverse concurrent regulation' (Sanwal and Lata, 1962a) of glutamate dehydrogenases, a model is proposed that appears to be compatible with the biochemical and genetic evidence. This model is based on that proposed by Jacob and Monod (1961a, 1961b).

The genetic model includes: the following features and assumptions:

(a) Operator genes which presumably are regions of deoxyribonucleic acid (DNA) at which messenger - ribonucleic acid (m-RNA) synthesis is initiated. Jacob and Monod (1964) have recently modified their concept and terminology of the operator region. They now consider this as a m-RNA "promoter region".

(b) Regulator genes that are responsible for the synthesis of aporepressor molecules (possibly a protein

(Garen, 1963, 1964)) which are capable of interacting at the promoter region and thereby inhibiting expression of the structural gene. The aporepressor molecule can undergo conformational changes in the presence of low molecular weight metabolites which function as inducer or repressors. Binding of an inducer to the aporepressor "deactivates" it so that it cannot interact with the 'promoter' region. The opposite is true for repressor molecules which, by binding to the aporepressor, activate it so that transcription of repressor genes is not possible.

The presence of the structural gene for NADP-specific GDH (*am*$^-$) has been previously demonstrated (Fincham, 1954). Twelve different *am*$^-$ mutants arising from independent mutational events have been mapped in a narrow region on the Vth linkage group in the middle of *spray* and *inos* loci (Figure 54).

Due to difficulties in screening for structural gene mutants of NAD-specific GDH (*d*$^-$), the precise location of this gene could not be established. The results (Table 22) do however, suggest that *d*$^-$ is not closely linked to *am inos* region. If it is on the same chromosome, its location would be distal to the *am* and *inos* loci. The behaviour of *i* gene, which is linked to *am* by eight recombination units (Figure 54), is particularly relevant to a discussion of the repressor locus (RG_1). Evidence presented

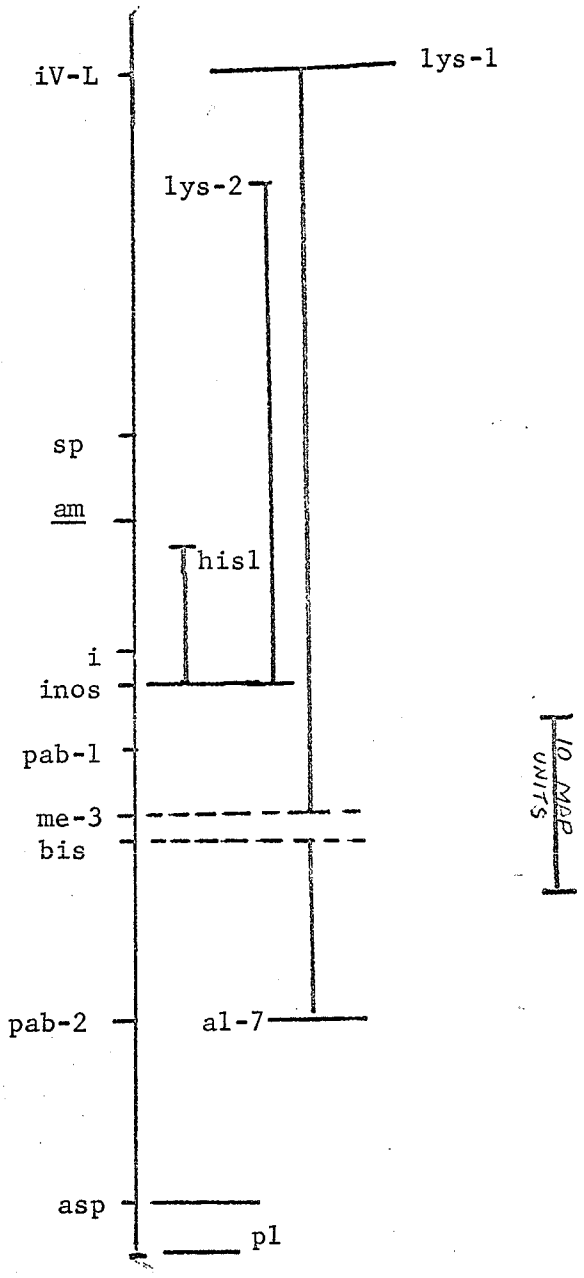


Figure 54. Genetic map of the Vth chromosome of Neurospora crassa. sp = spray gene; am = structural gene for NADP-GDH; i = regulator gene; inos - inositol gene.

here with forced heterokaryons of i^+/i^- type indicates a behaviour similar to that obtained with heterogenates (i^+/i^-) of β-galactosidase repressor (Jacob and Monod, 1961b) locus of *E. coli*, i.e., the effect of i^+ gene is in the *trans* position. Nutritional experiments in conjunction with the heterokaryon studies suggest that the *i*$^-$ strain produces an altered repressor of low affinity for the inducers ammonia and glutamate. Under such conditions ammonia is unable to induce the NAD-specific GDH enzyme by a release of repression.

The proposed model accounts for concurrent regulation in the following manner. The regulator gene (*i*) produces an aporepressor which interacts with the 'promoter' (Pd). Both the *d* and RG_2 linked genes of the NAD-dependent GDH operon are rendered inactive because m-RNA transcription is blocked at the promoter gene (Pd) by the repressor. Since the RG_2 gene (responsible for production of an aporepressor specific for the NADP-specific GDH operon) is not expressed, the NADP-specific GDH is synthesized at a maximum rate. No NAD-specific GDH is, however, synthesized. This situation corresponds to *Neurospora* in the conidial or germination stage of growth. NADP-dependent GDH is at its optimal level whereas the NAD-specific enzyme is not detected either spectrophotometrically or serologically. As the conidia develope the inducers (I) of NAD-specific

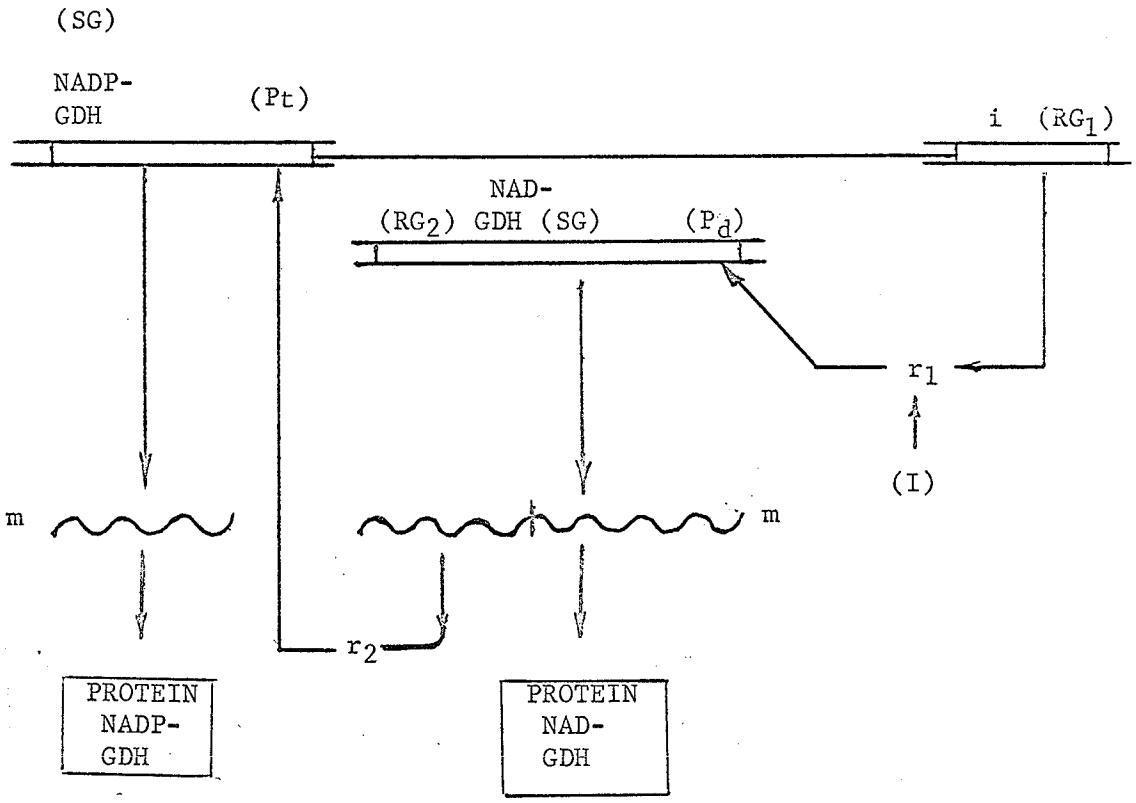


Figure 55. Genetic model proposed for inverse concurrent regulation of glutamate dehydrogenases of Neurospora. (SG) = structural gene; (RG) = regulator gene; (P) = promoter gene; r = repressor; (I) = inducer; (M) = messenger RNA.

GDH, ammonia (exhibiting low affinity for aporepressor) or glutamate (exhibiting high affinity for aporepressors), attain a certain level which on combining with the apo-repressor r_1, produced by the i gene, prevents the repressor from interacting with the promotor (Pd) gene. Hence, the whole NAD-specific GDH operon is expressed, both NAD-specific GDH and RG_2 gene-specific aporepressor are produced. The latter (r_2 in Figure 55), by combining with the pro-motor region (Pt) prevents the synthetic activity of the whole NADP-GDH operon. Thus, the rate of NADP-specific GDH synthesis decreases as the rate of synthesis of the NAD-specific enzyme increases.

The model also offers a possible explanation for the behaviour of $\underline{am^-i^-}$ strains. Mutation of the $\underline{i}$ gene to $\underline{i}^-$ genotype is assumed to produce an altered apo-repressor which has a very low or no affinity for the weak inducer, ammonia. Since the ammonia is no longer able to combine with the altered aporepressor, the NAD-GDH operon is not expressed. Therefore, mutants lacking the NADP-specific glutamate dehydrogenase enzyme but possessing an altered i locus ($\underline{am^-i^-}$) are unable to grow in the presence of ammonia because of glutamate starvation.

If certain assumptions (based on experimental data given in results) are made, this model also explains glycine

inhibition of *am* mutants. It will be recalled that methylamine and glycine (along with other analogues) both inhibit the growth of *am* mutants. Since methylamine, and to a certain extent glycine, are structurally related to ammonia it does not seem unreasonable to assume that all these compounds can bind to the aporepressor, r_1 (Figure 55). However, while binding of ammonia leads to a 'deactivation' of r_1 and a consequent transcription of NAD-GDH operon, binding of methylamine, glycine and other analogues does not lead to a 'deactivation', but has the effect that less aporepressor is available for binding with ammonia. Because glutamate has, perhaps, a higher affinity for r_1, it can effectively displace glycine and other analogues from the binding site and 'deactivate' the aporepressor. Thus glycine has no inhibiting effect in the presence of an endogenous source of glutamate (i.e., in prototrophs due to the activity of NADP-specific GDH) or an exogenous source of amino acids which can generate glutamate easily *in vivo*.

The genetic model discussed here is not expected to represent a conclusive solution to the problem of glutamate dehydrogenase regulation in *Neurospora*, rather, it may be considered only as a working hypothesis. It has the virtue that it predicts certain types of mutations and their phenotypes so that a diligent search for such mutants is

possible. For example, mutation of the RG_2 gene would lead to a constitutive non-repressible synthesis of the NADP-glutamate dehydrogenase and in such mutants, furthermore, no inverse concurrent regulation would occur. Their growth habit would, however, be identical to the wild-type strains.

Another mutation predicted by the model is the complete loss of the i gene. This would result in the constitutive, uninducible synthesis of NAD-specific glutamate dehydrogenase and a complete lack or very low levels of the NADP-linked enzyme. Since the aporepressor would not be synthesized, glycine would not inhibit its growth. Therefore, such mutants would again be phenotypically identical to wild-type strains.

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