THE UNIVERSITY OF MANITOBA

MOLECULAR ANALYSIS OF AN NAD-SPECIFIC GLUTAMATE DEHYDROGENASE AND AN ANTIGENICALLY CROSS-REACTING POLYPEPTIDE.

BY

BAIHUA YANG

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

DEDICATED TO

ZHAOBING

AND

THE MEMORY OF

MY MOTHER

WHO ALWAYS TAUGHT ME AND LOVED ME.

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ABSTRACT

An L-glutamine/L-glutamate inducible NAD-specific glutamate dehydrogenase (NAD-GDH) found in *Achlya klebsiana*, has been purified to electrophoretic homogeneity (with silver stain detection) by combined use of $(NH_4)_2SO_4$ fractionation, A25 DEAE-Sephadex columns, and Cibacron blue 3GA-agarose. At all stages of its purification, the enzyme retained its capacity to be allosterically activated by $NADP^+$ or NADPH. Purified, the enzyme was unstable, but was stabilized by 50% glycerol when stored at -20°C. The native enzyme, seemingly, aggregates and disaggregates. Molecular mass (M_r) of the enzyme was estimated as 470,000 by Sepharose 6B gel filtration and 500,000 by polyacrylamide gradient gel electrophoresis. When denatured by SDS-PAGE, the enzyme migrated as a single subunit of 125,000 M_r. The enzyme consists of 4 identical subunits. $NADP^+$ activation of the enzyme was negated by limited subtilisin treatment. Subtilisin altered the subunit size of the enzyme from 125,000 to 115,000 M_r leaving an enzyme that (a) was unresponsive to $NADP^+$ and (b) was twice as active catalytically as the unmodified enzyme without the activator. Prolonged subtilisin treatment (more than 60 min) degraded the enzyme into smaller peptide fragments.

Polyclonal antibody against the 125,000 M_r enzyme subunit recovered from SDS-PAGE slabs was raised in rabbits and then used to

immunochemically study the induction and repression processes of the enzyme. L-Glutamine was shown to be the most effective inducer, being about twice as effective as L-glutamate. Ammonia did not induce the enzyme. Enzyme synthesis was repressed by D-glucose. The polyclonal antibody interacted with a polypeptide of M_r 74,000 which showed the same kinetics of repression by glucose and *in vivo* decay as the 125,000 M_r enzyme subunit. Under normal conditions of growth and nutrient starvation, the 125,000 M_r subunit level was much higher than the 74,000 M_r polypeptide level. When proteinase inhibitors were used to deter the turnover of proteins in starving cells that were simultaneously fed L-glutamine so as to induce NAD-GDH synthesis, the two polypeptides accumulated in the opposite way with the 74,000 M_r polypeptide level being much more abundant than the 125,000 M_r enzyme subunit. The 74,000 M_r polypeptide was shown to be a component of a protein with an M_r of 220,000. What physiological relationship, if any, the 220,000 M_r protein has with the NAD-GDH is yet to be elucidated.

A cDNA library using mRNA from NAD-GDH induced cells was constructed in λgt11. The cDNA library was screened with purified polyclonal anti-GDH antibody that was free of anti-*E. coli* antibodies. Four positive cDNA clones were isolated. cDNA inserts in these clones were amplified by the PCR method and the longest one (λBY2) was sequenced by Dr. H. B. LéJohn.

An *Achlya* genomic DNA library was constructed in λEMBL3 with an average insert size of 15-20 kb. The library was screened with the cDNA that was sequenced. Nine positive clones were obtained. All of the genomic clones were related in that they bore overlapping sequences. One, λBYG3, was used for enzyme mapping and hybridisation analysis. All 4 cDNA inserts

strongly hybridised to overlapping regions of this genomic DNA. The gene in the genomic DNA fragment recognized by the cDNAs may not, however, be NAD-GDH. There is a strong likelihood that it is the gene for the 74,000 M_r protein.

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ABBREVIATIONS

ADP	---	adenosine 5′-diphosphate
AMP	---	adenosine 5′-monophosphate
ATP	---	adenosine 5′-triphosphate
BIS	---	N, N′-methylene bisacrylamide
bp	---	base pair
BSA	---	bovine serum albumin
cDNA	---	complementary deoxyribonucleic acid
cpm	---	counts per minute
Da	---	Dalton
dATP	---	2′-deoxyadenosine 5′-triphosphate
DEAE	---	diethylaminoethyl
DMF	---	dimethylformamide
DMS	---	dimethyl sulfate
DMSO	---	dimethyl sulphoxide
DNA	---	deoxyribonucleic acid
DNase	---	deoxyribonuclease
dNTP	---	2′-deoxynucleoside 5′-triphosphate
DTT	---	dithiothreitol
2,4-D	---	2,4-dichlorophenoxyacetic acid
EDTA	---	ethylene diamine tetra-acetic acid
ELISA	---	enzyme-linked immunosorbent assay

GDH --- glutamate dehydrogenase

GTC --- guanidine thiocyanate

GTP --- guanosine 5′-triphosphate

h --- hour

HEPES --- N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid

IAA --- indole-3-acetic acid

Ig --- immunoglobulin

IPTG --- isopropyl-1-thio-ß-D-galactoside

kDa --- kilodalton

Km --- Michaelis constant

min --- minute

MOPS --- morpholinepropanesulfonic acid

M_r --- molecular mass

mRNA --- messenger ribonucleic acid

NAD^+ --- nicotinamide adenine dinucleotide

NAD-GDH --- NAD-specific glutamate dehydrogenase

NADH --- reduced nicotinamide adenine dinucleotide

$NADP^+$ --- nicotinamide adenine dinucleotide phosphate

NADPH --- reduced nicotinamide adenine dinucleotide phosphate

NMN --- nicotinamide mononucleotide

Oligo --- oligonucleotide

Oligo(dT) --- oligodeoxythymidylic acid

PEG --- polyethylene glycol

pfu --- plaque forming unit

PMSF --- phenylmethylsulphonyl fluoride

$poly(A)^+$ --- polyadenylated mRNA

RNA --- ribonucleic acid

RNase --- ribonuclease

rpm --- revolutions per minute

SDS --- sodium dodecyl sulfate

TAME --- N-α-tosyl-L-arginyl methylester

TCA --- trichloroacetic acid

TCAc --- tricarboxylic acid cycle

TEMED --- N, N, N′, N′-tetramethylethylene diamine

TEPPD --- 50 mM Tris-acetate, pH 7.3, 1 mM EDTA, 10 mM potassium phosphate, 1 mM DTT, 0.1 mM PMSF

TEPPDG --- 50 mM Tris-acetate, pH 7.3, 1 mM EDTA, 10 mM potassium phosphate, 1 mM DTT, 0.1 mM PMSF, 20% glycerol

TMPO --- 2,2,6,6-tetramethyl-4-oxo-piperidine-1-oxyl

TPCK --- N-α-tosyl-L-phenylalanyl chloromethyl ketone

Tris --- trihydroxymethylaminomethane

Tris-Cl --- Tris hydrochloride

URS --- upstream regulatory sequences

uv --- ultraviolet

X-gal --- 5-bromo-4-chloro-3-indolyl-ß-D-galactoside

INTRODUCTION

Glutamate dehydrogenase (GDH) is a significant enzyme in the metabolism of organisms as the reaction it catalyses links the glycolytic and tricarboxylic acid cycle sequences with nitrogen metabolism. It catalyses either the reductive amination of α-ketoglutarate to yield glutamate or the oxidative deamination of glutamate to produce α-ketoglutarate. Various forms of glutamate dehydrogenase have been found in a number of organisms. Some organisms possess two forms of the enzyme, one using NAD^+ and the other specific for $NADP^+$ (Sanwal and Lata, 1961; DeCastro *et al.*, 1970); while other organisms have just one enzyme that is specific for $NADP^+$ (Halpern and Umbarger, 1960; Hooper *et al.*, 1967) or for NAD^+ (Goldin and Frieden, 1971; LéJohn, 1971). Still other organisms have an enzyme that can use either coenzyme (Snoke, 1956).

It was discovered that the catalytic activities of NAD-specific glutamate dehydrogenases among the fungi are modulated by a wide range of metabolic substances. For example, the enzyme from *Blastocladiella emersonii* is unidirectionally inhibited by Ca^{2+} and Mn^{2+} (LéJohn and Jackson, 1968) while those from the *Oomycota* (now regarded as *Protoctista*

rather than fungi, Margulis *et al.*, 1990) utilize $NADP^+$ as an activator, not as a coenzyme, and NAD^+ as coenzyme (LéJohn and Stevenson, 1970). Another interesting feature of this enzyme is its apparent induction by L-glutamine (Braithwaite, 1987). When vegetatively growing cells of *Achlya klebsiana*, a member of the *Protoctista*, were transferred from a nutrient-sufficient medium to a nutrient-free medium except for L-glutamine which could double as a carbon and nitrogen source, NAD-GDH activity was greatly induced. Nutrient-free medium induces sporulation in the cells, but L-glutamine represses this (LéJohn, 1983). L-Glutamate does not repress sporulation and the *in vivo* NAD-GDH inducing agent is believed to be L-glutamate. Therefore, NAD-GDH induction is not linked to sporulation.

To understand the molecular mechanism of induction and repression of this enzyme in *Achlya klebsiana*, my task was to (a) completely purify the enzyme, (b) raise antibody to it, (c) use the antibody to isolate a cDNA for the enzyme, (d) screen the organism's genomic library with the cDNA as a probe to isolate the NAD-GDH gene and (e) analyse the genomic positive DNA clones. The mechanism by which L-glutamine or L-glutamate induced the enzyme at the gene level can only be elucidated through analysis of the gene structure and its interaction with modulating proteins. It is shown here that the enzyme is rather large with an M_r of 500,000 consisting of four subunits of identical M_r values of 125,000. The capacity of $NADP^+$ to activate the enzyme is lost with limited proteolysis by subtilisin. This proteinase converts the enzyme subunit from 125,000 to 115,000 M_r. As well, the subtilisin-modified enzyme becomes more reactive to its substrate, NAD(H). Polyclonal antibody, which was raised against the NAD-GDH (125,000 M_r) subunit obtained from SDS-PAGE, was able to detect

its own antigen as well as that of another protein of 220,000 M_r native molecular mass which has the 74,000 M_r subunit as a component. This 74,000 M_r polypeptide and the 125,000 M_r subunit have the same kinetics of glucose-mediated repression and decay. The polyclonal antibody that recognized both protein subunits and native proteins was used to screen a cDNA library made in λgt11. Surprisingly, the 4 cDNA clones recovered were from the gene for the 74,000 M_r polypeptide. Using the cDNA clones to screen the organisms's genomic library yielded 9 genomic clones; all of which were products of the same region of the genome. Neither the cDNAs nor the genomic DNAs isolated with the aid of the polyclonal anti-GDH antiserum hybridised to other than a 2.2-kb poly(A)$^+$-rich RNA band and an RNA band of similar size in total cell RNA preparations. An mRNA of this size can only encode information for a protein that is no greater than 75,000 M_r. Thus, none of the 4 cDNAs or the 9 genomic clone DNAs is likely to harbour the gene for *Achlya* NAD-GDH which must have a transcript that is not less than 3.5-kb long. Because neither the cDNAs nor the genomic clone DNAs of *Achlya* could hybridise to NAD-GDH genes from *Neurospora crassa* and *Saccharomyces cerevisiae*, NADP-GDH gene of *Chlorella* and NAD(P)-GDH gene of human, the conclusion is that the gene isolated here is that for the 74,000 M_r protein and not that for NAD-GDH. Characterization of the isolated gene has been pursued with that possibility foremost in mind.

HISTORICAL

Glutamate dehydrogenase (GDH) is generally found in animals, plants, and microorganisms. The enzyme is located in the mitochondria of mammalian cells and is abundant in liver, heart, and kidney (Duve *et al.*, 1962). But it is found in the cytoplasm of microorganisms (Hollenberg *et al.*, 1970) and also in the chloroplast in plants (Stewart *et al.*, 1980). It is a key enzyme in nitrogen metabolism because it directly connects, reversibly, amino acid and carbohydrate metabolism (Marzluf, 1981) (see Fig 1).

1. Molecular Properties of Glutamate Dehydrogenase

In the early days, the structure of glutamate dehydrogenase was extensively studied by various groups (Olsen and Anfinsen, 1952; Strecker, 1953; Frieden, 1958; Kubo *et al.*, 1959). The enzyme used in these studies was bovine glutamate dehydrogenase. They showed that the enzyme is an oligomer consisting of 8 identical monomers. The monomers are in rapid

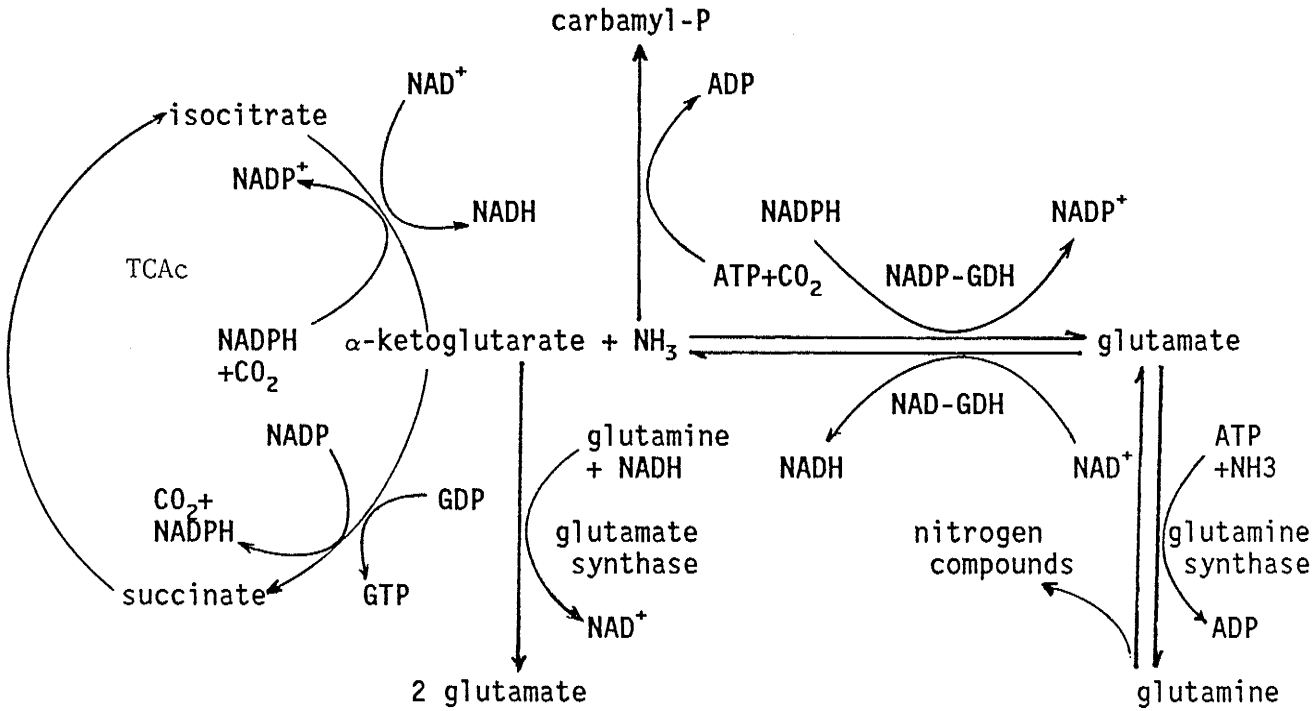
Figure 1. Interconversions of Glutamate and Glutamine Catalysed by Glutamate Dehydrogenase, Glutamate Synthase and Glutamine Synthetase.

NAD-GDH --- NAD-specific glutamate dehydrogenase

NADP-GDH --- NADP-specific glutamate dehydrogenase

TCAc --- Tricarboxylic acid cycle.

The figure was modified from Stadtman (1966).


carbamyl-P
ADP
NAD+
isocitrate
NADP+
NADH
TCAc
NADPH
NADP+
ATP+CO2
NADP-GDH
NADPH
+CO2
α-ketoglutarate + NH3
glutamate
NAD-GDH
NADP
glutamine
+ NADH
NADH
NAD+
ATP
+NH3
CO2+
NADPH
GDP
glutamate
synthase
nitrogen
compounds
glutamine
synthase
succinate
GTP
NAD+
ADP
2 glutamate
glutamine

equilibrium with the oligomer. The smallest enzymatically active monomeric form consists of six identical subunit polypeptide chains, each with a molecular mass of approximately 56,000 (Stadtman, 1966; Sund and Burchard, 1968; Krause *et al.*, 1974).

The ability of glutamate dehydrogenases to undergo reversible aggregation and disaggregation was first recognized by Olsen and Anfinsen (1952). Their finding was subsequently confirmed by other workers through light scattering experiments (Eisenberg and Tomkins, 1968; Markau *et al.*, 1971), x-ray small angle measurements (Sund *et al.*, 1969), and sedimentation coefficient determination (Frieden, 1959 a; Cassman and Schachman, 1971; Cohen and Mire, 1971). Yamamoto *et al.* (1987 b) reported that the dual pyridine nucleotide-specific glutamate dehydrogenase had an M_r of approximately 300,000 and that oligomeric forms with M_rs of 590,000 and 920,000 were observed on polyacrylamide gel disc electrophoresis. The M_r of the subunit was 48,000 (Yamamoto *et al.*, 1987 b).

The aggregation and disaggregation of glutamate dehydrogenase is affected by many factors. High concentration of the enzyme favours aggregation (Frieden, 1958; Kubo *et al.*, 1959; Fisher *et al.*, 1962 a). The optimum pH for enzyme activity (pH 8-9) also favours association of the enzyme (Fisher *et al.*, 1962 b; Fisher *et al.*, 1962 c; Ashby *et al.*, 1974; Neumann *et al.*, 1976; Syed and Engel, 1986; West and Price, 1988). In examining the possibility that ligand binding might preserve the active form of the enzyme from *Blastocladiella emersonii*, it was found that dissociation at pH 9.0 can indeed be prevented by the substrates NAD^+ and NADH, and the activators AMP, ADP, and Ca^{2+} (LéJohn *et al.*, 1969 b). NADH, NADP and GTP prevent bovine GDH from aggregating (Koberstein and Sund,

1973; Barbotin and Breuil, 1978). SDS (Tashiro *et al.*, 1982; Bell and Bell, 1984), urea (Kubo *et al.*, 1959; Karabashian *et al.*, 1988) and guanidine hydrochloride (Inone *et al.*, 1984; Tsou, 1986) are capable of denaturing glutamate dehydrogenase and cause the molecule to dissociate into subunits. Zn^{2+}, not Eu^{3+}, (Bell *et al.*, 1987) results in the formation of very large aggregates of the enzyme. Ca^{2+} (Jung *et al.*, 1973), Mg^{2+} (McCarthy and Tipton, 1984), ammonium sulphate (LéJohn and Stevenson, 1970), ADP (Frieden, 1959 b; Tomkins *et al.*, 1963), steroids (Yielding and Tomkins, 1960), and certain amino acids (Yielding and Tomkins, 1961) also produce effects on the protein aggregation and stability of the enzyme from different organisms. Frieden (1959, a; b) and Tomkins *et al.* (1961; 1963; 1965) suggested that the dissociated form of the enzyme catalyzed the alanine dehydrogenase reaction, while the tetramer form of the enzyme had glutamate dehydrogenase activity. ADP preserved the aggregated form of the enzyme, while GTP and steroids promoted its disaggregation (Tomkins *et al.*, 1961).

Unlike bovine glutamate dehydrogenase, the enzyme isolated from chicken liver shows little tendency to aggregate, while the enzyme from frog liver generally occurs as a dimer (Frieden, 1962; Fahien *et al.*, 1965). In yellow lupin root nodules, GDH has the property of random association of subunits to produce different oligomeric forms of the enzyme (Ratajczak *et al.*, 1989).

Differences between the reported M_r values of bovine glutamate dehydrogenase, vary from 280,000 (Sund and Burchard, 1968) to 400,000 (Colman and Frieden, 1966 b) and this emphasizes the difficulty in determining the M_r value of proteins that aggregate. It is now believed

that the true molecular mass of bovine glutamate dehydrogenase is 332,000 through determination of the amino acid sequence of the monomeric subunit (Moon and Smith, 1973).

Gronostajski *et al.* (1978) estimated that the NADP-specific glutamate dehydrogenase of *Chlorella sorokiniana* had an M_r of between 420,000 and 400,000 by using gradient polyacrylamide gel electrophoresis, but an M_r of 290,000 by sedimentation equilibrium. The latter value was closer to the M_r of 270,000 reported by Talley *et al.*(1972). Differences in M_r estimates of the magnitude observed with the NADP-glutamate dehydrogenases have been suggested for proteins that are "cigar-shaped" (Solomonson *et al.*, 1975). The subunit M_r of bovine GDH was calculated from the polypeptide sequence to be 55,390 which agrees with values obtained by sedimentation-equilibrium (Cassman and Schachman, 1971; Landon *et al.*,1971) and by light scattering measurements (Eisenberg and Tomkins, 1968). Other M_r values of 45,000-50,000 were obtained by gel chromatography in the presence of SDS (Page and Godin, 1969). M_r values of 313,000 and 320,000 have been obtained for the smallest, enzymatically active unit. Thus, the number of the subunits in the enzyme is six. The six subunits were demonstrated to be identical by the fact that alanine is the sole NH_2-terminal amino acid and threonine is the only COOH-terminal amino acid of the enzyme (Jirgensons, 1961; Appella and Tomkins, 1966). Physical and chemical studies on the enzyme completely denatured by high concentrations of guanidine hydrochloride indicated that the subunits are of identical size (Marler and Tanford, 1964; Landon *et al.*, 1971).

Generally speaking, glutamate dehydrogenases from animals, plants, fungi and bacteria have a molecular mass of 300,000 and are composed of

six identical subunits whose M_r values range from 43,000 to 50,000 (Smith, 1975). However, a molecular mass of 230,000 for an active NADP-glutamate dehydrogenase in the soluble fraction of *Trichomonas vaginalis* (Turner and Lushbaugh, 1988), and an M_r of 180,000 in *Euglena gracilis* (Javed and Merrat, 1986), were found. The smallest M_r of 98,000 was reported for NAD-glutamate dehydrogenase isolated from *Phycomyces* spores with a subunit of 54,000 (Van-Laere, 1988). The differences in M_r values of active glutamate dehydrogenases and their subunits in different organisms are listed in Table 1.

The NADP-specific glutamate dehydrogenase from *Neurospora* has 50 amino acid residues less than the bovine enzyme, but chicken liver glutamate dehydrogenase possesses three additional residues in its polypeptide chain (Moon *et al.*, 1973). The subunit size of the NADP-specific enzyme from *Neurospora* appears similar to those of the NADP-specific enzymes from *S. cerevisiae* (Vanard and Fourcade, 1972), *Escherichia coli* and the enzyme of dual coenzyme specificity from *Mycoplasma laidlawii* (Yarrison *et al.*, 1972). The subunit size of bovine glutamate dehydrogenase is larger than that of most other dehydrogenases (with notable exceptions mentioned below), but close to glucose-6-phosphate dehydrogenase of human erythrocytes (Ratazzi, 1968).

The M_r of NAD-specific glutamate dehydrogenases from *Neurospora crassa* (M_r, 464,000, Austen *et al.*, 1977; 1980) and *S. cerevisiae* (M_r, 450,000, Uno *et al.*, 1984) are very different from others that have been reported. The native enzymes possess four identical subunits. Each subunit is almost twice the size of other glutamate dehydrogenase subunits (Veronese *et al.*, 1974).

Table 1. Molecular Mass of GDHs

Organism	Coenzymes	Native enzyme M_r $X10^5$	Subunit M_r $X10^4$	References
Rat liver	NAD(P)	3.3-3.7	4.3-5.3	King & Frieden, 1970
Neurospora crassa	NAD	4.53-5.07	11.1-12.1	Veronese et al., 1974
	NADP	2.8-2.96	4.84	Wootton, 1973
Saccharomyces cerevisiae	NAD	4.5	10.0	Doherty, 1970
Chicken liver	NAD(P)	3.06-3.46	5.56	Anderson & Johnson, 1969
soybean SB3	NAD	2.63±0.12	4.1±0.2	Shargool & Jain, 1989
Bacillus fastidiosus	NADP	2.88	4.8	Op den Camp et al., 1989
Streptomyces fradiae	NADP	2.0	4.9	Vancurova et al., 1989
Hypho-microbium	NADP	3.8±0.1	6.34±0.4	Duchars & Attwood, 1987
Phycomyces spores	NAD	0.98	5.4	Vanlaere, 1988
mealworm fat body	NAD	3.4	5.7	Teller, 1988
sea anemone	NADP	3.25±0.5	4.9	Male & storey, 1983
Synecho-cystis PCC 6803	NADP	2.08	5.2	Florencio et al., 1987
Plasmodium falciparum	NADP	2.94	4.9	Ling et al., 1986
Pseudomonas aeruginosa	NADP	2.8	4.5	Joannou et al. 1988
P. sp AM1	NADP	1.9	5.0	Bellion & Tan, 1984

It has been demonstrated that isozymes of glutamate dehydrogenase appear in beef heart mitochondria (McDaniel *et al.*, 1986). Purified beef heart glutamate dehydrogenase subjected to SDS-PAGE had a protein corresponding to beef liver glutamate dehydrogenase and a smaller molecular mass protein. Agarose gel electrophoresis with activity staining resolved heart glutamate dehydrogenase activity into two fractions (with or without protease inhibitors) while liver GDH activity had only one fraction. One of the heart glutamate dehydrogenase fractions moved with liver glutamate dehydrogenase upon electrophoresis; the other activity band moved faster than beef liver GDH. It was concluded that beef heart glutamate dehydrogenase activity consists of two isozymes; one is the same as beef liver glutamate dehydrogenase, while the other is smaller (McDaniel *et al.*, 1986).

2. Coenzyme Specificity of Glutamate Dehydrogenase

There are three kinds of glutamate dehydrogenases based on their coenzyme specificity; they are NAD-specific glutamate dehydrogenase (NAD-GDH), NADP-specific glutamate dehydrogenase (NADP-GDH) and dual coenzyme specific glutamate dehydrogenase (NAD(P)-GDH). It is generally believed that the enzyme specific for NAD^+ is responsible for oxidation of glutamate to provide ammonia, whereas the one specific for $NADP^+$ is involved primarily in biosynthesis of glutamate providing the link between carbon and nitrogen metabolism. This belief is strengthened in studies that have both NAD-GDH and NADP-GDH in the same cell, but one or the other is made mutant. For *N. crassa* (Fincham, 1957), *S. cerevisiae* (Grenson and Hou,

1972), and *Aspergillus nidulans* (Kinghorn and Pateman, 1973), when the organism was made mutant (lacking one or the other GDH activity), the mutant enzyme form could not be detected in cells grown with NH_4^+ and glutamate as the nitrogen sources. Usually, when an organism could use both coenzymes in the glutamate dehydrogenase reaction, two distinct forms of the enzyme could be isolated, as in the case of *N. crassa* (Sanwal and Lata, 1961) and yeast (DeCastro *et al.*, 1970); each enzyme being specific for one coenzyme.

GDHs in different organisms are specific for different coenzymes (e.g. NAD^+ or $NADP^+$). The specificities for the two coenzymes among species are different (Fahien *et al.*, 1965; Corman and Kaplan, 1967). Frieden (1965) noted that all the non-animal enzymes are specific for just one or the other of the two coenzyme forms used by the bovine enzyme. Organisms in the "lower fungi" belonging to the *Acrasiales* and *Phycomycetes* possess only an NAD-specific glutamate dehydrogenase (LéJohn, 1971; 1974). In these cases, the more complex role required of this enzyme to carry out both the catabolic and biosynthetic reactions is reflected in the evolution of a variety of allosteric controls. LéJohn (1971) used the regulatory properties of glutamate dehydrogenases and other enzymes as one of the parameters to investigate the evolutionary relationships of fungi. Members of the *Ascomycetes*, *Discomycetes*, *Basidiomycetes*, and *Deuteromycetes* have both NAD-specific and NADP-specific glutamate dehydrogenases (LéJohn, 1974). In bacteria, many species such as *Escherichia coli*, *Salmonella typhimurium* possess only the biosynthetic enzyme which is specific for NADP. These organisms may metabolize glutamate for supply of energy by other routes (Smith *et al.*, 1975).

Table 2. Coenzyme Specificity of GDH

Organism	NAD-specific	NADP-specific	non-specific	NAD-specific NADP-activated	References
Salmonella typhimurium	-	+	-	-	Coulton & Kapoor, 1973
Aerobacter aerogenes	-	+	-	-	Meers & Pedersen, 1972
Bacillus cereus	-	+	-	-	Phibbs & Bernlohr, 1971
Rhodospirillum rubrum	+	-	-	-	Bachofen & Neeracher, 1968
Hydrogeno-monas H16	+	+	-	-	Kramer, 1970
Micrococcus aerogenes	+	+	-	-	Johnson & Westlake, 1972
Brevibacterium flavum	-	+	-	-	Shiio & Ozaki, 1970
Bacillus anthracoides	-	+	-	-	Hong et al., 1959
Mycoplasma laidlawii	-	-	+	-	Yarrison et al., 1972
Brassica rapa	-	-	+	-	Itagaki et al., 1988
Bacteroides fragilis	-	-	+	-	Yamamoto et al., 1987 a,b
Azospirillum brasilense	-	-	+	-	Maulik & Ghosh, 1986
Oomycetes	-	-	-	+	LéJohn & Stevenson, 1970
rat liver	-	-	+	-	Seyama et al., 1973
chicken liver	-	-	+	-	Snoke, 1956
bovine liver	-	-	+	-	Frieden, 1959 a, b,c

+, present; -, absent.

Interestingly, some bacteria have both kinds of enzymes (see Table 2). In vascular plants, there are two distinct glutamate dehydrogenases, a mitochondrial enzyme which uses NAD as the coenzyme and a chloroplast enzyme that uses NADP as the coenzyme (Stewart *et al.*, 1980). The dual specificity glutamate dehydrogenase is found mostly in animal tissues such as human liver, rat kidney, bovine liver (see Table 2). These enzymes can use either NAD or NADP as the coenzyme.

There is an interesting feature in the *Oomycetes* which possess an NAD-specific glutamate dehydrogenase; NADP-GDH could not be detected in the cells. Although NAD^+ is the coenzyme used by the GDHs of these "fungi", NADP(H) interacts with the enzyme as an allosteric activator (LéJohn and Stevenson, 1970; LéJohn *et al.*, 1970). Glutamate dehydrogenase activity is enhanced three to four-fold with $NADP^+$. NADPH also activates the enzyme but to a lesser extent. Furthermore, while small molecules such as ATP and GTP activated these enzymes, they inhibited the activity of many other GDHs (LéJohn and Stevenson, 1970).

3. Glutamate Dehydrogenase in Nitrogen Metabolism

Glutamate and glutamine play important roles in donating ammonia in the flow of nitrogen into all nitrogenous compounds of a cell. All cellular nitrogen is incorporated into macromolecules by two forms, the amino group of glutamate and the amide group of glutamine. Glutamate provides approximately 85% of the nitrogen source which is used for the synthesis of amino acids. Glutamine provides 15% of the remaining nitrogen source, which is used for the synthesis of purines, pyrimidines, amino

sugars, histidine, tryptophan, asparagine, NAD^+, and p-aminobenzoate (Wohlheuter *et al.*, 1973).

There are four enzymes of central importance which are involved in the metabolism of glutamate and glutamine: NADP-specific glutamate dehydrogenase, NAD-specific glutamate dehydrogenase, glutamate synthase and glutamine synthetase. NAD-glutamate dehydrogenase catalyses the NAD-specific oxidative deamination of glutamate to yield α-ketoglutarate and ammonia, as follows:

glutamate + NAD^+ -----> α-ketoglutarate + NH_4^+ + NADH

Although this reaction is reversible, it is believed that NAD-glutamate dehydrogenase serves chiefly a catabolic role (Sanwal and Lata, 1961; Grover and Kapoor, 1973). Why? First, under normal growth conditions, the concentration of NAD^+ in the cytoplasm is much greater than the concentration of NADH, forcing the equilibrium of the reaction toward the degradation of glutamate (Lehninger, 1982). Second, NAD-GDH levels in cells grown with glutamate as a nitrogen source are much higher than NAD-glutamate dehydrogenase levels in cells grown with ammonia as a source of nitrogen (Hierholzer and Holzer, 1963; Drillien *et al.*, 1973; Dubois and Grenson, 1974; Dubois *et al.*, 1977; Mitchell and Magasanik, 1984; Courchesne and Magasanik, 1988). NAD-GDH from *Bacillus stearothermophilus* can function in both directions i.e. amination and deamination and is strictly specific for NAD^+ (Mantsala, 1985). Glutamate dehydrogenase from *Azospirillum brasilense* which shows a dual coenzyme specificity is different. The NADP-linked activity of glutamate dehydrogenase showed K_m values of 2.5×10^{-4} M and 1.0×10^{-2} M for α-ketoglutarate and glutamate in the amination and deamination reactions, respectively, but the NAD-

linked activity of glutamate dehydrogenase could be demonstrated only for the amination of α-ketoglutarate but not for the deamination of glutamate. NADP-specific glutamate dehydrogenase catalyses the same reversible reaction as NAD-GDH; it functions in the biosynthesis of glutamate from ammonia and α-ketoglutarate (Maulik and Ghosh, 1986; Mora and Lara, 1988). Grenson *et al.* (1974) found that *S. cerevisiae* mutants that lacked NADP-GDH grew slowly with ammonia as a source of nitrogen indicating that this enzyme is involved in the biosynthesis of glutamate. Glutamate synthase catalyses the reductive amination of α-ketoglutarate by glutamine and NADH to yield two molecules of glutamate (Tempest *et al.*, 1970; Elmerich and Aubert, 1971; Tempest *et al.*, 1973; Roon *et al.*, 1974) as follows.

α-ketoglutarate + glutamine + NADH -----> 2 glutamate + NAD^+

Glutamine synthetase, the only amide synthetase found in living organisms, catalyses the synthesis of glutamine by incorporating ammonia into glutamate after being activated by ATP (Meister, 1980; Legrain *et al.*, 1982). The regulations catalyzed by these enzymes are shown in Fig 1.

In the metabolism of glutamate and glutamine, the functions of these enzymes are closely related. It is common for NADP-GDH and glutamate synthase to generate glutamate at the same time in some microorganisms (Kanamori *et al.*, 1987; Senior, 1975). In *Klebsiella aerogenes*, however, Brenchley *et al.* (1973) reported that the function of NADP-GDH and glutamate synthase was to provide glutamate for the synthesis of glutamine under high and low ammonium conditions, respectively. The enzymes glutamine synthetase-glutamate synthase allow the cell to incorporate ammonia from low concentration of exogenous sources because of the high affinity of glutamine synthetase for ammonia (Miller and Stadtman, 1972;

Kanamori *et al.*, 1987). In contrast, NADP-GDH has a low affinity for ammonia. The step of NADP-GDH-glutamine synthetase functions more rapidly, but only with a higher exogenous concentration of ammonia (Wootton, 1983). In *S. cerevisiae*, all four of these enzymes were found. The activities of these enzymes vary depending on what nitrogen source is present in the growth medium (Hierholzer and Holzer, 1963; Dubois and Grenson, 1974; Roon *et al.*, 1974; Legrain *et al.*, 1982; Mitchell and Magasanik, 1984). The levels of glutamine synthetase and NAD-GDH are low in wild-type cells grown with glutamine or ammonia as the source of nitrogen and are greatly increased while substituting glutamate as the source of nitrogen (Dubois and Wiame, 1976; Courchesne and Magasanik, 1983; Courchesne and Magasanik, 1988). Additional evidence was obtained for this view by using proline, which is exclusively converted to glutamate, as the source of nitrogen. The activities of NAD-GDH and glutamine synthetase in cells grown with proline as the source of nitrogen were higher than those of cells grown with glutamine as the source of nitrogen, but they were not as high as those cells grown with glutamate as the source of nitrogen (Courchesne and Magasanik, 1988). This confirms that NAD-GDH and glutamine synthetase in *S. cerevisiae* are induced by glutamate and repressed by glutamine and ammonia.

The role of NAD-GDH in *S. cerevisiae* is reversible. In the normal growth condition, NAD-GDH degrades glutamate into α-ketoglutarate and ammonia. However, a strain which lacks both NADP-GDH and NAD-GDH grows with ammonia as nitrogen source as well as a strain which lacks only NADP-GDH. In this case, glutamine synthetase is responsible for ammonia assimilation. Nevertheless, a greatly increased intracellular

concentration of NAD-GDH enables a mutant which lacks both NADP-GDH and glutamate synthase to grow with ammonia as the only source of nitrogen (Miller and Magasanik, 1990). Apparently, in this case, the high level of NAD-GDH makes it possible to generate glutamate from α-ketoglutarate, ammonia, and NADH.

In *N. crassa*, it was demonstrated that the main supply of glutamate is through NADP-GDH with a high or low exogenous ammonium concentration. In the ammonium limited chemostat cultures, NADP-GDH and glutamate synthase both contribute to the synthesis of glutamate (Lomnitz *et al.*, 1987). A glutamate synthase negative mutant with an altered structural gene (Hummelt and Mora, 1980 a; Romero and Davila, 1986) accumulated large amounts of glutamine in the chemostat. When supplied with an excess amount of ammonium in the growth medium, a slightly decreased glutamate pool and an increased glutamine pool were detected in this glutamate synthase-negative mutant, resulting in glutamine synthetase repression (Lomnitz *et al.*, 1987). Thus, some of the function of glutamate synthase is to return some of the nitrogen incorporated into glutamine by the NADP-GDH and glutamine synthetase to glutamate. Consequently, glutamate represses glutamate synthase and NADP-GDH activities (Hummelt and Mora, 1980 a; b; Hernandez *et al.*, 1983).

Generally, glutamate dehydrogenases (NAD and NADP-linked) play important roles in nitrogen metabolism. Their functions vary among different organisms (e.g., supply glutamate or use it). Glutamate dehydrogenase, glutamate synthase and glutamine synthetase together are therefore the key enzymes in nitrogen metabolism.

4. Kinetics and Regulation of Glutamate Dehydrogenase

Studies of the kinetics of glutamate dehydrogenase have been complicated because of the enzyme's aggregation-disaggregation properties, and because of the allosteric effects of the coenzymes. Some ligands, such as GTP (Wolff, 1962 b), thyroxine (Wolff and Wolff, 1957), diethylstibestrol (Tomkins *et al.*, 1961), and ADP (Frieden, 1959 b), affect polymerization and allostery which can influence the rate of the catalytic reactions of the enzyme. However, kinetic studies have still been carried out extensively.

Olsen and Anfinsen (1953) showed that NAD^+ is used more effectively than $NADP^+$ in bovine GDH but NAD^+ (or NADH) exerts different allosteric effects from $NADP^+$ on the glutamate dehydrogenase reaction, making it rather difficult to interpret the kinetic experiments. As a result, most of the knowledge concerning the mechanism was derived from studies utilizing NADP(H) (Smith *et al.*, 1957).

Kinetic studies of the glutamate dehydrogenase reaction mechanism of bovine liver glutamate dehydrogenase have been used in attempts to determine whether reaction with binding of the substrates is ordered or random. In the early studies, it was felt that the glutamate dehydrogenase reaction mechanism involved a compulsory order of binding with enzyme-coenzyme being the first complex formed followed by ammonium ion, and then α-ketoglutarate (Frieden, 1959 c). In Frieden's studies, NADPH was used as the coenzyme because at high concentrations, the more reactive NADH produces complex kinetics and inhibition, apparently due to binding at a second, non-active site (Goldin and Frieden, 1971). Fahien and Strmecki

(1969) found that in arsenate buffer, NADH gave linear plots, and in initial velocity studies with this coenzyme, a sequential order of addition was found, with the binding of NADH followed by α-ketoglutarate, and then NH_4^+. With Tris buffer and NADPH, ammonium ion was added before α-ketoglutarate. Engel and Dalziel (1969) studying the oxidative deamination of glutamate, reported that while their data failed to establish random binding, they could exclude certain compulsory order mechanisms while concluding that the mechanism probably involves partially random order binding (Engel and Dalziel, 1970). Limuti and Bell (1983) later described it as a rapid equilibrium random order binding.

Results from the studies of exchange rates of enzyme and substrate agreed with the concept of either partially random or ordered binding of substrates and coenzyme to the enzyme (Silverstein and Sulebele, 1973). Substrates and coenzymes can freely bind to the enzyme in any order. Their dissociation is easier from binary than from ternary complexes. These agreed with the earlier reported results (Colen *et al.*, 1972). It was later reported that kinetic data are consistent with an ordered addition of coenzyme prior to glutamate for NAD-GDH from *Peptostreptococcus asaccharolyticus*. However, the reverse of the mechanism (glutamate prior to coenzyme) is derived with ox glutamate dehydrogenase in the presence of ADP (Hornby and Engel, 1984 b). Recently, it was reported that the kinetic mechanism of NADP-dependent glutamate dehydrogenase in *Halobacterium halobium* was deduced as ordered, with NADPH, α-ketoglutarate and ammonia added in that order, and L-glutamate release preceding $NADP^+$ release (Bonete *et al.*, 1990).

It was reported that α-iminoglutarate was an intermediate in the

reaction catalyzed by glutamate dehydrogenase (Hochreiter and Schellenberg, 1969; 1972). The essential amino group of Lys-126 involved in substrate binding is considered as playing some role in a reaction involving α-iminoglutarate as an intermediate (Brown *et al.*, 1973). This Lys-126 was later blocked by 2, 2, 6, 6-tetramethyl-4-oxo-piperidine-1-oxyl (TMPO) leading to the cooperative inactivation of glutamate dehydrogenase (Karabashian and Agadzhanian, 1988). Lys-126 was also modified by pyridoxal-5-phosphate (Agadzhanian and Karabashian, 1986 a) resulting in blocking the epsilon-amino group of Lys-126 residue. The hypothesis of α-iminoglutarate as intermediate was supported by the observed difference in pH optima for the forward and reverse reactions (Sanner, 1971; Smith and Piszkiewiez, 1973). The optimal pH favoured by most GDH in the amination reaction is pH 7.6-8.0 (di Prisco and Garofano, 1974), but in the deamination reaction it is 8.5-9.0 (Strecker, 1953). As the higher pH results in deprotonation of the α-amino group of Lys-126 and partial deprotonation of the amino group of glutamate, this will favour the reaction of deamination since electrostatic attraction between the NAD^+ and the enzyme amino groups could exist if the α-amino group of Lys-126 was negatively charged. Lysine-128 is also found intimately involved in either direct or indirect interactions with all the substrates and also in catalysis (McPherson *et al.*, 1988).

The kinetic mechanism of NAD-GDH from lower fungi was described in detail by LéJohn *et al.* (1968; 1969 a). Kinetic studies were carried out on NAD-GDH using different kinds of substrates, optimal pH values and influence of temperature (Stevenson and LéJohn, 1971). $NADP^+$ (or NADPH) was an activator of NAD-GDH, but not a coenzyme in *Achlya* (LéJohn *et al.*,

1970; LéJohn and Stevenson, 1970) which, like other glutamate dehydrogenases, is an allosteric enzyme (LéJohn *et al.*, 1969 b; LéJohn and Stevenson, 1970). The influence of pH values on the allosteric activation was discussed (LéJohn and Jackson, 1968; LéJohn *et al.*, 1969 b). The effects were more evident at high pH values, only slightly effective at neutral pH, and ineffective below pH 6.0 (LéJohn and Jackson, 1968). Similarly, it was found that the enzyme activation by AMP was dependent on pH in the same manner. AMP was found to increase the affinity of the enzyme for NAD^+ and glutamate, and to decrease the affinity for NADH. The kinetic patterns for AMP activation with NAD^+ as substrate were complex, showing cooperative interactions (LéJohn and Jackson, 1968). In the studies of the *Blastocladiella* enzyme, NAD^+ and NADH show competitive inhibition patterns with each other, indicating that they bind to the same enzyme form (LéJohn *et al.*, 1969 a).

Glutamate dehydrogenase is a model of a regulatory enzyme. This enzyme has been found to be modulated by coenzymes, purine nucleotides, substrate, leucine and certain steroids (Goldin and Frieden, 1971; Couee and Tipton, 1989 a; b; Erecinska and Nelson, 1990). Regulation of NAD-specific glutamate dehydrogenase from the mitochondria of *Blastocladiella* has been found to occur by means of inhibition at high pH values by cations, particularly calcium and manganese (LéJohn, 1968 b) and by adenylates and guanylates (LéJohn and Jackson, 1968), and also by compounds connected with the tricarboxylic acid cycle and glycolysis (LéJohn, 1968 a). NAD-linked glutamate dehydrogenase from *Thiobacillus novellus* which also contains NADP-linked glutamate dehydrogenase (LéJohn and McCrea, 1968) is allosterically activated by AMP in the direction of

α-ketoglutarate formation. LéJohn *et al.* (1968) suggested that AMP could be acting as a signal pertaining to a requirement for NADH for use in ATP synthesis. The enzyme from the cytoplasmic fraction of rabbit liver was activated by ADP to 160%, whereas the enzyme from the mitochondrial fraction was activated by 230-240% (Kazaryan *et al.*, 1986). The other properties of these two enzymes were the same (Kazaryan *et al.*, 1985). The enzyme from ox liver and brain is activated by L-leucine in a similar way (Couee and Tipton, 1989 a; b). The enzyme in cell extract is activated by L-leucine to a much greater extent than the purified enzyme (Couee and Tipton, 1989 a; b). The reason that leucine activates glutamate dehydrogenase is due to the fact that leucine enhances binding of Mg^{2+} and decreases binding of, and the effect of, Zn^{2+} on the enzyme. Thus, since both metal ions enhance enzyme-enzyme interaction and Zn^{2+}, not Mg^{2+}, is a potent inhibitor of glutamate dehydrogenase, the addition of leucine in the presence of both metal ions results in activation of glutamate dehydrogenase without disruption of the enzyme-enzyme complex (Fahien *et al.*, 1985). By contrast, it was reported that glutamate dehydrogenase is a zinc containing protein and in a low concentration range externally added zinc ions induced an increase in the activity of the enzyme (Wolf and Schmidt, 1983). Leucine is also able to abolish the inhibition of rabbit glutamate dehydrogenase by phosphoenolpyruvate (Bryla and Matyaszczyk, 1983). NAD-GDH from *Oomycetes* is allosterically modulated positively by $NADP^+$, phosphoenolpyruvate, GTP, ATP, UTP and short chain acyl-CoA derivatives but negatively by AMP, citrate and long chain CoA derivatives (LéJohn *et al.*, 1970). Glutamate dehydrogenase from meal worm fat body is strongly inhibited by guanine nucleotides and ATP. The enzyme

was partially protected from the inhibition by addition of ADP to the assay medium (Teller, 1987). In contrast, it was reported that ADP inhibits ox liver glutamate dehydrogenase, except when the concentrations of both glutamate and coenzyme were high (Hornby *et al.*, 1984). NAD-GDH from *Trypanosoma cruzi* epimastigotes is also strongly inhibited by ADP, ATP, GDP and GTP (Urbina and Azavache, 1984). But AMP, ADP, ATP, citrate, isocitrate, pyruvate, L-proline and L-arginine did not have any effect on the NADP-linked glutamate dehydrogenase activity of this same organism (Carneiro and Caldas, 1983).

It was demonstrated that glutamate was involved in the induction of conformational changes of glutamate dehydrogenase (Bell *et al.*, 1985), resulting in enhancing the enzyme activity. Glutamate induces a different conformation of the enzyme-coenzyme complex. Although glutamate and α-ketoglutarate both tighten the binding of reduced coenzyme to the active site, the effect is much greater with glutamate. It was also suggested that two carboxy groups on the substrate were required to allow synergistic binding of coenzyme and substrate to the active site of glutamate dehydrogenase (Bell *et al.*, 1985). Glutamate dehydrogenase from *Peptostreptococcus asaccharolyticus* was reported not to be regulated by purine nucleotides, but was subject to strong inhibition with increasing ionic strength (Hornby and Engel, 1984 a).

The coenzyme binding is divided into two subsites, (Cross and Fisher, 1970), one specific for the amide segment of NMN and the other specific for the ADP moiety. When coenzyme is bound to the amide subsite, the tryptophan absorption regions become perturbed (Cross *et al.*, 1972). It was demonstrated that the 3-acetylpyridine and pyridine-3-aldehyde analogs

of NAD^+ are utilized by bovine glutamate dehydrogenase more effectively than NAD^+ itself (Kaplan *et al.*, 1956). Gore and Greenwood (1972) reported that the NADP-GDH of *Neurospora* utilizes both the 3-acetylpyridine and deamino analogs of $NADP^+$ with a lower V_{max} than with $NADP^+$. This enzyme is rapidly inactivated upon reaction with tetranitromethane (Blumenthal and Smith, 1975). The inactivation is completely prevented by the presence of coenzyme $NADP^+$ but not by the substrate NADH. It was surmised that the primary modification was nitration of one tyrosine residue per polypeptide chain (Blumenthal and Smith, 1975).

Since the observation (Frieden, 1963 b) that GTP inhibits, and ADP activates, glutamate dehydrogenase, and because both nucleotides are found in human serum (Holt *et al.*, 1983), attempts have been made to clarify the mechanism and to detect the interacting sites. Colman and Frieden (1966 a) reported that acetylation of bovine glutamate dehydrogenase increases the dissociation constant for GTP and decreases that for ADP. Subsequently, it was found that amino groups are the primary sites of acetylation (Colman and Frieden, 1966 b). Price and Radda (1969) found that N-acetylimidazole could acetylate up to six tyrosine residues without loss of activity for substrate. However, reaction of about one tyrosine per subunit results in desensitization toward GTP, but the response to ADP is not abolished. The inhibitory GTP and the activatory ADP sites are close but not identical (Jacobson and Colman, 1983). The tyrosine is an essential residue in the GTP binding site of glutamate dehydrogenase, while the lysine that is modified is not involved in the inhibitory action of GTP (Jacobson and Colman, 1983). The primary site of reaction is tyrosine-406 in the linear sequence (Smith and Piszkiewicz, 1973). It was

reported that other substrates had the capacity of desensitization to both GTP inhibition and ADP activation (Malcolm and Radda, 1970; Nishida and Yielding, 1970; DiPrisco, 1971). For example, when bovine liver GDH was incubated with 8-[(4-bromo-2, 3-dioxobutyl) thio] adenosine 5′-diphosphate (8-BDB-TA-5′-DP) and 5′-triphosphate (8-BDB-TA-5′-TP) to yield enzyme with about 1 mol of reagent incorporated/mol of enzyme subunit, the modified enzyme was catalytically active but had decreased sensitivity to inhibition by GTP, reduced extent of activation by ADP, and diminished inhibition by high concentrations of NADH (Ozturk *et al.*, 1990).

It appears that the primary effect of both GTP and ADP is on binding of the coenzyme. Yielding and Holt (1967) reported that binding of NADH is weakened in the presence of ADP and slightly strengthened with GTP. This was confirmed by other workers (Dalziel and Egan, 1972; Malencik and Anderson 1972). Markau *et al.* (1972) suggested that the effect of ADP on coenzyme dissociation may result from competition for a portion of the coenzyme site. Related to the effects of the purine nucleotides are the allosteric effects of NAD(H). Glutamate dehydrogenase in some organisms displays nonlinear kinetics with respect to NAD and NADH (Fahien *et al.*, 1965; Corman and Kaplan, 1967). Ox liver glutamate dehydrogenase was inhibited in the direction of glutamate deamination by NADH and NADPH with both NAD and NADP as coenzymes (Popova and Sugrobova, 1983). An exception is the glutamate dehydrogenase of *Drosophila*, which is activated by NADH (Bond and Sang, 1968). NADP(H) was shown not to bind at the regulatory site, presumably because of the presence of the 2-phosphate (Pantaloni and Dessen, 1969). It was later reported that NADP(H) could bind to the regulatory site weakly. ADP and GTP can also bind to the coenzyme

regulatory site (Cross and Fisher, 1970; Jallon and Iwatsubo, 1971; Koberstein *et al.*, 1973). In the absence of NADH, every promoter of this glutamate dehydrogenase can bind one molecule of GTP; in the presence of NADH the additional binding site for GTP was induced. To achieve the inhibitory action of GTP, the binding of the effector to only one (NADH-induced) site is enough (Agadzhanian and Karabashian, 1986 b). The location of the binding site was widely studied (Coffee *et al.*, 1971; Fisher 1973; Prough *et al.*, 1973). Each of the binding sites probably consists of some sort of "pocket" on the enzyme surface. Therefore, many different types of residues have been found to be involved (Smith *et al.*, 1975). Banerjee *et al.* (1987) reported that the oxidized coenzyme NAD^+ binds to two sites per subunit of bovine liver glutamate dehydrogenase with equal affinity in the absence of dicarboxylic acid coligands. In the presence of glutamate or α-ketoglutarate, the affinity of one site is unchanged, but the affinity of the other (presumed to be the active site) is considerably increased and now requires two dissociation constants to describe its saturation (Banerjee *et al.*, 1987). The presence of an allosteric site for $NADP^+$ in glutamate dehydrogenase which uses NAD^+ as coenzyme but with an NADP-type mechanism makes the enzyme an interesting subject of study in kinetic, regulatory and evolutionary terms (LéJohn and Stevenson, 1970). The treatment of bovine liver glutamate dehydrogenase with chymotrypsin generates a proteolytic derivative that is activated 3 to 4-fold over the native enzyme. The activated glutamate dehydrogenase shows altered kinetic parameters and altered response to allosteric modulation by GTP and ADP. The proteolytically cleaved glutamate dehydrogenase was less responsive to GTP and was no longer activated by

ADP, although ADP binding sites remained intact on the enzyme (Place and Beynon, 1983). Others have reported that GTP and NADH together can provide the best protection for beef liver GDH against the proteolytic effects of chymotrypsin (Aitchison and Engel, 1983).

5. Purification of Glutamate Dehydrogenase

The purification of glutamate dehydrogenase was originally described in 1951 (Olson and Anfinsen, 1951; Strecker, 1951) with bovine liver. Subsequently, these workers reported that a highly purified crystalline glutamate dehydrogenase was obtained. Since it was required to do the compositional studies and detailed investigations of the relationship of structure to enzyme activity, glutamate dehydrogenase has been purified from various sources.

The degree of GDH purification from different organisms varies greatly. Fahien *et al.* (1969) reported that a reasonably high specific activity of the enzyme was obtained from bovine liver. A specific activity (nmoL NADH consumed/mg protein) of 1900 was obtained from yeast NAD-GDH (Miller and Magasanik, 1990). Sometimes, the degree of purification was very low; specific activity (U/mg protein) of 1.2 from bovine brain and 0.48 from bovine intestinal mucosa was reported (Grisolia *et al.*, 1964). After purification, the purity of the enzyme was examined by electrophoresis and Coomassie Blue staining and it was reported that electrophoretically homogeneous enzyme was obtained (Yeung *et al.*, 1981).

Some of the glutamate dehydrogenases have been found to be thermo stable (Barratt and Strickland, 1963; Ahmad and Hellebust, 1989), but

others are not very stable (LéJohn and Stevenson, 1970). The activity of NAD-GDH was observed to be quite stable in *Chlorella sorokiniana*, whereas the activity of NADP-GDH in the organism decayed very rapidly (Gronostajski *et al.*, 1978). The same thing was reported for *Chlorella pyrenoidosa* (Shatilov and Kretovich, 1977). Hynes (1974) reported the rapid loss of NADP-specific glutamate dehydrogenase from *Aspergillus* when glucose is absent from the medium, suggesting the presence of protease in the absence of glucose. In *Candida utilis*, inactivation of NADP-specific glutamate dehydrogenase has been observed to occur in response to changes in the nitrogen compounds available (Ferguson and Sims, 1971). Induction of *Achlya NAD-GDH* by glutamate shows an oscillatory pattern of synthesis and degradation of the enzyme (Smaluck, 1971). Each succeeding period of degradation is followed by a phase of synthesis of the enzyme from a higher starting point. Balinsky *et al.* (1970) reported that the enzyme isolated from frog and tadpole liver was unstable. Beef glutamate dehydrogenase has also been found to be unstable with latent proteolytic activity (Cassman and Schachman, 1971). The instability in terms of the state of association of the enzyme and the influence of allosteric effectors has also been examined (Eisenkraft and Veeger, 1970; LéJohn *et al.*, 1969 a). This instability of the enzyme makes its purification much harder. The procedures that have been commonly used to purify the enzyme from bovine liver (Olson and Anfinsen, 1952; Fahien *et al.*, 1969) were found to be unsuitable with bovine brain as a starting material (McCarthy *et al.*, 1980). It is often possible to maintain enzyme activity in 50% glycerol when stored at -20°C (Deutscher, 1990), but glycerol could interfere in further purification of enzyme. The use of dithiothreitol at

about 0.1-1.0 mM is preferred because it does not form mixed disulfides with proteins, as 2-mercaptoethanol does (Cleland, 1964). It was reported (Gronostajski *et al.*, 1978) that although dithiothreitol stabilized the activity of the NADP-GDH during preparative electrophoresis, this reducing agent caused a loss in activity of the enzyme in the purified state or in crude cell extracts during storage at -20° C. The stabilities of NAD- and NADP-specific glutamate dehydrogenase in *Chlorella pyrenoidosa* and *Chlorella sorokiniana* were found to be quite different; the NADP enzyme is more stable (Shatilov and Kretovich, 1977). In *Clostridium symbiosum*, it was found that various salts promoted re-activation of inactivated GDH to a limited extent (Syed and Engel, 1990). This enzyme appeared to be reversibly converted from a high-activity form at low pH to a low-activity form at high pH (Syed and Engel, 1990).

An affinity column formed by the reaction of the amino group of L-glutamic acid with 1-N-bromoacetyl-6-N-sepharose hexyldiamine has been employed as a purification tool (Blumenthal and Smith 1973). $NADP^+$ affinity column has been successfully used in NADP-GDH purification in *Chlorella* (Yeung *et al.*, 1981). Bovine liver glutamate dehydrogenase could not be recovered from a column obtained by attaching NAD or NADP to ϵ-aminocaproyl sepharose by treatment with a carbodiimide. This enzyme could be eluted, however, from a derivative formed by linking AMP through its amino group and a six carbon spacer molecule to sepharose (Lowe *et al.*, 1972). This AMP attached sepharose 4 B has also been successfully used in *Neurospora* NADP-GDH purification (Watson and Wootton, 1977). Some coenzyme analogues were immobilized in a chemically defined way to a spacer arm and successfully used in affinity chromatography of

dehydrogenases (Mosbach *et al.*, 1972; Craven *et al.*, 1974; Trayer *et al.*, 1974). The use of such adsorbents as an analytical tool has also been investigated (Harvey *et al.*, 1974 a; b; Lowe *et al.*, 1974 a.b; Trayer and Trayer, 1974). These workers attempted quantitatively to relate ligand-specific chromatographic behaviour to ligand-binding parameters derived from studies of enzymes in free solution. The effectiveness of such analyses has been limited by the occurrence of non-specific binding of dehydrogenases to the adsorbents (Barry and O' Carra, 1973).

A variety of reactive triazine-based textile dyes immobilized to agarose and other matrices have been widely used in affinity chromatography for enzyme purification (Thompson *et al.*, 1975; Baird *et al.*, 1976; Lowe *et al.*, 1981). In particular, immobilized Cibacron Blue F3 G-A appears to be an especially effective adsorbent for the purification of pyridine nucleotide-dependent oxidoreductases, phosphokinases, coenzyme A-dependent enzymes, hydrolases, RNA and DNA nucleases, polymerases, restriction endonucleases, synthetases and serum lipoproteins (Dean and Watson, 1979; Lowe, 1979; Gianazza and Arnaud, 1982). It was shown that active Cibacron Blue F3G-A possesses the general ability to bind a wide range of NAD- and NADP-dependent dehydrogenases (Lowe *at al.*, 1980; Atkinson *et al.*, 1981; 1982; Farmer and Easterby, 1982; Hughes *et al.*, 1982). However, the NADP-specific glutamate dehydrogenase purified from epimastigotes of the *Tulahuen* strain, *Tul 2 Stock*, of *Trypanosoma cruzi*, was inhibited by Cibacron Blue FG3A (Orellano *et al.*, 1985). Nevertheless, the task of selecting a suitable immobilized dye remains very much an empirical process involving the testing of a number of dyes for the ability to bind the protein in question. Often it

is adequate simply to test one or two of the more commonly used dyes, since these are found to give satisfactory results in a surprisingly large number of cases (Lowe and Pearson, 1984). Affinity precipitation is also used to purify glutamate dehydrogenase. Graham *et al.* (1985) demonstrated that in the presence of glutaric acid, N2, N2'- adipodihydrazido-bis (N6-carbonylmethyl-NAD^+) (bis-NAD^+) forms cross-links between molecules of glutamate dehydrogenase, resulting in precipitation and purification of glutamate dehydrogenase.

Due to the differences in properties of GDHs from different organisms, the procedures reported for enzyme purification varied greatly. In many cases, affinity chromatography is of great help in enzyme purification. Because of the instability of GDHs in some organisms, it is wise to find ways of protecting these enzymes against (i) inactivation, (ii) denaturation and (iii) irreversible adsorption in purification and storage.

6. Induction and Repression of Glutamate Dehydrogenase

Synthesis of glutamate dehydrogenase has been found to be under the control of certain induction-repression mechanisms. Synthesis of NAD-specific glutamate dehydrogenase in *Oomycetes* and *Hypochytridiomycetes* is repressed when the organisms are grown in the presence of either glucose or sucrose (LéJohn and Stevenson, 1970). Glutamate and other amino acids which serve as the precursors of glutamate can act as inducers of the repressed NAD-GDH in *Oomycetes* and *Hypochytriomycetes* (LéJohn and Stevenson, 1970). NAD-GDH in *Apodachlya brachynema* is also repressed by

glucose as a sole carbon source and induced with glutamate, proline, alanine or ornithine plus aspartate as sole carbon sources (Price and Gleason, 1972). NAD-GDH of *Physarum polycephalum* has a 50% increase in specific activity when grown on a medium containing 0.5 M mannitol instead of glucose (Huettermann *et al.*, 1971; Huettermann and Cheft, 1971). NAD-GDH is repressed by NH_4^+ in *S. cerevisiae* (Hierholzer and Holzer, 1963; Holzer and Hierholzer, 1963) in the presence and absence of glutamate, whereas NADP-GDH activity is similar in the cells grown in either NH_4^+ or glutamate but reduced in cultures containing both nitrogen sources. NAD-GDH is repressed in normal yeast cells grown with high concentration of sugars (Polakis and Bartley, 1965; Beck and Meyenburg, 1968) but NADP-GDH activity is induced (Brown and Johnson, 1970; Nunez de Castro *et al.*, 1970). In *Saccharomyces carlsbergensis*, NAD-GDH is also repressed by NH_4^+ and induced by glutamate; while NADP-GDH responds in the opposite manner (Westphal and Holzer, 1964; Holzer, 1966; Hollenberg *et al.*, 1970). The activity of NADP-GDH in *S. cerivisiae* is also decreased under conditions in which intracellular ammonia concentrations increase (Bogonez *et al.*, 1985).

NADP-GDH in *Chlorella sorokiniana* is also induced by ammonia (Prunkard *et al.*, 1986a). The induction produced a larger precursor protein (M_r = 58,500) which was subsequently processed to the subunit (M_r = 53,000) of NADP-GDH (Prunkard *et al.*,1986 b). In uninduced cells, NADP-GDH is rapidly synthesized, covalently modified and then degraded. Therefore, it was surmised that the NADP-GDH mRNA was present, but the enzyme protein itself was absent in the uninduced cells (Turner *et al.*, 1981). In *Chlorella vulgaris*, the activity of NADP-GDH increased after

nitrogen starvation (Morris and Syrett, 1965).

In algae, NADP-GDH activity is regulated by the availability of a nitrogen source (Molin *et al.*, 1981; Everest and Syrett, 1983; Tischner, 1984; Fayyaz-Chaudhary *et al.*, 1985). In *Euglena*, NADP-GDH is induced under conditions of nitrogen stress or glutamate availability and the induction is regulated primarily at the post-transcriptional level (Parker *et al.*, 1985).

In *Neurospora*, the activity of NADP-GDH increases after nitrogen starvation (Barratt, 1963). Ammonia was suggested as a repressor of NADP-specific glutamate dehydrogenase, and an inducer of NAD-specific glutamate dehydrogenase (Barratt, 1963; Stachow and Sanwal, 1967). In *N. crassa*, the levels of NAD-GDH and NADP-GDH are also regulated by the repression-induction mechanism. In the presence of glutamate or a nitrogenous precursor (ammonia, alanine, aspartate etc.), NAD-GDH activity is induced while NADP-GDH activity is repressed (Sanwal and Lata, 1962 a; b; Kapoor and Grover, 1970), resulting in an inverse relationship between these two enzymes (Sanwal and Lata, 1962 b; c). Urea, metabolically derived from either glutamate or ammonia, was suggested as a regulator for the concurrent synthesis of the enzyme forms.

It was suspected that the increase in enzyme activity is actually due to synthesis of new enzyme protein rather than activation of a pre-existing pro-enzyme. It was demonstrated with *Neurospora* that the increased activity was indeed due to new synthesis of the protein (Sanwal and Lata, 1961). Huettermann *et al.* (1971) demonstrated that changes in glutamate dehydrogenase levels during differentiation in *Physarum polycephalum* were due to *de novo* synthesis of new enzyme. Newly

synthesized NADP-GDH was also detected in the induction of *Chlorella pyrenoidosa* (Talley *et al.*, 1972). These studies indicated that the process of new enzyme synthesis is under control at the transcriptional and/or translational levels.

The actual agent *in vivo* for regulation was suggested to be NH_4^+ (Stachow and Sanwal, 1967). Pateman (1969) used mutants of *Aspergillus nidulans* which could not convert nitrite or nitrate to ammonia. The fact that the mutants, in contrast to the parent strain, did not produce an increase in NADP-specific glutamate dehydrogenase when nitrate was added to the growth medium indicates that nitrate alone did not act as an inducer for NADP-GDH. Urease-less mutants also lost the ability to lower the activity of glutamate dehydrogenase when urea could not be converted to ammonia. Thus, the apparent effects of nitrate and urea are actually due to their conversion to ammonia. Induction of NAD-GDH and repression of NADP-GDH are proportional to the concentration of NH_4^+ in the growth medium. In addition glutamate at high concentration can repress the NADP-specific glutamate dehydrogenase (Barratt, 1963). The high concentration of NH_4^+ may result in a general ammonia toxicity (Strickland, 1969).

Polakis and Bartley (1965) reported that disppearance of sugars from the medium resulted in loss of activity of biosynthetic enzymes such as NADP-GDH in *S. cerevisiae*. Tuveson *et al.* (1967) also demonstrated that sucrose is required for NADP-GDH induction in *Neurospora*, but the results did not support the reciprocal induction-repression of the two enzyme forms as suggested by Sanwal and Lata (1962 c). A reciprocal relationship of induction-repression in the presence of catabolites was obtained by Kapoor and Grover (1970) in *N.* crassa.

NAD-GDH activity diminishes and NADP-GDH activity is elevated by increasing the concentration of sucrose or glucose in the growth medium (Kapoor and Grover, 1970). After cells were grown in the medium containing carbon which repressed NAD-GDH activity, the enzyme was derepressed rapidly by treating the repressed cells with antibiotics such as polymyxin B or amphotericin B. The derepression at the transcriptional level occurred very rapidly in response to polymyxin B but more slowly to amphotericin B (Vierula and Kapoor, 1987). The induction of NAD-GDH also occurred at the transcriptional level within 30 minutes of transfer of cells to a carbon-free medium (Vierula and Kapoor, 1986). The evidence for this is that a brief shift (5 min) to induction medium followed by a return to 1% sucrose medium is sufficient to trigger synthesis of abundant NAD-GDH transcripts but low levels of the active enzyme (Vierula and Kapoor, 1986). It was earlier suggested that the repression-induction of the two glutamate dehydrogenases is controlled by some balance between the internal amino acids and glucose metabolites (Strickland, 1971).

The effects of glucose has been examined in *Coprinus lagopus* (Fawole and Casselton, 1972) and *Aspergillus nidulans* (Hynes, 1974). In general, glucose was found to repress NAD-specific glutamate dehydrogenase production and induce the synthesis of NADP-GDH. Fawole and Casselton (1972) suggested that this is actually a response to α-ketoglutarate as a product of glucose metabolism. Hynes (1974) reported that growth on a medium containing glutamate resulted in a rapid loss of the NADP-specific glutamate dehydrogenase, but the enzyme level was maintained when the medium also contained glucose. Therefore, it appears that the induction and repression of the two enzyme forms is affected both by nitrogen

metabolism and by catabolite effects of carbon sources. However, glutamate dehydrogenase activity was not significantly altered by varying the concentration of glucose in the culture medium when culturing pancreatic islets (MacDonald *et al.*, 1991).

Induction and repression of glutamate dehydrogenase synthesis in higher fungi which contain both NAD-specific and NADP-specific glutamate dehydrogenase have also been studied in *Fusarium oxysporum* (Sanwal 1961), *Coprinus lagopus* (Fawole and Casselton, 1972) and *Schizophyllum commune* (Dennen and Niederpruem 1964). In *Aspergillus niger* (Galbraith and Smith 1969) and *Fusarium oxysporum* (Sanwal 1961), the enzyme activity of both dehydrogenases were related to the age of the cultures. NH_4^+ also induced NADP-GDH and repressed NAD-GDH. In the old culture, NAD-GDH activity increased but NADP-GDH activity decreased. The activity of NADP-GDH in *Schizophyllum commune* was depressed in glucose-containing medium with NH_4^+ as the sole nitrogen source and increased when glutamate was the source of nitrogen (Dennen and Niederprume, 1964). NAD-GDH was induced by NH_4^+ and unaffected by glutamate (Dennen and Niederpruem, 1967) which usually induce NAD-GDH. NAD-GDH activity was repressed by glutamate in *Aspergillus aerogenes* (Meers *et al.*, 1970).

The induction-repression mechanism has been studied in several bacteria. NADP-GDH was repressed by glutamate in *Bacillus anitratum* (Jyssum and Jones, 1965), *Rhodospirillum japonicum* (Mooney and Fottrell, 1968; Fottrell and Mooney, 1969) and induced by NH_4^+ in *Hydrogenomonas H16*. In the last organism, this enzyme activity was repressed by nitrogen starvation (Kramer, 1970) but unaffected by several nitrogen sources (Truper, 1965; Joseph and Wixom, 1970). Neither glutamate nor NH_4^+ had any

regulatory effect on this enzyme in *Neisseria meningitidis* (Jyssum and Borchgrevink, 1960). NAD-GDH was induced by glutamate in *T. novellus* (LéJohn, 1967; LéJohn and McCrea, 1968), by nitrogen starvation in *Hydrogenomonas H16* (Kramer, 1970), and by NH_4^+ as the only nitrogen source in *Neisseria meningitidis* (Jyssum and Borchgrevink, 1960).

It was found that NH_4^+ induced NADP-GDH activity but glutamate repressed enzyme synthesis in a number of *Bacilli* under the same growth conditions (Phibbs and Bernlohr, 1971; Meers and Pedersen, 1972).

The induction and repression of glutamate dehydrogenase have been investigated in a few higher plants. NADP-GDH in wheat grains was induced by 2,4-D, kinetin and IAA (Gil'manov and Sultanbaev, 1989). NAD-GDH was induced by glutamine and glutamate in the roots of pea seedlings. It was also induced by NH_4^+ in oat leaves (whereas glutamine, glutamate, urea, or α-ketoglutarate did not induce or repress the enzyme), and in rice plant roots (Barash *et al.*, 1973). In both cases, a new enzyme band was detected by gel electrophoresis (Kanamori *et al.*, 1972; Barash *et al.*, 1973). Both NAD-GDH and NADP-GDH are induced by glucose in grape leaves (Gordeziani *et al.*, 1972).

Glutamate dehydrogenase activity is markedly influenced by starvation of shrimps. A 50% decrease of glutamate dehydrogenase activity was observed following 7 days of fasting but subsequently no further decrease in glutamate dehydrogenase activity was noticed during starvation up to a maximum of 17 days (Batrel and Regnault, 1985).

From the above, one can see that GDHs are regulated by nitrogen and carbon sources in growth media. The activity of most GDHs are induced by nitrogen sources such as ammonia and glutamate but are repressed by carbon

sources such as glucose and sucrose. The induced activity of the enzyme is due to new synthesis of the enzyme protein.

7. Gene Analysis of Glutamate Dehydrogenase

The genes encoding glutamate dehydrogenase have been studied in a wide variety of organisms including humans (Banner et al., 1987; Mavrothalassitis *et al.*, 1988), fungi (Bascomb *et al.*, 1986; Vierula and Kapoor, 1989; Miller and Magasanik, 1990), and bacteria (Valle *et al.*, 1983; 1984; Riba *et al.*, 1988). The NADP-specific glutamate dehydrogenase gene in *Escherichia coli* was located at 38.6 min on the genetic map (Helling, 1990; Kim *et al.*, 1990) and 1,860 kb on the physical map (Helling, 1990).

Isolation of the GDH gene was accomplished by using a variety of methods, viz; Amuro *et al.* (1988) and Mavrothalassitis *et al.* (1988) synthesized oligonucleotides based on the amino acid sequence information of glutamate dehydrogenase to screen a human λgt11 cDNA library that was constructed from human liver poly(A)$^+$ RNA (Cladaras *et al.*, 1986). An antibody to glutamate dehydrogenase was used successfully to screen λgt11 expression cDNA and genomic libraries (Vierula and Kapoor, 1989; Miller and Magasanik, 1990) by the methods described by Young and Davis (1983 a; b; 1985). In *Chlorella sorokiniana*, the NADP-GDH translatable mRNA is only 0.074 % (Turner *et al.*, 1981) of the total cellular translatable poly(A)$^+$ RNAs in fully induced cells (Turner, 1980). Therefore, Bascomb *et al.* (1986) employed purified anti-NADP-GDH IgG to immunoselect polysomes translating mRNA which was used for synthesis of cDNA and screening of

glutamate dehydrogenase cDNA. An NADP-specific glutamate dehydrogenase gene of *Neurospora crassa* which contains two introns (Fincham and Kinnaird, 1984) was used successfully as a heterologous probe to screen two independently constructed *Aspergillus nidulans* genomic libraries (Gurr *et al.*, 1986). The isolation of the *Saccharomyces cerevisiae* gene for NADP-dependent glutamate dehydrogenase was carried out by cross hybridisation using the *Neurospora crassa am* gene known to encode NADP-GDH, as a probe (Nagasu and Hall, 1985).

In human liver, evidence was obtained which indicated the existence of more than one glutamate dehydrogenase-related gene (Mavrothalassitis *et al.*, 1988). Differences between the amino acid sequences deduced from a variety of cDNA isolates suggested that there may be at least two active human glutamate dehydrogenase genes (Banner *et al.*, 1987). But only one mRNA of 3.7 kb that hybridised to a GDH probe was detected in human brain (Banner *et al.*, 1987). The homologous regions of three human liver and three human brain cDNAs had identical sequences over more than 2 kb including 3'-untranslated regions. This suggests that identical glutamate dehydrogenase mRNAs are present in human liver and human brain (Nakatani *et al.*, 1987). Although only one gene appears to be expressed, human genomic DNA blots show the existence of more than one glutamate dehydrogenase gene (Nakatani *et al.*, 1987). Amuro *et al.* (1990) obtained two genomic clones of the glutamate dehydrogenase gene, both of which have a restriction map distinctly different from one another. Although the nucleotide sequences of three exons were virtually the same in both clones, Southern blotting analysis of the genomic DNAs revealed that at least two DNA fragments of different sizes exist. This implies that the

two clones are generated from two different gene loci for glutamate dehydrogenase on the human chromosome (Amuro *et al.*, 1990). In *Chlorella*, a single gene encodes the α- and ß-subunits of NADP-glutamate dehydrogenase which is regulated by light and dark (Schmidt, 1988). In *Drosophila melanogaster*, only one copy of the GDH gene is present (Papadopoulou and Louis, 1990). Rat liver and kidney each contain two glutamate dehydrogenase mRNAs of 3.5 kb and 2.8 kb. The 3.5 kb transcript is prominent in rat brain, whereas the 2.8 kb transcript is hardly detectable. This result suggests that glutamate dehydrogenase gene expression is differentially controlled in rat brain (Banner *et al.*, 1987 b). Blotting analysis of RNA from human, monkey and rabbit showed that glutamate dehydrogenase mRNA is present in various amounts in all tissues tested (Mavrothalassitis *et al.*, 1988).

GDH genes from different organisms can be very homologous. The cDNA of NADP-GDH in *Chlorella* contains a highly conserved sequence common to NADP-GDH genes of *Escherichia coli* and *Neurospora crassa* (Schmidt, 1988). A human glutamate dehydrogenase cDNA is homologous enough to isolate a corresponding glutamate dehydrogenase cDNA in a rat liver cDNA library (Das *et al.* 1989). The DNA sequences of upstream regulatory sequences (URS) of the NADP-GDH gene in *N. crassa* were found to be homologous to yeast URS sequences (Frederick and Kinsey, 1990 a). The amino acid sequences of the yeast and *N. crassa* enzyme (NADP-GDH) are 63% conserved (Moye *et al.*, 1985). The former is 51% homologous to *Escherichia coli* and 24% to bovine NADP-GDH, (Nagasu and Hall, 1985). The polypeptide sequence of NADP-GDH in *Aspergillus nidulans* contains regions of homology with glutamate dehydrogenase proteins from a range of organisms (Hawkins *et*

al., 1989). The nucleotide sequence of the N-terminal coding region of *Klebsiella aerogenes* glutamate dehydrogenase gene was determined and found to be strongly homologous with that of *Escherichia coli* (Mountain *et al.*, 1985).

In human glutamate dehydrogenase gene, two introns were found (Amuro *et al.*, 1990). The NADP-GDH gene in *Aspergillus nidulans* contains two putative introns of 53 nucleotides (Hawkins *et al.*, 1989). In *Drosophila melanogaster*, the transcript includes at least one large intron and matures to an approximately 2.4 kb long polyadenylated RNA whose expression is under developmental control (Papadopoulou and Louis, 1990). In *Neurospora crassa*, introns were detected in the gene for NADP-GDH (Fincham *et al.*, 1985). There are two introns of 66 and 61 bases respectively. They interrupt sequences that are conserved between *Neurospora crassa* and *Escherichia coli* and the two introns show some striking internal similarities to each other (Kinnaird and Fincham, 1983). It is surmised that many introns exist in the coding region of the NADP-GDH gene (Cock and Schmidt, 1989) of *Chlorella sorokiniana*. The gene of NADP-GDH in yeast has no introns (Moye *et al.*, 1985).

The length of non-coding sequence in any glutamate dehydrogenase gene differs in different organisms. The human glutamate dehydrogenase gene has a 3′-untranslated sequence of 1,261 nucleotides (Nakatani *et al.*, 1987). Mavrothalassitis *et al.* (1988) also reported that a 3′-untranslated region of 1,262 bp was detected. A cDNA from a rat liver contains a 5′-untranslated region of 60 bp, a protein coding region of 1,674 bp and a 3′-untranslated region of 1,129 bp (Das *et al.*, 1989). The NADP-GDH gene in *Aspergillus nidulans* is only 1,485 bp long, is interruped

by two putative introns of 53 bp, and has a protein encoding region of 1,380 bp (Hawkins *et al.*, 1989).

Studies on the regulation of gene expression have been carried out for the *am* (GDH) gene of *Neurospora crassa*. Two distant upstream regulatory sequences were found controlling the level of expression of the *am* gene (Frederick and Kinsey, 1990 b). The closer of these two sequences (URS*am* α) is approximately 1.4 kb upstream of the transcriptional start site. The second sequence (URS*am*5 ß) is located between 2.1 and 3.2 kb upstream of the transcription start site. Deletion of either of these two sequences reduces *am* expression to about 50% of the wild-type level. Deletion of both elements reduces the expression of *am* to 5-16% of wild-type level. Deletion of other sequences (e.g. 1.1 kb just downstream of URSam α or 3.5 kb upstream of URSam ß) had no effect on gene expression. Overall, knowledge about the structure of GDH genes, especially the NAD-specific one, is in its infancy and hardly anything is known about their mode of regulation at the DNA level.

METHODS AND MATERIALS

I. METHODS

1. CELL CULTURES AND GDH INDUCTION

1.1. Cell Cultures

1.1.1. Culture for cell stock. Approximately 1 mL of *Achlya Klesbsiana* spore suspension containing approximately 10^5 spores was inoculated onto each agar plate. After incubating at 22°C for 3 days, when the mycelia could be seen by the naked eye, the plates were sealed and stored at 5°C. The plates were kept for 3 months before new plates were prepared.

1.1.2. Cultures for inoculation. Cultures were prepared by inoculating 1 mL of spore suspension (10^5 spores/mL) to each plate which contained 20 mL GYCaMg medium (5.0 g/L glucose, 0.5 g/L yeast extract, 1 mM $CaCl_2$ and 1mM $MgCl_2$ in distilled water) and incubating at 22°C. After 2 days, the culture was filtered through 0.61 μm nylon cloth and the spore suspension

was used as inoculum. Alternatively, the culture was transferred every 3-5 days to ensure the vegetative growth of the cells.

In defined medium (Barksdale 1963), the inoculation method was the same as in GYCaMg medium except that the cultures were incubated for 5-7 days at 22°C. The inoculum was taken directly from a defined medium culture without filtering the spores.

For large scale cell cultures, spores from 50 plates containing a total of 10^8 spores were required to inoculate 10 litres of medium in each carboy. If defined medium was used in the carboy, spores were obtained from cells grown in defined medium and incubated at 22°C for 48 h. If GGY medium (10 g/L glutamate, 0.5 g/L glucose, 0.5 g/L yeast extract) was used in carboys, cultures providing spores for inoculation contained GYCaMg medium and the cells had been grown at 22°C for 45 h.

1.1.3. Culture for small scale assay. Two Erlenmeyer flask containing 1 L medium were each inoculated with 100 mL spore suspension containing 10^7 spores from plates by filtering the cells through 0.61 μm nylon cloth. The medium was either GYCaMg medium, defined medium or GGY medium. The flask was agitated (170 rpm) at 28°C for 18 h.

1.1.4. Culture for enzyme purification. For enzyme purification, large quantities of mycelia were required. A carboy containing 10 litres of medium was inoculated aseptically with spore suspension (containing 10^8 spores) prepared from 50 plate liquid cultures of *Achlya klebsiana*. Sterile air was forced through the culture by means of 2 spargers which extended to the bottom of the carboy. The air was strong enough to agitate

the culture vigorously. The cells were grown at 28°C for 18 h when the cells were ready for additional enzyme induction. If the medium was GGY, the enzyme induction could be carried out in the same culture system by simply adding glutamine suspended in 50 mL sterile distilled water to bring the culture to 5 mM glutamine (0.7 g/L) and incubating at 28°C for 3 h. If the growth medium was defined medium, the cells were transferred to starvation medium (1 mM Tris-acetate, pH 6.5, 0.1 mM $CaCl_2$,) by the technique described in "1.1.5. Nutritional stress condition" and then induced with 5 mM glutamine (as sterile filtered 100 mM stock solution in 1 mM Tris-acetate, pH 6.5,) at 28°C for 3 h.

1.1.5. Nutritional stress condition. After incubation in flasks or carboys, the cells were filtered through Whatman No. 1 filter paper by vacuum ensuring that the mat was kept moist at all times. The cells were washed 3 times with twice the original volume of starvation medium after which they were resuspended in starvation medium and brought to the original volume.

1.2 Glutamate Dehydrogenase Induction

1.2.1. Induction under different conditions. Cells were grown for 18 h in defined medium at 28°C with agitation (170 rpm), and then transferred to starvation medium and induced with either 5 mM L-glutamine, 5 mM L-glutamate, 5 mM NH_4Cl, or 5 mM L-glutamate + 5 mM NH_4Cl for 5 h at 28°C with agitation (170 rpm). Cells were also grown either in GGY medium for 12, 15, and 18 h at 28°C with agitation, grown in GGY medium for 18 h and

induced with 5 mM L-glutamine, or grown in defined medium with or without the inducer (5 mM L-glutamine).

1.2.2. Time-course of induction. When the cells grown in defined medium were transferred to starvation medium, L-glutamine (100 mM stock solution in 1 mM Tris-acetate, pH 6.5) was added to a final concentration of 5 mM. The cultures (200 mL each) were agitated (170 rpm) at 28°C for 0, 1, 2, 3, 5 and 8 h. Cultures were harvested by filtering through Whatman No. 1 filter paper with a vacuum pump after each incubation period. After the cells were collected, they were washed with enzyme extraction buffer TPEDGP consisting of 50 mM Tris-acetate, pH 7.3, 10 mM K_2HPO_4, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF (phenylmethyl sulphonyl fluoride) and 20% (v/v) glycerol. After vacuum drying, the paper-like cells were used fresh or stored at -70°C until required.

1.2.3. Induction by different concentrations of L-glutamine.
When cells grown in defined medium were transferred to starvation medium, L-glutamine was added to each culture (200 mL each) to the final concentration of 0 mM, 1 mM, 2mM, 3 mM, 5 mM and 8 mM. The cultures were agitated (170 rpm) at 28°C for 3 h.

1.2.4. Repression and induction with a combination of glucose and glutamine. When cells grown in defined medium were transferred to starvation medium, L-glutamine and glucose were added to each culture (200 mL each) at different concentrations. The final organic nitrogen and glucose carbon concentrations in each culture were:

(a) 5 mM glutamine: 30 mM glucose; (b) 1.0 mM glutamine: 30 mM glucose; (c) 0.5 mM glutamine:30 mM glucose; (d) 0.1 mM glutamine:30 mM glucose; (e) 0 mM glutamine: 30 mM glucose; (f) 5 mM glutamine: 3 mM glucose; (g) 5 mM glutamine: 0.3 mM glucose; (h) 5 mM glutamine: 0.1 mM glucose; (i) 5 mM glutamine: 0 mM glucose.

The cultures were agitated (170 rpm) at 28°C for 3 h.

1.2.5. Halflife of induced enzyme. Cells were grown in GGY medium for 18 h at 28°C with agitation (170 rpm). The culture was directly induced with 5 mM L-glutamine for 3 h in the same growing condition. The cells were collected and transferred to starvation medium and brought to the original culture volume. The cells were agitated (170 rpm) at 28°C for 0, 1, 2, 3, and 5 h.

2. PURIFICATION OF GLUTAMATE DEHYDROGENASE

The purification of NAD-GDH is described under RESULTS.

3. GLUTAMATE DEHYDROGENASE

3.1. Glutamate Dehydrogenase Assay

Assays of glutamate dehydrogenase activity were performed either spectrophotometrically or colourimetrically in gradient or non-gradient polyacrylamide gels and agarose gels.

3.1.1. Spectrophotometric assay. Glutamate dehydrogenase reductive amination reaction was performed routinely during enzyme purification. The method used has been described (Stevenson, 1974). Sometimes, the oxidative deamination reaction was carried out. The reaction mixture was composed of 67 mM Tris-acetate, pH 8.0, 100 mM L-glutamate, 2.67 mM NAD^+ with or without $NADP^+$ (0.167 mM). In all cases, initial velocity measurements were carried out with a Gilford model 2400 multiple recording spectrophotometer using a 3 mL silica cuvette (1 cm light path) at room temperature. Enzyme activity is expressed as nmol NADH formed or consumed per min at 340 nm. When possible, the quantity of the enzyme used per assay was sufficient to produce a continuous linear change in optical density for at least 2 min.

3.1.2. Colorimetric assay. Non-denaturing gradient polyacrylamide gels were prepared with a gradient maker from Buchler by mixing 2 mL solution A (Tris-base 36.3 g, 1 N HCl 48 mL, TEMED 0.23 mL, distilled water to a total volume of 100 mL, pH was adjusted to 8.9 with 1 N HCl), 18 mL solution C (Acrylamide 28 g, BIS 0.735 g, distilled water to a total volume of 100 mL. The solution was filtered through Whatman No. 1 filter paper), 160 μL 10% ammonium persulphate, in the container connected to the outlet, and 2 mL solution A, 2 mL solution C, 15.9 mL distilled H_2O, and 160 μL 10% ammonium persulphate in another container for gel slabs of dimensions 180 mm x 110 mm x 1.5 mm. As soon as the gel flowed to the right position a layer of alcohol was loaded on top and the gel allowed to polymerize for 30 min. This gel contained an acrylamide gradient from 3% to 25%. An agarose solution of 1% was added on top to make wells

following polymerization. The electrophoresis buffer consisted of 124 mM Tris-base, 27 mM barbituric acid, 1 mM EDTA, pH 8.7 at 4°C. The buffer and gel were pre-chilled to 4°C before pre-electrophoresis that was carried out at 100 V for 60 min in a cold room (4°C). Samples were brought to 15% sucrose before loading. High molecular weight protein markers from Pharmacia Fine Chemicals were used to compute the M_r of the native enzyme. Electrophoresis was carried out at 200 V (20 mA approximately) for 60 h in the cold room. The gel was stained in 50 mL activity staining solution containing 100 mM Tris-HCl, pH 9.0, 50 mM sodium glutamate, 1.2 mM NAD^+, 0.26 mM $NADP^+$, 0.07 mM phenazine methosulphate, 0.5 mM nitroblue tetrazolium dye at room temperature in the dark for 1 h. The reaction was stopped by rinsing the gels exhaustively with distilled water.

The non-denaturing, non-gradient polyacrylamide gels were prepared by mixing 2.5 mL solution A, 2.86 mL solution C, 0.2 mL 10% ammonia persulphate, 14.44 mL H_2O to make gel rods. The electrophoresis buffer was the same as that used in "gradient gel". Electrophoresis was carried out at 5 mA/gel for 4 h at 4°C. The enzyme activity staining was carried out in small test tubes with the same solution as "gradient gel".

Agarose gel (1%) was prepared as slabs of dimensions (140 x 110 x 7 mm). After pre-electrophoresis at 100 V for 30 min at 4°C, samples were loaded and electrophoresis was carried out at 150 V for 3 h at 4°C. Following electrophoresis, the plate was taped and activity staining was carried out directly on the plate.

Samples used for glutamate dehydrogenase activity assay were either crude cell extracts, or partially purified or purified glutamate dehydrogenase solutions. In the enzyme induction experiments, cell-free

extracts were prepared by sonicating the cells in a Sonic Dimembrator Model 300 at 60% of maximum speed in TPEDGP buffer (3 mL buffer for each gram of wet weight cells) at 0°C for 4, 30 s cycles. The sample were then centrifuged at 25,000 x g for 20 min at 4°C. The supernatant was used to assay enzyme activity spectrophotometrically and/or colorimetrically and to determine protein concentration.

3.2. Protein Concentration Determination

Protein concentration of most samples was determined routinely by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as the standard. In some cases, protein concentration of protein markers in Sepharose 6B column effluent was determined spectrophotometrically at 280 nm, following the method of Layne (1957), originally described by Kalcker (1947). Protein concentration in the samples from late steps of enzyme purification was too low to be determined by the method of Lowry *et al.*. Therefore, the protein concentrations were estimated by electrophoresing the samples in SDS-polyacrylamide gel with bovine serum albumin as the standard and staining with silver as described later.

3.3. Molecular Mass (M_r) Estimation

The molecular mass (M_r) of the subunit of glutamate dehydrogenase was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 4% polyacrylamide stacking gel and 12% polyacrylamide separating gel by vertical electrophoresis, a method initially reported by Shapiro *et al.*

(1967) and further experimentally verified by Weber and Osborn (1969) and others (DunKer and Rueckert, 1969; Neville, 1971). The separating gel (180 mm x 110 mm x 0.7 mm) was prepared by mixing 5.0 mL solution A, 8.4 mL solution C, 100 μL 20% SDS, 250 μL 10% ammonium persulphate, 6.25 mL distilled water. Alcohol was layered on top of the gel while it was polymerizing. The stacking gel was prepared by mixing 1.25 mL solution B (Tris-basae 5.98 g, 1 N HCl 35 mL, TEMED 0.46 mL, distilled to a total volume of 100 mL, pH was adjusted to 6.7 with 1 N HCl.), 0.7 mL solution C, 30 μL 20% SDS, 80 μL 10% ammonium persulphate, and 3.0 mL distilled water. Protein samples were dissolved in sample buffer (330 mM Tris-base, 2.0% SDS, 3.2 mM ß-mercaptoethanol, 5.4 M glycerol, 30 mM bromophenol blue) and boiled for 2 min. The running buffer contained 192 mM glycine, 25 mM Tris-base and 0.1% SDS. The following protein markers were used as references: myosin H-chain M_r 200,000 (prestained, M_r 211,400); phosphorylase b M_r 97,400 (prestained, M_r 107,000); bovine serum albumin, M_r 68,000 (prestained, M_r 69,300); ovalbumin M_r 43,000 (prestained, M_r 45,800), carbonic anhydrase, M_r 29,000 (prestained, M_r 28,700), ß-lactoglobulin M_r 18,400 (prestained, M_r 18,200) and lysozyme, M_r 14,300 (prestained M_r 15,400). After running at 250 V for 4 h or after the dye ran out of the gel, it was fixed with 12.5% TCA-4% sulphosalicylic acid-30% methanol solution. Protein staining was carried out with 0.2% Coomassie Blue R 250 for 2 h. The gel was destained with methanol:acetic acid:water solution (5:10:85).

Silver stain was also carried out on SDS-PAGE for samples from late steps of enzyme purification with silver staining kits from Bio-Rad and Sigma, by methods described by Merril et al. (1981) and destaining methods

described by Switzer et al. (1979).

Molecular mass of the native glutamate dehydrogenase was determined using non-denaturing gradient polyacrylamide gel electrophoresis as described under "Glutamate Dehydrogenase Assay" with the following protein markers as references: thyroglobulin, M_r 669,000; ferritin, M_r 440,000, catalase, M_r 232,000; lactate dehydrogenase, M_r 140,000, and bovine serum albumin, M_r 69,000. The sample-containing gel was cut out and stained for glutamate dehydrogenase activity. The markers-containing gel strip was stained with Coomassie Blue R250.

Molecular mass of native glutamate dehydrogenase was also determined using a Sepharose 6 B column. Sepharose 6 B in the original suspension was used to pack the column directly (1.5 cm x 40 cm, 70 mL). After column packing, three bed volumes of enzyme column buffer (TED), pH 8.3, containing 0.5 M KCl was run through the column at 4°C. Blue Dextran 2000 was used to determine the void volume. Mixed protein markers (thyroglobulin, M_r 669,000, bovine liver GDH, M_r 336,000; BSA, M_r 69,000) in 1 mL containing 20% sucrose were applied to the column underneath the buffer. This was done by loading the sample with a syringe connected to a piece of fine capillary tubing that was held at one end a few mm above the gel bed surface. The flow rate was 5 mL/h. Each fraction collected was 0.5 mL. Protein concentration in the effluent was monitored spectrophotometrically at 280 nm by the method described by Layne (1957). Enzyme samples (1 mL) containing 20% sucrose were also applied to the column in the same way. The flow rate was the same as that used in protein markers elution. The glutamate dehydrogenase elution curve was determined by measuring GDH activity spectrophotometrically at 340 nm

using the reductive amination reaction with $NADP^+$ activation.

3.4. Glutamate Dehydrogenase Stability Analysis

The stability of glutamate dehydrogenase was tested with and without glycerol and $(NH_4)_2SO_4$ at 0°C (see RESULTS). The stability of glutamate dehydrogenase was also assayed under the protection of different reagents at 37°C for a fixed time (see RESULTS).

4. IMMUNOLOGICAL TECHNIQUES

4.1. Polyclonal Antibody Preparation

Antiserum against the glutamate dehydrogenase subunit excised from SDS-PAGE slabs was raised in a New Zealand white rabbit (ca. 6 months old, 3 kg) by intramuscular injection of 100 μg glutamate dehydrogenase together with complete Freund adjuvant at six positions of both legs. A second subcutaneous injection of 50 μg enzyme protein, together with incomplete Freund adjuvant, was given 3 weeks later at four positions in each leg. Eleven days later, 1.2 mL blood sample was removed from the rabbit and 400 μL serum was obtained. This serum was used as a source of anti-glutamate dehydrogenase antibody in Western transfer blotting experiments (see the following part) and ELISA tests to check the quality of the antiserum. As the antiserum was active in Western transfer blotting experiments, 50 μg glutamate dehydrogenase, together with incomplete Freund adjuvant was injected at four positions of each leg to

enhance the antibody. Two weeks after the last injection, 75 mL blood sample was obtained from the rabbit and stored in glass tubes. The sample was allowed to clot at room temperature for 4 h. As soon as the blood clotted, the edges of the clot were rimmed with a pasture pipet. Antiserum was obtained by centrifuging at 121 x g for 5 min and stored at -20°C in 1 mL aliquots in Eppendorf tubes. The pellets were kept at 4°C overnight and some additional antiserum was obtained by centrifuging at 121 x g for 10 min. A total of 35 mL of antiserum was recovered by this method.

4.2. Antibody Property Test

The properties of the antiserum were tested quantitatively and qualitatively.

To obtain an equivalence point in quantitative precipitation of antibody and antigen, a procedure described by Jamieson *et al.* (1972) was used and modified in some steps. Reaction mixtures were prepared (total volume 0.45 mL) to contain 100 μg glutamate dehydrogenase, 0.15 M NaCl, 1 mM sodium azide, and 4.7% dextran T70. Antiserum (50 μL) was then added to each mixture tube. The mixtures were incubated at 37°C for 2 h and then allowed to stand at 2°C for 24 h. The precipitate was collected by centrifuging at 4°C for 10 min and washed with 1 mL of 0.15 M NaCl. The precipitate was dissolved in 1 mL 0.1 M NaCl. A portion of 700 μL from each sample was removed for protein concentration determination by the method of Lowry *et al.* (1951).

An ELISA test was carried out by the method described by Douillard

and Hoffman (1983). A series of antiserum dilutions were incubated against different antigen dilutions proportionately according to the equivalence point obtained above. Each well of an ELISA plate was initially coated with a suitable amount of antigen in 100 μL 0.05 M carbonate buffer, pH 9.5. The plates, coated with a blocking buffer composed of 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20, 1 mM sodium azide and 5% skimmed milk powder (TNTSS) containing 3% bovine serum albumin, were then incubated with a series of dilutions of antigen (100 μL) obtained above and then incubated with 100 μL of enzyme-labelled second antibody (anti-rabbit IgG) diluted at 1:3,000. The enzyme substrate (100 μL) was incubated for 30 min at room temperature and the absorbance was determined at 490 nm spectrophotometrically.

Achlya klebsiana cell-free extract and partially and completely purified glutamate dehydrogenase were electrophoresed in 7% acrylamide SDS-PAGE at 65 V overnight. The immunostaining was slightly modified from the method described by Timmons and Dunkar (1990). Proteins were electroblotted (Biorad Trans-Blot) onto nitrocellulose using a buffer consisting of 0.248 M Trizma base and 1.87 M glycine, pH 8.5. Electroblotting was carried out at 4°C for 10 h at 350 mA (15 V approximately). Immediately after protein transfer, the nitrocellulose membrane was incubated in a blocking buffer (TNTSS) at room temperature overnight with agitation (100 rpm). The membrane was washed briefly twice with TNTSS devoid of skimmed milk and sodium azide (TNT). Anti-glutamate dehydrogenase serum was diluted 5,000-fold with a volume of TNTSS sufficient to cover the membrane and incubated for 4 h or overnight with gentle agitation (100 rpm). The membrane was washed three times in TNT

(30 min each) with agitation (120 rpm), then immunostained by incubating with 7,500x diluted anti-rabbit IgG (H and L chains) alkaline phosphatase conjugate (Promega Corp. Madison, WI) for 2 h in TNTSS devoid of sodium azide. The membrane was washed three times as before and mopped dry by filter paper. The protein bands were colour developed with the substrate nitroblue tetrazolium (NBT, 50 μg/mL in 70% dimethylformamide) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 50 μg/mL in 70% dimethylformamide) by mixing 200 μl NBT stock solution in 30 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), and the solution was then mixed with 100 μl BCIP. When the colour developed to the desired intensity, the reaction was stopped by incubating the membrane in 5% acetic acid solution. The result was compared to a silver stained SDS-PAGE gel showing glutamate dehydrogenase position and protein markers.

5. cDNA LIBRARY TECHNIQUES

5.1. Preparation of Total RNAs

Spores obtained from 5 plates were grown in 1 litre of defined medium at 28°C for 18 h with agitation (170 rpm). The stationary cells were transferred to 1,000 mL starvation medium in flasks by the techniques described earlier for enzyme induction in the presence of 5 mM L-glutamine and agitated (170 rpm) for 0, 1, 2, 3, 5 h at 28°C. Cells were collected by filtering with a vacuum pump. Cells were also grown in GGY medium for 18 h at 28°C with agitation (170 rpm) and then further induced for NAD-GDH

activity for 3 h with 5 mM L-glutamine in the same growing condition. In both cases, immediately after filtering, cells were frozen with liquid nitrogen and then either stored at -70°C or used immediately.

Total RNA was isolated by the guanidinium thiocyanate : phenol : chloroform extraction method (Chomczynski and Sacchi, 1987). When two phases did not appear after centrifugation, the procedure was modified by adding 0.15 volume of chloroform-isoamyl alcohol instead of one-tenth volume of chloroform-isoamyl alcohol. The extracted RNA was dissolved in RNase-free distilled water.

5.2. mRNA Purification and Its Translation *In Vitro*

Two oligo(dT)-cellulose columns were prepared by loading two 5-mL hypodermic syringes with presoaked oligo(dT)-cellulose (Pharmacia), each with a bed volume of 2 mL. The columns were washed with 10 bed volumes of sterile distilled water, 5 bed volumes of 0.1 M NaOH, and 10 bed volumes of binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl, 0.5% SDS). RNA solution (1 mL) was incubated at 65°C for 10 min and allowed to cool to room temperature. An equal volume of loading buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 M NaCl, 0.5% SDS) was added to the RNA solution. This mixture was applied to one of the oligo(dT)-cellulose columns at room temperature. The effluent was collected and reapplied to the column which was then incubated for 20 min and washed with 5 bed volumes of wash buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl). The sample was eluted with sterile TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and collected in 0.5 mL fractions. The RNA-containing fractions were

identified by mixing 2 μL of each fraction with 20 μL ethidium bromide solution (1 μg/mL) and exposing the mixture to ultraviolet light. Fractions containing most of the poly(A)$^+$ RNA were pooled together. The pooled sample was heated at 65°C for 10 min and brought to room temperature before applying to the second oligo(dT)-cellulose column. Sample application, reapplication, elution, fraction collection and mRNA identification were the same as previously described. The concentration of the pooled mRNA sample was estimated by comparing it to RNA standards using the ethidium bromide staining technique. The mRNA sample was distributed into small aliquots in microfuge tubes and precipitated by adding 0.2 volume 2 M NaCl and 3 volumes cold (-20°C) absolute ethyl alcohol. RNA was stored under ethyl alcohol at -20°C. Just before use, the mRNA was collected by centrifuging at 14,000 x g at 4°C for 15 min, washed with 70% alcohol, dried by vacuum and dissolved in 20 μL TE buffer, pH 7.5.

A Poly(A) Quik mRNA Purification Kit from Stratagene was also used for mRNA purification. The procedure provided by the manufacturer was followed.

A kit purchased from Promega was used for translation *in vitro* following the manufacturer's method. Brome mosaic virus RNA was used as control. The translation products were analyzed in a standard scintillation counting machine.

5.3. cDNA Synthesis and Ligation

Two systems for synthesising cDNA were used; one from a Pharmacia

cDNA synthesis kit, the other from a Promega kit.

In the Pharmacia system, five μg mRNA was used to synthesise double-stranded cDNA according to the manufacturer's instruction. The effluent recovered from the last step was used for cDNA ligation.

cDNA for ligation was recovered by mixing 1 μg poly(A)$^+$ RNA:cDNA equivalent (62 μL), 2.5 μg λgt11 vector, 6 μL 3 M sodium acetate, pH 5.2, 120 μL absolute ethanol and chilled at -70°C for 15 min. The mixture was spun for 15 min in a microcentrifuge and the pellet was dried. The ligation reaction was carried out according to the manufacturer's instructions.

For the Promega system, the procedure was a modification of the manufacturer's instructions. 3 μg mRNA was used to synthesize the first strand of the cDNA in a total volume of 35 μL, which contained 1.6 μg oligo(dT) primer, and 30 units reverse transcriptase. The second strand reaction was the same as described in the manual. The precipitated and dried double-stranded cDNA was dissolved in 30 μL TE buffer, pH 7.5, and size-fractionated (using Sepharyl S-400 columns provided in the kit) to eliminate small size cDNA. The cDNA solution was brought to 30 μL and then ligated to EcoRI adaptors in a total volume of 60 μL at 14°C overnight. The next morning, 6 additional units of T4 DNA ligase was added and the reaction incubated for a further 3 h, followed by a kinase reaction in a volume of 80 μL containing 20 units of T4 polynucleotide kinase. The kinased product was extracted with phenol:chloroform followed by the removal of unligated adaptor using a Sepharyl S-400 column. The cDNA solution was concentrated to 10 μL.

Ligation of cDNA and vector was carried out by mixing 2 μL of the

cDNA with adaptor, 1 μg λZap II vector in a volume of 10 μL containing 1 mM ATP, pH 7.5, and 2 units of T4 DNA ligase. The reaction was incubated at 14°C overnight. Another 2 units of T4 DNA ligase was added and again, the reaction was incubated at 14°C overnight followed by *in vitro* packaging (Stratagene Cloning System).

5.4. cDNA Library Generation, Titration and Amplification

The packaging procedure was carried out according to the instructions in the packaging kits from either Stratagene or Promega. The cDNA library was stored at 4°C.

In library titration, a series of dilutions of the library was plated on LB plates (10 g tryptone/L + 5 g yeast extract/L + 10 g NaCl/L + 15 g agar/L, pH 7.5) containing ampicillin (10 mg/mL). The diluted phage was absorbed directly to 100 μL indicator host bacteria *E. coli* Y1090(r^-) (Snyder *et al.*, 1987), which had been grown overnight at 37°C in LB medium with shaking, at 37°C for 25 min. Following the addition to 3 mL melted top agar (50°C) of 20 μL IPTG (20 mg/mL in H_2O), 20 μL X-gal (50 mg/mL in dimethylformamide) and 10 mM $MgCl_2$ (Calos *et al.*, 1983), the phage-infected culture was mixed with the top agar and immediately plated onto an LB agar plate. The cDNA library titration and the percentage of recombination were determined using the same plates.

The amplification of the λgt11 library was carried out by absorbing 63,000 pfu to 1.6 m overnight culture *E. coli* Y1090 (r^-) at 37°C for 20 min. The culture was plated onto 4 large Petri plates (150 mm) and incubated at 42°C for 6 h. Twelve mL of phage buffer was added to each

plate. After incubation at room temperature for 2 h, the phage suspension was recovered and extracted with 0.3 volumes of chloroform. A series of dilutions (10^{-4} to 10^{-9}) were plated out to titer the amplified library.

5.5. cDNA Library Screening with Anti-Glutamate Dehydrogenase Antibody

A portion of the cDNA library containing 1.0×10^4 pfu of original library was incubated with 200 μL of overnight culture 1090(r-) for 25 min at 37°C. The mixture was spread onto LB plates (90 mm). The plates were incubated at 42°C for 5 min without the lid, then for 3.5 h with the lid and then inverted. The plate was moved to 37°C and carefully overlaid with a dry nitrocellulose membrane which had been saturated previously with 10 mM IPTG in water and on which 200 ng GDH was dotted as a positive control. The plate was incubated at 37°C for 4 h. Most of the following screening procedure was the same as in " Antibody Property Test ". Labelled at 3 positions, the membranes were saturated with TNTSS buffer for 4 h and incubated in the purified *E. coli* 1090(r^-) protein-free anti-NAD-GDH antibody for 2 h at room temperature. The membranes were washed three times (1 h each) with TNTS buffer and then incubated with anti-rabbit antibody conjugate for 2 h. The membrane was washed three times (1 h each) with TNTS buffer before colour development. Positive plaques were confirmed and purified further twice by the same screening procedure.

5.6. Purification of Anti-GDH Antibody

As the polyclonal antiserum obtained contained *E. coli* protein

antibodies, they had to be removed from the polyclonal antiserum before it could be used to immunoscreen *Achlya* cDNA:λgt11 libraries expressed in *E. coli*.

IgG of the polyclonal antiserum was purified by a modification of the method of Palmiter *et al.* (1971). Polyclonal antiserum (5 mL) was diluted with an equal volume of phosphate buffer (10 mM sodium phosphate, pH 7.3, 15 mM NaCl). The diluted antiserum was brought to 40% saturation with saturated $(NH_4)_2SO_4$, pH 7.0, (neutralised with NH_4OH). After thorough mixing, IgG was recovered as a precipitate by centrifuging at 15,000 x g for 10 min at room temperature and dissolved in 6 mL phosphate buffer. The solution was brought to 40% saturation with $(NH_4)_2SO_4$ and incubated at 4°C overnight. The precipitate was pelleted at 1,000 x g for 15 min and then dissolved in 5.5 mL phosphate buffer; each mL contained 2.4 mg IgG. One and a half mL of this solution was used for anti-GDH antibody purification by affinity chromatography.

The method of purifying an antibody by affinity chromatography has been described by de Wet *et al.* (1984). *E. coli* 1090(r^-) was grown in LB medium at 28°C overnight with agitation (170 rpm). The cells were recovered and resuspended in 100 mL 0.1 M $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl. The suspension was incubated with 200 mg lysozyme for 20 min at room temperature followed by the addition of 1 mg pancreatic DNase I and 200 μL Triton X-100 and incubation for 1.5 h at 4°C. The supernatant was obtained by centrifuging at 8,000 x g for 20 min at 4°C, the pH was adjusted to 9.0 with 1 N NaOH, and the supernatant was kept on ice until used. Cyanogen-bromide-activated Sepharose 4B (1 gram) was washed with 1 mM HCl on a sintered glass filter and then mixed with the supernatant. The mixture was

mixed while incubating in an end-over-end machine at 4°C overnight. The gel was packed into a column. The unbound active sites of the gel were blocked by flushing the column with 100 mL 0.1 M $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl (50 mL/h) followed with 100 mL 1 M Tris-Cl, pH 8.0 (50 mL/h). Unbound proteins were washed off with 3 cycles (50 mL each) of 0.1 M $NaHCO_3$ buffer, pH 8.3, and 0.1 M acetate buffer, pH 4.0, each containing 0.5 M NaCl. After equilibration with 50 mM Tris-Cl, pH 8.0 buffer, containing 150 mM NaCl, the gel was incubated with 1.5 mL of the purified IgG in an end-over-end machine at 4°C overnight. The slurry was packed into a column and washed with 25 mL 50 mm Tris-Cl buffer, pH 8.0, containing 150 mM NaCl. The effluent was distributed in 1.5 mL aliquots into Eppendorf tubes and stored at -20°C.

Further purification of anti-GDH antibody was carried out by immunoadsorption to *E. coli* proteins on λgt11 plaques on filters as follows. λgt11 (10^5 pfu) was absorbed to 400 μL overnight culture *E. coli* 1090(r^-) at 37°C for 25 min and then plated onto 2, 90-mm nutrient agar plates. The plates were incubated at 42°C for 4 h. Two nitrocellulose membranes were overlaid onto the two plates and left overnight at 37°C. The membranes were taken out and overlaid in reverse onto another two plates prepared as above. Following an incubation of 5 h at 37°C, the membranes were incubated in blocking buffer overnight at room temperature. The membranes were repeatedly incubated in 25 mL blocking buffer containing 50 μL affinity chromatography purified anti-GDH antibody at room temperature for 4 h each so as to effectively adsorb *E. coli* protein antibodies. The 25 mL antibody-containing blocking buffer was recovered and stored at 4°C at the presence of 1 mM NaN_3.

Purification of the antibody was monitored by using it to immunostain a plaque-blotted nitrocellulose membrane on which 200 ng NAD-GDH from *Achlya* was dot blotted. An acceptable purity was shown by the disappearance of plaque colouration and the maintenance of GDH dot colouration.

5.7. Fusion Protein Preparation

Fusion proteins were prepared (Snyder *et al.*, 1987) from 4 positive recombinants and 1 negative recombinant by infecting 1.6×10^9 phages to 3.2×10^8 *E. coli* Y1089 cells in a volume of 1 mL. The cultures were incubated at 37°C for 1 h when the bacteria lysed. The lysates were collected by centrifuging at 20,000 rpm for 20 min. The supernatants were frozen at -70°C and then lyophilized overnight. The powder containing the fusion proteins was resuspended in 100 μL H_2O.

5.8. Amplification of λgt11 Recombinant and Purification of Its DNA

Small scale amplification was carried out by infecting 200 μL *E. coli* Y1090(r^-) culture with 5×10^4 pfu from purified plaques at 37°C for 25 min. The infected cells were plated on LB agar (90 mm) and incubated overnight at 37°C. Five mL of phage buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01 % gelatin) was added to each plate. The plates were agitated (80 rpm) at room temperature for 2 h. The suspension was collected, extracted with 0.3 volume of chloroform and clarified by centrifuging at 5,000 rpm for 10 min. The titer was determined by the

described above without the addition of IPTG and X-gal.

Large scale amplification was carried out by adsorbing 2 X 10^8 phages to 3 x 10^{10} *E. coli* Y 1090 (r-) directly obtained from an overnight culture. The infected cells were incubated at room temperature for 25 min and then used to inoculate 1 litre of prewarmed (37°C) LB medium containing 10 mM $MgSO_4$. The infected cultures were agitated vigorously for 7 h until the bacteria lysed.

The purification of phages and recombinant DNA was the same as described by Maniatis *et al.* (1982).

5.9. cDNA Insert Investigation and Generation by PCR

Proof that the λgt11 positive cDNA recombinants contained inserts was accomplished by *in vitro* cloning using the PCR technique (Sambrook *et al.*, 1989). Two oligonucleotide sequences close to the insert sites on λgt11 arms were synthesized as primers for insert generation by PCR (polymerase chain reaction). The forward primer (20 bp) was 5'd GGT GGC GAC GAC TCC TGG AG 3'. The reverse primer (20 bp) was 5'd TTG ACA CCA GAC CAA CTG GT 3'. Extraction of synthesized oligonucleotide primer was according to the method provided by the company. In the PCR amplification, 5 ng of template DNA (λBY2 DNA), 200 ng each of the forward and reverse primers and 2 units of Taq DNA polymerase were used. The denaturing,annealing, and reaction temperatures were 93°C, 50°C and 72°C respectively. The reaction was repeated for 35 cycles. An aliquot of 5 μL was removed from each PCR product and electrophoresed in agarose gel to check the quality of the products. The rest of the products were precipitated with alcohol and

resuspended in 300 μL TE buffer.

5.10. RNA Hybridisation

Total RNAs were prepared by the methods described above from cells grown in defined medium and induced with 5 mM L-glutamine for 0, 1, 2, 3, 5 h. Total RNAs were electrophoresed in 1% agarose by the methods described in "membrane transfer and detection methods" (Amersham, 1985). The RNAs were then transferred to nylon Hybond N^+ with 20 x SSPE (3.6 M NaCl, 0.2 M NaH_2PO_4, pH 7.7, 0.02 M Na_2EDTA) or with 0.05 M NaOH overnight at room temperature. The Hybond N^+ was then fixed by the method described in "protocols for nucleic acid blotting and hybridization" (Amersham, 1988) and stored at 4°C wrapped in Saran Wrap.

The fixed membrane was prehybridised in a solution composed of 50% formamide, 10% dextran sulphate, 1% SDS, 20 μg denatured non-homologous salmon sperm DNA/mL at 42°C for 5 h. A probe was prepared by using 0.2 μg PCR product of λBY2 and ^{32}p dATP (10 mci/mL) as described by Sambrook *et al*. (1989). The synthesized probe was denatured by incubation in a boiling water bath for 10 min and chilling in ice water for 5 min. The probe was added by injecting it into the prehybridisation bag using a hypodermic syringe. Hybridis ation was carried out at 42°C for 15 h. The membrane was then washed in 5 X SSPE, 2 X SSPE, 2 X SSPE + 0.1% SDS, 1 X SSPE, 1 X SSPE + 0.1% SDS (15 min each at 42°C) until the background of radioactivity was low. The membrane was blotted dry, covered with saran wrap and exposed to x-ray film at -70°C with an intensifying screen overnight.

6. GENOMIC LIBRARY TECHNIQUES

6.1. Genomic DNA Preparation and Its Partial Digestion

Achlya klebsiana spore suspension containing 10^7 spores was inoculated into 50 plates of GYCaMg medium, grown for 2 days to produce fresh spores (amplified) which were then transferred to 4 litres of PYG medium (peptone 1.0 g/L, yeast extract 1.0 g/L, glucose 5.0 g/L) in 4 flasks. Cultures were agitated (170 rpm) at 28°C for 24 h. Cells were suction filter dried (52 g) and ground with liquid nitrogen to a fine powder. The powder was suspended in 200 mL buffer composed of 0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl, 0.1 M EDTA and 1% sarkosyl. The suspension was digested with 4 mg RNAse at 37°C for 1 h and then 20 mg proteinase K at 37°C for 3 h. High molecular weight genomic DNA was prepared by Dr. H. B. LéJohn by published procedures (LéJohn, 1989).

The restriction enzyme MboI was used to partially digest the genomic DNA. Partial digestion with 9.2 units/mg DNA at 37°C for 1 h gave DNA fragments primarily in the range of 15-23 kb. After partial digestion, 10 μL of the sample (containing 780 ng DNA) was electrophoresed in a 0.7% agarose gel. The rest (3,200 μL containing 249 μg DNA) was extracted once with water saturated phenol:chloroform (1:1) and once with chloroform:isoamylalcohol (24:1). The supernatant (3 mL) was precipitated with 0.1 volume of 3M sodium acetate, pH 5.2, 3 volumes absolute ethanol overnight at -20°C. The DNA was collected by centrifuging in a Micro-MB centrifuge for 20 min and washed with 75% ethanol. The DNA was dissolved in 100 μL TE buffer, pH 8.0.

6.2. DNA Fractionation and Ligation

A sucrose gradient (5 - 25%) was made in a 12 mL tube. The partially MboI-digested DNA was brought to 200 μL and incubated at 68°C for 10 min before loading on top of the sucrose gradient. DNA was fractionated by centrifuging at 40,000 x g at 20°C for 18 h using a Beckman L8-80 ultracentrifuge with a Ti 41 rotor. Fractions were collected in 0.5 mL approximately. Ten μL from each fraction was electrophoresed in a 0.4% agarose gel at 160 V for 3 h to determine the DNA size range. Those fractions containing DNA between 15 - 23 kb were pooled and precipitated with ethanol by the techniques described above and resuspended in 100 μL TE buffer. Again, a small amount of the sample was electrophoresed in a 0.7% agarose gel to check the quality of the DNA for ligation.

Ligation occurred in a mixture consisting of 0.5 μg *Achlya* DNA, 1.35 μg λEMBL3 arms, 0.5 mM $MgCl_2$, and 2 units of T4 DNA ligase in a total volume of 10 μL. Ligation mixture (10 μL) was incubated at 14°C overnight. Next morning, another 2 units of T4 DNA ligase was added followed by further incubation at 14°C overnight. Three μL of the mixture was then electrophoresed in a 0.7% agarose gel to ascertain whether ligation had occurred.

6.3. Genomic DNA Library Generation, Titration and Amplification

The packaging systems used were from Stratagene, Promega and Amersham. Ligation mixture (3 μL) was used for each packaging reaction. The procedures provided by the manufacturers were followed.

Titration of the genomic library was carried out on a series of library dilutions. A mixture composed of 100 μL diluted library, 100 μL freshly grown (5 h) or overnight *E. coli* $P_2$392 culture was incubated at 37°C for 30 min before plating on LB plates. The plates were incubated at 37°C overnight followed by titering.

The amplification of *Achlya* genomic library was carried out by absorbing 141,000 pfu to 1.6 mL of an overnight *E. coli* P_2 392 culture at 37°C for 20 min. The culture was plated onto 4 large Petri plates (150 mm) and incubated at 37°C for 8 h. Twelve mL phage buffer was added to each plate, and incubated at 4°C overnight. The phage suspension was recovered and mixed with 0.3 volumes of chloroform. A series of dilutions (10^{-4} to 10^{-9}) were plated out to titer the amplified genomic library.

6.4. Genomic Library Screening

6.4.1. Random Primer Labelling of DNA. The procedure and methods were as described by Sambrook *et al.* (1989). However, to simplify the procedure, the method was modified as follows. Template DNA (200 ng) and random primer (75 ng) in 12 μL were mixed by centrifugation in a screw capped microfuge tube (1.5 mL) and incubated in boiling water for 5 min. The tube was immediately transferred to ice water for 5 min and then quick spun for a few sec to pellet the solution. The reaction was initiated by adding 1 mM DTT, 0.25 mM dNTP (dCTP, dGTP, dTTP), 2 μL 10 x RP buffer (900 mM HEPES, pH 6.6, and 100 mM $MgCl_2$), 3 μL fresh α-^{32}P dATP (NEN product, 10 mci/mL), 5 units E. coli DNA polymerase I Klenow fragment. The mixture was pelleted by a quick spin and incubated at room temperature for 4 h.

The reaction was stopped by adding 20 μL stop buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, pH 8.0, 0.5% SDS). A suitable amount of water (300 μL approximately) was added to the reaction product. The labelled DNA was denatured by incubating in a boiling water bath for 5 min and plunging into ice water for 5 min before use in hybridisation.

6.4.2. Genomic library plating and blotting. Ten μL of original genomic library (4.7 X 10^4 pfu) was absorbed to 0.8 mL overnight culture of $P_2$392 in LB medium containing 10 mM $MgSO_4$ and 0.2% maltose. The mixture was incubated at 37°C for 30 min and plated on two 150 mm Petri plates. Following plaque development, the plates were blotted to Hybond N^+ nylon (Amersham Corp.) in duplicate using the method described in Maniatis *et al.* (1982).

6.4.3. Screening of *Achlya* Genomic library with other GDH genes.

The genomic library was screened with probes obtained from various sources of the GDH gene; *Chlorella* NADP-GDH cDNA from plasmid pGDc23 (this transformed plasmid was kindly supplied by Dr. R. R. Schmidt at The University of Florida), pλZ9 containing human GDH cDNA (kindly supplied by Dr. N. Moschonas, University of Crete); part of the NAD-GDH gene of *N. crassa* (plasmid pVG1 containing part of the structural gene was kindly supplied by Dr. M. Kapoor at the University of Calgary). The whole NAD-GDH gene of yeast, (plasmid pSM2 containing this gene was kindly supplied by Dr. B. Magasanik at the Massachusetts Institute of Technology). From amplified plasmids, the probes were made by random primer labelling and nick translation. The temperature for prehybridisation and hybridisation

was 55°C. The condition used for washing the hybridised filters depended on the level of radioactivity. Washing was brief if counts were relatively low, or extensive if the counts were relatively high. Putative positive colonies were selected and rescreened to a level of consistency compared to controls.

6.4.4. Genomic library screened with cDNA insert. Genomic library blotted membranes were prehybridized in the solution described in "Hybond-N^+, Protocols for Nucleic Acid Blotting and Hybridization" (Amersham) for 3 h. λBY2 insert cDNA was used as a probe. Hybridisation was carried out in a plastic container at 63°C overnight with gentle shaking. The washing condition was as follows: 2 X SSPE at 63°C for 10 min, 2 X SSPE + 0.1% SDS at 63°C for 10 min, 1 X SSPE at 63°C for 10 min, 1 X SSPE + 0.1% SDS at 63°C for 10 min. Positives were confirmed by rescreening the isolated clones (density of 200-300 plaques/90 mm Petri plate). Confirmed positives were purified by a third screening density of 10-100 plaques/90 mm Petri plate.

6.5. Amplification of Positive Clones and DNA Purification

Small scale amplification of positive genomic clones was done the same way as described for amplification of the λgt11 cDNA expression library. Seven of the positives were amplified in large scale reactions by the same method used for amplifying the λgt11 cDNA expression library. The recombinants of λEMBL3-GDH genomic DNA were purified by the method described by Maniatis *et al.* (1982).

6.6. Restriction Enzyme Mapping of Genomic Clones.

The method described by Legerski et al. (1978) was used for restriction enzyme mapping.

II MATERIALS

1. CHEMICALS.

Most of the chemicals used in this research were obtained from Sigma Chemical Company, St. Louis, MI and Fisher Scientific Company. Finest quality was used whenever possible.

Media were made from general laboratory reagents except for yeast extract which was purchased from Difco Laboratories.

Chemicals for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories.

Chemicals and vials for liquid scintillation counting were obtained from Kent Laboratories, Fraser Medical Supplies Ltd., Vancouver, B.C.

X-ray film was manufactured by Kodak.

2. MEMBRANES

Membrane filters (0.45 μm) were obtained from Millipore Corp. and Gelman Instrument Co.

Nitrocellulose membranes were obtained from Bethesda Research Laboratories and Bio-Rad Laboratories.

Hybond-C, Hybond C extra, Hybond-N, and Hybond-N^+ were purchased from

Amersham.

3. RESINS.

DEAE-cellulose, TEAE-cellulose, Cibacron Blue 3 GA-agarose, NAD-agarose, ß-nicotinamide-adenine dinucleotide-agarose, and cyanogen bromide activated Sepharose 4B were purchased from Sigma.

Sephadex G150, Sephadex G200, DEAE-Sephadex A25, Sepharose 6B, Dextran T70, Blue Dextran 2000 were obtained from Pharmacia.

Oligo(dT) cellulose was obtained from Sigma and Pharmacia.

4. KITS.

The *in vitro* translation kit, ligation kit, λgt11 system(kit), nick translation kit, and λEMBL3 system (kit) were obtained from Promega.

The Poly(A) Quik mRNA Purification Kit and predigested λZapII/EcoRI cloning kit were obtained from Stratagene.

The Random Primer labelling Kit, Standard RNA Kit, Standard DNA Kit and Standard Low Molecular Mass Protein Markers were obtained from BRL.

The Immunoscreening Kit was obtained from Proto Blot.

Standard High Molecular Protein Marker is a product of Pharmacia. Standard Prestained Low Molecular Protein Marker is a product of Bio-Rad.

The Silver Staining Kit was obtained from both Bio-Rad and Sigma.

The cDNA Synthesis Kit was obtained from both Pharmacia and Promega.

The *In vitro* Packaging Kit was obtained from Promega, Stratagene and Amersham.

5. ENZYMES AND RADIOISOTOPES

Enzymes were obtained from BRL, Pharmacia and Promega. Radioisotopes were obtained from Du Pont.

RESULTS

1. PURIFICATION AND CHARACTERIZATION OF ENZYME

1.1. Enzyme Protection and Purification.

1.1.1. Enzyme Protection. The enzyme, NAD-linked glutamate dehydrogenase of *Achlya klebsiana*, is quite stable in cells stored at -70°C. The cells were therefore stored in this manner until a sufficient quantity of cells was obtained before initiating the isolation and purification of the enzyme. Once purified, however, the enzyme was unstable even when stored frozen at -70°C. It was necessary, therefore, to develop conditions that stabilized the enzyme during and after purification. What follows, is an account of that attempt.

The stability of glutamate dehydrogenase was tested by incubating unfractionated crude cell extract (obtained by the method of sonication mentioned earlier) of NAD-GDH induced cells in a solution containing 50 mM Tris-HCl, pH 7.3, 10 mM K_2HPO_4, 1 mM EDTA, 1 mM DL-dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride (TPEDP) at 0°C for the following times: 5,

10, 15, 25, 35, and 40 days. The results (Fig 2) show that the half life of the enzyme was 6.3 days when stored in TPEDP at 0°C. The activity decreased by 90% after 23 days of storage. After 40 days, no enzyme activity could be detected. In the presence of 20% glycerol, the enzyme was more stable. Its half life doubled (13 days), and after 23 days, the enzyme activity had diminished by 74%. In the presence of 20% glycerol and 42% $(NH_4)_2SO_4$, the half life of the enzyme increased to 21 days. After 23 days, the enyzme activity had diminished by 54% at 0°C. Thus, both $(NH_4)_2SO_4$ and glycerol could be incorporated into a purification protocol.

The concentrations of glycerol and ammonium sulphate used were arbitrarily selected. To determine the ideal condition that would provide maximum protection against enzyme inactivation, kinetic studies were conducted in which a wide concentration range of the protective agents was used. Enzyme in cell free extract was incubated with either glycerol (0-50%), NaH_2PO_4 (0-0.3M), KCl (0-0.8M), $(NH_4)_2SO_4$ (0-0.8M), or combinations of glycerol (20%) and KCl (0-0.8M), glycerol (20%) and $(NH_4)_2SO_4$ (0-0.8M) or glycerol (20%), KCl (0.4M) and $(NH_4)_2SO_4$ (0.4M). After incubation at 37°C for 20 min, the samples were chilled on ice for 5 min, then brought to room temperature before the enzyme activity was determined. The results show that, except for NaH_2PO_4 (results not shown), all agents effectively protected the enzyme against denaturation (Figs. 3-5). Less than 10% of the enzyme activity was lost in 50% glycerol (Fig. 3), 0.8M KCl (Fig. 4) and 0.8M $(NH_4)_2SO_4$ (Fig. 4). Less than 5% of the activity was lost when glycerol and KCl were used simultaneously (Fig 5), while the enzyme was fully protected in the presence of glycerol (20%), KCl (0.8 M) and $(NH_4)_2SO_4$ (0.8 M) (results not shown).

Figure 2. Stability of Unpurified NAD-GDH in the Presence and Absence of Glycerol and $(NH_4)_2SO_4$

NAD-specific glutamate dehydrogenase was obtained from glutamine induced cells by grinding with liquid nitrogen, and extracting with TPEDP buffer (3 mL/g of wet weight cells). The enzyme was recovered in the supernatant by centrifuging at 20,000 X g for 20 min. Stability analysis of NAD-GDH was carried out by incubating the extract with and without 20% glycerol and 42% $(NH_4)_2SO_4$ at 0°C for 40 days. Enzyme activity of the extract was determined at different times as described. The data in the figure are the results for a single experiment.

1: with 20% glycerol and 42% $(NH_4)_2SO_4$

2: with 20% glycerol

3: control

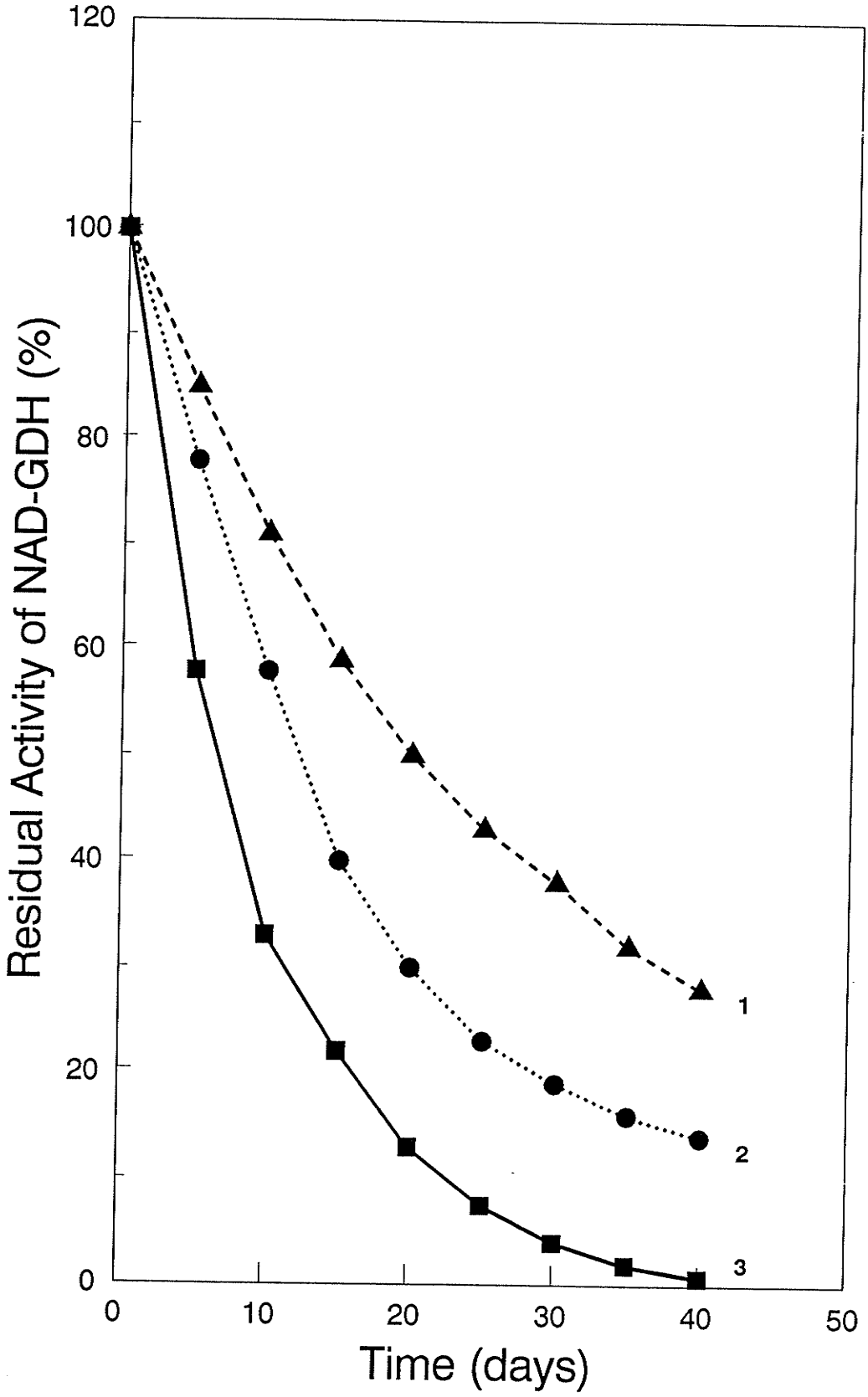

120
100
80
60
40
20
0
Residual Activity of NAD-GDH (%)
0
10
20
30
40
50
Time (days)
1
2
3

Figure 3. Stability of NAD-GDH in the Presence of Glycerol

NAD-specific glutamate dehydrogenase was obtained from glutamine induced cells by grinding with liquid nitrogen and extracting with TPEDP buffer (3 mL/g of wet weight cells). The enzyme was recovered in the supernatant by centrifuging at 20,000 X g for 20 minutes. Determination of the stability of NAD-GDH was carried out by adding different concentrations of glycerol to the extract, heating to 37° C for 20 min and then assaying for residual enzyme activity.

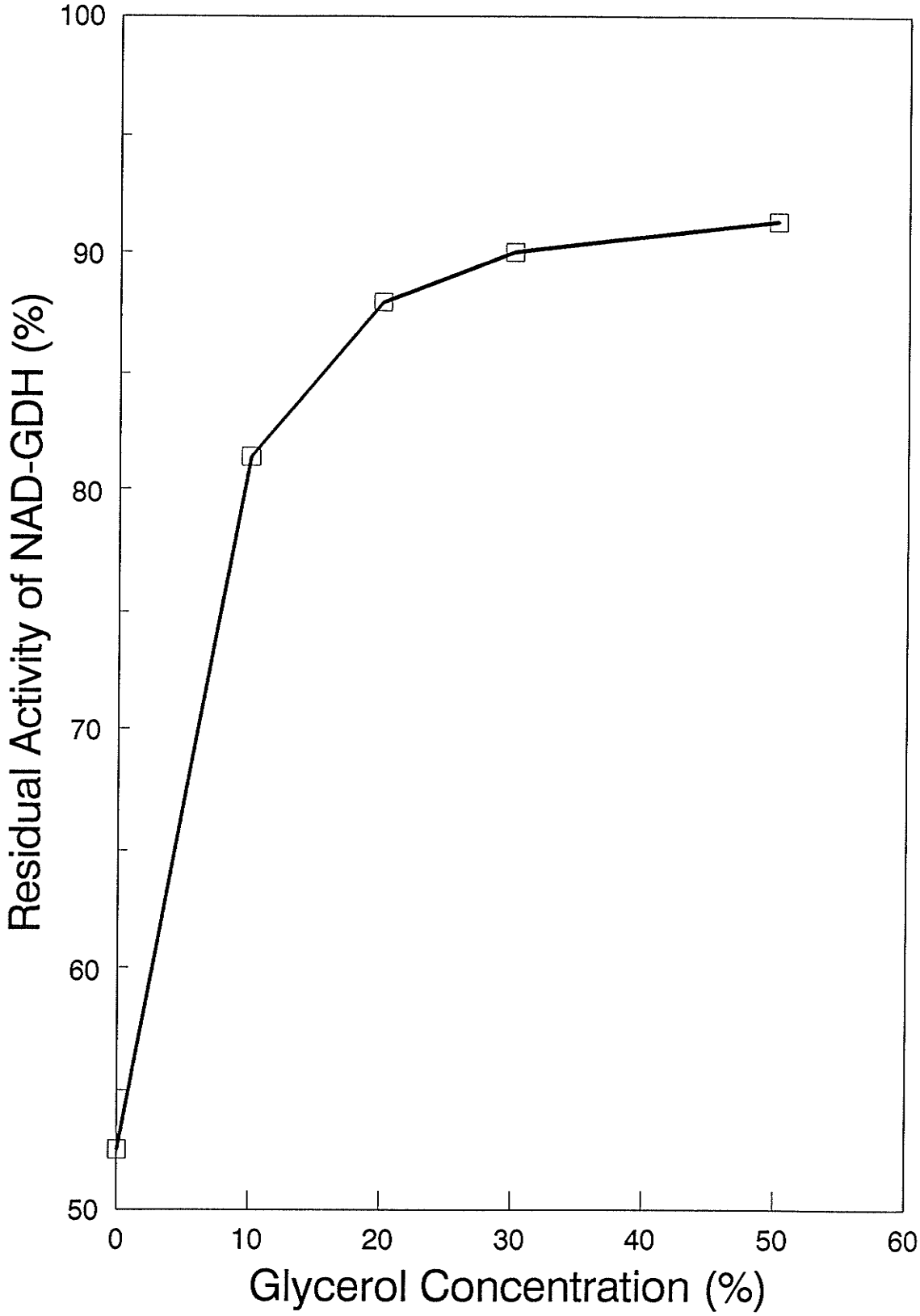

100
90
80
70
60
50
Residual Activity of NAD-GDH (%)
0
10
20
30
40
50
60
Glycerol Concentration (%)

Figure 4. Stability of GDH Activity in the Presence of KCl and $(NH_4)_2SO_4$

NAD-specific glutamate dehydrogenase was obtained from glutamine induced cells by grinding with liquid nitrogen and extracting with TPEDP buffer (3 mL/g of wet weight cells). The enzyme was recovered in the supernatant by centrifuging at 20,000 X g for 20 min. The stability of NAD-GDH was studied by incubating the cell extract with different concentrations of (1) $(NH_4)_2SO_4$ and (2) KCl, heating to 37°C for 20 min, then determining the residual enzyme activity. The data in the figure are the means for three independent experiments.

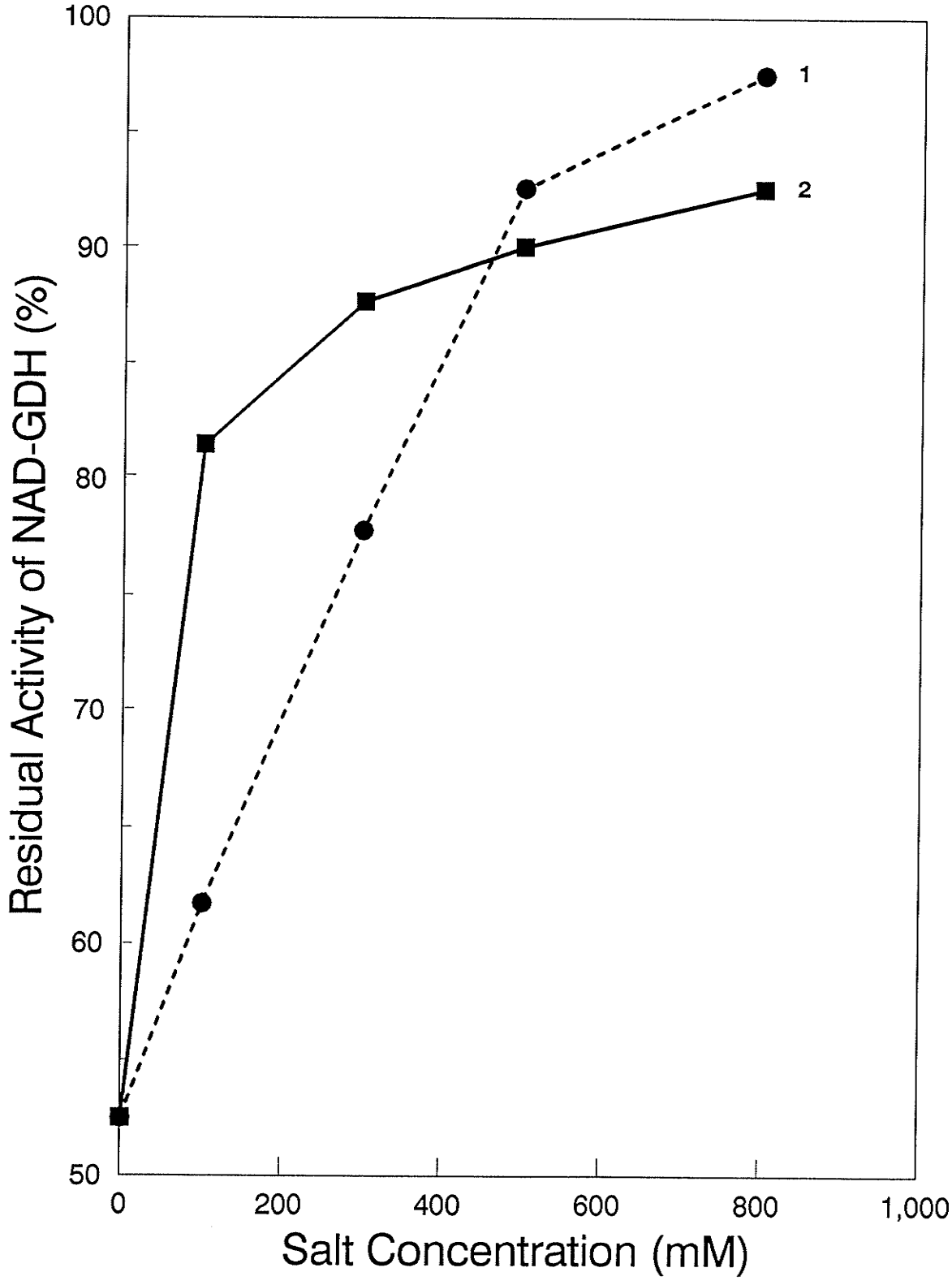
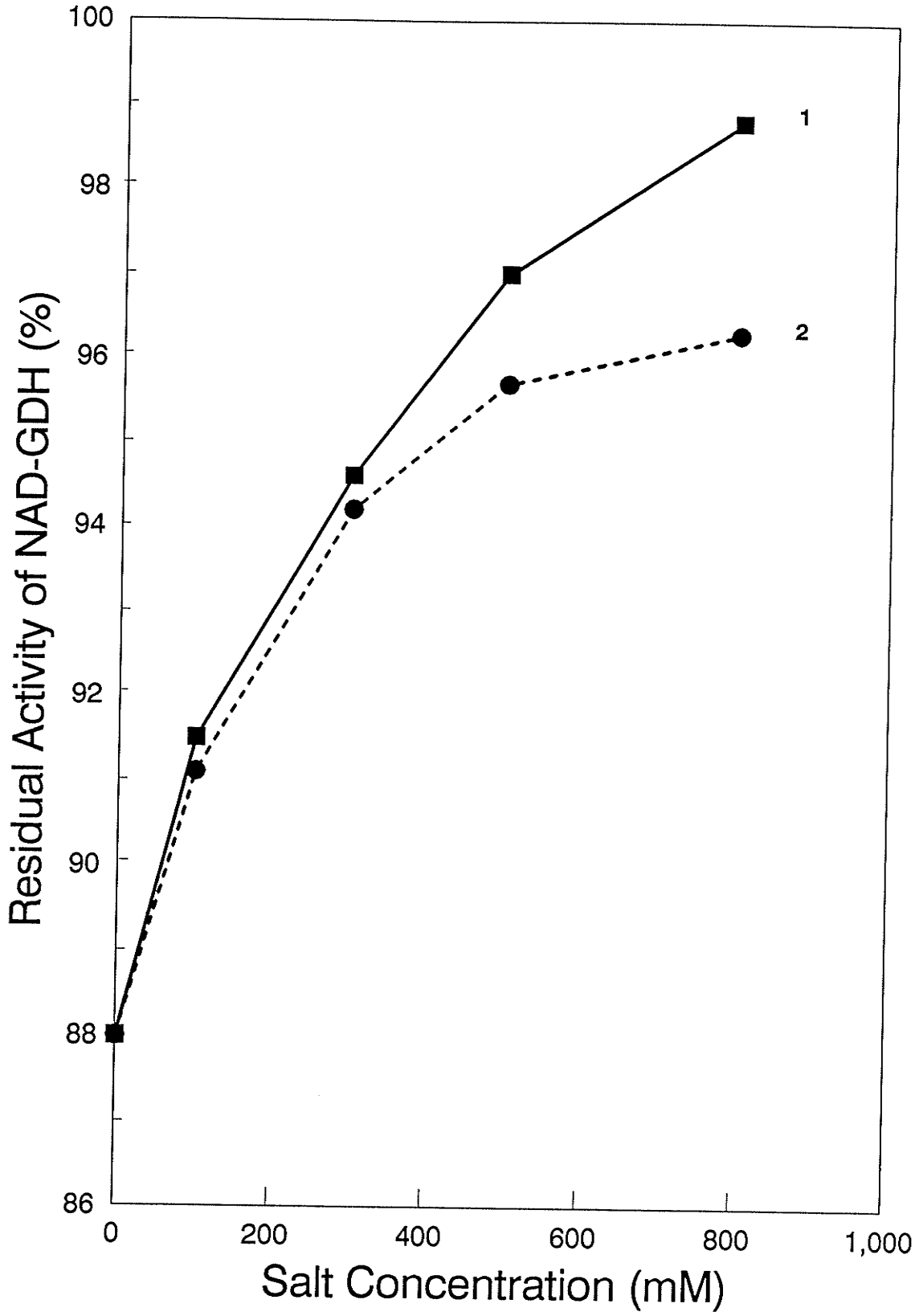

100
90
80
70
60
50
Residual Activity of NAD-GDH (%)
0
200
400
600
800
1,000
Salt Concentration (mM)
1
2

Figure 5. Stability of GDH Activity in the Presence of KCl, $(NH_4)_2SO_4$ and Glycerol

NAD-specific glutamate dehydrogenase was obtained from glutamine induced cells by grinding with liquid nitrogen and extracting with TPEDP buffer (3 mL/g of wet weight cells). The enzyme was recovered in the supernatant by centrifuging at 20,000 X g for 20 min. The stability of NAD-GDH was determined by adding either (1) KCl or (2) $(NH_4)_2SO_4$ at different concentrations in the presence of 20% glycerol, heating to 37°C for 20 min and determining the enzyme activity.


100
98
96
94
92
90
88
86
Residual Activity of NAD-GDH (%)
0
200
400
600
800
1,000
Salt Concentration (mM)
1
2

Once agents that protected the enzyme against thermal denaturation were found, it became possible to develop procedures that allowed the enzyme to be purified with high yield. The purpose of the purification was to have an absolutely pure glutamate dehydrogenase to raise antibody for use in isolating the gene and to analyse the mode of induction of the enzyme.

1.1.2. Enzyme Purification. Following the preparation of cell-free extract through disruption of cells by grinding with liquid nitrogen, all further operations were carried out at 0°C (ammonium sulphate fractionation and sample storage for short periods) and 4°C (enzyme purification in columns).

Step 1. Cells induced for the production of high levels of NAD-specific glutamate dehydrogenase were frozen and disrupted by grinding in liquid nitrogen to a fine powder using a mortar and pestle. The fine powder obtained after grinding was transferred to a centrifuge bottle and suspended in enzyme extraction buffer containing 50 mM Tris-HCl, pH 7.3, 10 mM K_2HPO_4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM PMSF and 20% glycerol (TPEDPG). A ratio of 2 mL TEPPDG for every gram of wet weigh cells was used. The suspension was incubated on ice for several hours with gentle occasional shaking, to effectively release soluble proteins from the cell debris. Shortly after the slurry was completely thawed, the cell suspension was centrifuged at 16,000 x g for 20 min at 4°C to clarify it. The clear yellow supernatant was saved for glutamate dehydrogenase isolation.

Step 2. The cell-free extract was brought to 42% saturation with ammonium sulphate powder added slowly with constant stirring on ice.

After complete dissolution of the salt, the extract was incubated at 0°C for 2 h, with slow stirring, then centrifuged at 16,000 x g for 20 min at 0°C. The supernatant was saved and more powdered ammonium sulphate added to bring it to 60% saturation. After incubating at 0°C for 2 h with slow stirring, the suspension was centrifuged as before and the pellet was collected and dissolved in 10 mL of buffer composed of 25 mM Tris-HCl, pH 8.3 at 0°C, 1 mM EDTA, 1 mM DTT. The enzyme activity and protein concentration were determined from the supernatant and pellets of 42% and 60% ammonium sulphate saturation, respectively.

Step 3. A column of DEAE-Sephadex A25 (50 x 2.6 cm, 265 mL bed volume) was prepared in the following way as described by Pharmacia Fine Chemicals, but with modifications. Thirty grams of DEAE-Sephadex A25 beads were soaked in 500 mL of a buffer containing 250 mM Tris-HCl, pH 8.0 at 22°C, 1 mM EDTA (TE) with slow stirring. The buffer was changed several times during a one day swelling period until the pH of the buffer approached 8.0 when the column was deemed ready for packing. The operating pressure was 1 cm H_2O/cm height of gel bed. The column was packed at room temperature and then transferred to 4°C. Five bed volumes of the same buffer was run through the column to stabilize the gel bed and then one bed volume of TE buffer containing 1 mM DTT (TED) at 4°C. The enzyme sample (resuspension of 60% $(NH_4)_2SO_4$ precipitation) from step 2 was applied to this column by layering it on top of the gel bed underneath the buffer. This was done by using a syringe tube fitted with a piece of fine capillary tubing, an end of which was held a few cm above the gel bed surface. After the sample was deposited on the gel bed and absorbed, the column was incubated for 30 min and then washed with 2 bed column volumes

with the TED buffer at the flow rate of 15 mL/h. The enzyme was eluted with 150 mL of 0.1 to 0.8 M KCl gradient buffer with TED at the flow rate of 10 mL/h. Fractions containing high enzyme activity were identified, their protein concentration determined, individually analyzed by SDS-PAGE and silver staining and then pooled for application to Cibracon Blue 3GA-agarose column. The elution curve is presented in Fig 6.

Step 4. Cibacron Blue 3 GA-agarose, (attached to 4% beaded agarose, Sigma product) suspended in 0.5 M NaCl, described by Heyns and Demoor (1974), and Scawen *et al.* (1982), was used to pack a column (49 x 1.5 cm, 86 mL bed volume) at 4°C. The column was equilibrated with five bed volumes of TE buffer, pH 8.3, containing 0.5 M KCl and then one bed volume of TED buffer containing 0.5 M KCl (TEDK). Enzyme samples containing 90% of enzyme activity collected from step 3 were directly applied to the column underneath the buffer as described previously. The column was incubated for 10 min, and then washed with three bed volumes of the TEDK buffer at a flow rate of 12 mL/h. The column was eluted with a combination of glycerol and KCl gradients buffered with TEDK. The gradients were 0.5 M to 1.5 M KCl and 0 to 20% glycerol. The flow rate was 8 mL/h. Fractions containing high enzyme activity were used to (i) determine the protein concentration (Fig 7), and (ii) analyze the protein components by SDS-PAGE and silver staining.

Step 5. Samples from the last step were pooled, brought to 65% saturation with ammonium sulphate powder as described above, and suspended in 10 mL TED buffer, pH 8.6 at 0°C. The suspension was applied to a second DEAE-Sephadex A25 column. Most of the techniques in this step were the same as in step 3 except for a pH change that affected the binding

Figure 6. Elution Profile of GDH in A25 DEAE-Sephadex Column

Procedures were as described in Step 3 in the RESULTS section. The experiment was repeated five times but data in the figure is (and has to be) from a single experiment.

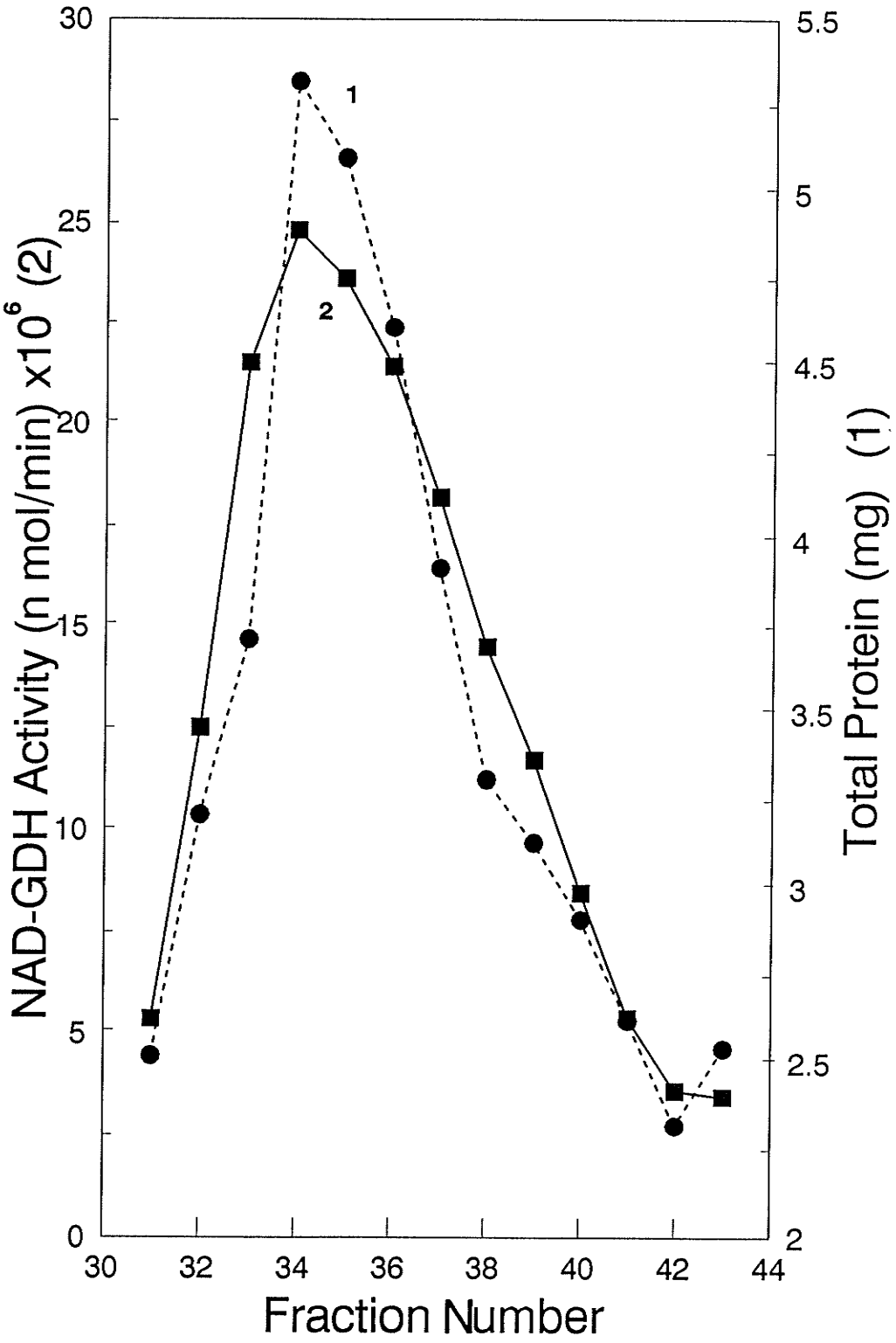
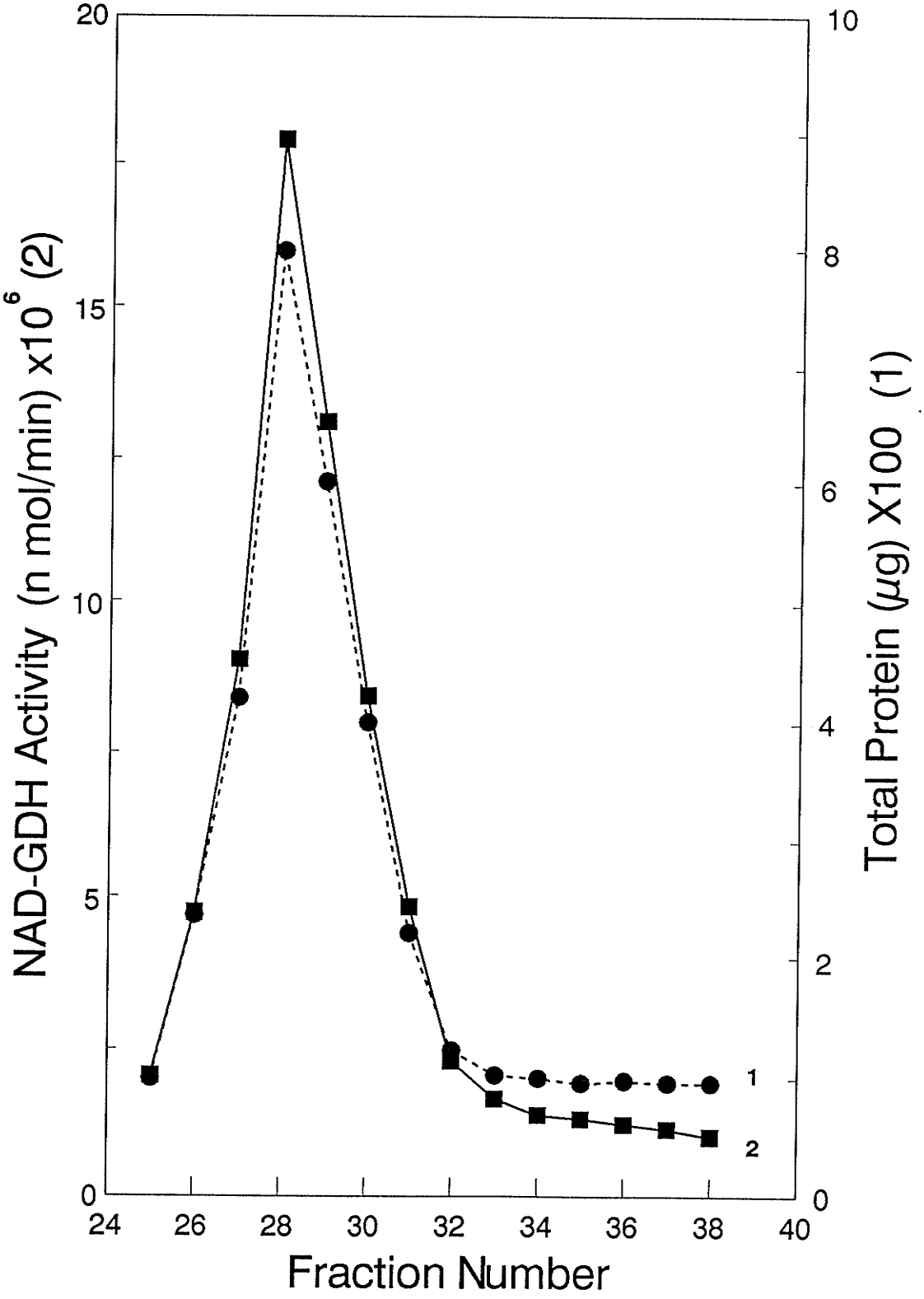

30
25
20
15
10
5
0
5.5
5
4.5
4
3.5
3
2.5
2
30
32
34
36
38
40
42
44
1
2
NAD-GDH Activity (n mol/min) x10^6 (2)
Total Protein (mg) (1)
Fraction Number

Figure 7. Elution Profile of GDH in Cibacron Blue 3 GA-Agarose Column.

Procedures were as described in Step 4 in the RESULTS section. The experiment was repaeted five times but data in the figure is (and has to be) from a single experiment.


NAD-GDH Activity (n mol/min) x10^6 (2)
Total Protein (μg) X100 (1)
Fraction Number
20
15
10
5
0
10
8
6
4
2
0
24
26
28
30
32
34
36
38
40
1
2

behaviour of the enzyme to the column. The pH of the buffer was increased to 8.6 from pH 8.3. The enzyme was eluted with TED, pH 8.6, which buffered two gradients; one gradient was 0.1 to 0.8 M KCl, while the other was 0 to 20% glycerol. The flow rate was 8 mL/h. Fractions containing high enzyme activity (Fig 8) were used to (i) estimate the protein concentration with BSA as a standard and (ii) determine purity of the enzyme by analysis on SDS-PAGE and silver staining (Fig 9). Because the sensitivity of the staining was very critical in deciding the purity of the sample, the staining protocols provided by different manufacturers were modified in the following way. Gels were fixed overnight, and most importantly, developed for at least 30 min instead of 3-8 min. This lengthy staining period highlighted artifacts that could be seen in the control part of the gels. Moreover, the gels were stained with two cycles of silver reagents to increase the sensitivity.

Samples from different stages of purification were analyzed on SDS-PAGE and stained with Coomassie Blue and silver (Fig 10).

The enzyme purification procedure is summarized in Table 3.

1.2. Determination of M_r of Enzyme and Enzyme Subunit

The molecular mass (M_r) of native glutamate dehydrogenase was estimated by Sepharose 6B filtration. Data from the column filtration were used to calculate the M_r for each protein standard and enzyme sample from the equation $K_{av} = (V_e - V_o) / (V_t - V_o)$,

where V_e = elution volume for the protein standards and enzyme sample,

V_o = elution volume for blue dextran 2000,

Figure 8. Elution Profile of GDH in A25 DEAE-Sephadex Column

Procedures were as described in Step 5 in the RESULTS section. The experiment was repeated five times but data in the figure is (and has to be) from a single experiment.

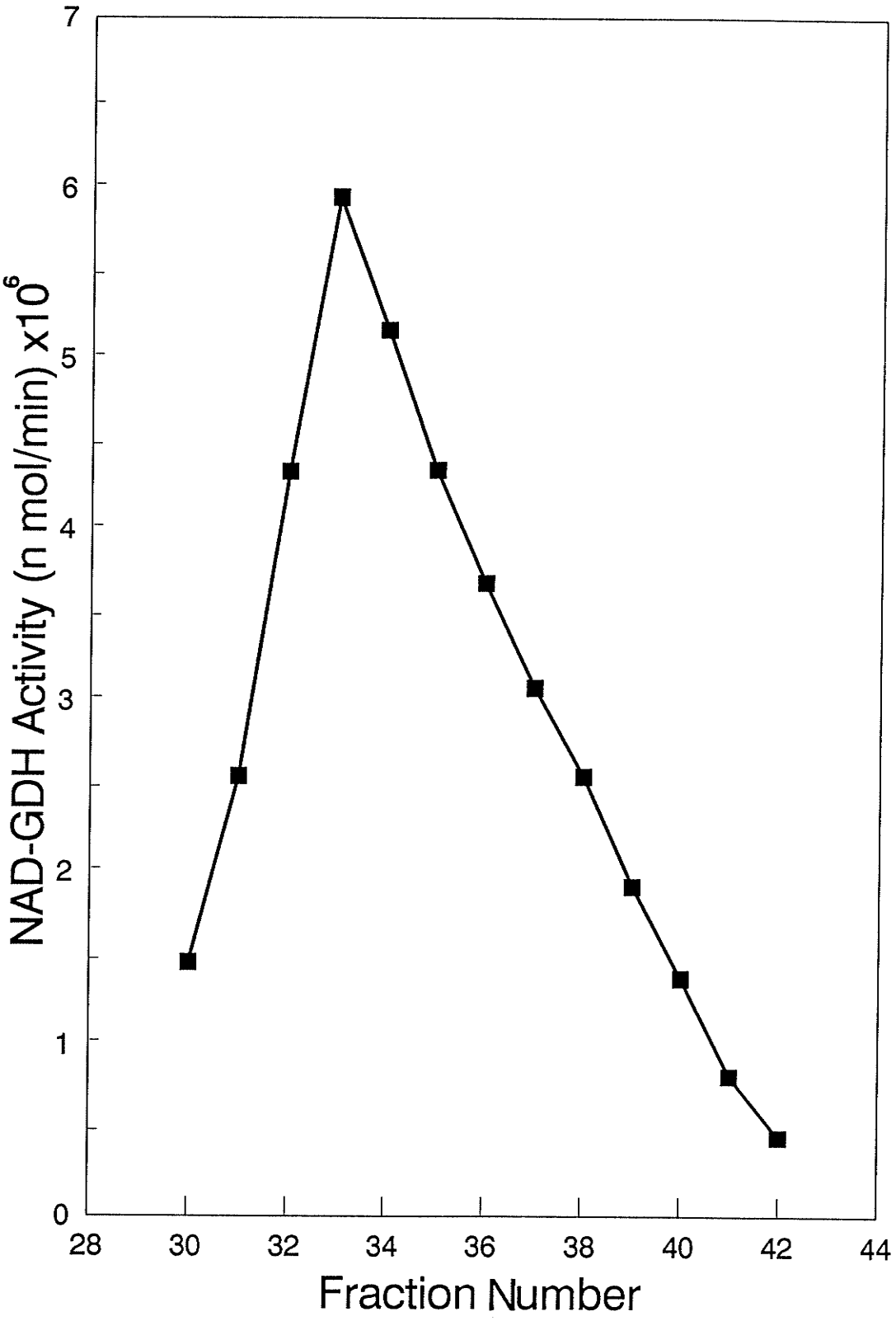

NAD-GDH Activity (n mol/min) x10^6
0
1
2
3
4
5
6
7
28
30
32
34
36
38
40
42
44
Fraction Number

Figure 9. Silver-stained SDS-PAGE Electrophoregram of Protein Fractions Recovered from A25 DEAE-Sephadex Column

Four different protein fractions in Fig 8 (#32-35 from left to right in this figure) containing high NAD-GDH activity recovered from the second A25 DEAE-Sephadex column (see step 5 in "Methods and Materials") in the purification of *Achlya* NAD-GDH were analyzed on SDS-PAGE. Protein bands shown were detected after two cycles of silver staining.

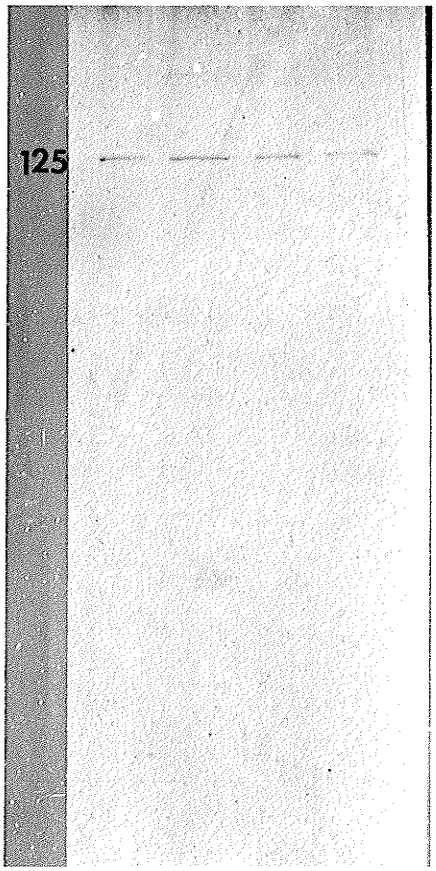

125

Figure 10. Silver and Coomassie Blue stained SDS-PAGE Electrophoregram of Protein Samples from Different Stages of Enzyme Purification

Protein samples collected from different steps in the purification of *Achlya* NAD-GDH were denatured by SDS-PAGE and analyzed for NAD-GDH subunit. The sole band in lane 7 is the monomer of NAD-GDH marked 125 (kDa). Protein standards (lane 1) and samples from cell-free extract (lane 2), 42% $(NH_4)_2SO_4$ precipitate (lane 3), and 60% $(NH_4)_2SO_4$ precipitate (lane 4) were stained with Coomassie Blue R-250, while those of samples from A25 DEAE-Sephadex column (lane 5), Cibacron Blue 3 GA-agarose column (lane 6), and the second A25 DEAE-Sephadex column (lane 7), were stained with silver. M_r values (x 10^3) of protein markers are presented at the left margin.

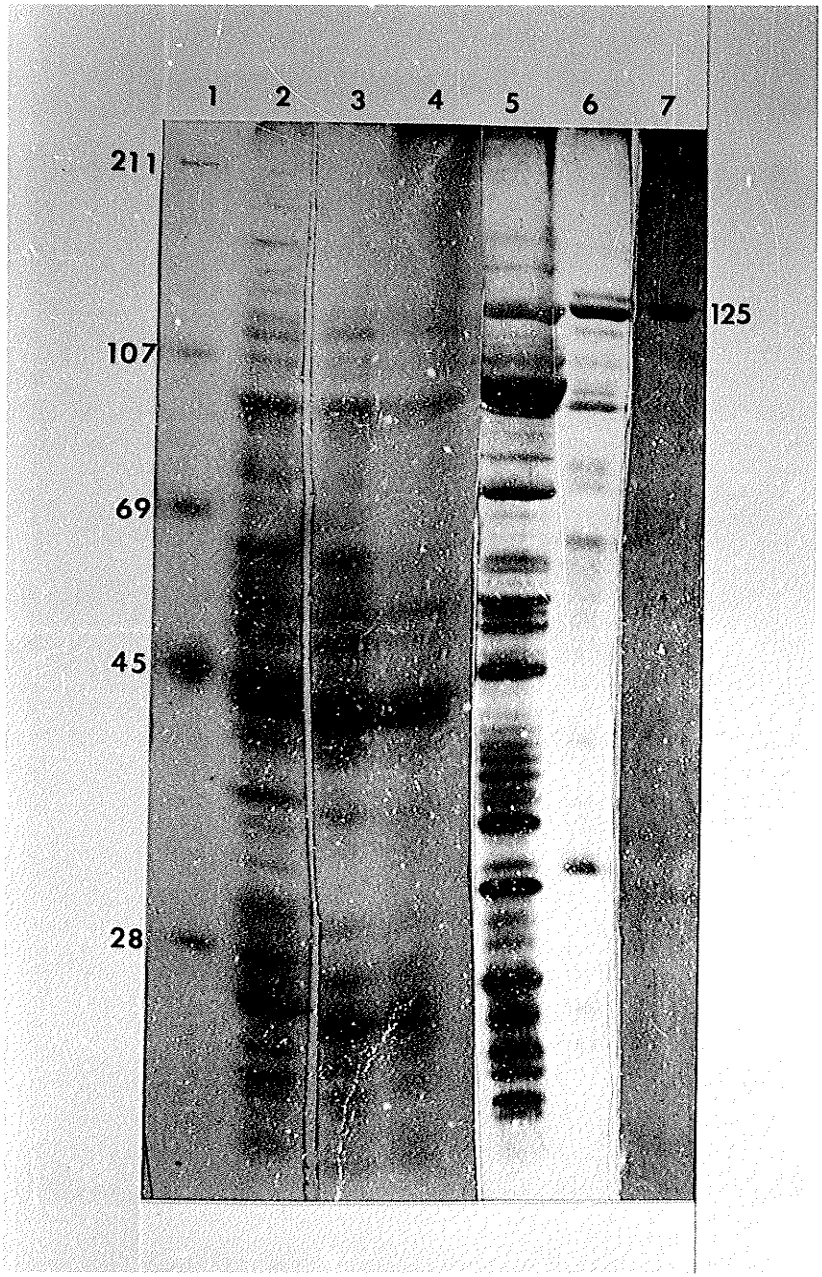

1
2
3
4
5
6
7
211
107
69
45
28
125

Table 3. Summary of purification of *Achlya* glutamate dehydrogenase.

Steps	Volume (ml)	Activity nmol/min	Protein (mg)	Specific activity*	Purification (fold)	Yield (%)	Purification factor **
Extract	300	3.84×10^8	1874	2.05×10^5	1.00	100	---
42% $(NH_4)_2SO_4$ saturation	336	3.36×10^8	689	4.88×10^5	2.38	87.5	2.08
60% $(NH_4)_2SO_4$ saturation	12	3.04×10^8	356	8.54×10^5	4.17	79.2	1.59
A_{25} DEAE-Sephadex	40	1.76×10^8	45.2	3.89×10^6	19.0	45.8	2.63
Cibacron blue 3GA-agarose	45	7.04×10^7	3.50	2.01×10^7	98.0	18.3	2.06
65% $(NH_4)_2SO_4$ saturation	10	6.88×10^7	3.30	2.08×10^7	101	17.9	1.01
A_{25} DEAE-Sephadex	38	3.79×10^7	0.820	4.62×10^7	225	9.87	1.23
65% $(NH_4)_2SO_4$ saturation	4	3.76×10^7	0.800	4.70×10^7	229	9.79	1.01

* specific activity is nmol NADH oxidated/min/mg protein.
** purification factor = (fold purified / step) x (yield / step).

V_t = total bed volumes.

K_{av} was plotted against the corresponding M_r of each protein standards. From the plot, M_r of the native enzyme was estimated as 470,000 (Fig 11).

M_r of the native enzyme was also determined by polyacrylamide gradient (3-25%) gel electrophoresis (Fig 12). The relative mobilities of the 5 protein standards were plotted against the log of their corresponding molecular masses. M_r of the enzyme was determined by its relative mobility as 500,000 (Fig 13).

The subunit structure of the enzyme was analyzed by SDS-PAGE (Fig 10). Only a single polypeptide band was seen after two cycles of silver staining, and from this, the M_r of the subunit was estimated as 125,000 (Fig 14). On the basis of this estimation, the native enzyme must consist of 4 subunits of identical M_r values.

The subunit M_r of most of the NAD-specific glutamate dehydrogenases studied so far, vary between 45,000 and 58,000 (Table 1), except for *Neurospora crassa*, *Saccharomyces cerevisiae* and *Achlya klebsiana* which have subunit M_r values of 116,000 115,000 and 125,000 respectively.

1.3. $NADP^+$ Activation of Enzyme Modulated by Limited Subtilisin Action

An attempt was made to dissociate the $NADP^+$ modulation effect of NAD-GDH from its catalytic activity by limited proteolysis. Subtilisin was chosen as the proteinase to conduct the study. It was prepared at a concentration of 1 $\mu g/\mu L$ in 100 mM Tris-HCl, pH 8.0, and stored at - 20°C. Glutamate dehydrogenase which had an activity of 649 nmol NADH consumed/min/μL when activated with $NADP^+$, was brought to room temperature

Figure 11. M_r Estimation of *Achlya* NAD-GDH in Sepharose 6B Column.

The procedure is described in " METHODS 3.3 Molecular Mass Estimation". Protein markers used to calibrate the system are specified.

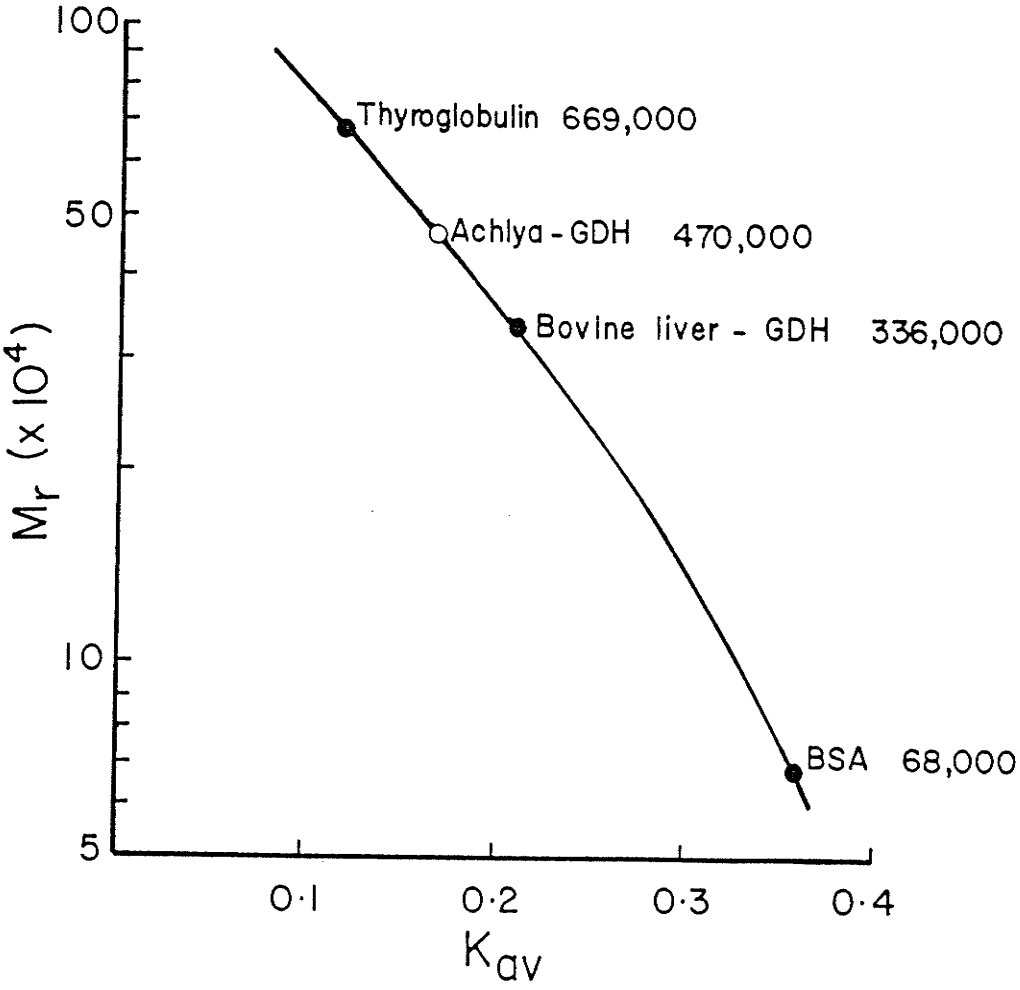

100
50
10
5
M_r ($\times 10^4$)
Thyroglobulin 669,000
Achlya - GDH 470,000
Bovine liver - GDH 336,000
BSA 68,000
0·1
0·2
0·3
0·4
K_{av}

Figure 12. Electrophoregram of Purified *Achlya* NAD-GDH in a 3-25% Polyacrylamide Gradient Gel.

The procedure of electrophoresing the enzyme in a non-denaturing gradient gel is described in "3.1 Glutamate Dehydrogenase Activity Assay". M_r values (x 10^3) are given at the left margin. Lane 1; protein markers: thyroglobulin, 669; ferritin, 440; catalase, 232; LDH (lactate dehydrogenase), 140 and BSA, 68. Lane 2; *Achlya* NAD-GDH, M_r, 500 (x 10^3). NAD-GDH was detected by activity staining.

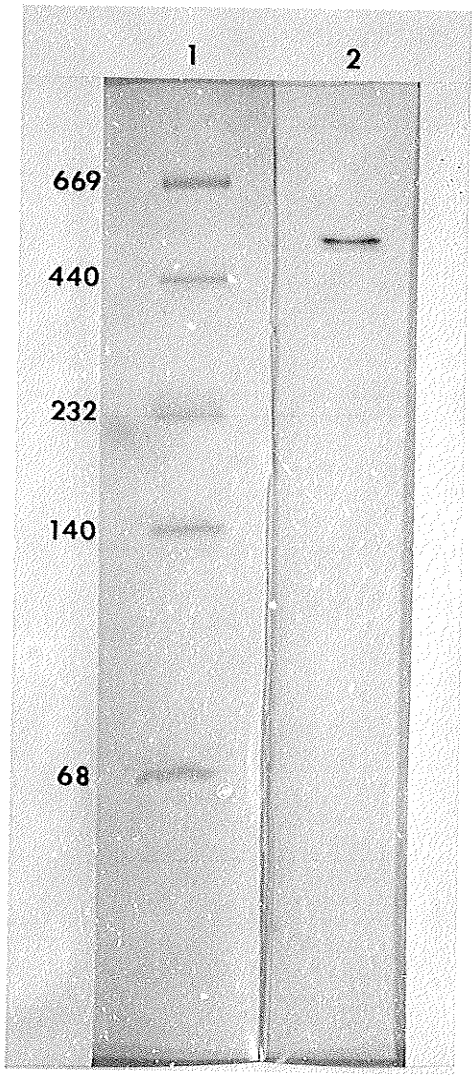

1
2
669
440
232
140
68

Figure 13. M_r Estimation of *Achlya* NAD-GDH in 3-25% Polyacrylamide Gradient Gel.

The M_r values of protein markers are given in the figure.

LDH --- lactate dehydrogenase

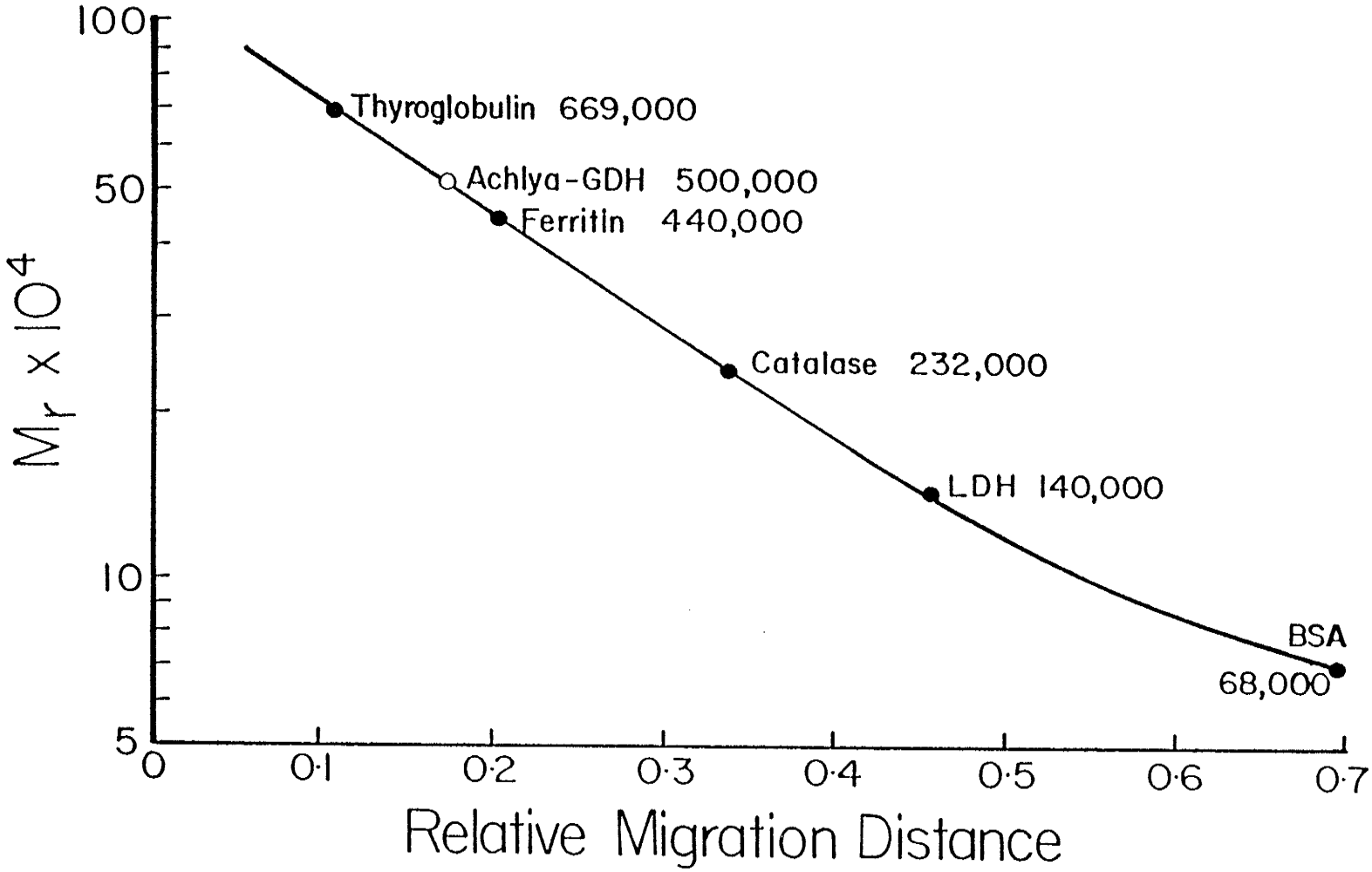
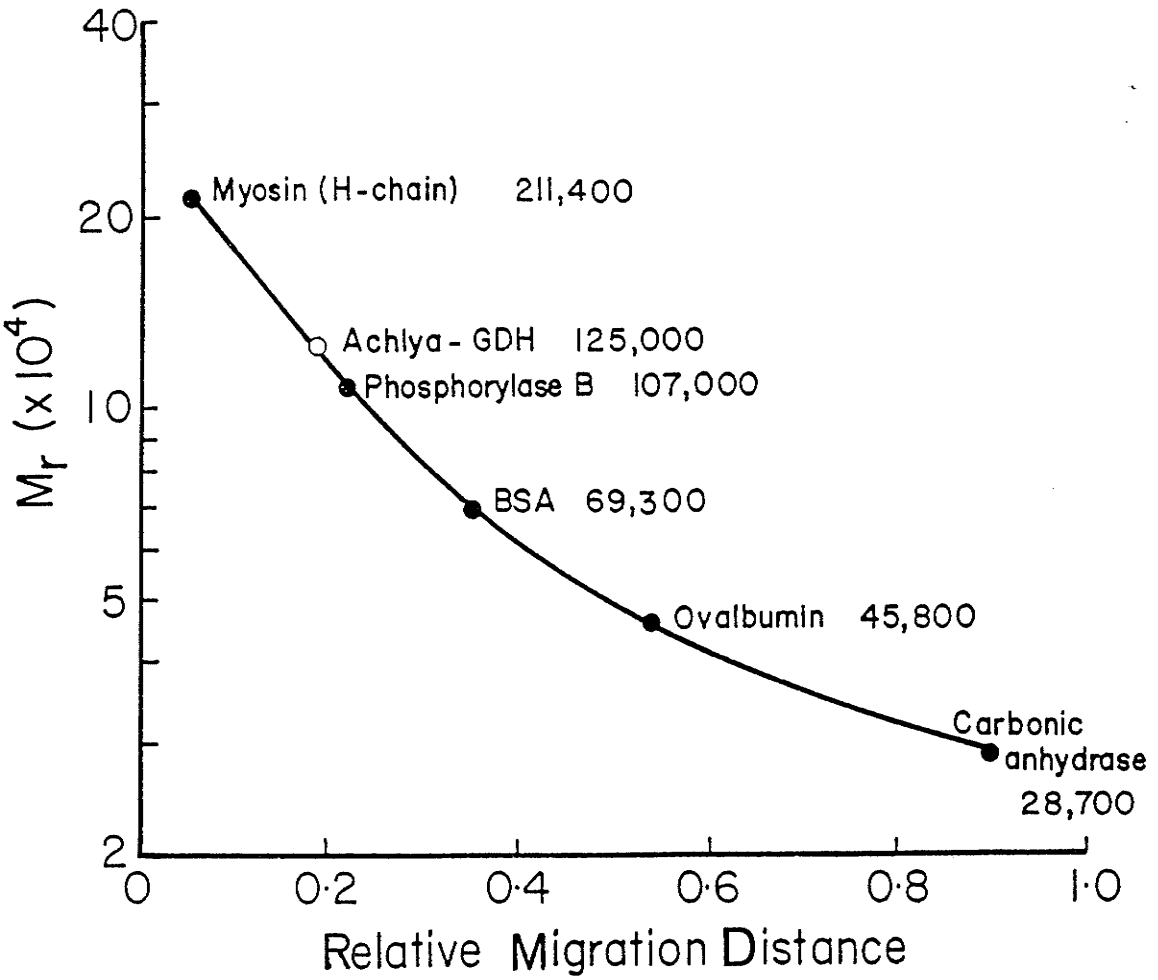

100
50
10
5
$M_r \times 10^4$
Thyroglobulin 669,000
Achlya-GDH 500,000
Ferritin 440,000
Catalase 232,000
LDH 140,000
BSA
68,000
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
Relative Migration Distance

Figure 14. M_r Estimation of the Subunit of *Achlya* NAD-GDH by SDS-PAGE.

The procedure is described in "3.3 Molecular Mass Estimation". The M_r values of protein markers are given in the figure.

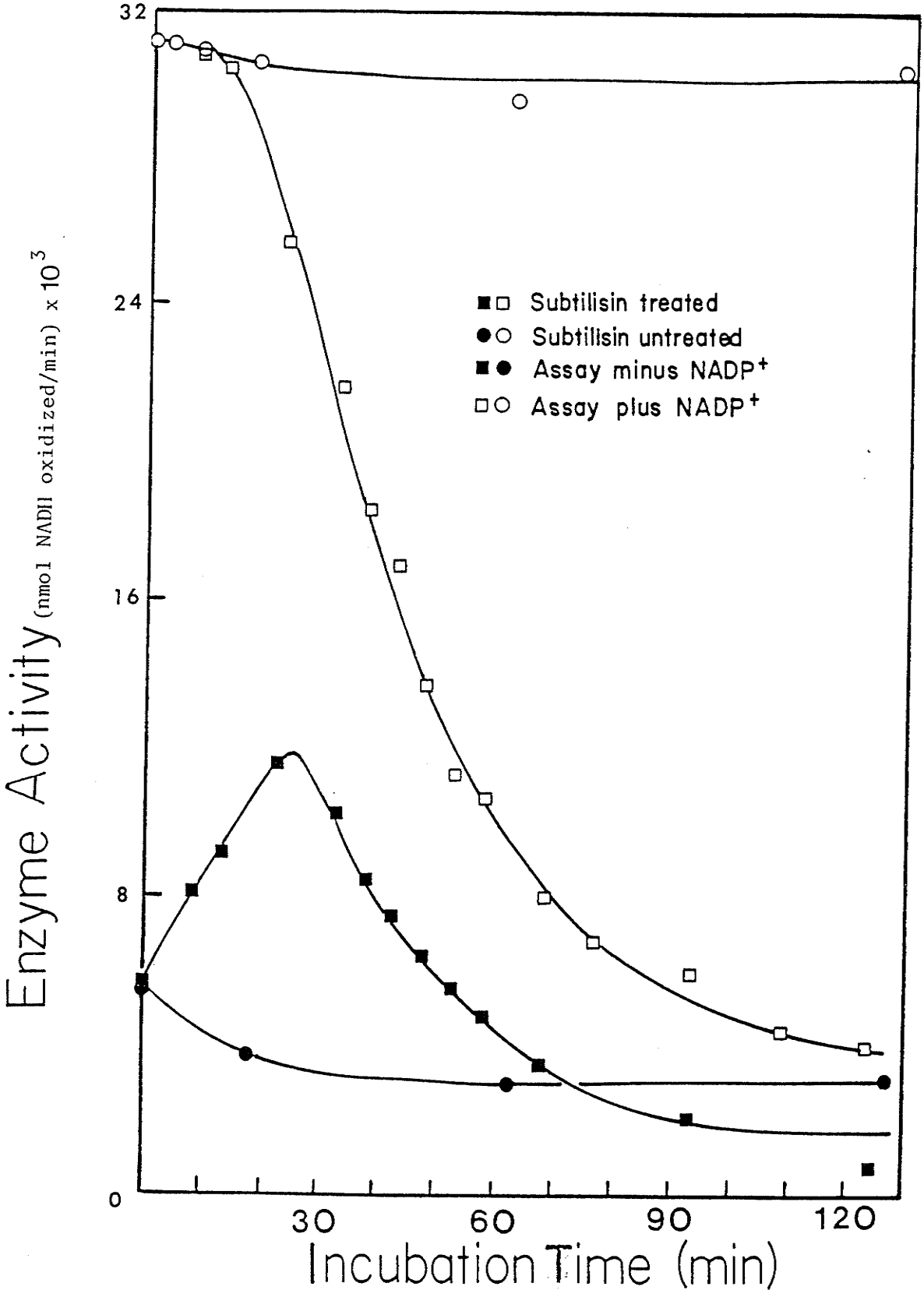

40
20
10
5
2
M_r (x 10^4)
Myosin (H-chain) 211,400
Achlya - GDH 125,000
Phosphorylase B 107,000
BSA 69,300
Ovalbumin 45,800
Carbonic anhydrase 28,700
0
0·2
0·4
0·6
0·8
1·0
Relative Migration Distance

just before subtilisin treatment in a buffer composed of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and 0.5 M KCl. To each mL of the enzyme solution 30 μL (30 μg) of the subtilisin stock solution was added and the mixture left at room temperature for different periods. The high concentration of subtilisin was required in the experiments as the enzyme sample used was partially purified. A portion of the mixture was removed and glutamate dehydrogenase activity determined spectrophotometrically at 5 min intervals in the first 100 min and 15 min intervals in the second 100 min. The activity of subtilisin treated glutamate dehydrogenase was determined with and without $NADP^+$. As well, the activity of untreated glutamate dehydrogenase was determined with and without $NADP^+$ to serve as controls. The results (Fig 15) demonstrate several features. First, there was an exponential loss, with time, of the $NADP^+$ activation property of the enzyme which became fully desensitized to $NADP^+$ in 90 min. Second, during the initial phase (30 min) of subtilisin treatment, the enzyme activity was enhanced by about 100% in the absence of $NADP^+$. This implied that the initial subtilisin effect did not destroy the catalytic function of the enzyme but modified it in a way that uncoupled the catalytic sites(s) from the $NADP^+$ modulating (allosteric) site. Third, without subtilisin treatment, the enzyme activity diminished about 30% within 30 min and then remained constant over the next 2 to 3 h (not shown). This partial loss in activity is observed whether or not the enzyme is activated by $NADP^+$ (open and closed circles in Fig 15). Presumably, the enzyme becomes partially inactivated by dilution during the long incubation period at room temperature.

To determine what effect subtilisin had on the structure of NAD-GDH

Figure 15. Influence of Subtilisin on the Allosteric and Catalytic Activities of Purified *Achlya* NAD-GDH.

The allosteric and catalytic activities of purified *Achlya* NAD-GDH were compared after treatment with subtilisin. Subtilisin (30 μg/ml) and purified *Achlya* NAD-GDH (1.5 μg/mL) were incubated in TEDK buffer at room temperature. At the specified times, 100 μL of the incubation mixture was removed and spectrophotometrically assayed for glutamate dehydrogenase activity in the absence (■) and presence (□) of $NADP^+$. In a parallel experiment, NAD-GDH was incubated without subtilisin and aliquots assayed in the absence (•) and presence (◦) of $NADP^+$ at specified times. Assays were carried out by the reductive amination process as summarized in "Materials and Methods". The data in the figure are the means for three independent experiments.


32
24
16
8
0
30
60
90
120
Enzyme Activity (nmol NADH oxidized/min) x 10^3
Incubation Time (min)
Subtilisin treated
Subtilisin untreated
Assay minus NADP+
Assay plus NADP+

subtilisin-treated glutamate dehydrogenase was recovered temporally and analyzed by gel electrophoresis. The conditions of glutamate dehydrogenase treatment with subtilisin were the same as described above. A portion (20 μL) was removed from the proteinase treated mixture at 0 min, 1 min, 2 min, 3 min, 5 min, 10 min, 20 min and 30 min respectively. The sample removed was mixed with 10 μL of 5x protein sample buffer and incubated in a boiling water bath for 10 min, then chilled on ice. After the treatment, all samples were incubated in a boiling water bath for 1 min and brought to the bottom of the tubes by a brief centrifugation. Samples were electrophoresed in a 7% SDS-PAGE gel in the presence of protein markers as described in "Methods and Materials". Untreated glutamate dehydrogenase was used as control (see Fig 16 legend). The gel was silver-stained. The results (Fig 16) show a time-dependent change in the subunit M_r of the enzyme from 125,000 to 110,000. After 1 min of subtilisin treatment, only a small fraction of the native subunit had been converted to 110,000; but almost 50% of the native protein was in the 110,000 M_r form after 30 min. After 60 min, only a small portion of the 125,000 and 110,00 subunit polypeptides remained; most of the enzyme had been cleaved into smaller polypeptides. No further studies were carried out with the subtilisin modified NAD-GDH.

The purified enzyme still utilized NAD(H) as coenzyme and $NADP^+$ as an activator (Fig 17) as did partially purified enzymes (LéJohn and Stevenson, 1970; LéJohn *et al.*, 1970; Stevenson and LéJohn, 1971).

Figure 16. SDS-PAGE Analysis of *Achlya* NAD-GDH Treated with Subtilisin for Different Times.

The treatment of *Achlya* NAD-GDH with subtilisin is described in the legend to Fig 15. Twenty microliters of the reaction mixture was removed at the specified time, subtilisin was inactivated by boiling for 10 min, then proteins were precipitated by 20% trichloroacetic acid at 0° C for 1 h followed by centrifuging in a microfuge. The pellet was dissolved in 0.1 N NaOH and mixed with an equal volume of 2% SDS, 5 mM mercaptoethanol, 9.5 M urea and 0.1 M Tris-HCl, pH 9.0 solution. The sample was electrophoresed in an SDS-PAGE (7% gel) system. Polypeptides on the gel were detected by silver staining. The M_r value signified by the "upper" arrow head is 125,000. The one signified by the "lower" arrow head is 115,000.

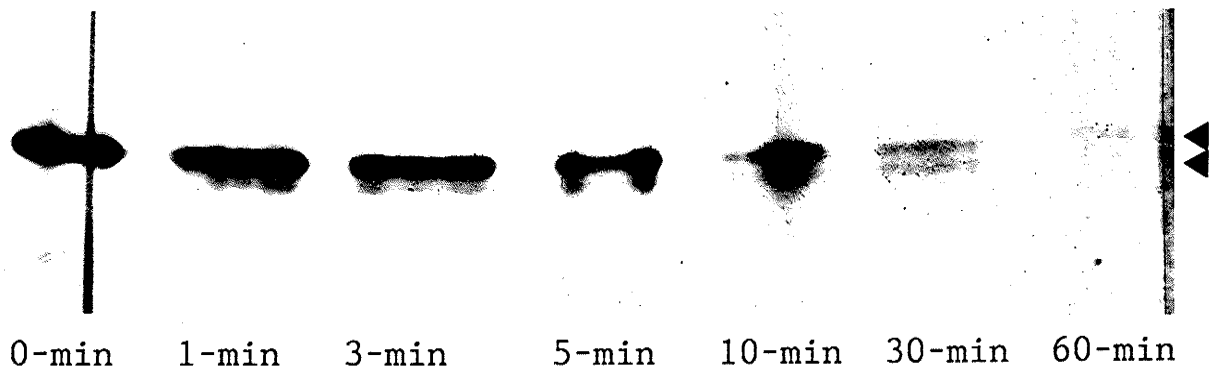
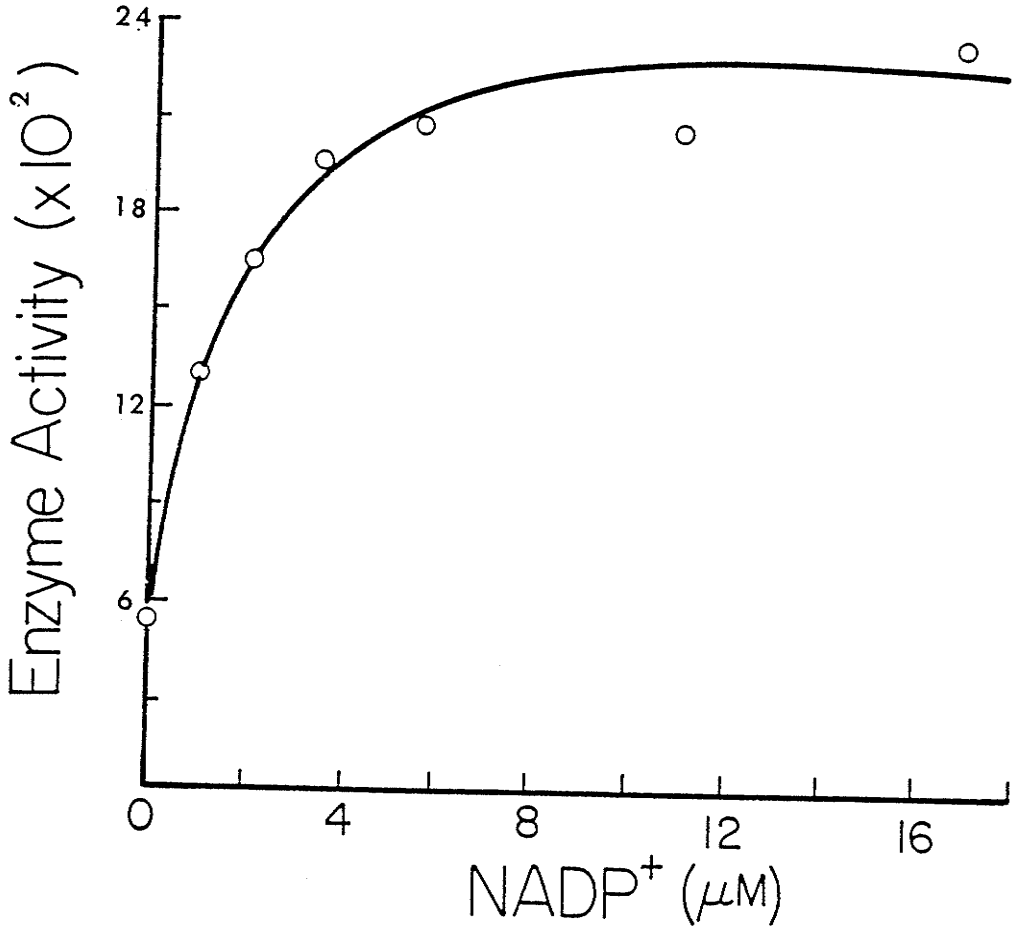

0-min
1-min
3-min
5-min
10-min
30-min
60-min

Figure 17. $NADP^+$ Activation of *Achlya* NAD-GDH

Purified *Achlya* NAD-GDH activity was assayed spectrophotometrically in a reaction system (3 mL) containing 67 mM Tris-acetate, pH 9.0, 6.7 mM α-ketoglutarate, 0.1 M ammonia and 0.167 mM NADH. A constant amount of enzyme (see Methods and Materials, 3.1.1. Spectrophotometric assay) was used. $NADP^+$ concentration is indicated in the figure. The experiment was repeated once but the data are from only one of the two experiments.

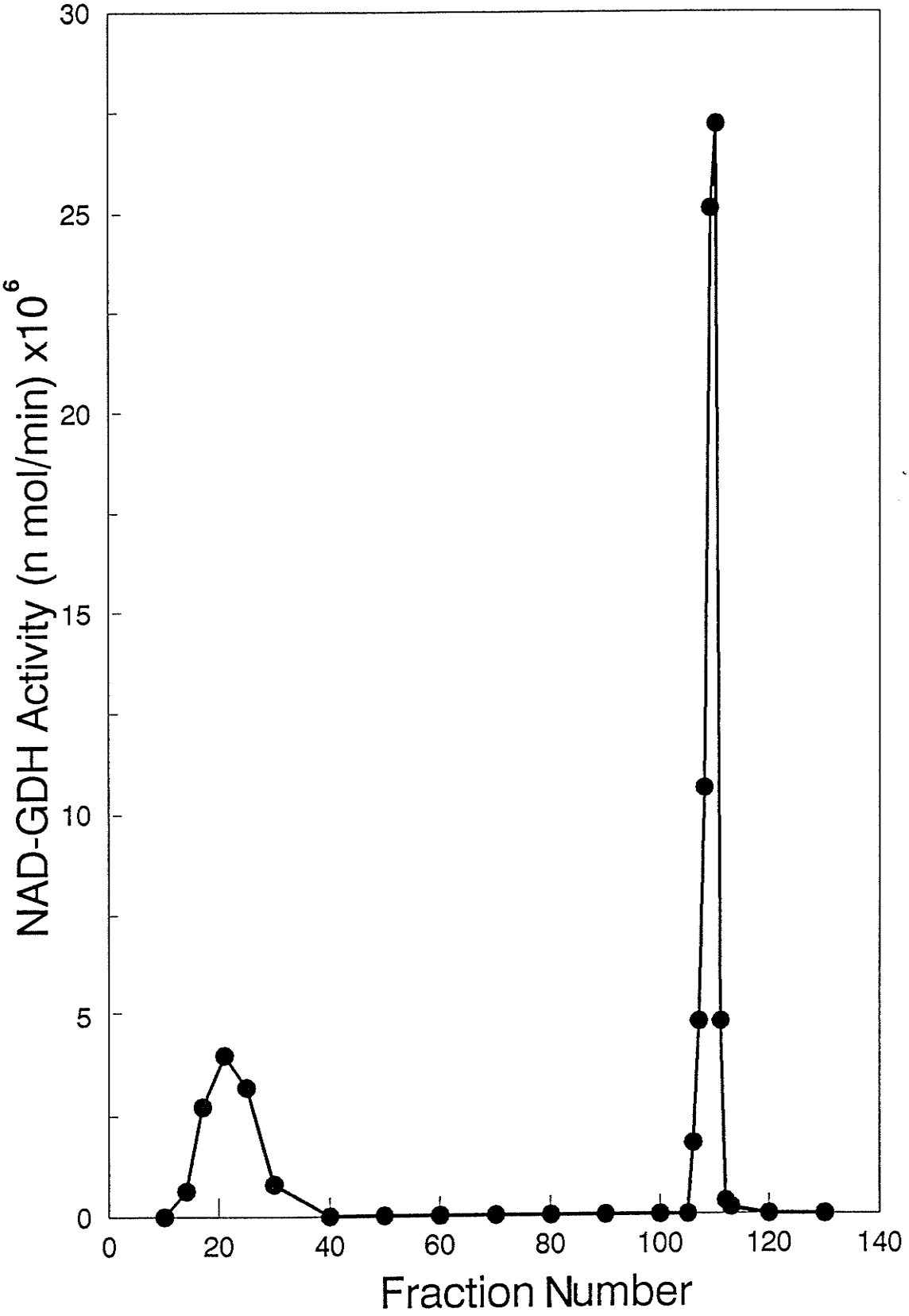

Enzyme Activity (x 10²)
24
18
12
6
0
4
8
12
16
NADP⁺ (μM)

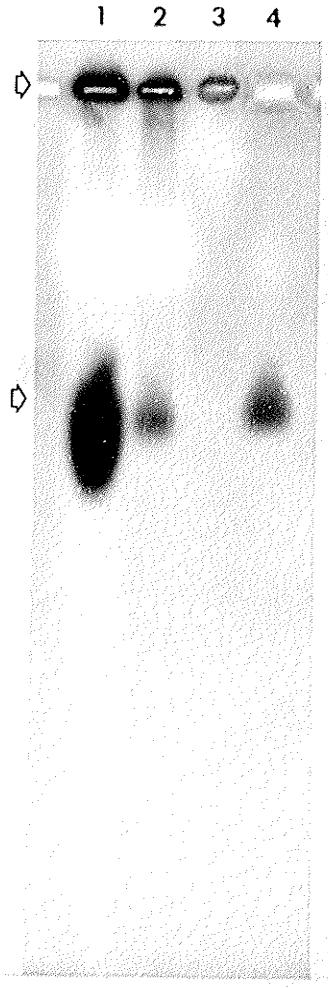
1.4. Aggregation of the Enzyme

The purified enzyme may lose the property of aggregation which may happen while the enzyme is in its unpurified state. The result in Fig 18 shows that when the enzyme sample was purified in the A25 DEAE-Sephadex column, two enzyme peaks were detected. To determine whether the enzyme activities in the two peaks were due to the same protein, they were tested in several ways. First, the enzyme activity was tested in the presence of NADH + NADPH, only NADH, and only NADPH. Results showed that enzyme in both peaks uses NAD(H) as coenzyme and is activated by NADP(H) (data not shown). Second, double diffusion test showed that enzyme from the first peak formed a single immuno-precipitation reaction line with the antibody raised against the enzyme from the second peak (Fig 20). Third, when both peaks of enzyme were used for further purification, both of them contained the same enzyme subunit with M_r of 125,000 (data not shown). What is the reason then for the two enzyme peaks? It is conceivable that the protein in the first peak is aggregated because it is bound to other cell components. This possibility was examined in the following way. Samples from the following: (a) cell-free extract, (b) effluent of DEAE-cellulose column, and (c) first and second peak of A25 DEAE-Sephadex column were electrophoresed in an agarose gel and stained for enzyme activity. The results (Fig 19) show that there are two enzyme activity bands detected in the cell-free extract and in the sample from DEAE-cellulose; one enzyme moving into the gel and the other retained in the wells. Only one protein band was detected in the sample from the first peak, and it did not migrate from the well, whereas the protein in the second peak migrated

Figure 18. A Fast and Slow Moving Enzyme Activity Peak Eluted from A25 DEAE-Sephadex Column.

Post DEAE-cellulose treatment, fractions (6.3 mL/fraction) containing the enzyme were applied to A25 DEAE-Sephadex column stabilized by TED buffer, pH 7.3. After washing with one bed volume of this buffer containing 10 mM KCl, the column was eluted with the same buffer containing a KCl gradient from 100 mM to 300 mM. NAD-GDH activity appeared in fraction number 17-26 and 106-111 separately. The experiment was repeated five times but the data in the figure are from one experiment.


30
25
20
15
10
5
0
NAD-GDH Activity (n mol/min) x10^6
0
20
40
60
80
100
120
140
Fraction Number


1
2
3
4

from the well. It is likely that the enzyme retained in the well was an aggregated form of NAD-GDH. The enzyme moving into the gel was considered as the "monomeric" native GDH because its M_r was 500,000.

2. IMMUNOLOGICAL ANALYSIS OF ENZYME

2.1. Analysis of NAD-GDH Antiserum

Preparation of anti-GDH antiserum was as described in "Materials and Methods". The antiserum was quantitated by ELISA and its activity determined in the following ways.

A double diffusion test was carried out on a 1.25% agar plate containing 0.15 M NaCl with 0.01% thimerosal. A strong reaction band was discernible between *Achlya* cell-free extract and anti-GDH antiserum after incubating the plate at room temperature overnight (Fig 20). This primary test showed that anti-GDH antibody was present in the antiserum. The specificity of the antiserum was tested further.

Achlya cell-free extract and partially purified and fully purified NAD-GDH were electrophoresed in SDS-PAGE (7% gel) and electroblotted onto a nitrocellulose membrane. Proteins on the membrane were immunologically stained with anti-glutamate dehydrogenase antiserum and detected by the anti-rabbit IgG alkaline phosphatase conjugate system (see "Materials and Methods" for details). Two immunoreactive bands were detected in the cell free extract, one of M_r 125,000 and the other of M_r 74,000. Only the 125,000 M_r immunoreactive polypeptide was detectable in the partially purified enzyme sample and in the fully purified preparation (Fig 21). The purified NAD-GDH was activated by $NADP^+$ and had retained all the kinetic properties deduced for the enzyme in crude cell-free extract, viz: thermal instability, K_m values for substrates and molecular mass. Thus, the 74,000 M_r polypeptide may not be a constituent of the NAD-GDH.

Figure 20. Double Diffusion Test of GDH and Its Antibody.

The double diffusion test was carried out on a 1.25% agar plate containing 0.15 M NaCl and 0.01% thimerosal. After sample loading, the plate was incubated at room temperature overnight. 1. purified anti-GDH IgG, 2. anti-GDH antiserum, 3. cell-free extract of *Achlya*, 4. NAD-GDH from fast moving peak of A25 DEAE-Sephadex column, 5. NAD-GDH from slow moving peak of A25-DEAE-Sephadex column.

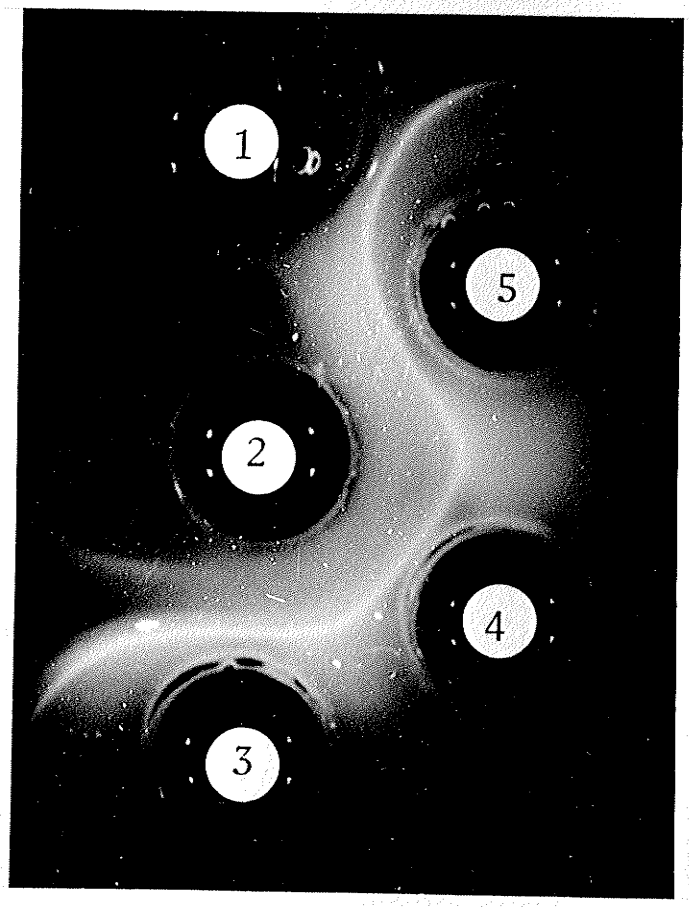
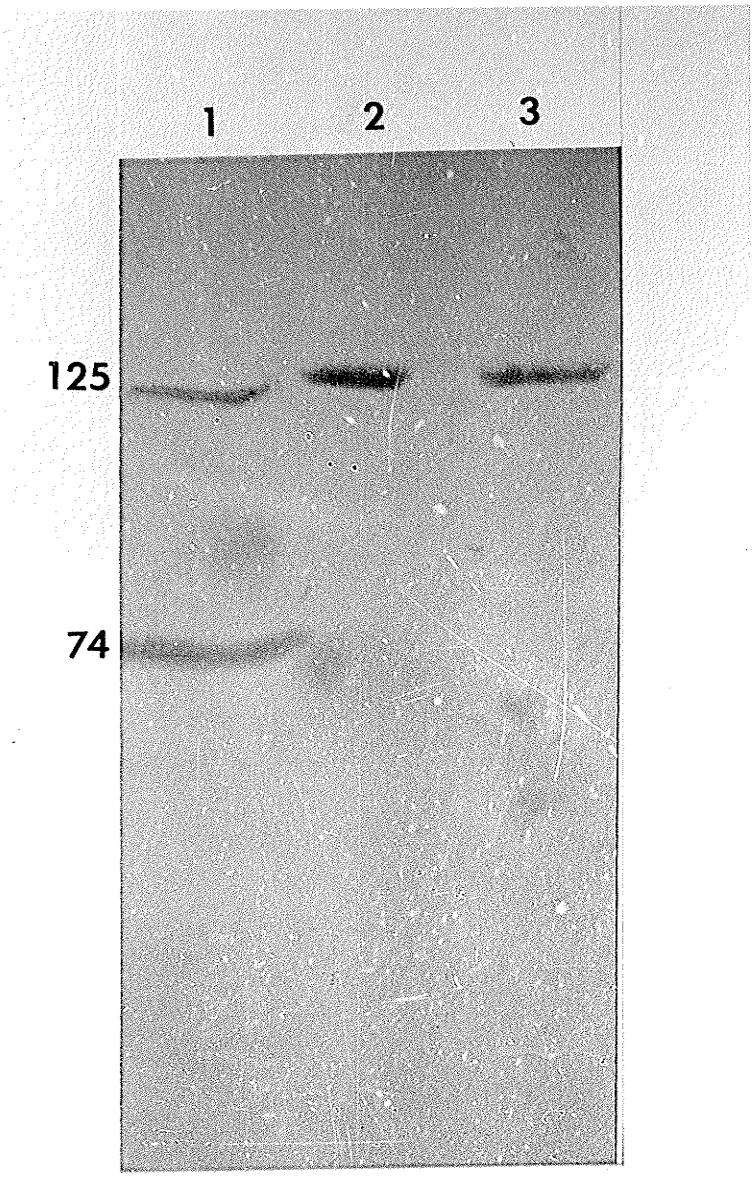

1
5
2
4
3

Figure 21. Immunostaining of NAD-GDH in Different Protein Fractions during Purification

Protein samples from cell free extract of induced cells (lane 1), partially purified NAD-GDH (step 3 of NAD-GDH purification) (lane 2) and purified NAD-GDH (lane 3) were fractionated by SDS-PAGE, electroblotted and immunostained. M_r values (x 10^3) are given in the ordinate axis. The M_r were determined by comparing the immunostained Western blot to the protein gel containing the markers (the same markers as used in Fig 10). The M_r in the following immunostained Western blots were determined in the same way.


1
2
3
125
74

The antiserum was quantitatively tested by antigen-antibody precipitation and ELISA procedure. An equivalence point at which the maximum precipitation is obtained from the antigen-antibody interaction was first obtained by the method described by Jamieson *et al.* (1972). Results in Fig 22 show that when 36 μg partially purified glutamate dehydrogenase was treated with 50 μL of antiserum, maximum precipitation was achieved. In the ELISA procedure, a series of antiserum dilutions was incubated against different antigen dilutions proportionately according to the equivalence point. Although the purpose of the experiment was to find out at which dilution the antiserum was be used for Western blot staining, different concentrations of antigen were used according to the regular ELISA test. Each well of a special plate was initially coated with a suitable amount of antigen in 100 μL 0.05 M carbonate buffer, pH 9.6. After saturating with blocking buffer containing 3% BSA, a series of antiserum dilutions (100 μL) were distributed in the wells of the plates, then incubated with 100 μL enzyme labelled second antibody (anti-rabbit IgG) diluted at 1:3,000. The enzyme-substrate (100 μL) was incubated for 30 min at room temperature and the absorbancy determined at 490 nm. The results are shown in Table 4. Although some abnormal results could be seen in the table, the O. D. values become constant decrease at the dilutions higher than 3,200. This suggested that the data at higher dilutions of antiserum may be more reliable. Different concentrations of NAD-GDH (antigen) had no significant effect on antibody titration. This was probably due to the fact that the amount of antigen first adsorbed to the test plate was so excessive that the additional NAD-GDH did not constitute a variable parameter. So the antibody concentration was the crucial factor

Figure 22. Determination of Equivalence Point for NAD-GDH and Its Antibody.

The precipitation occurred under the condition described in "Materials and Methods". Protein concentrations were determined by the method of Lowry *et al.*(1951). The data in the figure are the means for three independent experiments.

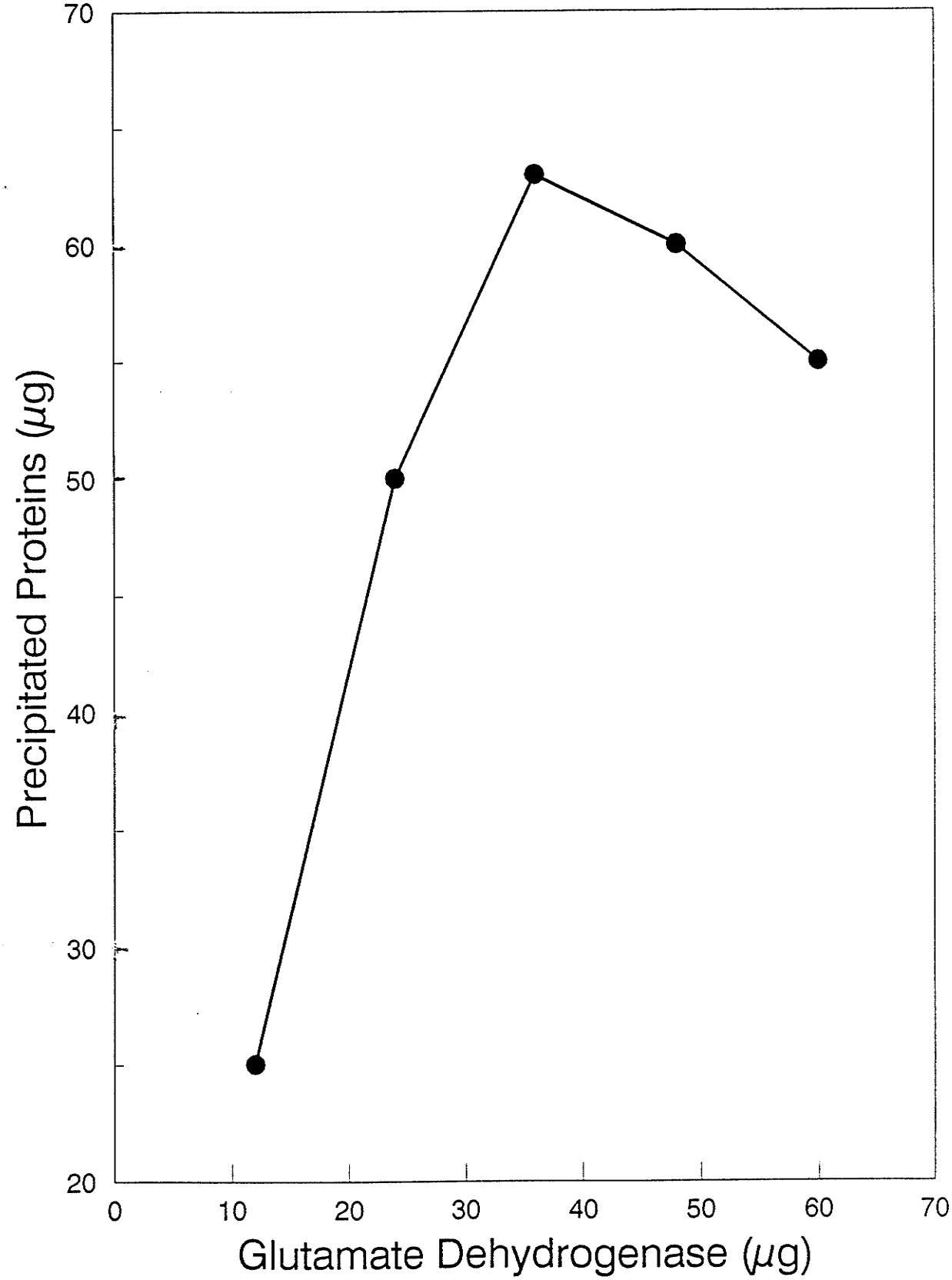

70
60
50
40
30
20
0
10
20
30
40
50
60
70
Precipitated Proteins (μg)
Glutamate Dehydrogenase (μg)

Table 4. ELISA Test of NAD-GDH with Its Antibody

Antiserum dilution	Antigen (GDH) (μg)					
	0	7.2	1.44	0.72	0.36	0.18
10*	0	1.334	1.318	1.257	1.238	1.264
50	0	1.314	1.286	1.291	1.236	1.260
100	0	1.300	1.230	1.238	1.367	1.294
200	0	1.377	1.225	1.247	1.201	1.342
400	0	1.420	1.296	1.319	1.364	1.387
800	0	1.260	1.245	1.297	1.323	1.323
1,600	0	1.302	1.278	1.273	1.211	1.191
3,200	0	1.187	1.130	1.003	1.139	1.083
6,400	0	1.009	1.126	0.975	0.852	0.898
12,800	0	0.919	0.737	0.704	0.749	0.719
25,600	0	0.755	0.533	0.520	0.525	0.521
51,200	0	0.511	0.453	0.394	0.406	0.381

* The figures in this lane signify antiserum dilution.

The data are the means of two experimental results.

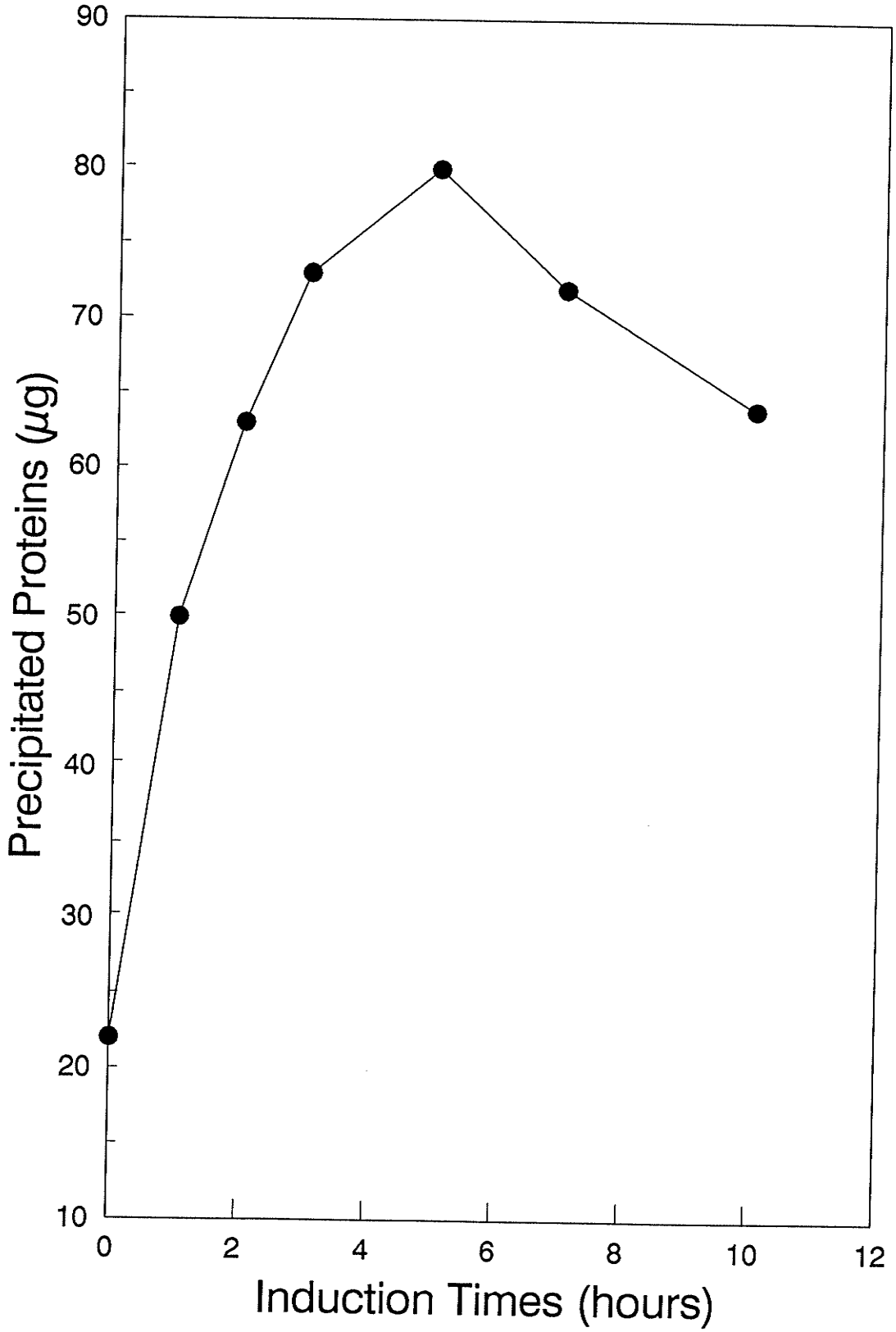
in this test. This experiment indicated that a high dilution of antibody could be used in immunostaining of Western blot to lower the background.

In the antigen-antibody precipitation, the amount of antigen and antiserum used was according to the result of the equivalence point for NAD-GDH and its antiserum. Cells were induced with 5 mM glutamine for 0, 1, 2, 3, 5, 7, and 10 h respectively. Enzyme was extracted as described in Methods and Materials. Antiserum was incubated with suitable amounts of enzyme protein (the same amount of protein, 100 μg, from each sample) by preparing a mixture (total volume 0.5 mL) containing 0.15 M NaCl, 1 mM sodium azide, and 4.7% Dextran T70 (Jamieson *et al.*, 1972). The mixtures were incubated at 37°C for 2 h and then allowed to stand at 4°C for 24 h. After centrifuging, the precipitates were washed with 1 mL 0.15 M NaCl, then dissolved in 0.5 mL 0.1 M NaOH to determine the protein concentration. The results in Fig 23 indicate that when cells were induced with 5 mM L-glutamine for 5 h, maximum precipitation of the NAD-GDH and its antibody was obtained.

Based on the high activity of antiserum obtained in the ELISA test, a high dilution of antiserum was used in Western blotting (see "Methods and Materials"). While antisera are usually used at 1/200 to 1/1,000 dilutions, the anti-GDH antiserum made above was sufficiently active that a dilution of 1/5,000 could be used.

Figure 23. Quantitative Immunoprecipitation Analysis of NAD-GDH during Induction

NAD-GDH was induced in cells incubated with 5 mM glutamine for different times as described in "Methods and Materials" and cell-free extracts prepared. An excessive amount of antiserum was used to precipitate glutamate dehydrogenase as described under "Investigation of Induced Glutamate Dehydrogenase with Its Antibody". The precipitated proteins were quantitated according to Lowry *et al.* (1951). The data in the figure are the means for three independent experiments.


90
80
70
60
50
40
30
20
10
Precipitated Proteins (μg)
0
2
4
6
8
10
12
Induction Times (hours)

2.2. Basis of Enzyme Induction

2.2.1. Induction with different reagents.

Ammonia, glutamate, sucrose (or glucose) serve as inducers or repressors of various glutamate dehydrogenases (NAD- or NADP-specific) (see Historical). In *Achlya klebsiana*, preliminary studies had shown that cells grown in a medium containing abundant glucose (5 g/L) and limited quantities of amino acids (0.5 g yeast extract/L) (GY), produce low levels of NAD-GDH (Stevenson and LéJohn, 1970). When GY medium was supplemented with either glutamate (5 mM) or glutamine (5 mM), the cells were induced to produce copious amounts of NAD-GDH (Braithwaite, 1987). Similar results were obtained if the cells were first grown vegetatively in GY medium, then transferred to a medium of Tris-acetate, pH 6.5 and Ca^{2+} (starvation medium) supplemented with either glutamate or glutamine. Glutamine was a more effective inducer of NAD-GDH than glutamate under these conditions. One possible explanation for this is that glutamine was being metabolised to glutamate and ammonia, both of which could be serving as inducers. To test this hypothesis, the level of induction of NAD-GDH by L-glutamine, L-glutamate and ammonium ions was compared. The results (Table 5) show that ammonium ions had an insignificant effect on NAD-GDH induction. Thus, ammonium ions may not be an inducer of the enzyme. Glutamate and glutamine induced the enzyme 2.9-fold and 5.1-fold, respectively. The inefficacy of ammonium ions as an inducer may, in part, be due to poor uptake and/or the known inability of the cells to metabolise inorganic nitrogen (Dr. H. B. LéJohn, personal communication).

Table 5. Level of NAD-GDH in Cells Prior to and After Starving for 3 h in the Presence of Inorganic and Organic Nitrogen Sources

Condition	Concentration (mM)	Specific activity* (NAD-GDH)	Fold change
uninduced:		3.8×10^4	----
induced:			
+ NH_4^+	(5)	4.6×10^4	1.2-fold
+ L-glutamate	(5)	1.1×10^5	2.9-fold
+ L-glutamate, NH_4^+	(5+5)	1.15×10^5	3.0-fold
+ L-glutamine	(5)	1.92×10^5	5.1-fold

* Specific activity is expressed as nmol NADH oxidized/min/mg protein.

The data are the means of two independent experiments.

2.2.2. Glucose and L-glutamine mutually control NAD-GDH level.

It has been reported that L-glutamate induces and glucose represses glutamate dehydrogenase (LéJohn and Stevenson, 1970). The results in Table 5 support the idea that directly or indirectly, L-glutamine induces the enzyme. Because only an NAD-GDH has been found in *Achlya klebsiana*, it seemed likely that other enzymes of nitrogen metabolism, glutamine synthetase and glutamate synthase should exist in the organism. Otherwise, the sole NAD-GDH left to control carbon and nitrogen flow at this metabolic branchpoint would be subjected to extremely complex regulatory systems. As will be seen later, immunostaining indicates that a protein with M_r of 220,000 is related to the NAD-specific GDH. For the moment, the objective was to demonstrate that the induction and repression of NAD-GDH were mediated *de novo*. In other words, that induction was due to actual protein synthesis and repression was due to lack of that synthesis. The analysis was done by immunodetection of electrophoresed and electroblotted enzyme as well as determination of the specific activity of the enzyme. The results (Figs 24 and Fig 25) show that glutamine is an effective inducer even in the presence of abundant amounts of glucose. Reduction of enzyme activity occurred when glucose was supplied at relatively low concentrations, but little additional reduction could be seen at much higher glucose concentrations. This could be explained on this basis; that starving cells have limited metabolic capacity to use glucose for constant regeneration of the repressor. L-glutamine, on the other hand, may be metabolised much better and, thereby, produce sufficient amounts of the inducer.

The enzyme extracts were electrophoresed in SDS-PAGE and electro-

Figure 24. Influence of Glutamine and Glucose on Induction and Repression of NAD-GDH

Cells grown in defined medium were transferred to starvation medium containing 30 mM glucose and glutamine in different concentrations (0, 0.1, 0.5, 1.0 and 5.0 mM) for 3 h at 28°C with agitation (170 rpm) (see "Materials and Methods"). Cell-free extracts were prepared by sonication. GDH activity was determined by the reductive amination and oxidative deamination reactions with and without $NADP^+$. The experiment was repeated once but the data are from only one of the two experiments.

1: assayed with NADH + NADP

2: assayed with NADH

3: assayed with NAD + NADP

4: assayed with NAD

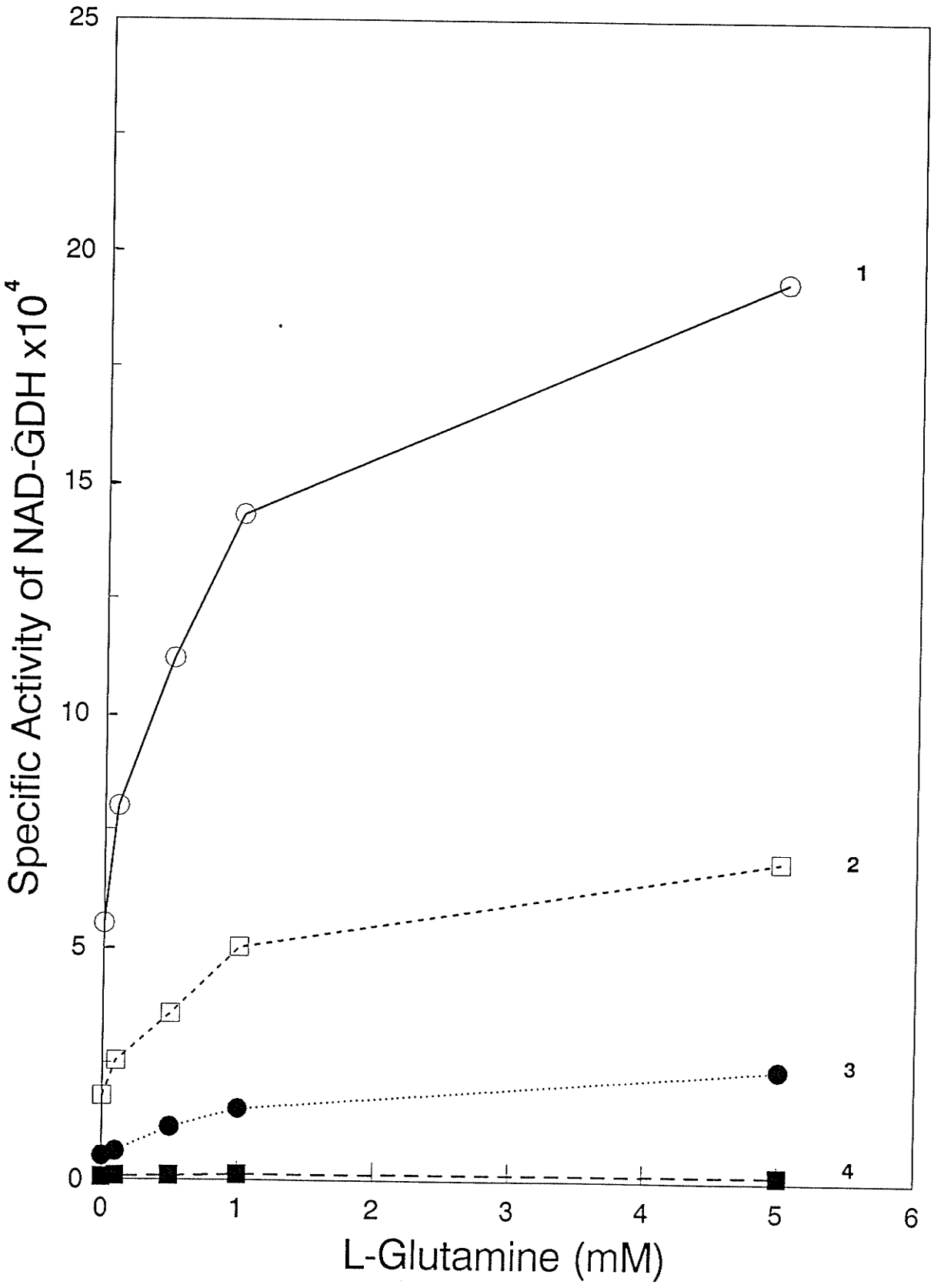

Specific Activity of NAD-GDH x10^4
25
20
15
10
5
0
0
1
2
3
4
5
6
L-Glutamine (mM)
1
2
3
4

Figure 25. Influence of Glucose on the Induced Level of NAD-GDH

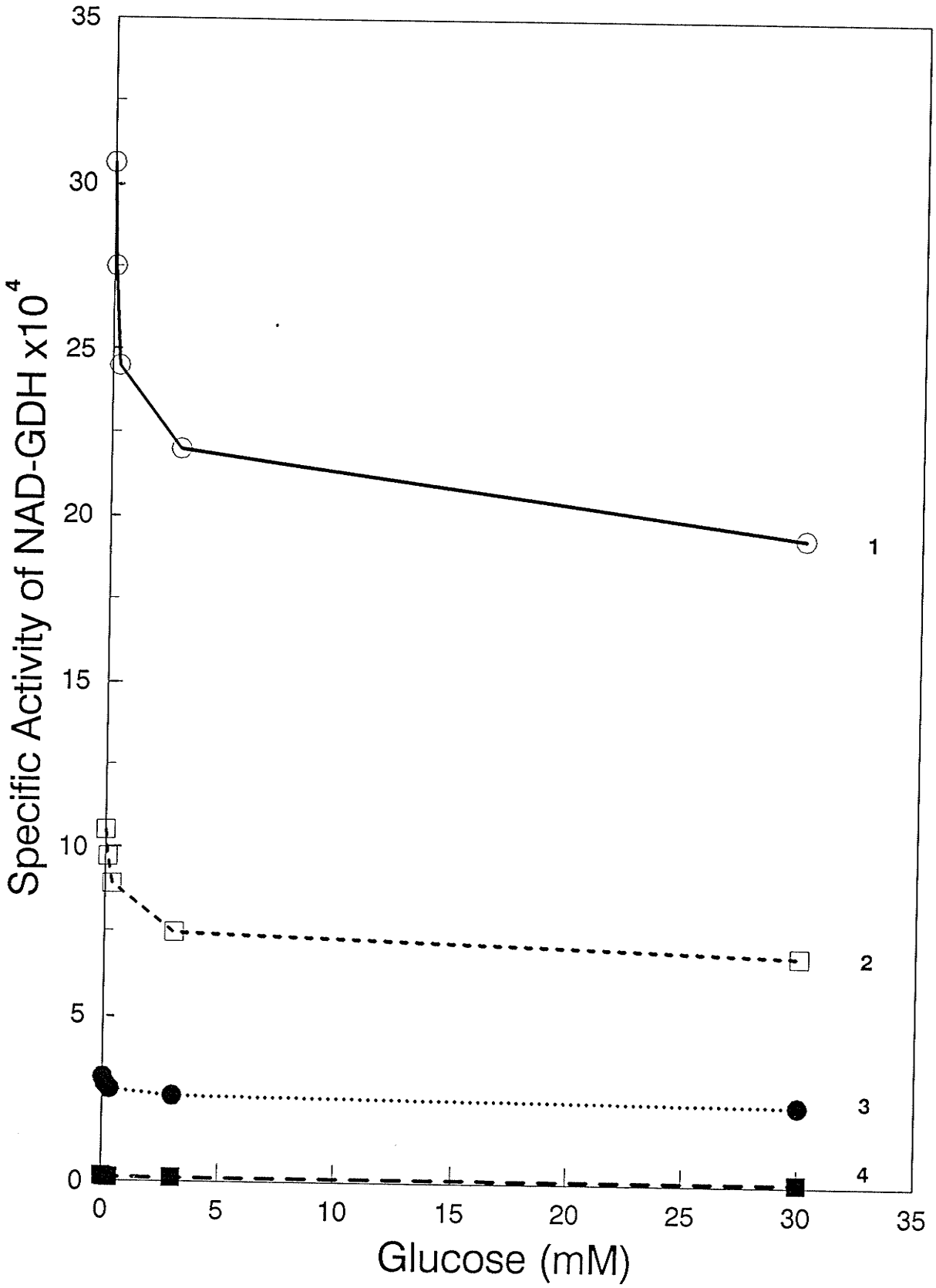
Cells grown in defined medium were transferred to starvation medium containing 5 mM glutamine and different amounts of glucose (0, 0.1, 0.3, 3.0 and 30 mM) for 3 h at 28°C with agitation (170 rpm). Cell-free extracts were prepared by sonication. GDH activity was determined by reductive amination and oxidative deamination reactions with and without $NADP^+$. The experiment was repeated once but the data in the figure are from only one of the two experiments.

1: assayed with NADH + NADP

2: assayed with NADH

3: assayed with NAD + NADP

4: assayed with NAD


Specific Activity of NAD-GDH x10^4
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
Glucose (mM)
1
2
3
4

blotted onto nitrocellulose membranes. The membranes were immunostained with 1:5,000 diluted anti-GDH antiserum and two polypeptides of M_r 125,000 and 74,000 were detectable (see Figs 26 and 27). The immunostained 125,000 M_r polypeptide was the NAD-GDH subunit, but the 74,000 M_r polypeptide was unassigned at this stage, and subsequent studies showed that it is a component of a native protein with an M_r of 220,000.

2.2.3. Time and concentration dependency of L-glutamine in enzyme induction. Following the determination that glutamine induces NAD-GDH, it was necessary to quantitate the process. The optimum concentration of glutamine required for NAD-GDH induction was determined in the following way. Cells were grown vegetatively for 20 h as stationary cultures in defined medium with glucose as the carbon source and then transferred to starvation medium. The cells were incubated with various concentrations of glutamine (0-8 mM) for 3 h at 28°C. Cell free extracts were analyzed electrophoretically, immunologically and spectrophotometrically. The results show that the concentration of L-glutamine required for maximum induction of NAD-GDH was 5 mM (Figs 28 and 29). The increase in enzyme activity was reflected quantitatively in the intensity of immunostained protein. The optimum concentration of 5 mM glutamine was used to deduce the maximum time required for induction. Incubation was followed for a total time of 8 h (Fig 30). It took no longer than 5 h to attain optimum induction (15 to 17-fold increase).

Worthy of note is the fact that the 74,000 M_r protein was present in these cell extracts (Fig 26-27). The central question was why? Is the 74,000 M_r polypeptide a breakdown product of the 125,000 M_r protein, or is

Figure 26. Immunodetection of NAD-GDH Levels in Fig 24

The five different protein samples from Fig 24 were used. The same amount of protein (60 μg) from each sample was electrophoresed in 7% polyacrylamide gel at 75 V overnight. Proteins were electro-blotted onto nitrocellulose membrane and immunostained with anti:NAD-GDH antibody. Bands were shown after colour development. The colour development was stopped when bands were clear to prevent high background of colour. All Western blot staining in the following figures was done this way. This result does not show the expected intensity increase of the 125,000 M_r protein. This may be due to technique problems. The M_r was determined the same way as in Fig 21. M_r values (x 10^3) of immunostained bands in Western blotted membrane are presented on the left margin.

lanes 1-5 contain protein from the samples that were collected after treatment of cells with 0, 0.1, 0.5, 1.0 or 5.0 mM glutamine, respectively.

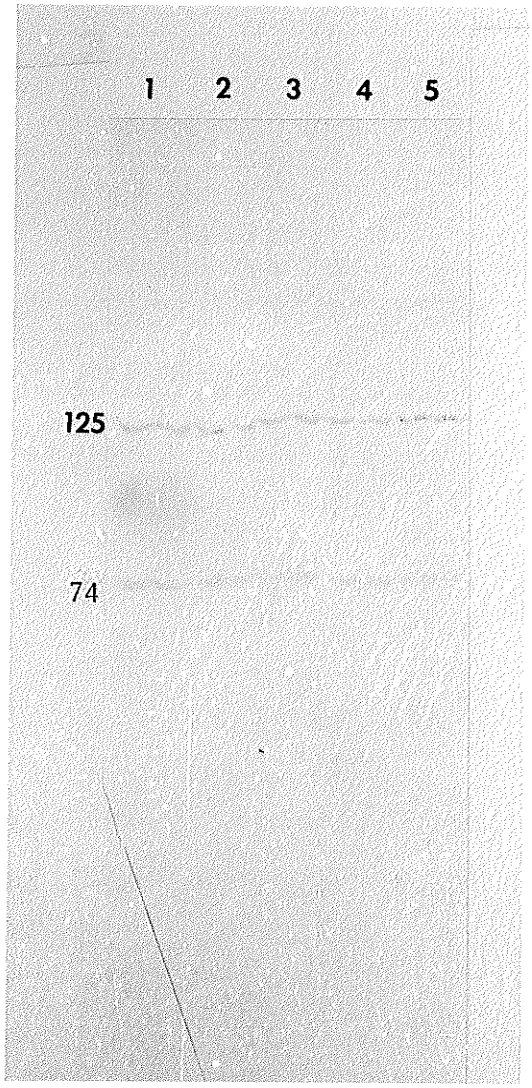

1
2
3
4
5
125
74

Figure 27. Immunodetection of NAD-GDH Levels in Fig 25.

Protein samples from Fig 25 were used. The same amount of protein (60 μg) from each sample was electrophoresed in 7% polyacrylamide gel at 75 V overnight. Proteins were electro-blotted onto nitrocellulose and immunostained. The M_r was determined in the same way as in Fig 21. M_r values (x 10^3) of immunostained bands are presented on the left margin.

lanes 1-5 contain protein from the samples that were collected after treatment of cells with 0, 0.1, 0.3, 3.0 or 30 mM glucose, respectively.

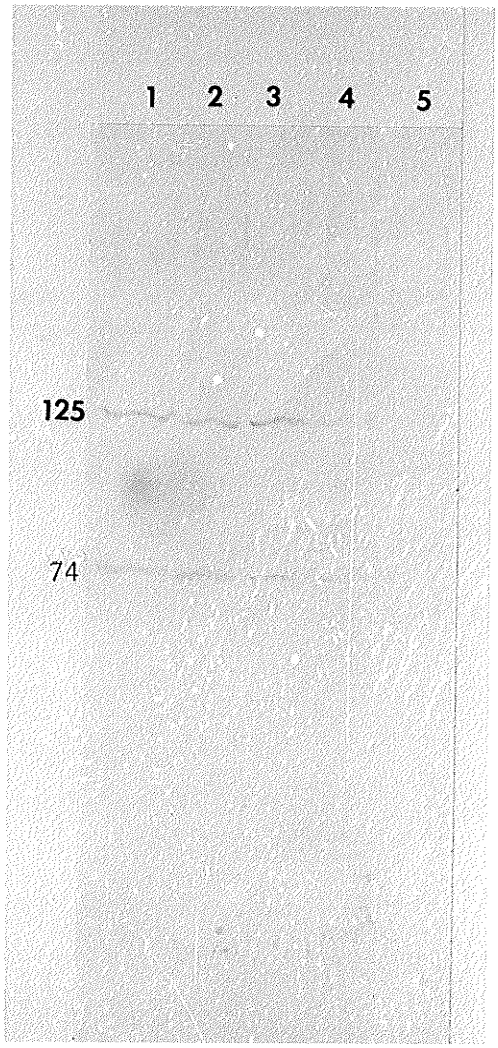

1
2
3
4
5
125
74

Figure 28. NAD-GDH Induced as a Function of Glutamine Concentration

Cells grown in defined medium were transferred to starvation medium and induced with different concentrations of L-glutamine for 3 h. Enzyme was extracted by sonication. Enzyme activity was assayed by the reductive amination reaction with $NADP^+$. Protein concentration was determined by the method of Lowry *et al.* (1951). The experiment was repeated once but the data in the figure are from only one of the two experiments.

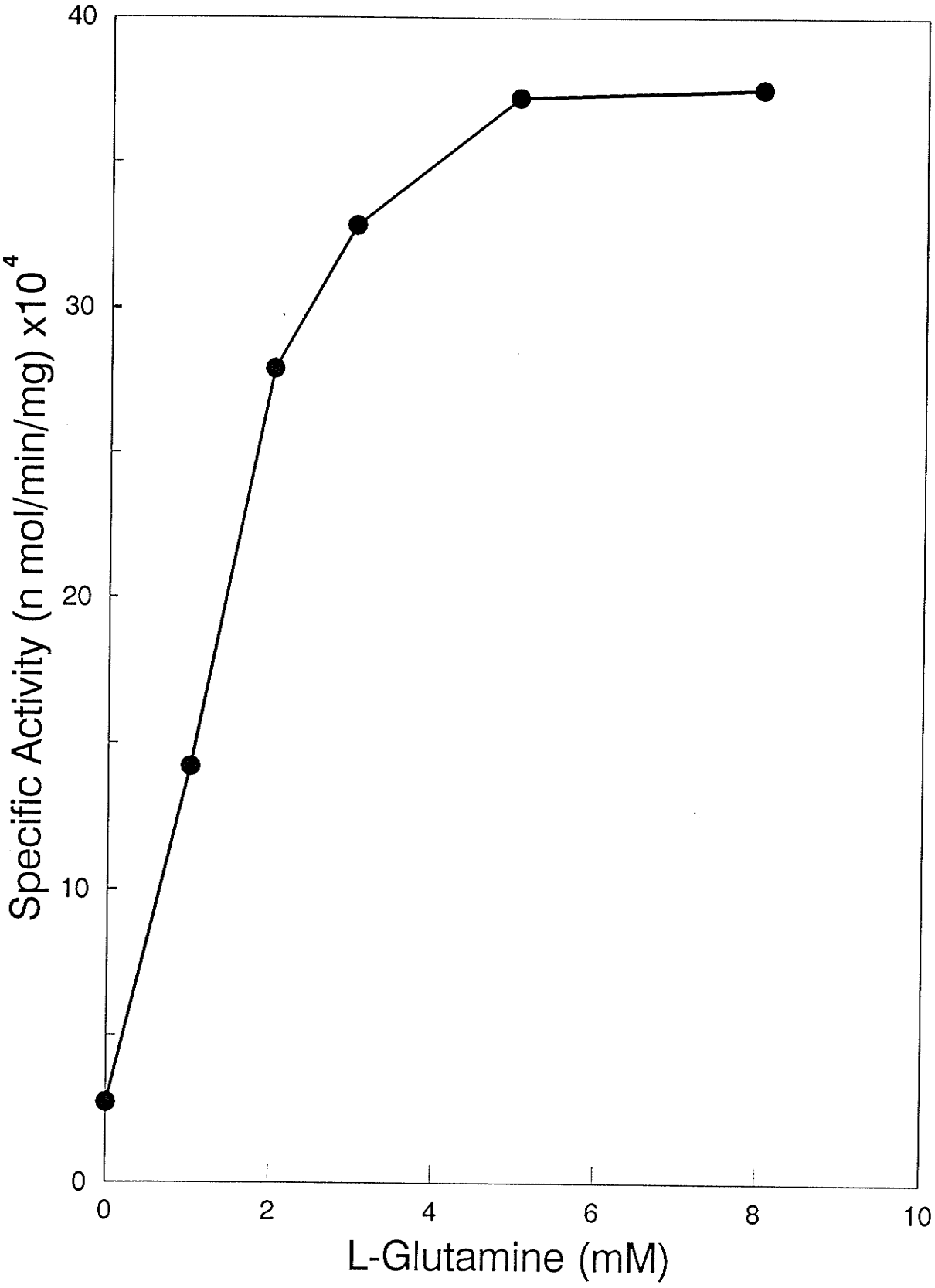

40
30
20
10
0
Specific Activity (n mol/min/mg) x10^4
0
2
4
6
8
10
L-Glutamine (mM)

Figure 29. Immunodetection of NAD-GDH Levels in Extracts of Cells Exposed to Different Concentrations of Glutamine

To see the induction of NAD-GDH protein, I just used the first 5 samples which showed induction of the enzyme in Fig 28 (0, 1, 2, 3 and 5 mM L-glutamine). The same amount of protein (60 μg) from each sample was electrophoresed in 7% polyacrylamide gel at 75 V overnight. Proteins were blotted onto nitrocellulose and immunostained. The M_r was determined in the same way as in Fig 21. M_r (x 10^3) of immunostained bands are presented on the left margin.

lanes 1-5 contain protein from the samples that were collected after treatment of cells with 0, 1, 2, 3 or 5 mM glutamine, respectively.

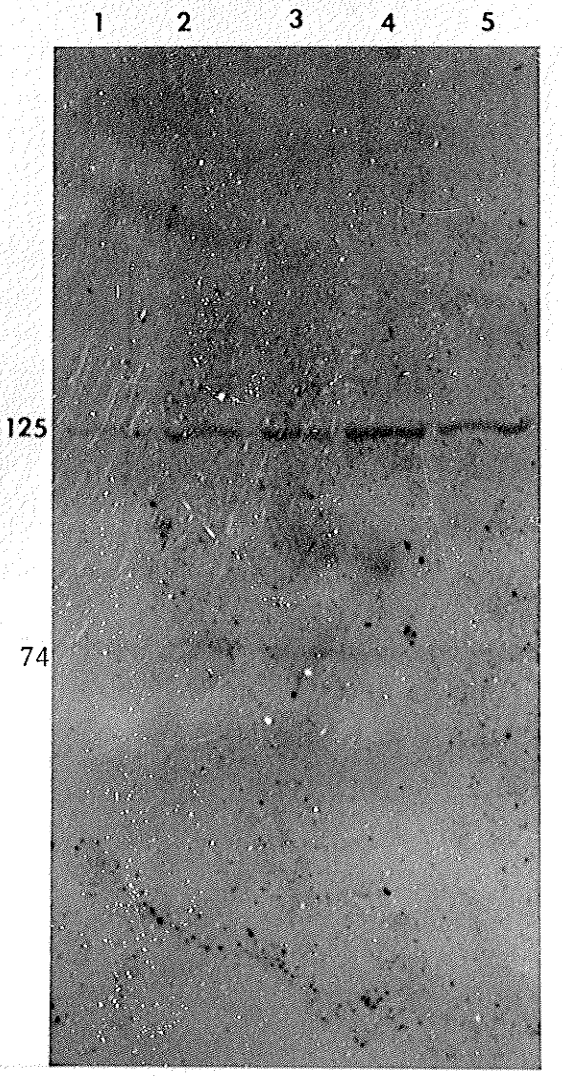
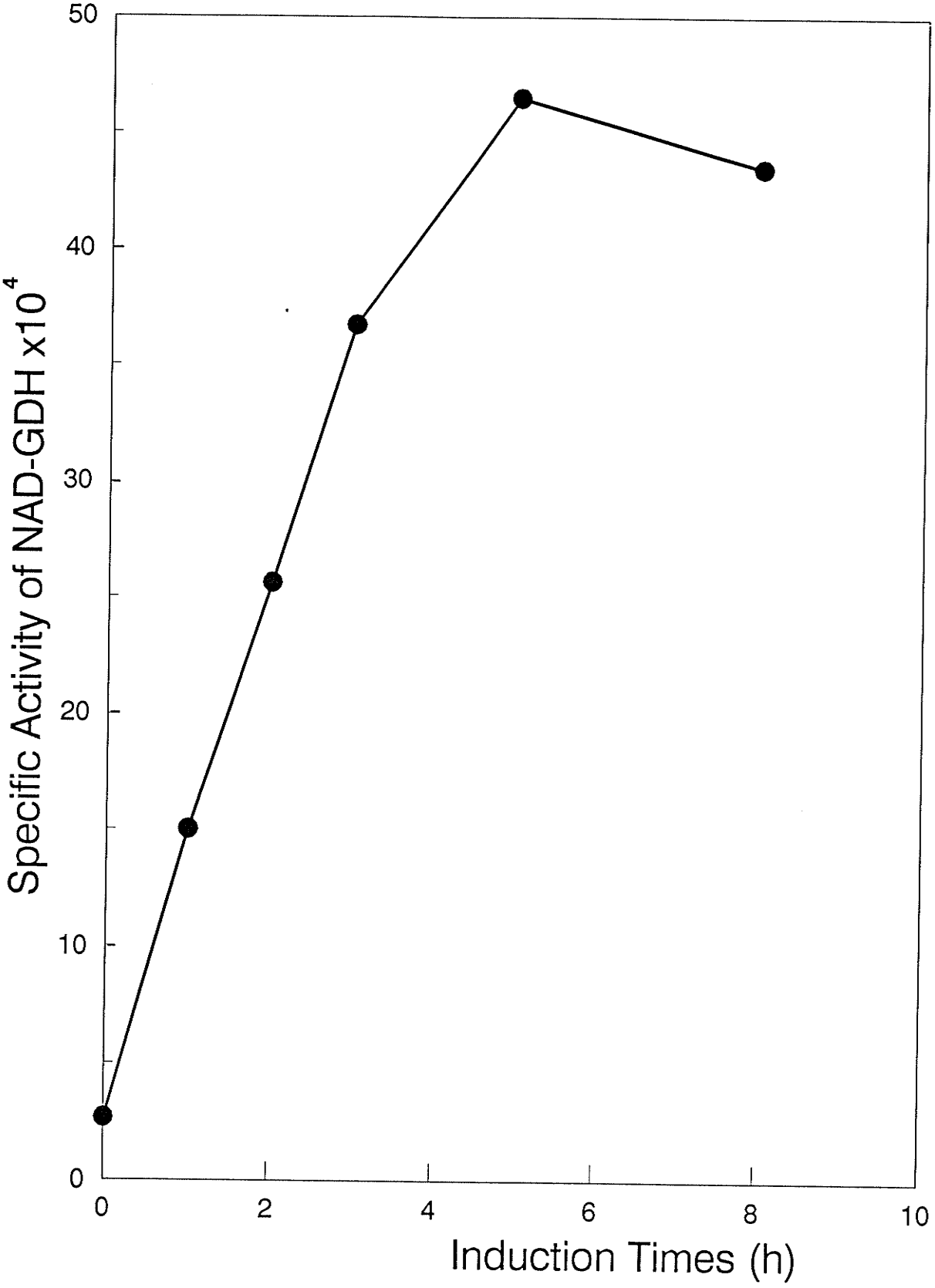

1
2
3
4
5
125
74

Figure 30. NAD-GDH Induction by Glutamine as a Function of Time

Cells grown in defined medium were transferred to starvation medium and NAD-GDH induced with 5 mM L-glutamine for different periods. Cell-free extracts were obtained by sonication. Enzyme activity and protein concentration were determined as in the legend to Fig 28. The experiment was repeated once but the data in the figure are from only one of the two experiments.


Specific Activity of NAD-GDH x10^4
50
40
30
20
10
0
0
2
4
6
8
10
Induction Times (h)

it a partially synthesized enzyme subunit? Alternatively, is the 74,000 M_r protein a distinct protein that is simply antigenically related to NAD-GDH, or is the antiserum a composite of 2 distinct set of antibodies to *Achlya* proteins? These questions are re-addressed experimentally later. What was clear, at this stage,is that the 74,000 M_r polypeptide was not a component of the native NAD-GDH, since the purified enzyme which was specific for NAD^+ and responsive to $NADP^+$ activation had been shown physically to consist of four identical M_r 125,000 subunits (Fig 9, Figs 11-14). To eliminate the possibility that unrelated polyclonal antibodies were reacting with two unique polypeptides even though the antibody was raised against the SDS-PAGE fractionated 125,000 M_r protein band, the anti-GDH polyclonal antiserum was made to interact with the 125,000 M_r GDH subunit affixed on nitrocellulose by electrophoresing the purified NAD-GDH in SDS-PAGE gel and Western blotting onto nitrocellulose membrane before testing its effectiveness on the 74,000 M_r polypeptide. This was carried out as described in the Western blot procedure except the GDH-treated antiserum (7.5 mL) was recovered in order to test its activity on fractionated cell-free extract. Polyclonal antiserum that had, presumably, been depleted of its anti-125,000 M_r polypeptide antibody showed little reactivity against the 74,000 M_r polypeptide. No band could be detected on fractionated cell-free extract when it was stained with NAD-GDH-adsorbed antiserum. It appears from this that the 125,000 M_r protein and the 74,000 M_r polypeptide share the same group of antigenic determinants, but the results are not unequivocal.

2.2.4. Decay of the 125,000 M_r subunit and the 74,000 M_r polypeptide.

It was shown that L-glutamine could arrest sporogenesis in *Achlya klebsiana* while L-glutamate does not (LéJohn *et al.*, 1978; LéJohn, 1983). Both amino acids induce NAD-GDH in *Achlya*, although L-glutamine is more effective. It seems unlikely, therefore, that the induction of NAD-GDH would play a central role in sporogenesis. But sporogenesis is accompanied by extensive degradation of preformed cell proteins (LéJohn, 1983). Therefore, it is expected that NAD-GDH, like other cell proteins, would be a victim of protein turnover in a starving cell. But the individual decay kinetics of the cell proteins may not necessarily be the same. The relative stability of induced NAD-GDH and the 125,000 and 74,000 M_r polypeptides in a starving cell was therefore studied to gauge how tightly coordinated the two were physiologically. Cells were grown in GGY medium for 18 h and then induced with 5 mM L-glutamine for 3 h. The induced cells were transferred to starvation medium and starved for 0, 1, 2, 3, and 5 h. The level of enzyme in the cells was determined at those intervals. The results in Fig 31 show that more than 50% of the induced enzyme activity was lost within 5 h of starvation. Loss of enzyme activity was matched by a decline in the level of the 125,000 M_r and 74,000 M_r polypeptides (Fig 32). These two polypeptides, therefore, exhibited the same properties of decay.

The next question to answer is whether the 125,000 M_r protein is a precursor of the 74,000 M_r polypeptide. Are they products of the same or similar genes (because of the antibody data)? An investigation of the possible precursor-product relationship of the two polypeptides was carried out first in the next series of experiments. This was followed by

Figure 31. Loss of NAD-GDH Activity with Time, *In Vivo*

Cells were grown in GGY medium with agitation (170 rpm) at 28°C for 18 h and then NAD-GDH induced with 5 mM L-glutamine in the same growth medium. The induced cells were transferred to starvation medium and incubated for 0, 1, 2, 3, and 5 h. Cell-free extracts were prepared by sonication. GDH activity was assayed reversibly, spectrophotometrically, with and without $NADP^+$. The experiment was repeated once but the data in the figure are from only one of the two experiments.

1: assayed with NADH + $NADP^+$

2: assayed with NADH

3: assayed with NAD^+ + $NADP^+$

4: assayed with NAD^+

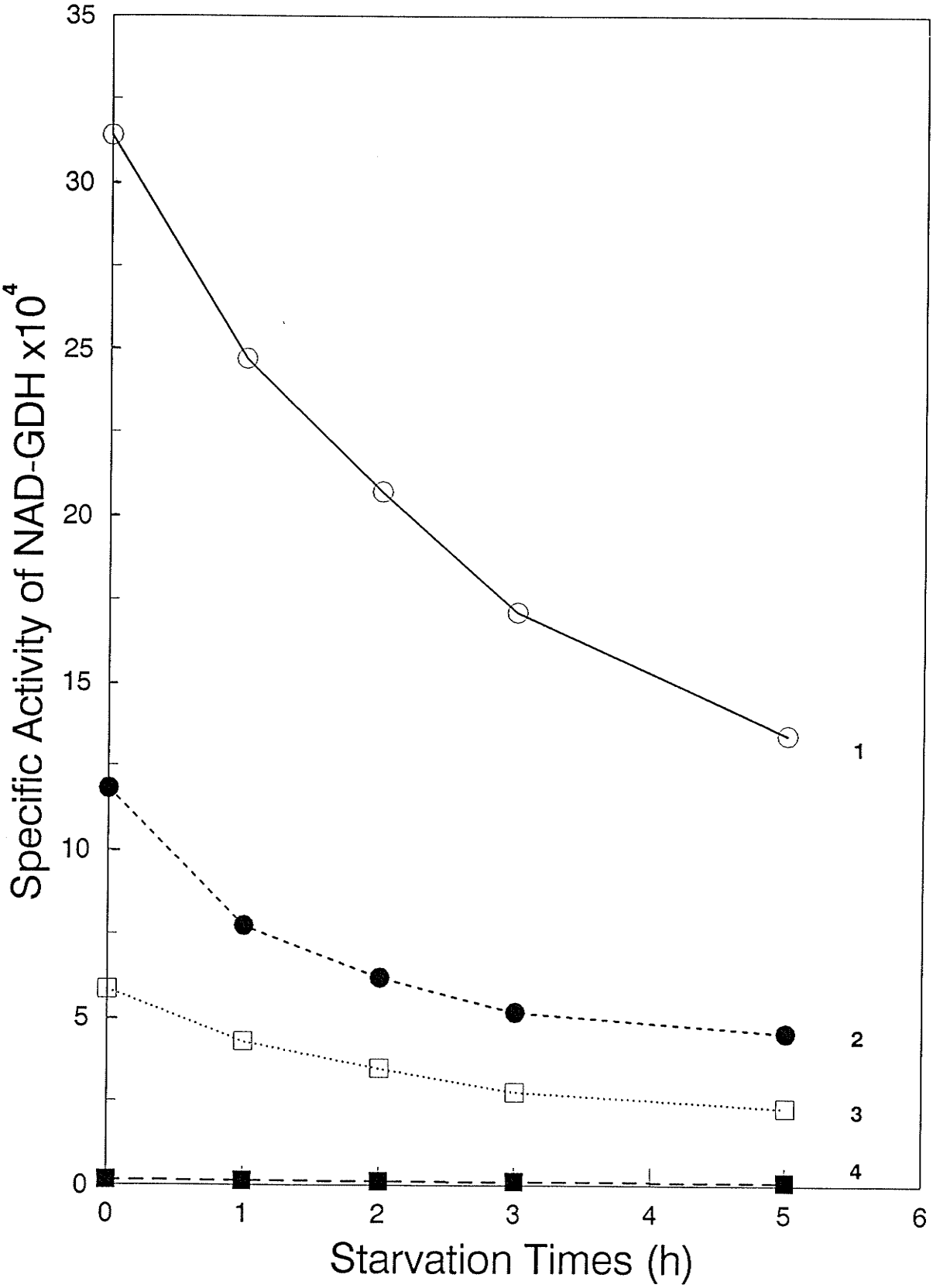
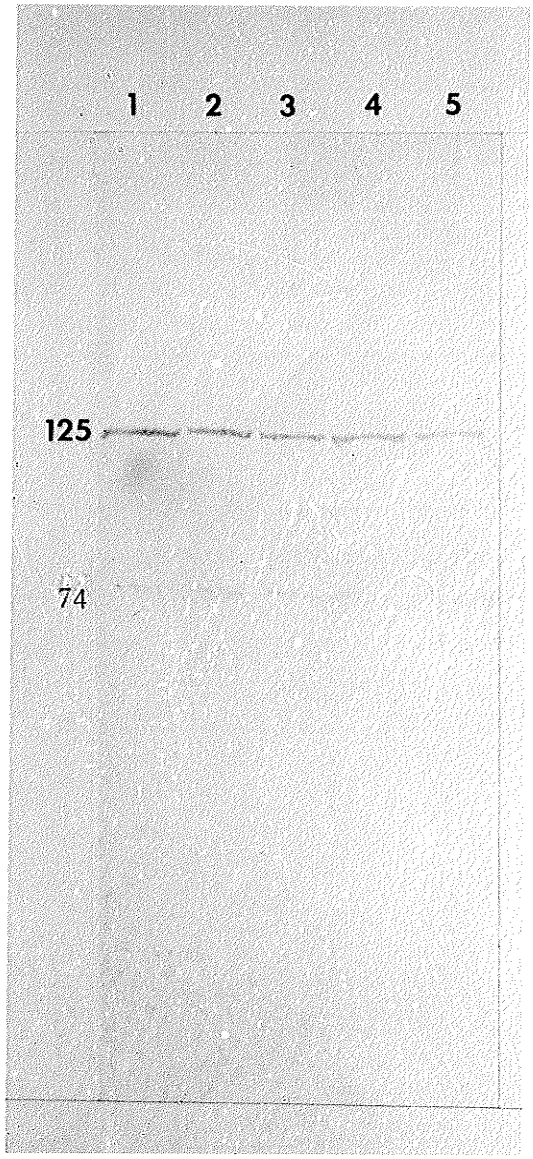

Specific Activity of NAD-GDH x10^4
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
Starvation Times (h)
1
2
3
4

Figure 32. Determination of NAD-GDH Protein Level in Cells during Loss of Enzyme Activity by Immunostaining

The same amount of protein (60 μg) from each sample of Fig 29 was electrophoresed in a 7% polyacrylamide gel at 75 V overnight. Proteins were blotted onto nitrocellulose and immunostained. The M_r was determined in the same way as in Fig 21. M_r (x 10^3) of immunostained bands are presented on the left margin.

lanes 1-5 contain protein from the samples that were collected after cells were starved for 0, 1, 2, 3 or 5 h, respectively.


1
2
3
4
5
125
74

an examination of the question whether there really are two distinct types of antibodies in the antiserum through DNA studies.

2.3. Use of Proteinase Inhibitors to Study Mode of Enzyme Induction

To investigate the possibility that the 74,000 M_r polypeptide is a product of the 125,000 M_r subunit, proteinase inhibitors were used to block degradation of cell proteins. Cells were grown in defined medium for 18 h and then transferred to starvation medium. NAD-GDH in starving cells was induced with L-glutamine and the cells were simultaneously exposed to proteinase inhibitors, such as TAME, TLCK, benzamidine and PMSF. In all cases, the level of the 74,000 M_r polypeptide was much higher than that of the 125,000 M_r protein (data not shown). TAME was arbitrarily chosen to conduct further experiments. In one case, TAME was added simultaneously with the inducing agent L-glutamine to starving cells, and enzyme activity, and levels of the 125,000 M_r protein and 74,000 M_r polypeptide were determined periodically. In another case, TAME was added 30 min before L-glutamine, and enzyme activity and the levels of the 125,000 and 74,000 M_r polypeptides determined periodically. When TAME and L-glutamine were added simultaneously, there was a large increase in the level of the 74,000 M_r polypeptide over that of the 125,000 M_r polypeptide (Fig 33). It is not known why TAME can prevent the degradation of the 74,000 M_r polypeptide, but not NAD-GDH, resulting in a low level of NAD-GDH and a high level of 74,000 M_r polypeptide. This can also be seen in Fig 34. It is deduced from the data that the proteinase inhibitor in preventing degradation of pre-formed cell proteins in nutrient-starved cells, was

Figure 33. Time Course Induction of NAD-GDH in the Presence of Proteinase Inhibitor

Cells were grown in defined medium for 18 h and then transferred to starvation medium containing 5 mM L-glutamine and 100 μg TAME/ml. At different periods, cells were harvested and proteins were analyzed for the NAD-GDH subunit by immunoblotting.

lane 1: protein from 15 min induction;

lane 2: protein from 30 min induction;

lane 3: protein from 45 min induction;

lane 4: protein from 60 min induction;

lane 5: protein from 90 min induction.

The M_r was determined in the same way as in Fig 21. M_r values (x 10^3) of immunostained bands are presented at the left margin. This experiment was to see the effect of proteinase inhibition on the relative levels of the 125,000 Mr and 74,000 Mr protein, and the 0 min sample was not included.

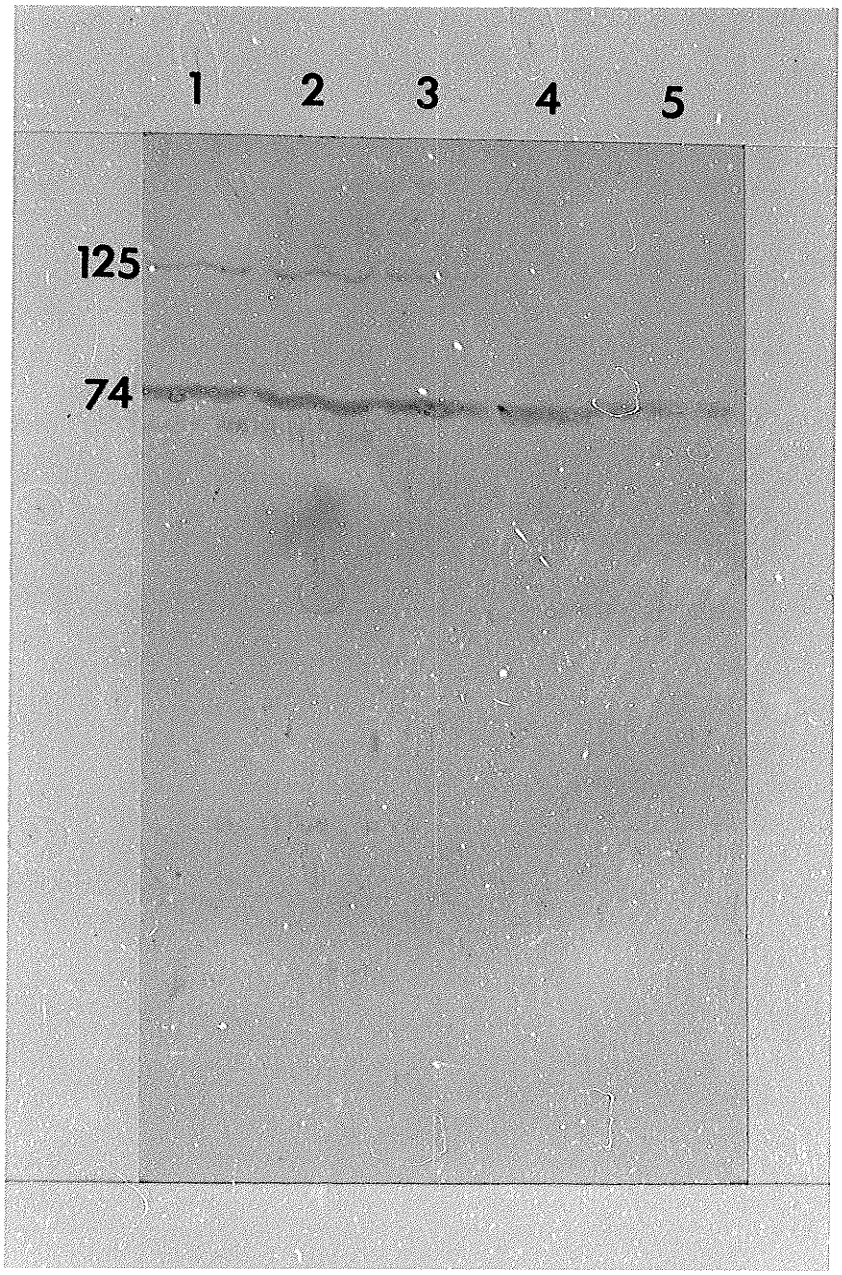
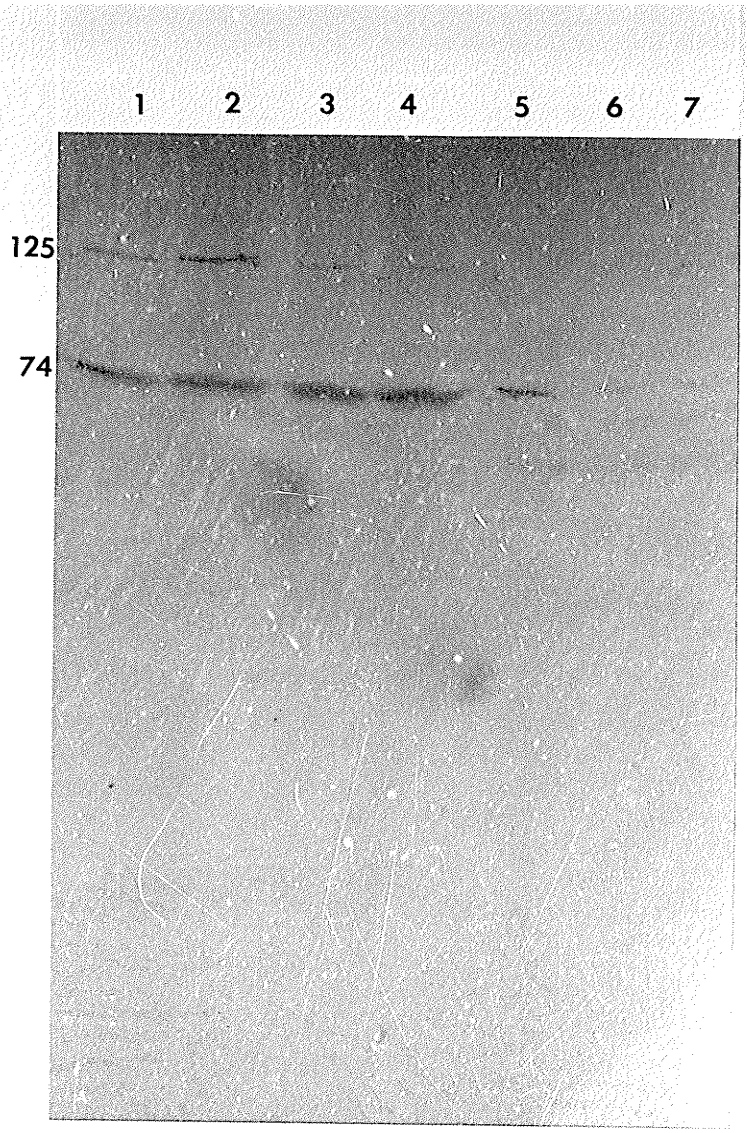

1
2
3
4
5
125
74

Figure 34. Effect of Pre-exposure of Cells to Proteinase Inhibitor before Induction of NAD-GDH

Cells were grown in defined medium for 18 h and then transferred to starvation medium containing TAME (100 $\mu g/\mu l$) for 30 min. Following this, 5 mM glutamine was added to the cultures and samples taken after 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5), 90 min (lane 6), and 120 min (lane 7). Cell extracts were immunoblotted and immunostained after electrophoresis. The M_r was determined in the same way as in Fig 21. M_r values (x 10^3) of immunostained bands are presented at the left margin.


1
2
3
4
5
6
7
125
74

indirectly hindering synthesis of the enzyme (hence the 125,000 M_r subunit). However, the amounts of both proteins decrease with TAME treatment, albeit the relative levels of the two proteins are reversed in comparison to induction under proteinase-free condition. The 74,000 M_r protein, it seems, is unlikely to be a product of the 125,000 M_r protein.

2.4. Immunodetection of Native Protein for 74,000 M_r Polypeptide

The proteinase inhibitor studies hint strongly that the 74,000 M_r polypeptide is not physically related to the 125,000 M_r subunit. Is the 74,000 M_r polypeptide part of a larger protein complex or is it in its native state already? This question was experimentally examined by (a) immunoanalysis of proteins electroblotted from non-denaturing electrophoresed gels, and (b) after recovering the undenatured immunostained proteins, denaturing them and analysing the denatured products electrophoretically and immunochemically. The procedure used is as follows. Proteins from NAD-GDH induced cells were electrophoresed in their native state in a 3-20% polyacrylamide gradient gel. The proteins were electroblotted and immunostained with the polyclonal antibody. The results (Fig 35) show that two bands of approximately 500,000 M_r and 220,000 M_r were stained. The 220,000 M_r protein was recovered from a duplicate unstained gel, denatured, electrophoresed, electroblotted and immunostained as described below. A gel strip around the region of 220,000 M_r protein was recovered by cutting, and eluting in an electroelutor containing 50 mM Tris-acetate, pH 8.9, 0.1% SDS. The protein solution was concentrated by 10% TCA precipitation, electrophoresed in SDS-PAGE (10%

Figure 35. Immunodetection of the Native Protein Containing the 74,000 M_r Polypeptide

Achlya cell-free extracts were electrophoresed in a non-denaturing gradient gel (3-19.6%) as described in "Methods and Materials". Proteins in the gel were electroblotted and immunostained.

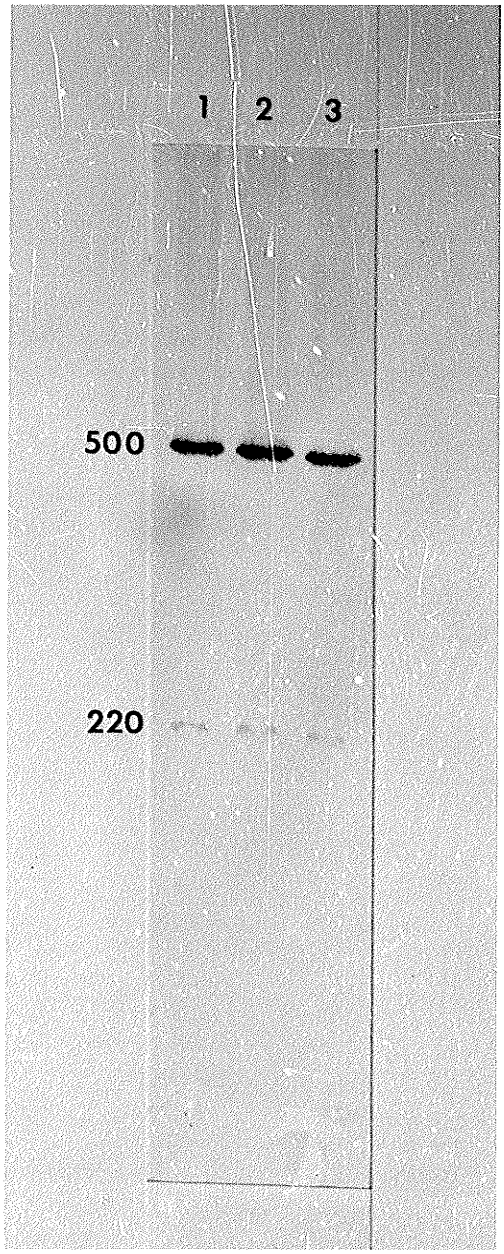
lane 1: cells fed with 10 mM glutamate;

lane 2: cells fed with 5 mM glutamine and 200 μg TAME/ml;

lane 3: cells fed with 5 mM glutamine.

The figures in the picture signify kDa.

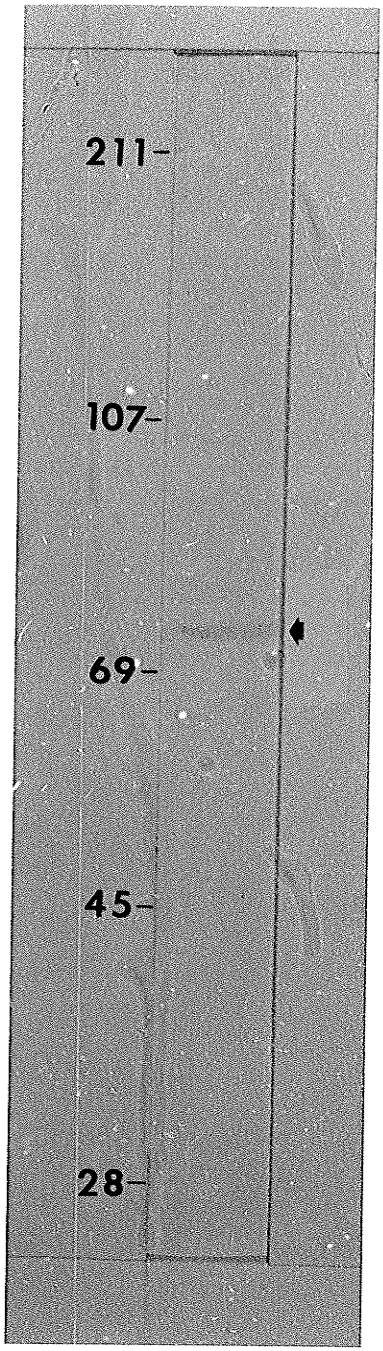
The M_r was determined in the same way as in Fig 21. M_r values ($\times 10^3$) of immunostained bands are presented at the left margin.


1
2
3
500
220

gel), electroblotted and immunostained with polyclonal anti-GDH antiserum. One band with a M_r of approximately 74,000 was detected after colour development (Fig 36). This band, therefore, is a product of the 220,000 M_r protein. The physical structure of this 220,000 M_r protein is not determined by this result, and the physiological significance of this protein is also unknown. It is, however, a protein that may or may not share chemical similarity to NAD-GDH. This depends entirely on the uniqueness of the anti:NAD-GDH antiserum prepared.

Figure 36. Detection of a 74,000 M_r Polypeptide Recovered from SDS Denatured 220,000 M_r Protein

The 220,000 M_r protein band obtained (see Fig 35) was electroeluted, concentrated, electrophoresed in SDS-PAGE, electroblotted onto nitrocellulose membrane and immunostained with anti:NAD-GDH antibody. After colour development, one band (signified by the arrow) with M_r of 74,000 was detected. The M_r was determined in the same way as in Fig 21. M_r values (x 10^3) of protein markers are presented at the left margin of the immunostained band.


211-
107-
69-
45-
28-

3. ISOLATION AND ANALYSIS OF cDNA CLONES

Because the antiserum against NAD-GDH was immunoreacting with two polypeptides of vastly different molecular masses (M_r 74,000 and 125,000) and nothing else, and the antiserum itself was raised against the 125,000 M_r polypeptide fractionated in SDS-PAGE gels, a tentative conclusion is that one species of antibody was cross-reacting with two proteins. The possibility, however, that the antiserum contains a second antibody species specific for the 74,000 M_r polypeptide cannot be summarily discounted. With this in mind, all succeeding experiments were designed and analysed for such a possibility.

The first of these experiments involved preparation of a cDNA library in λgt11 for the purpose of isolating cDNA(s) clones encoding the NAD-GDH gene(s). The cDNA library was to be screened by the polyclonal anti:NAD-GDH antiserum purified from anti:*E. coli* protein contaminations. Theoretically, if there are two antibody species, two types of cDNA clones corresponding to the genes for the 74,000 and 125,000 M_r proteins will be detected. If there is only one antibody species, then only one type of antigen determinant will be detected. However, different proteins might have the same type of antigen determinant resulting in the detection of more than one cDNA species. Such kinds of problem had to be kept in mind while screening the cDNA library.

3.1. Poly(A)$^+$ RNA Purification and Translation *In Vitro*

Total RNA was extracted by the method described by Chomczynski and

Sacchi (1987). The extracted RNA was stored stably at -70°C for several months.

Total poly(A)$^+$ mRNA was purified from total RNA by successive oligo(dT) cellulose column chromatographies. The purity of the mRNA was determined by electrophoresing in an agarose gel. When little rRNA contamination was evident, the concentration of mRNA was estimated by mixing 1 μL mRNA solution with 20 μL ethidium bromide solution (1 μg/mL) and compared to a series of known RNA concentrations using a uv light box. The yield of mRNA from different sources of cells is presented in Table 6. It shows that the yield of mRNA from induced cells was much higher than that from starved and stationary cells.

The integrity of the poly(A)$^+$ RNA was determined by mRNA translation *in vitro* (Promega Corp.). One microgram of mRNA from each sample was used. Brome mosaic virus RNA was used as standard. The results presented in Fig 37 indicate that mRNA from starved cells and from induced cells was more effectively translated *in vitro* than mRNA from stationary phase cells. Even though the yield was lower, mRNA from starved cells was as active as the mRNA from induced cells. Without an analysis of the relative amount of different mRNA species and the protein products, it is difficult to decide whether this is due to more active mRNAs in starved cells as a whole or whether it is a consequence of elevation of the level of a few active mRNA species.

3.2. Preparation of cDNA Library

For cDNA synthesis, commercially produced kits (Pharmacia Inc., and

Table 6. Yields of mRNA Purified from Cells Grown in Different Media

medium	cells (g)	mRNA (μg)
Uninduced condition:		
- starvation medium	5	14.0
- defined medium	5	16.6
Induced condition:		
- starvation medium	5	44.0

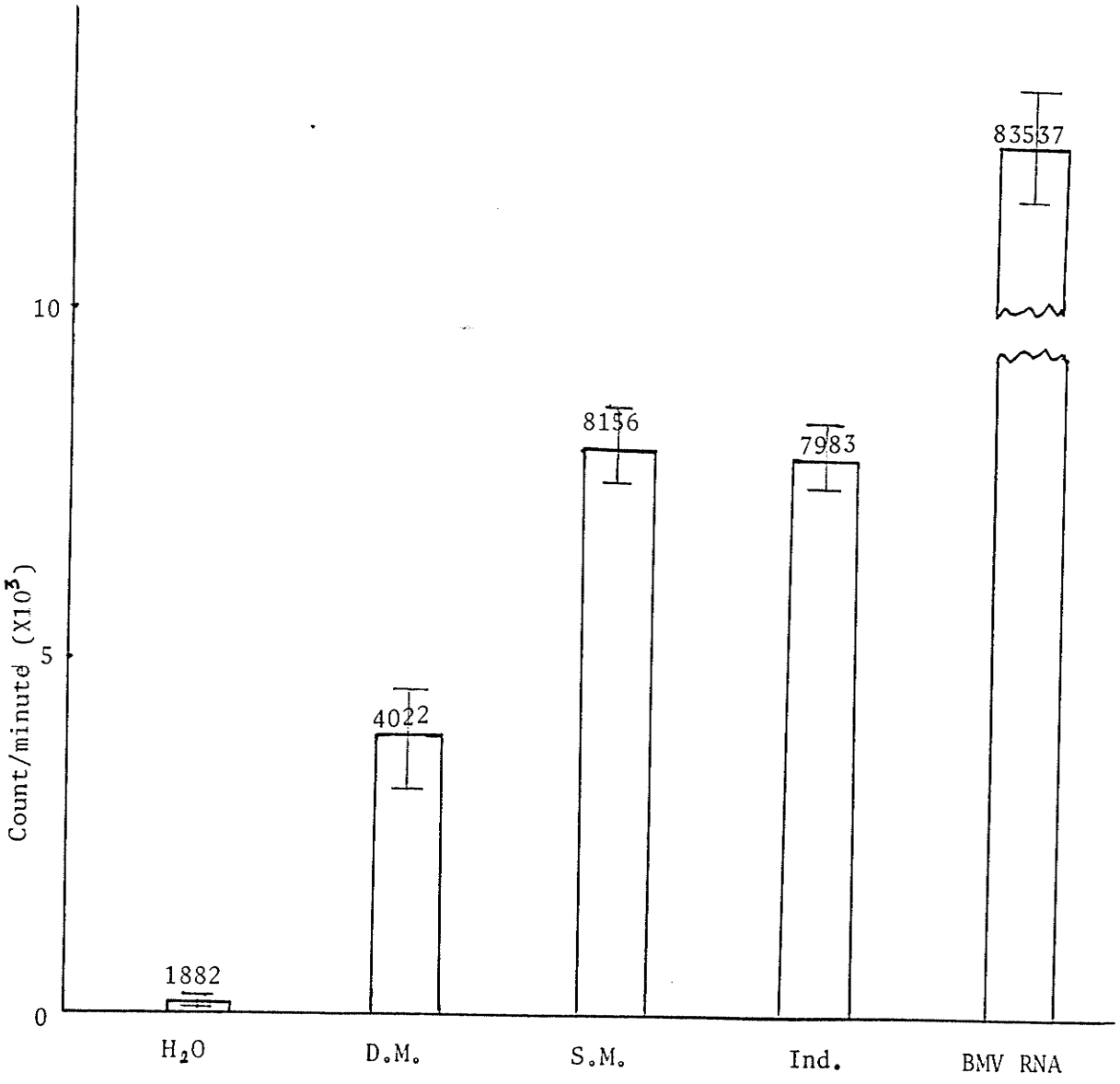
The data are from the actual experimental results. It was too expensive to repeat the mRNA purification. However, if the results are unreliable, the following experiments (i. e. in vitro translation and cDNA synthesis) can tell.

Figure 37. *In Vitro* Translation of Poly(A)$^+$ RNA Isolated from *A. klebsiana* Cells in Different Physiological Conditions

The mRNAs were obtained from cells (a) in defined medium (DM), (b) in starvation medium (SM), (c) induced with 5 mM L-glutamine for 3 h (Ind.). Control is brome mosaic virus (BMV) RNA. The procedure described in the instruction manual of Promega was followed. One microgram of mRNA was used for translation in a total volume of 50 μl. Each assay was repeated 3 times. The bars are standard deviations. They were calculated by the following formula:

$$\text{Standard deviation} = \sqrt{\frac{\sum(Si - \bar{S})^2}{n - 1}}$$

Si is the individual value, $\bar{S}$ is mean of the 3 values, n is the number of observations.


Count/minute (X10³)
10
5
0
1882
4022
8156
7983
83537
H₂O
D.M.
S.M.
Ind.
BMV RNA

Promega Corp.) were used. The cDNA clones selected were from the library generated by the Pharmacia kit. The procedures recommended by the manufacturers were followed. Five μg mRNA was used to synthesize double-stranded cDNA. An effluent of 180 μL (3 μg) was obtained from the last step of cDNA synthesis. One third of this product (1 μg) was ligated to 2.5 μg of λgt11 arms at 14°C overnight in a total volume of 10 μL. A control was made by replacing the cDNA insert with a standard DNA insert supplied in the kit. A 2 μL aliquot of the ligated product was electrophoresed in a 1% agarose gel. Because not all the cDNA ligated, another 1 μg of λgt11 arms and 2 units of ligase were added to the ligation mixture followed by another incubation at 14°C overnight. The occurrence of ligation was confirmed by electrophoresing in an agarose gel.

Three μL of the ligation mixture was used to generate a λgt11 library in a final volume of 500 μL using the *in vitro* packaging system of Stratagene. The library was titered in LB/ampicillin plates. In the presence of IPTG, X-gal and $MgCl_2$, the titer of the library and the percentage of recombinant phage could be determined in the same plate. It was determined that the library contained 1.84×10^5 pfu/mL. The percentage of recombinant phage was 93.5%. In the control, the titer was 2.54×10^6 pfu/mL. The percentage of recombinant phage was 97%.

3.3. Screening λgt11 cDNA Library with Anti-GDH Antibody

A portion of the original λgt11 cDNA library containing 1×10^4 pfu was directly absorbed to 200 μL of an overnight culture of *E. coli* Y1090

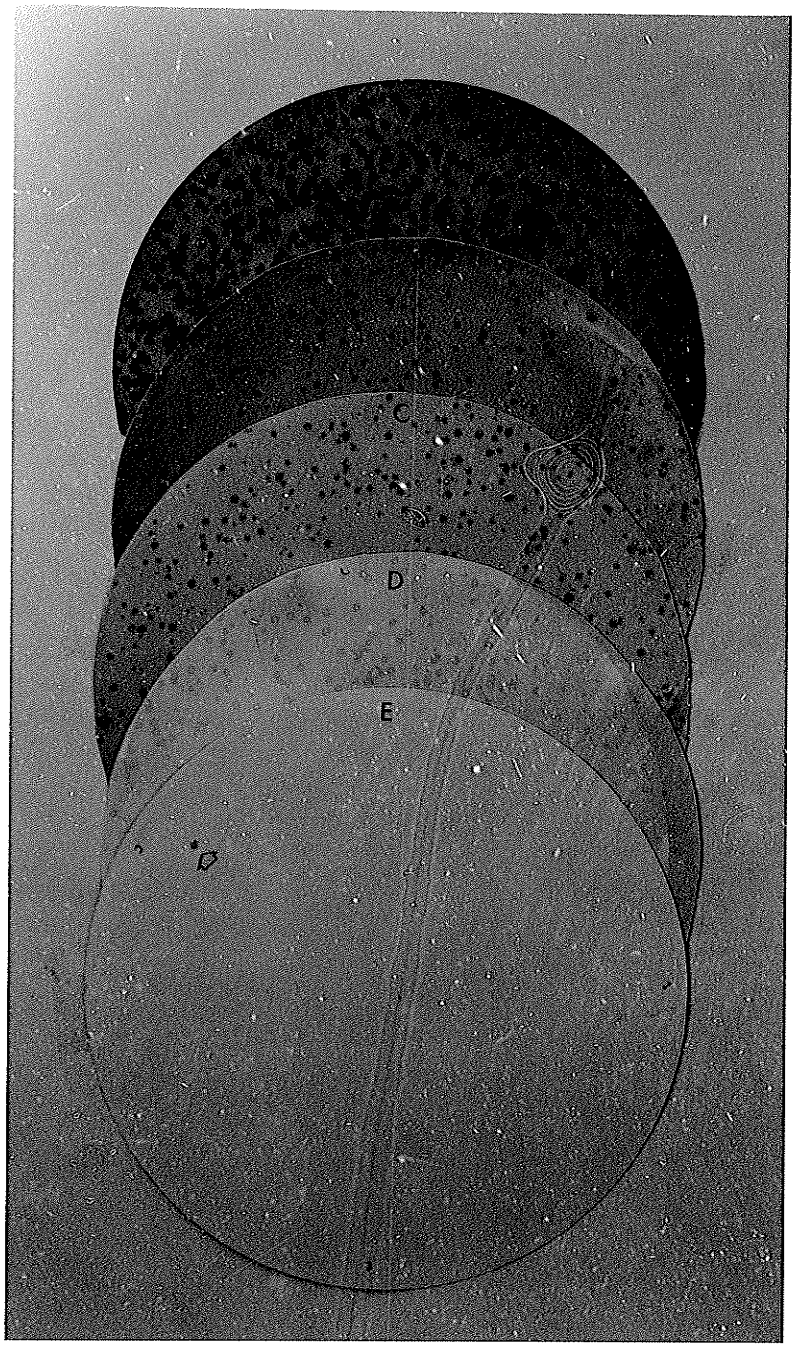
(r^-) for 25 min at 37°C. The amount of cells used was 10 times more than necessary for plaque formation; this improves the plaque forming capacity of the phages in cDNA library screening. The infected cells were grown in a 90-mm (dia.) Petri plate. The LB plate used was 2 days old. The plate was incubated at 42°C for 5 min without the lid. After covering, the plate was incubated for 3.5 h. The membrane treatment, membrane blotting, incubation, and screening procedures are in " Materials and Methods".

When the nitrocellulose membrane was screened with unpurified anti-GDH antiserum, the result (Fig 38 A) shows that all plaques were immunostained to dark purple, indicating that the antiserum was strongly contaminated with *E. coli* proteins. Results in Fig 38 B and Fig 38 C are membranes screened with antiserum purified by the procedures discussed under "Removing anti-*E. coli* antibodies by pseudoscreening" and "Preparation of *E. coli* lysates for absorption of anti-*E. coli* antibodies" by Sambrook *et al.* (1989). The result in Fig 38 D is a membrane screened with antiserum purified by affinity chromatography as described by de Wet *et al.* (1984).

When a membrane was screened with anti-GDH antiserum purified by the procedure described in Methods and Materials, one positive phage clone (λBY1) was obtained (see Fig 38 E). A small area on the plate identical to this positive was picked and suspended in 1 mL phage buffer. This clone was subcultured and rescreened; not all of the resulting plaques were positive. The rescreening result showed that approximately 1/5 of the plaques were positive. One positive clone was selected and purified a third time. At this stage, all plaques gave a positive reaction. By using the same procedure, a fraction containing 1 x10^4 pfu from the original cDNA

Figure 38. Immunoscreening of cDNA Library.

The original cDNA library was plated and immunoscreened with unpurified, partially purified and purified anti-GDH antiserum by the procedure described in "Methods and Materials". A: unpurified anti-GDH antiserum; B, C and D: partially purified anti-GDH antiserum; E: purified anti-GDH antibody. The open arrow identifies a positive cDNA clone.


C
D
E

library was screened. A second positive clone, λBY2, was obtained. A third fraction of 2 x 10^4 pfu from the original cDNA library was screened. Two more positive clones, λBY3 and λBY4, were obtained. The anticipation was that at least one of the 4 cDNA clones would encode a part of the gene for the 74,000 M_r polypeptide while the others would harbour part of the NAD-GDH gene (125,000 M_r protein).

3.4. PCR Regeneration of λgt11-cDNA Inserts

The four positive clones, λBY1, λBY2, λBY3 and λBY4, were amplified as small scale cultures to an approximate concentration of 10^{10} pfu/mL. Subsequently, they were amplified further in this way. Recombinant phages (10^8 pfu for each clone) were adsorbed to 2.4 x 10^{10} *E. coli* Y1090 (r^-) cells at 37°C for 20 min and then grown in one litre LB medium containing 10 mM $MgCl_2$ at 37°C with vigorous agitation for 7 h when the cells became completely lysed. Chloroform (50 mL) was added to each lysate to extract bacterial debris and some proteins. The succeeding steps, DNase and RNase treatment, NaCl salting, PEG precipitation, and chloroform extraction were as described by Maniatis *et al.* (1982). The phage solutions were used directly for ultracentrifugation in CsCl gradients (0.75 g CsCl per mL of phage solution) at 4°C and 120,000 x g for 20 h. Isolation of recombinant λDNA was as described by Maniatis *et al.* (1982). When digested with EcoRI, no insert of the four positive DNAs could be excised. Fusion protein from the λBY2 clone was the largest, based on electrophoretic size, and it was anticipated that this clone would contain the longest insert among the cDNAs. It was therefore used for further analysis. (λBY1 had the shortest

fusion protein). None of the 4 clones could relinquish their putative inserts by EcoRI digestion. Therefore, the DNA of λBY2 was digested with (i) KpnI + BamHI, (ii) KpnI + PvuI and (iii) KpnI + SacI, and electrophoresed in a 1.5% agarose gel. Theoretically, the sizes of λgt11 (no insert) digested products should be, respectively, (i): 11.6kb, 11kb, 7.4*kb, 6.5kb, 5.5kb, 1.5kb; (ii): 12kb, 11.3kb, 9.9kb, 5.1kb, 2.2*kb, 1.5kb, 0.7kb, 0.6kb, 0.4kb; and (iii): 18.5kb, 17.1kb, 4.4kb, 2.1*kb, 1.5kb. If an insert exists in λBY2, it should alter the sizes of the bands marked with an asterisk which contain the insertion site. While there is the existence of a band around the 7.4-kb position in lane 4 of Fig 39, implying that there might be a restriction enzyme site in the insert, cDNA and genomic sequencing results show that there is neither a BamHI site nor a KpnI site in the λBY2 insert. This argues against the idea that the band is of 7.4-kb length. DNA band size determination is not precise in the region where the band in question is located. Lanes 5 and 6 are missing the 2.2 and 2.1 kb bands, respectively, indicating the presence of an insert. Analysis of the other cDNA clones gave similar results. The next task was to recover the cDNA inserts from the four positive clones. This was carried out by the PCR (Polymerase Chain Reaction). Forward and reverse primers close to the EcoRI sites on the λgt11 arms were chemically synthesized. Five ng of template DNA, 0.2 μg of each primer and 2 units of Taq DNA polymerase were used in a 35 temperature cycle reaction. The temperature cycles were 93°C (30 sec), 50°C (30 sec) and 72°C (1 min). A portion of 5 μL from the PCR products (100 μL totally) was removed and electrophoresed in a 1% agarose gel. The results indicated that the insert of λBY2 is indeed the longest, and λBY1 the shortest (Fig 40).

Figure 39. Evidence for the Existence of An Insert in the cDNA Clone

DNA from λBY2 clone was digested with different combinations of restriction endonucleases and electrophoresed in a 1.5% agarose gel.

lane 1: λDNA;

lane 2: HindIII digested λDNA;

lane 3: 1-kb ladder;

lane 4: KpnI + BamHI;

lane 5: KpnI + PvuI;

lane 6: KpnI + SacI.

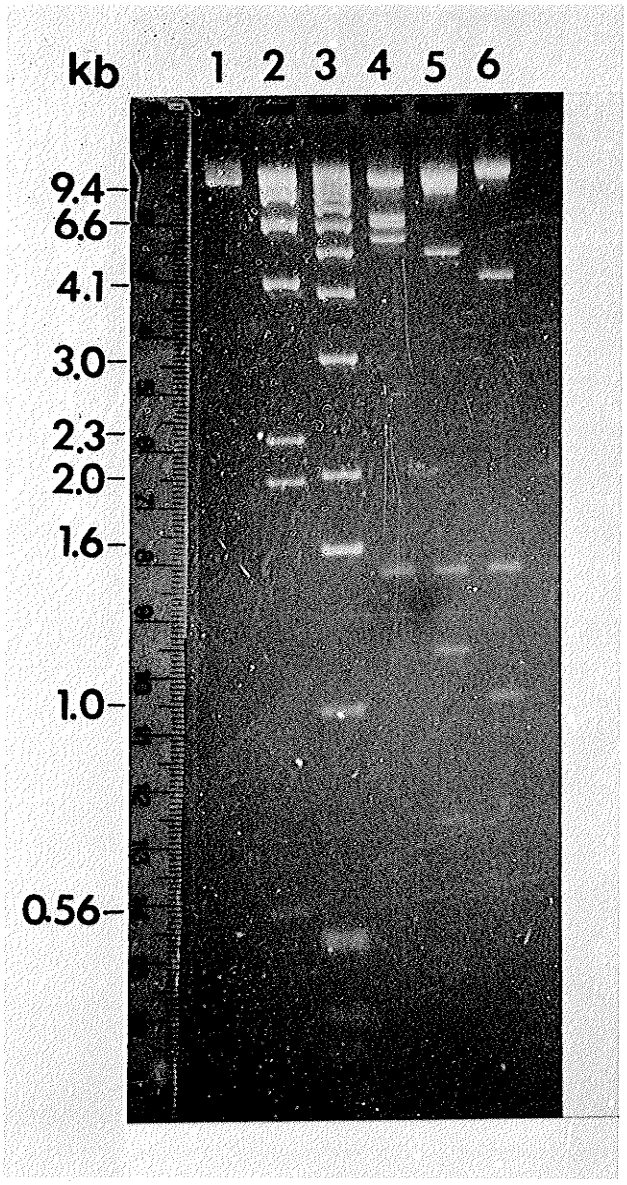

kb
1
2
3
4
5
6
9.4
6.6
4.1
3.0
2.3
2.0
1.6
1.0
0.56

Figure 40. Determination of the Size of the cDNA Inserts in the Four Positive λBY cDNA Clones

The cDNA inserts were generated by PCR (see Methods and Materials). The products were analysed by electrophoresis in a 1% agarose gel.

lane 1, Hind III digested λDNA, The sizes (kb) are from top to bottom: 23.1, 9.49, 6.56, 4.36, 2.32 and 2.03.

lane 2, 1 kb ladder DNA markers, The sizes are (kb): 12.22, 11.20, 10.08, 9.16, 8.14, 7.13, 6.11, 5.09, 4.07, 3.05, 2.04, 1.64, 1.02 and 0.52.

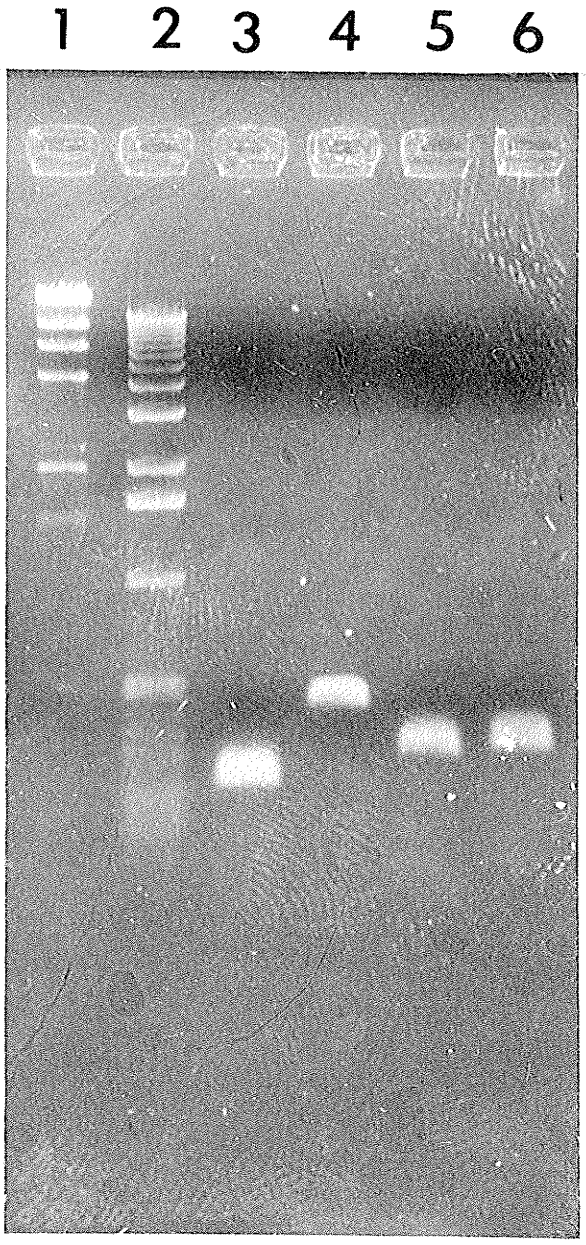
lane 3, PCR product of λBY1.

lane 4, PCR product of λBY2.

lane 5, PCR product of λBY3.

lane 6, PCR product of λBY4.

The results show that all of the inserts are 500 bp approximately. There are some factors affecting the size of synthesized cDNA. For example, contaminating RNase or endonuclease in the polymerase will make the synthesized cDNA shorter (Sambrook *et al.*, 1989). It is not unusual to obtain cDNA inserts of 500 bp.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3.5. Fusion Protein Detection

Additional support for the presence of insert cDNA in the λBY clones was obtained by *in vivo* synthesis of fusion proteins. The protocol used in generating a protein consisting of a hybrid between ß-galactosidase and the protein generated from the inserted cDNA has been described in "Methods and Materials".

Fusion protein-containing samples (4 positives and 1 control) were electrophoresed on SDS-PAGE (7% gel) in duplicate. One set of the samples was stained with Coomassie Blue R-250 (data not shown). The other was electroblotted onto nitrocellulose membrane and immunostained with anti-GDH antiserum. The results (Fig 41) show that the amount of fusion proteins produced by λBY1 and λBY2 were much more than that produced by λBY3 and λBY4. No fusion protein could be stained immunologically in the control. Besides the fusion proteins, large amounts of *E. coli* proteins were immunostained by the antiserum, since it was an unpurified polyclonal antiserum.

3.6. Hybridisation Analysis of GDH-Induced Cell Poly(A)$^+$ RNA with cDNA Clones

Poly(A)$^+$ RNA samples purified as described above were electrophoresed in a 1.2% agarose denaturing gel containing formamide and formaldehyde using the procedure described in "Membrane transfer method" (Amersham, 1985). RNAs were transferred by 0.05 M NaOH onto nylon membrane

Figure 41. Detection of Fusion Protein Expressed by cDNA Clones

Four cultures of *E. coli* Y1089 were infected independently with λBY1, 2, 3 and 4 clones by the method described in "Methods and Materials". Y1089 lysates were electrophoresed in a 7% polyacrylamide gel (SDS-PAGE), electroblotted and immunostained with anti-GDH antiserum.

lane 0: negative control;

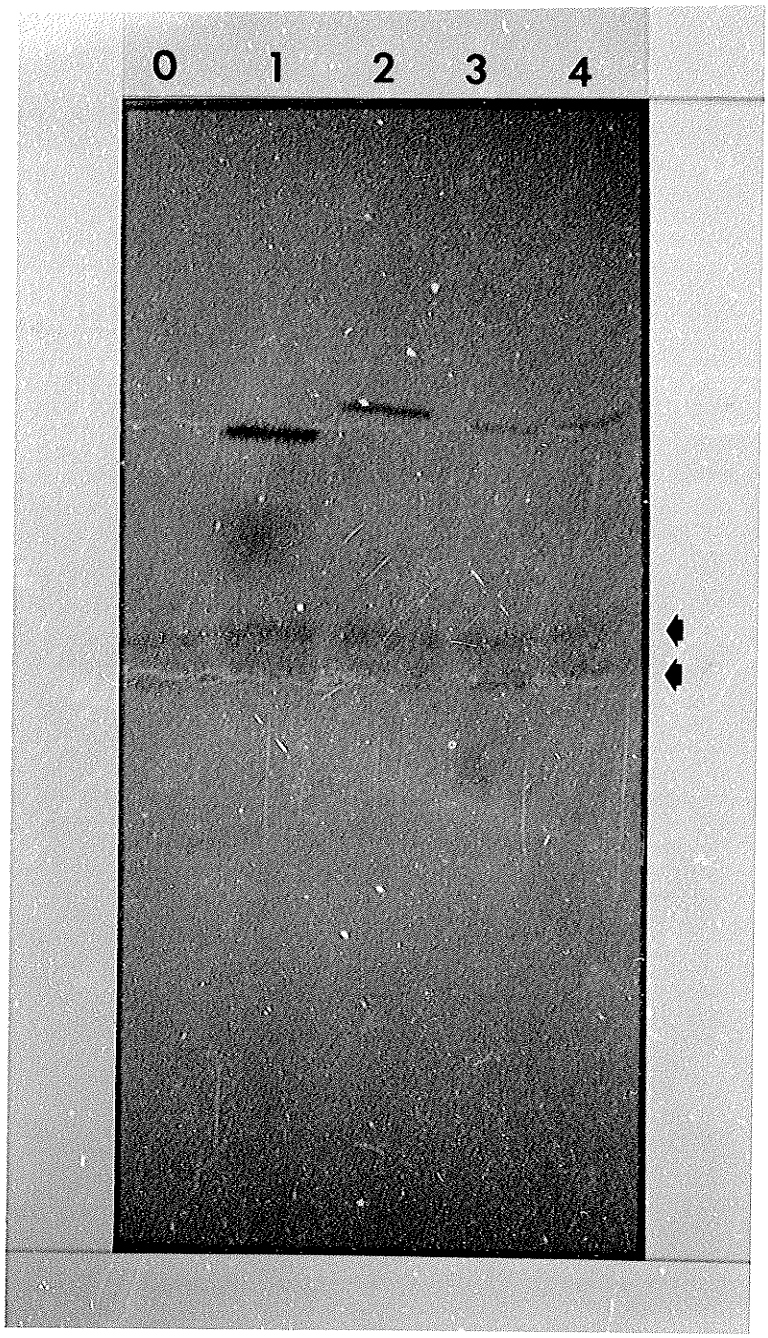
lane 1: λBY1 infection;

lane 2: λBY2 infection;

lane 3: λBY3 infection;

lane 4: λBY4 infection.

Arrow heads signify *E. coli* proteins immunostained by anti-GDH antiserum. Bands above the arrow heads are immunostained fusion proteins produced by λBY1, λBY2, λBY3 and λBY4 respectively.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Hybond-N^+. The membrane was prehybridized at 42°C for 3 h and hybridized with random primer labelled λBY2 cDNA insert in the same solution as described in "Membrane transfer methods" (Amersham, 1985) at 42°C overnight. The membrane was washed with 2 x SSPE at 42°C for 5 min, 2 x SSPE + 0.1% SDS at 42°C for 5 min, 1 x SSPE until the radioactivity was low, and exposed to Kodak X-Omat film at -70°C overnight. After film development, the results presented in Fig 42 (lane 4) show that intense hybridisation occurred with a poly(A)$^+$ RNA migrating around 2.2 kb. A minor hybridisation band around 3.5 kb was also seen (lane 2). Because the main and strongly hybridising band was 2.2 kb, this cannot be the mRNA for the NAD-GDH gene whose transcript length must be no less than 3.5 kb.

As the λBY2 cDNA probe was not hybridising well to any RNA that could be construed as mRNA for the NAD-GDH gene, would any of the other 3 cDNA clones hybridise well? As shown later, this was unlikely because the 4 λBY cDNA clones had overlapping sequences and may therefore derive from the same gene. Therefore, the 4 cDNA clones may encode sequences for the 2.2 kb transcript and possibly the 74,000 M_r polypeptide. If this is so, it implies that the cDNA library is devoid of NAD-GDH cDNAs because of poor representation of NAD-GDH mRNA among the poly(A)$^+$ RNA population.

Several questions spring to mind because of this observation. First, does this mean that two distinct species of antibody exist in the antiserum and the 4 λBY cDNA clones were being detected by one species? Second, could it be that there is really a single antibody species recognising antigenically-related proteins from two genes? In the first case, use of any of the 4 λBY cDNA inserts as probe of a genomic DNA library will detect only one gene. In the second case, one or two genomic

Figure 42. *Achlya* RNA Hybridized with cDNA Clones

Total RNAs and poly(A)$^+$ RNA were prepared from *Achlya klebsiana* in which NAD-GDH has been induced with 5 mM glutamine for 3 h as described in "Methods and Materials". The RNAs were electrophoresed in an agarose denaturing gel and blotted onto Hybond N$^+$ membrane. The membranes were probed with labelled PCR product of the λBY2 insert.

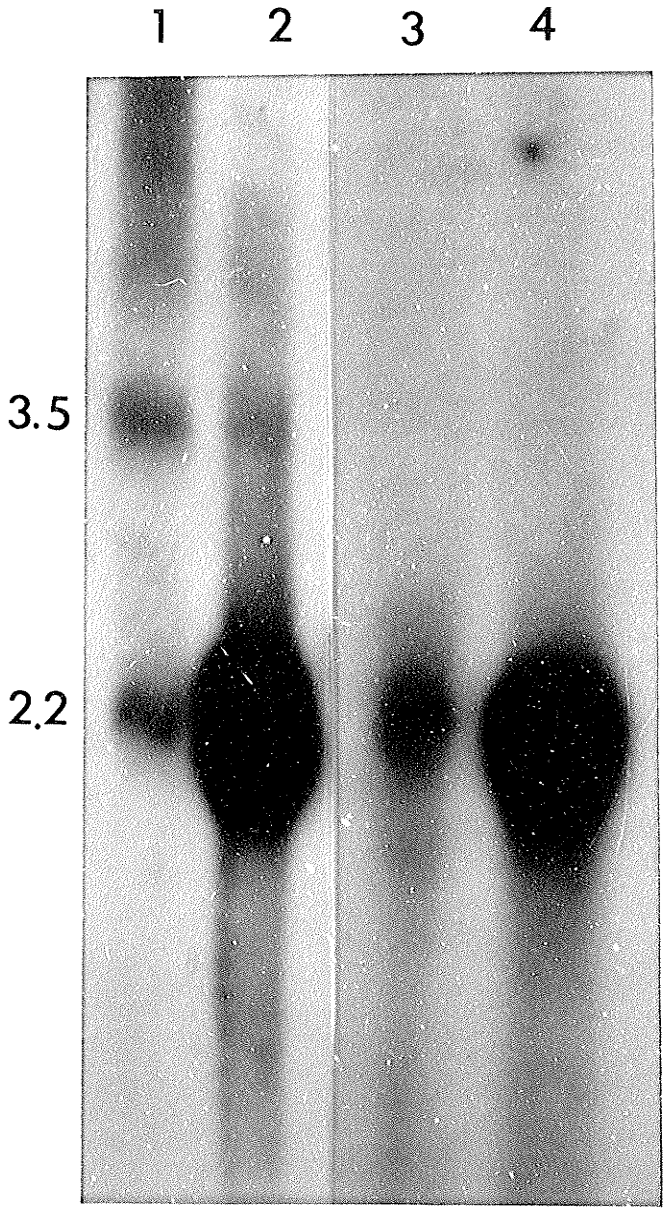
lane 1 and lane 3: total RNAs;

lane 2 and lane 4: poly(A)$^+$RNA;

lane 1 and lane 2: hybridization membrane washed with 1 x SSPE at room temperature;

lane 3 and lane 4: hybridization membrane washed with 1 x SSPE at 42°C.

RNA sizes (kb) (signified on the left margin) were determined by comparing the developed film to the agarose gel picture which contained RNA markers obtained from BRL.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3
4
3.5
2.2

DNA sequences for the two genes may be detectable depending on whether the cDNA sequences come from regions of homology in the two genes. Third, if the lack of hybridisation between cDNA and poly(A)$^+$ RNA in the expected region for NAD-GDH is due to poor representation of the correct transcript, then hybridisation between total unfractionated RNA and cDNA should show whether transcripts of 3.5 kb or greater exist in NAD-GDH induced cell extracts. This experiment was done first before attention was turned to the genomic clones.

Total RNAs were extracted from cells induced for NAD-GDH with 5 mM L-glutamine over a period of 3 h, by the method described by Chomczynski and Sacchi (1987). The RNA samples were electrophoresed, Northern blotted, and hybridized with the cDNA insert of λBY2 as a probe. When the hybridisation membrane was washed with 1 x SSPE at room temperature, the results (Fig 42, lane 1) showed that, besides the 2.2 kb band, the cDNA insert also hybridised to an RNA band of 3.5 kb but with less intensity than the 2.2 kb band at the higher stringent condition (lane 3), indicating that there may be some homology between the two RNAs. When the hybridisation membrane was washed with 1 x SSPE at 42°C, only the 2.2 kb band retained the probe (Fig 42, lane 3). In both cases (room temperature and 42°C), a 2.2 kb poly(A)$^+$ RNA strongly hybridised to the probe (Fig 42, lanes 3 and 4), indicating that the cDNA insert was probably derived from the 2.2 kb band.

4. ISOLATION AND ANALYSIS OF GENOMIC CLONES

4.1. Genomic DNA Library Preparation

Genomic *Achlya* DNA was partially digested with MboI using 9.2 units of the enzyme per mg DNA at 37°C for 1 h. The products were extracted with phenol:chloroform, precipitated with alcohol, resuspended in TE buffer and fractionated in a 5-20% sucrose gradient by ultracentrifugation. Fractions were collected and size-determined on a 0.4% agarose gel by electrophoresis. Two fractions (#12 and #13) contained DNA sizes between 15-23 kb (Fig 43). These fractions were recovered separately, DNA precipitated with ethanol, resuspended in 100 μL buffer and stored at -70°C.

One of the fractions, #12, was used for ligation; the other fraction, #13, was saved for an emergency. Half a microgram of genomic DNA was ligated to 1.35 μg λEMBL3 arms in a total volume of 12 μL. A portion (3 μL) from the ligation mixture was used for analysis by electrophoresis in a 0.7% agarose gel. The results (Fig 44) show complete ligation. Therefore, 3 μL of the ligation mixture was used to prepare a genomic DNA library. The *in vitro* packaged product was titered. The results showed that the genomic DNA library had a titer of 4.7×10^6 pfu/mL or 3.76×10^7 pfu per μg genomic insert DNA used. Some of the library was stored at -70°C in 7% dimethyl sulphoxide. A portion of the genomic library containing 1.4×10^5 pfu was used for amplification to a final titer of 10^{10} pfu/mL and then stored at -70°C in 7% dimethyl sulphoxide.

Figure 43. Size Determination of MboI Digested *Achlya* Genomic DNA Fragments by Agarose Gel Electrophoresis

Fractions 6-20 obtained from the bottom to the top of the sucrose gradient were size-determined on a 0.4% agarose gel by electrophoresis (lanes 2-15). Fractions 12 and 13 (lanes 8 and 9) containing DNA fragments in the size range of 15-20 kb were recovered and used in constructing a genomic DNA library. The DNA markers (lane 1) were HindIII digested λDNA with sizes (kb) from top to bottom of 23.1, 9.49, 6.56 and 4.36. The lower bands (see legend to Fig 40) were indiscernible.

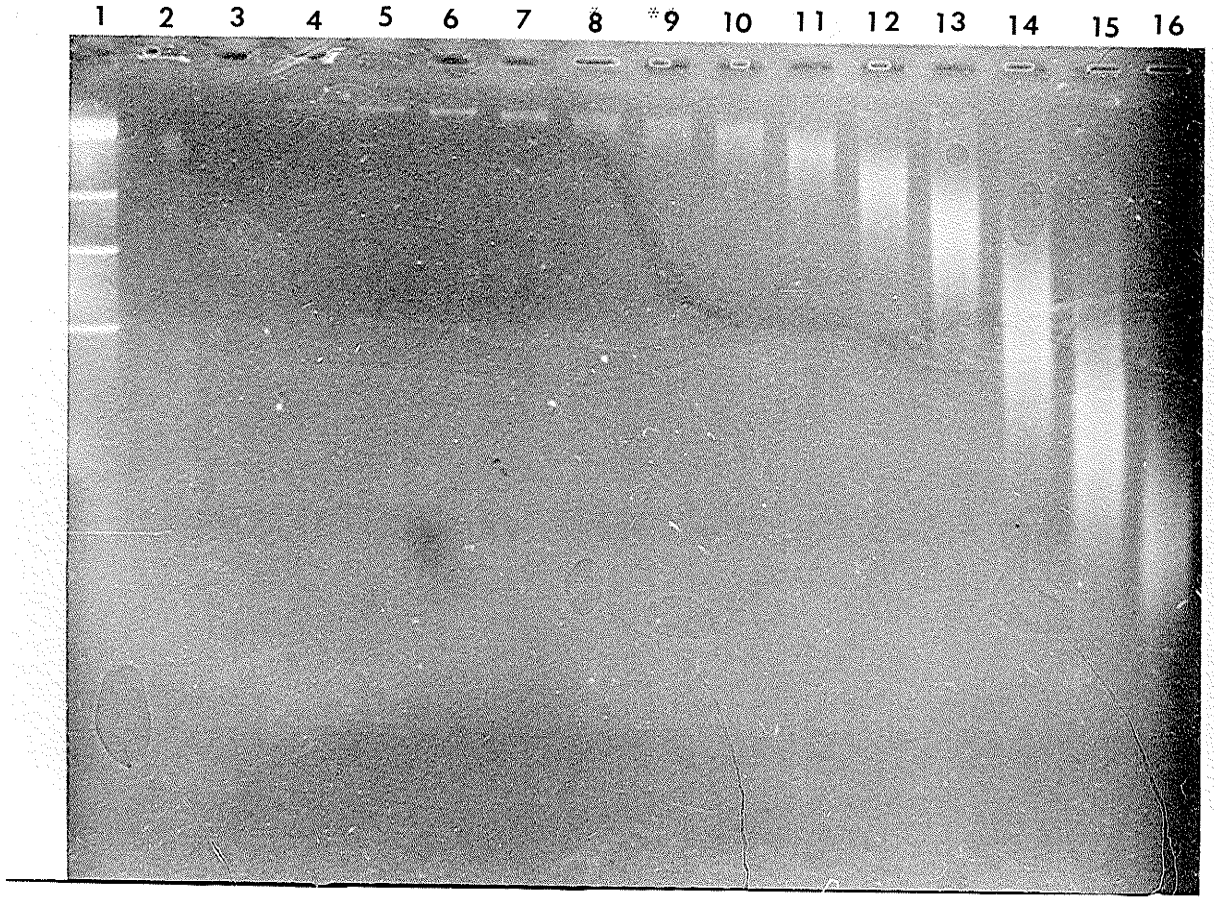
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Figure 44. Ligation of Fractionated Genomic DNA to λEMBL3

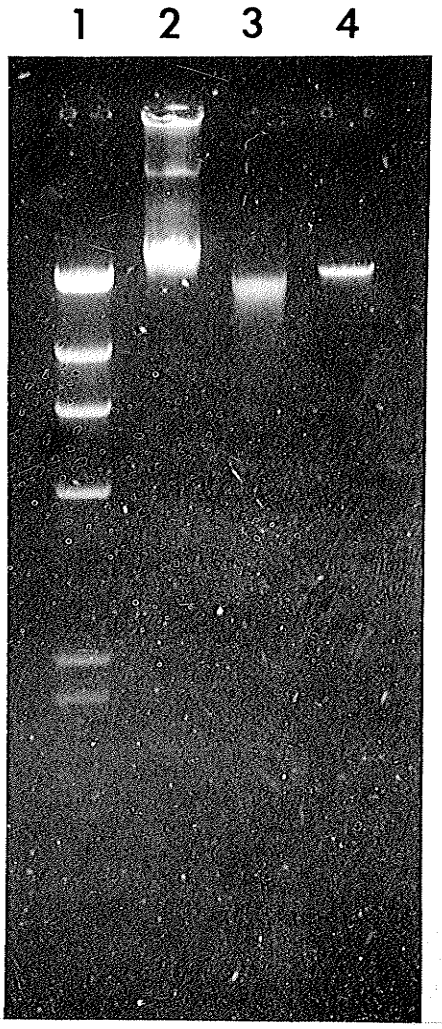
The procedure is described in Materials and Methods.

lane 1: Hind III digested λDNA (The sizes of the bands are 23.1 kb, 9.42 kb, 6.56 kb, 4.36 kb, 2.32 kb, 2.03 kb from top to bottom.);

lane 2: fractionated genomic DNA ligated to λEMBL3;

lane 3: fractionated genomic DNA;

lane 4: λDNA.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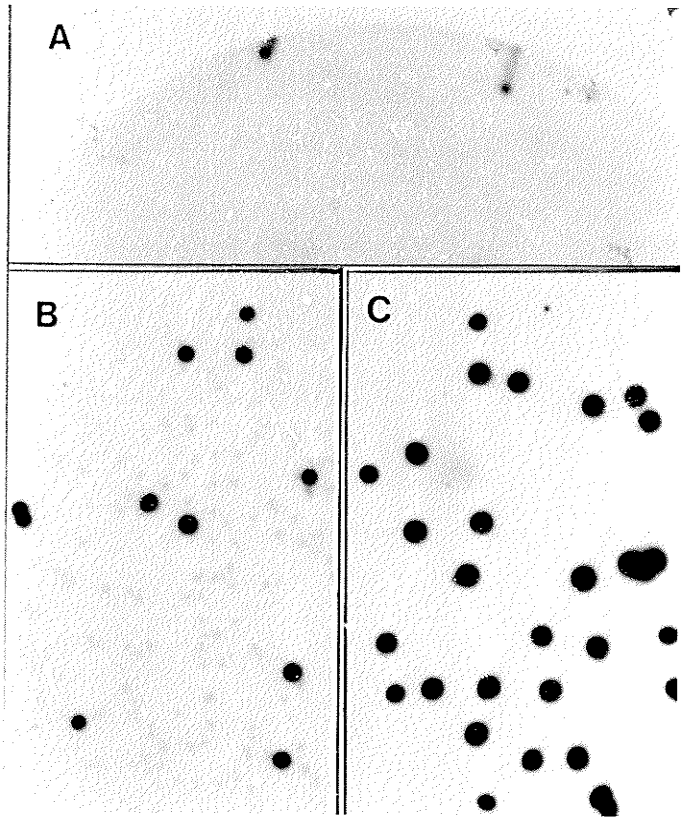
4.2. Screening *Achlya* Genomic Library with cDNA Insert

When the *Achlya* genomic library was screened with the insert of pGDc23 (*Chlorella* NADP-GDH cDNA), the insert of pλZ9 (human GDH cDNA), the insert of pVG1 (*Neurospora crassa* NAD-GDH structural gene), and the insert of PSM2 (yeast NAD-GDH structural gene), no positive clones were detected. The *Achlya* genomic library was then screened with λBY2 as a probe.

A portion of the original *Achlya* genomic library containing 4.7×10^4 pfu was absorbed to 0.8 mL overnight culture of *E. coli* P2.392 and the culture grown in two large 150 mm plates overnight at 37°C. Plaques were lifted to Hybond-N in duplicate. λBY2 PCR amplified insert was used as probe. Labelling was by the random primer technique. Hybridisation was carried out at 63°C overnight and the membranes were washed as described in "Methods and Materials". One set of membranes was washed to the stringency of 1 x SSPE at 63°C for 10 min, and the other set of membranes was washed similarly but the 1 x SSPE solution contained 0.1% SDS. The membranes were exposed to Kodak X-Omat film overnight at -70°C. After film development, the two sets of films were compared. Most of the intensely dark spots in one set had identical dark spots (with less intensity) in the other set. In all, 12 "plaque areas" deemed positive were removed and suspended in 1 mL phage buffer. Two such "plaque areas" are shown in Fig 45 A. The putative positive clones were rescreened at a plaque density of 200-300 pfu/80 mm (dia.) Petri plate culture. Nine of the 12 "clones" turned out to be true positives. They are designated λBYG1-λBYG9. Fig 45 B shows the true positives against the background of negative plaques.

Figure 45. Genomic DNA Library Screening

The original *Achlya* genomic DNA library was plated and screened with the PCR product of λBY2 as a probe by the procedure described in "Methods and Materials". Positives were confirmed by a second screening using a lower density of plaques. The true positives were purified by a third screening. A: original genomic library screening showing 2 positive clones, B: second screening showing true positives and negatives, C: third screening showing pure positive clones.


A
B
C

These nine positives were further purified by re-screening at a density of ~100 pfu/plate. Fig 45 C shows that all plaques were positive after this re-screening. All positive genomic DNA clones were amplified in small scale as described for the λgt11 cDNA library.

4.3. Preparation and Analysis of Recombinant Genomic DNA

Positive clones, λBYG1, λBYG2, λBYG3, and λBYG4, were grown in P2.392 in LB medium containing 10 mM $MgSO_4$. The procedure used was the same as in amplification of the λgt11 recombinant DNA. After CsCl ultracentrifugation, instead of one phage band, two bands were detected in each tube. Each phage band was recovered separately. DNAs from the phages were extracted and purified (Maniatis *et al.*, 1982).

A question raised was whether the DNAs in the top phage band and bottom phage band were the same or not. If they were different DNAs, which one was the right clone harbouring the gene sought? It became necessary to analyse the DNA preparations from all phage bands first. Subsequently, one of the DNA preparation could be chosen for analysis in detail. All 8 samples of DNA were electrophoresed in a 0.7% agarose gel, the sizes of which were close to λDNA. The DNA preparations were digested with SalI and SmaI and electrophoresed in a 1% agarose gel. Several of the DNAs had similar SalI DNA fragment digestion patterns (data not shown). When these DNA bands for SalI digestion were Southern blotted onto Hybond N^+ and probed with labelled PCR product of λBY2, there was a common hybridising band of 0.55 kb in all DNAs obtained from the phages from the top and

bottom bands (Fig 46). SmaI digestion products yielded common bands among DNAs from the top bands of the 4 clones, but DNAs from the lower phage bands could not be readily digested (data not shown). The lower phage bands may therefore be artifacts or abnormal progenies among the phage populations. It is worthwhile to note that amplification of the other 5 genomic clones failed to produce more than one homogeneous phage band in each case.

Now the aim was to find out whether the 9 genomic DNA clones were a mixture of recombinants carrying genes for the 125,000 and 74,000 M_r polypeptides. The genes could be on the same or on different inserts. As shown in Fig 46, DNA preparations from clones λBYG1, 2, 3, and 4 contain a 0.55 kb SalI DNA fragment which hybridized to the λBY2 insert. This implies that the 4 DNA clones may be derived from the same DNA fragment. A better understanding of the positive genomic inserts could be obtained by comparison of the restriction endonuclease maps of the 4 cloned inserts available at that moment. Restriction endonuclease maps (Fig 47) show that all 4 genomic clones contain a common region which has identical restriction endonuclease sites. This proves that the 4 genomic clones were derived from the same DNA fragment in the cells.

To extend the analysis, λBYG5-λBYG7 clones were amplified. This time, only one phage band was seen after CsCl ultracentrifugation. DNAs from all 7 clones were digested with EcoRI. The digested products were Southern blotted onto Hybond N^+ and hybridized with random primer labelled cDNA insert of λBY2. The results (Fig 48) show that a 1.1 kb hybridising fragment was present in every clone. Therefore, all 7 clones may be related. As all the positive genomic clones were isolated by using the

Figure 46. Hybridization of Restriction Enzyme Digested Genomic Clones with λBY2 cDNA

In phage amplification, two bands (labelled "Top" and "Bottom") were detected in every ultracentrifuge tube of λBYG1, λBYG2, λBYG3, and λBYG4. DNAs prepared from all bands were digested by SalI, electrophoresed in an agarose gel, Southern blotted onto nylon Hybond-N^+ and hybridized with random primer labelled PCR product of λBY2. A small fragment (0.55 kb) from the "Top" phage band from all positive clones strongly hybridized to the probe. 1: "Top" band of λBYG1, 2: "Bottom" band of λBYG1, 3: "Top" band of λBYG2, 4: "Bottom" band of λBYG2, 5: "Top" band of λBYG3, 6: "Bottom" band of λBYG3, 7: "Top" band of λBYG4, 8: "Bottom" band of λBYG4.

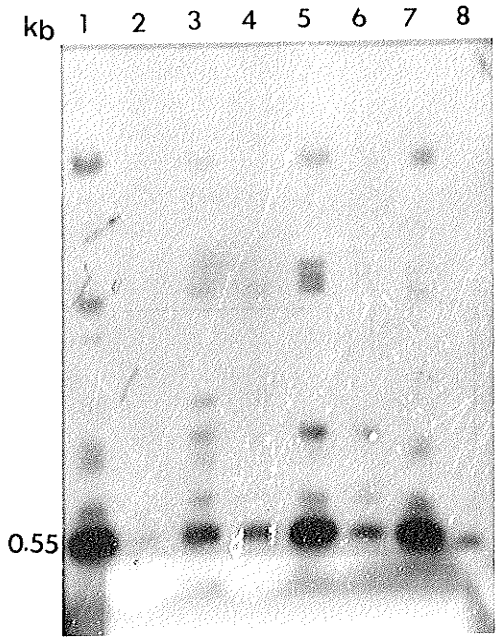

kb
1
2
3
4
5
6
7
8
0.55

Figure 47. Restriction Maps of Genomic DNA Inserts

■: cDNA hybridization region.

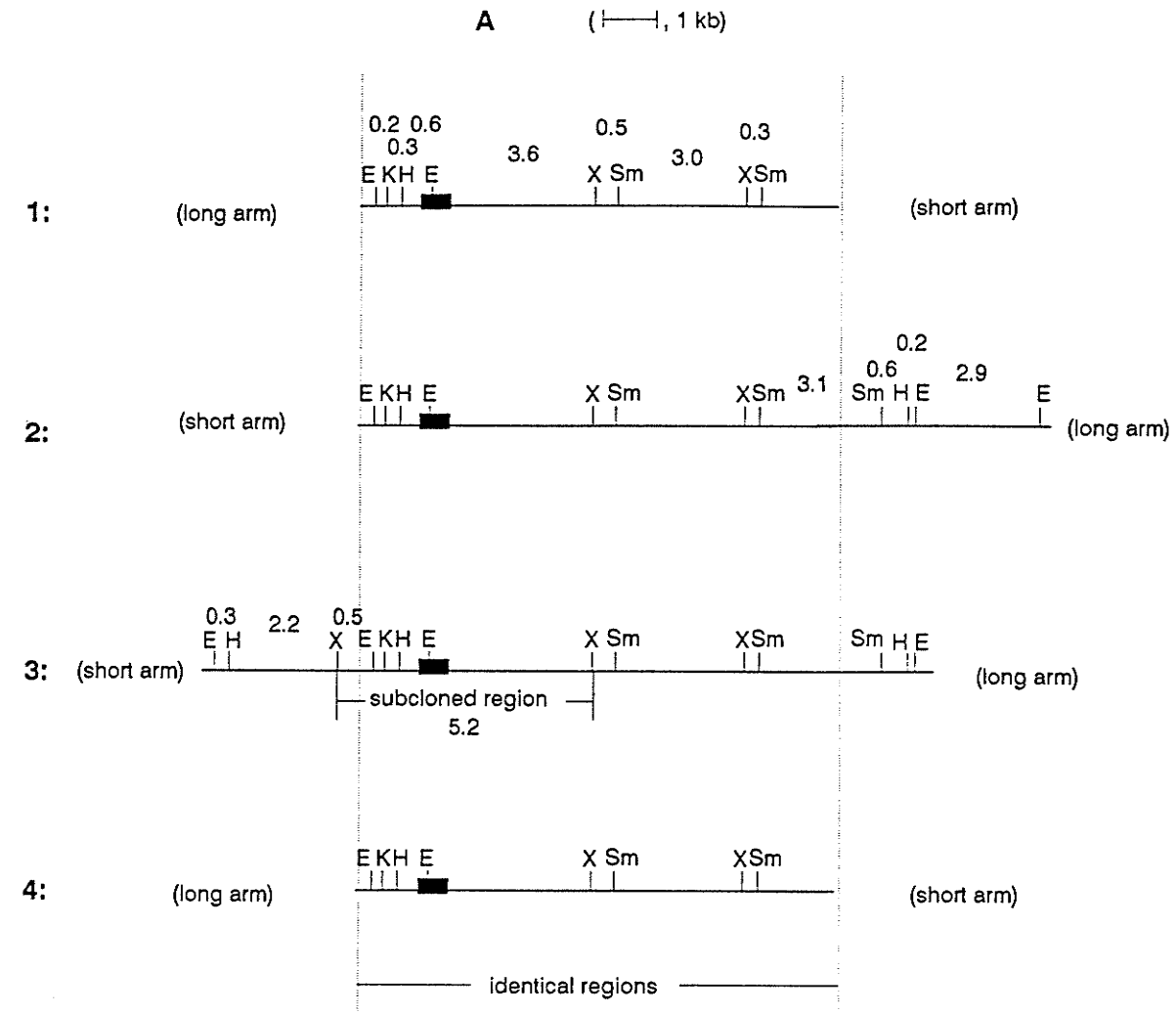
A: Restriction maps of genomic DNA clones λBYG1, λBYG2, λBYG3 and λBYG4.

The sizes of restriction endonuclease fragments are signified either above or below the DNA fragments.

1: λBYG1; 2:λBYG2; 3: λBYG3; 4: λBYG4.

E: EcoRI; H: HindIII; K: KpnI; Sm: SmaI; X: XbaI.

B: Restriction map of the 5.2 kb XbaI fragment from λBYG3.


A
(, 1 kb)
1:
(long arm)
0.2 0.6
0.3
3.6
0.5
3.0
0.3
E KH E
X Sm
XSm
(short arm)
2:
(short arm)
E KH E
X Sm
XSm 3.1
0.2
0.6
2.9
Sm H E
E
(long arm)
3:
(short arm)
0.3
2.2
0.5
E H
X E KH E
X Sm
XSm
Sm H E
(long arm)
subcloned region
5.2
4:
(long arm)
E KH E
X Sm
XSm
(short arm)
identical regions

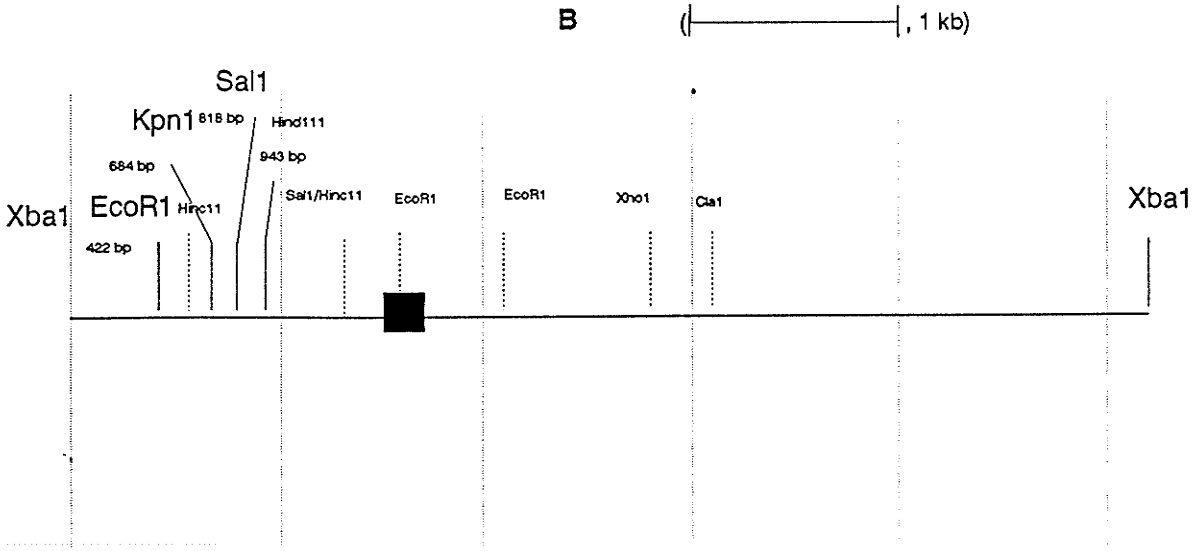
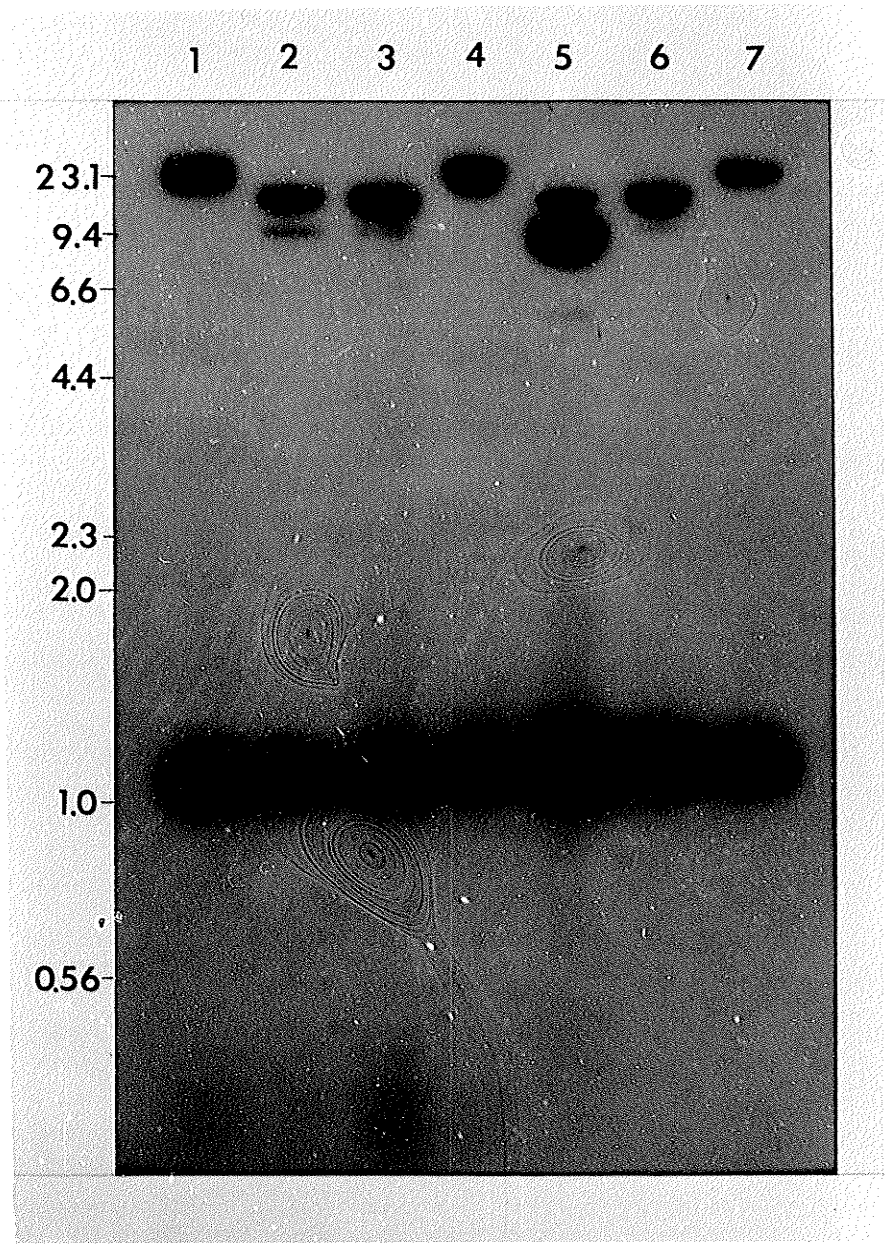

B
(, 1 kb)
Sal1
Kpn1
818 bp
Hind111
943 bp
684 bp
Xba1
EcoR1
Hinc11
Sal1/Hinc11
EcoR1
EcoR1
Xho1
Cla1
Xba1
422 bp

Figure 48. Hybridization of EcoRI Digested Genomic Clones with the λBY2 cDNA Insert

DNAs from genomic clones λBYG1-λBYG7 were digested with EcoRI. The products were electrophoresed in a 1% agarose gel overnight, Southern blotted onto Hybond N^+ and hybridized with the random primer labelled PCR product of λBY2.

DNA sizes (kb) are signified at the left margin.


1
2
3
4
5
6
7
23.1
9.4
6.6
4.4
2.3
2.0
1.0
0.56

cDNA insert of λBY2 as a probe, these results are not surprising.

4.4. Analysis of cDNA Insert and Genomic Positive λBYG3

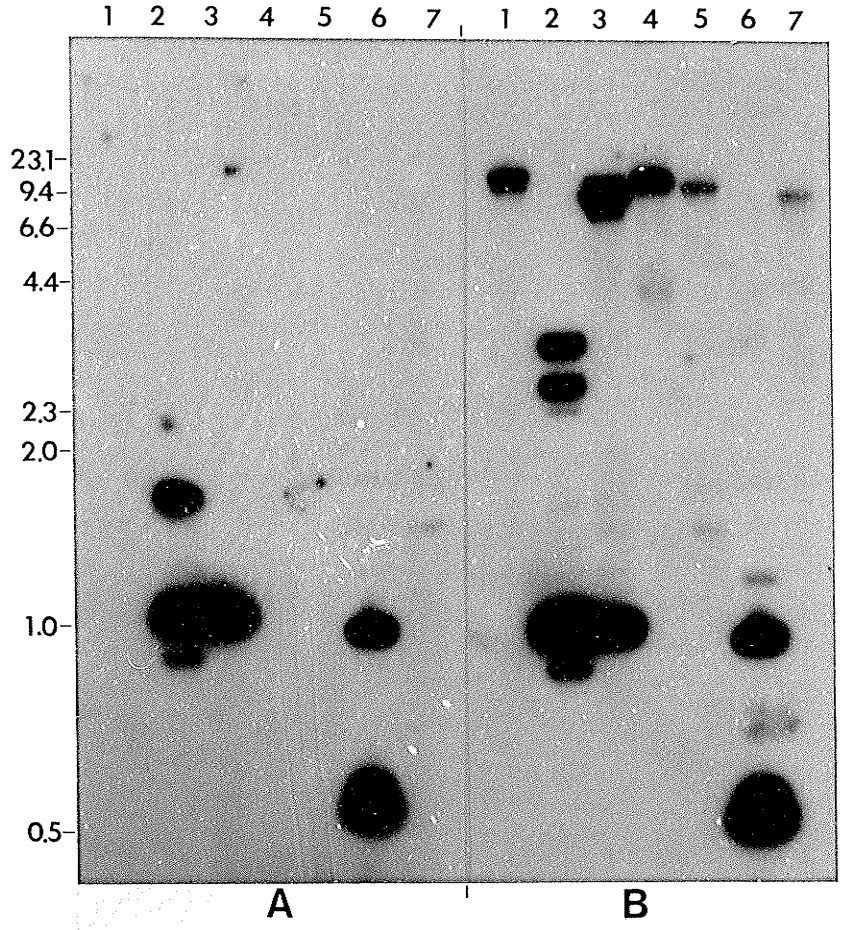
The recombinant DNA, λBYG3, was used to characterize the four positive GDH cDNAs. This was carried out by electrophoresing the 4 PCR products of λBY1-λBY4 clones which were Southern blotted onto Hybond-N^+ and then probed with random primer labelled λBYG3 under highly stringent conditions. All 4 cDNA inserts strongly hybridized to the genomic DNA (data not shown). Therefore, the 4 cDNA sequences derive from the genomic clone selected. As all the genomic clones λBYG1-λBYG7 are similar, they must carry the gene for the cDNA.

λBYG3 was digested by BamHI, ClaI, EcoRI, EcoRV, KpnI, SalI and SmaI. The digested products were electrophoresed and Southern blotted onto Hybond-N^+ in duplicate. One of them was hybridized with the PCR product of λBY2 as a probe. The other was hybridized with a mixture of the PCR products of λBY1 + λBY3 + λBY4 as mixed probes. The results (Fig 49) show that some of the bands were common in that they hybridized to λBY2 alone or to the mixture of λBY1, 3 and 4. By contrast, some bands hybridized to the mixed PCR products but not to the PCR product of λBY2 indicating that they are not commonly represented in the cDNAs. One band (1.6 kb) in the ClaI digestion hybridised to the PCR product of λBY2 but not to the mixed PCR products. This indicates that λBY2 shares some but not all of its sequences with the other cDNAs.

λBYG3 was also digested by ClaI, EcoRI, HindIII, SmaI, and XbaI respectively, electrophoresed and Southern blotted in quadruplicate. Each

Figure 49. Hybridization of Restriction Enzyme Digested Genomic DNA λBYG3 with λBY2-cDNA and Mixed cDNA (λBY1, 3 and 4) Inserts

Genomic DNA from clone λBYG3 was digested with different restriction enzymes, electrophoresed on an agarose gel in duplicate, Southern blotted onto nylon Hybond-N^+, and hybridized with random primer labelled PCR products of (A) λBY2, and (B) λBY1+λBY3+λBY4. 1: BamHI, 2: ClaI, 3: EcoRI, 4: EcoRV, 5: KpnI, 6: SalI, 7: SmaI. DNA sizes (kb) are signified at the left margin.


1 2 3 4 5 6 7
1 2 3 4 5 6 7
23.1
9.4
6.6
4.4
2.3
2.0
1.0
0.5
A
B

of them was hybridized with random primer labelled PCR products of λBY1, λBY2, λBY3, and λBY4 respectively. Certain of the hybridisation patterns are different in Fig 49 and Fig 50 even though the genomic DNA, restriction enzymes and probe were the same. This was due to partial digestion (for ClaI) or incomplete transfer of the DNA to the membrane (for EcoRI and SmaI). The purpose of these two experiments was to check for overlap region among the cDNA inserts. The results show that some small genomic DNA bands hybridized to all of the cDNA inserts indicating that overlap regions exist among the cDNA inserts. Results in Fig 50 show that one 5.2 kb band digested by XbaI hybridized to all of the cDNA inserts. This band, probably the smallest one containing the whole gene of interest, was subcloned into M13 plasmid and has been partially sequenced by Dr. Linda Cameron. The sequenced region of this 5.2 kb XbaI fragment shares identical sequence with the cDNA insert of λBY2 (sequenced by Dr. H. LéJohn) that is presented in Fig 51. This proves that the cDNA was derived from this genomic fragment.

Figure 50. Hybridization of Restriction Enzyme Digested Genomic DNA λBYG3 with Different cDNA Inserts

Genomic DNA from clone λBYG3 was digested with (1) ClaI, (2) EcoRI, (3) HindIII, (4) SmaI, and (5) XbaI, electrophoresed, Southern blotted in quadruplicate. Each blot was hybridized separately with random primer labelled PCR products of either (A) λBY1, (B) λBY2, (C) λBY3, or (D) λBY4.

DNA sizes (kb) are signified on the left margin.

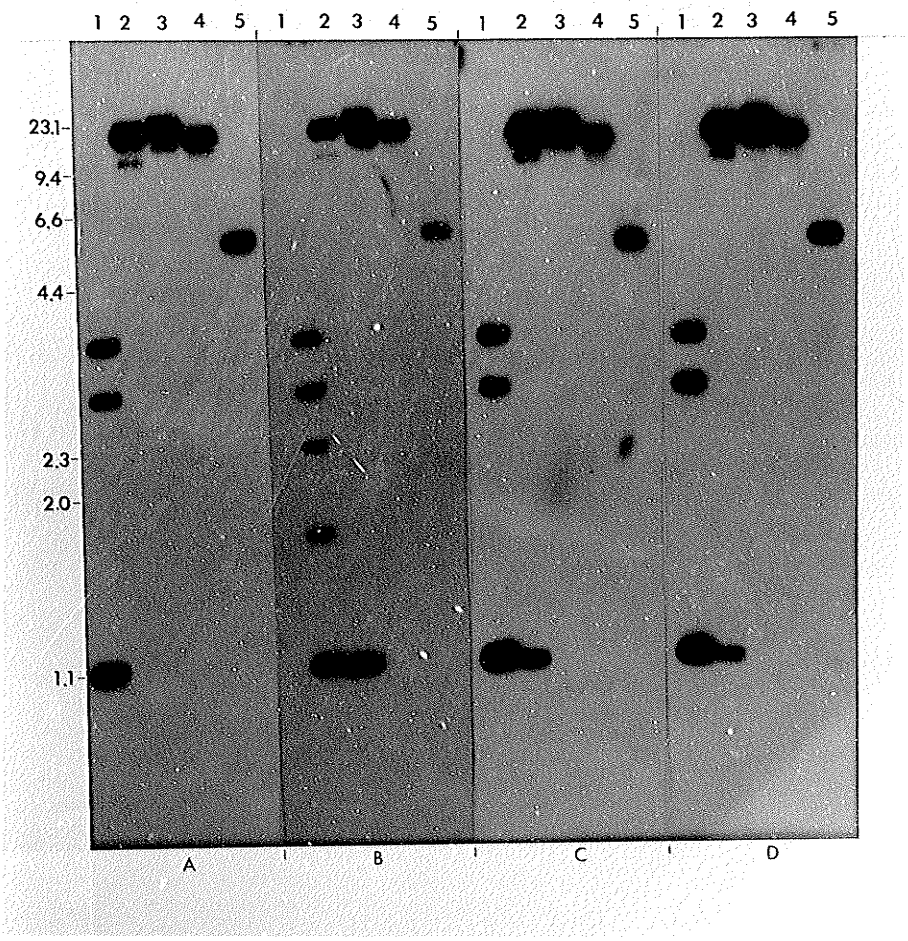

1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
23.1
9.4
6.6
4.4
2.3
2.0
1.1
A
B
C
D

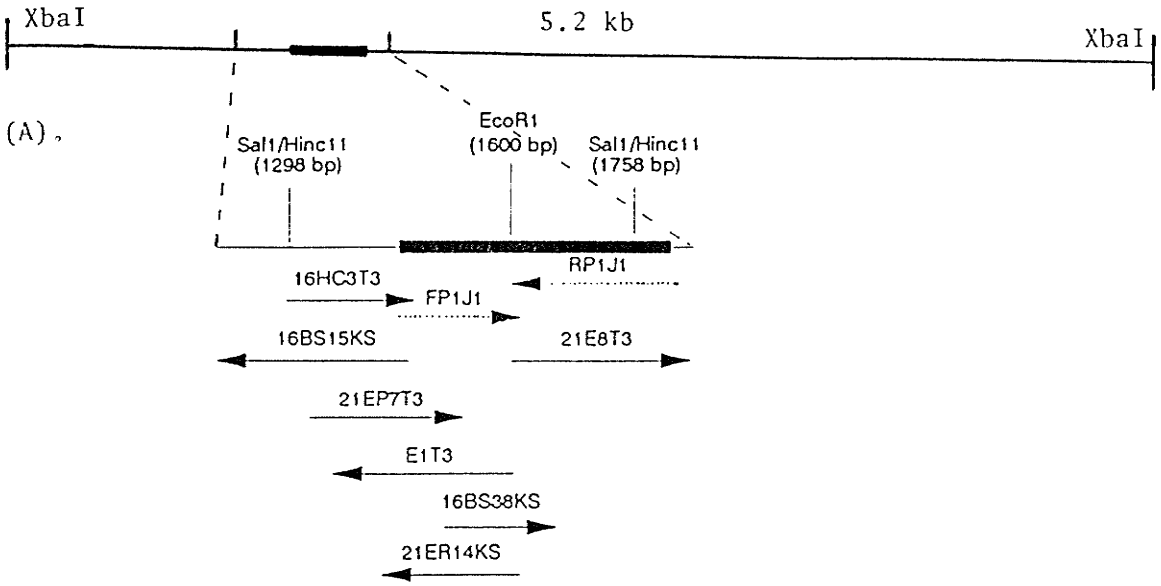
Figure 51. Sequence of the λBY2 cDNA Clone and Part of the 5.2 kb XbaI Genomic DNA Fragment.

(A). The XbaI fragment (5.2 kb) and an exploded view of the sequenced region that corresponds to the λBY2 cDNA sequence are shown. The number in bp refers to the length of sequence from the left end of the XbaI 5.2 kb fragment. The arrows show the sequencing strategy for the genomic DNA (solid lines) and the λBY2 cDNA (dotted lines). Arrows indicate the length and direction of sequencing. 16 and 21 refer to the clone number in M13. The designation given to each sequenced fragment refers in the following order to first, the clone number (e.g., 21); second, the flanking restriction sites (e.g., EP; see part B); third, the subclone number (e.g., 7) and the sequencing primers (e.g., T3). This describes 21EP7T3.

(B). Sequencing was carried out by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). In cDNA sequencing, the PCR product of the insert in λBY2 was used directly. The forward primer (FP) and reverse primer (RP) used in the sequencing are the same as those for PCR amplification of the cDNA insert (see "Methods and Materials", 5.8. Amplification of λgt11 Recombinant and Purification of DNA). Prior to sequencing, the double-stranded cDNA was denatured by boiling. Prior to boiling, the undenatured PCR generated DNA dissolved in TE pH 7.5 buffer was mixed with the appropriate primer in this ratio: 200 ng PCR DNA : 1 ng primer. After boiling for 5 min, the DNA solution was rapidly chilled by

plunging the tube in ethanol kept at -70°C. The tube was held at this temperature for 3 min. Without completely thawing the solution, all other components required for the Sequenase reaction were added drop-wise to the side of the tube, and mixed by a 20 sec forward-reverse quick-spin to initiate the reaction. In the genomic DNA sequencing, the 5.2 kb DNA was subcloned in M13 phage truncated to a variety of lengths by partially digesting with single or pairs of different restriction endonucleases such as HincII (HC), BamHI/Sau3A (BS), EcoRI (E), EcoRI partial digestion (EP), and EcoRI/RsaI (ER). The primers used for genomic DNA sequencing were T3 (5′ dATTAACCCTACTAAAGG) and KS (5′ dTCGAGGTCGACGGTATC) in the orientations shown in the figure. The different recombinant M13 DNAs, whose 5.2 kb insert had been partially deleted, were denatured by treatment with alkali as described by Chen and Seeburg (1985). DNA sequencing reactions were carried out as prescribed for the Sequenase system of United States Biochemicals from which a kit was obtained. Both cDNA and genomic DNA sequences were determined by the Sequenase system. Genomic DNA sequencing was carried out by Dr. L. Cameron, and cDNA sequencing was by Dr. H. B. LéJohn. The upper sequence is from the genomic DNA clone of λBYG3 and the middle sequence is from the cDNA insert in λBY2. The lower sequence is the deduced amino acid sequence.

(A).



(B).

```
1   GTC TCC TCG ATG TAC CTC AAT GCT CGT GTG TCC ACA ATT GAT
1   GTC TCC TCG ATG TAC CTC AAT GCT CGT GTG TCC ACA ATT GAT
    Val Ser Ser MET Tyr Leu Asn Ala Arg Val Ser Thr Ile Asp


43  GTC ACC TTC GAC ATT GAG TCC AAC GGT ATC TTG AAG TGT CTG
43  GTC ACC TTC GAC ATT GAG TCC AAC GGT ATC TTG AAG TGT CTG
    Val Thr Phe Asp Ile Glu Ser Asn Gly Ile Leu Lys Cys Leu


85  CCG TCG AAA AGT CGA CTG GTA AAG AAA ACA AGA TTA CCA TTA
85  CCG TCG AAA AGT CGA CTG GTA AAG AAA ACA AGA TTA CCA TTA
    Pro Ser Lys Ser Arg Leu Val Lys Lys Thr Arg Leu Pro Leu


127 CCA ACG ACA AGG GTC ACC TCA CCA AGG ATG ATA TTG AAC GCA
127 CCA ACG ACA AGG GTC ACC TCA CCA AGG ATG ATA TTG AAC GCA
    Pro Thr Thr Arg Val Thr Ser Pro Arg MET Ile Leu Asn Ala


169 TGG TGC AAG AAG CTG AAA AGT ACA AGT CGG AAG ATG AAG CCA
169 TGG TGC AAG AAG CTG AAA AGT ACA AGT CGG AAG ATG AAG CCA
    Trp Cys Lys Lys Leu Lys Ser Thr Ser Arg Lys MET Lys Pro


211 ACA AGC TCC GCA TTG AAG CCA AGA ACG GTC TTG AAA ACT ACG
211 ACA AGC TCC GCA TTG AAG CCA AGA ACG GTC TTG AAA ACT ACG
    Thr Ser Ser Ala Leu Lys Pro Arg Thr Val Leu Lys Thr Thr


253 CTA CAA CCC TCC GCA ACA CCC TCA TCG ACG AAA AGC TCC AAG
253 CTA CAA CCC TCC GCA ACA CCC TCA TCG ACG AAA AGC TCC AAG
    Leu Gln Pro Ser Ala Thr Pro Ser Ser Thr Lys Ser Ser Lys


295 GCA AGA TCG ATG AAA GCG ACA AGA AGG TCA TTG TCG ACA AGG
295 GCA AGA TCG ATG AAA GCG ACA AGA AGG TCA TTG TCG ACA AGG
    Ala Arg Ser MET Lys Ala Thr Arg Arg Ser Leu Thr Thr Arg


337 TCA CCG ACA TCA TTA ACT GGC
337 TCA CCG ACA TCA TTA ACT GGC
    Ser Pro Thr Ser Leu Thr Gly
```

DISCUSSION AND CONCLUSIONS

Metabolic Importance of *Achlya* NAD-GDH

NAD-specific glutamate dehydrogenase in *Achlya klebsiana*, a member of the *Oomycota* of the Protoctista Kingdom, has been studied extensively and shown to be interesting in several ways. The enzyme is markedly induced by L-glutamine, and L-glutamate. L-Glutamine is twice as effective as L-glutamate. However, NH_4^+ does not induce the enzyme. Therefore, it is likely that glutamate is the actual inducer (see below). The partially purified enzyme required NAD^+ and NADH as cofactors of the oxidative deamination and reductive amination reactions, respectively. Both reactions are activated by $NADP^+$ (and by NADPH to a lesser extent) (LéJohn and Stevenson, 1970; Stevenson and LéJohn, 1971). These properties of NAD-GDH in *Achlya* are unique features not observed in NAD-GDHs from other organisms.

Achlya has not been shown to contain an NADP-specific GDH (LéJohn, 1971 and personal communication). Therefore, the allosteric activating effect of NADP(H) on the enzyme must rely on the ready supply of these pyridine nucleotides from metabolically linked reactions in the cell. It

is possible that the absence of NADP-GDH and the activation of NAD-GDH by $NADP^+$ in *Achlya* are not entirely unrelated for we see that most organisms have either an NAD(P)-GDH (both coenzymes are effectively utilized) or the NAD- and NADP-specific forms of the enzyme (Smith *et al.*, 1975). When the two enzymes are present as in *Neurospora crassa* and *Saccharomyces cerevisiae*, the NAD-specific form is inducible by glutamate and repressed by sugars whereas the NADP-specific form is inducible by sugar and repressed by glutamate (Holzer and Hierholzer, 1963; Tuveson *et al.*, 1967; Beck and Meyenburg, 1968; Kapoor and Grover, 1970). Most investigators support the idea that NAD-GDH functions primarily to deaminate glutamate while NADP-GDH aminates α-ketoglutarate even though both enzymes are perfectly reversible *in vitro*. Thus, *Achlya* NAD-GDH must somehow fulfil both roles unless there is a compensating enzyme such as glutamate synthase to generate glutamate. But even that would require the synthesis of glutamine via glutamine synthetase which, in itself, utilizes glutamate. Underneath this is a concept of a "glutamate cycle", an idea that is explained later in this "Discussion". For *Achlya*'s NAD-GDH to operate efficiently at this branchpoint of carbon and nitrogen flow (organic acids connected to amino acids, reversibly), it must have evolved complex and subtle controls mediated by small molecules. Part of this complexity is what we are seeing in the effect of NADP(H) and other controlling ligands studied earlier (LéJohn, 1975). But because the enzyme is inducible by organic nitrogen and repressible by sugar carbon, a better understanding of the mode of regulation of the enzyme must involve an analysis of the protein and its gene. It is this complexity that made it critical to first understand, in molecular terms, the manner of expression

of the enzyme. But, with lack of classical genetic procedures (isolation of mutants, etc.), one must first analyse the protein, then its gene and all the physiological conditions that influence expression of the gene. The protein was isolated in pure form and studied physically and kinetically. It has yet to be studied chemically. The gene was to be isolated starting with the purified enzyme. First, by determining the amino acid sequence of appropriate peptide fragments of the enzyme, a probe was to be made from the known amino acid sequence to isolate the gene from a genomic library. Second, antibody to the enzyme was to be used as a probe to screen a cDNA expression library. Through misjudgment, I failed to carry out the first step. And by relying on the second step, I encountered an unforseen problem in that the gene of an immunologically related (i.e. to NAD-GDH) protein was isolated rather than NAD-GDH itself. More on this later.

<u>It was important to protect the enzyme during purification.</u>

Preliminary tests showed that the enzyme became progressively unstable with increased purification. It was stabilized by a combination of agents, notably glycerol and either KCl or $(NH_4)_2SO_4$. The reason is that glycerol can help to maintain stability of protein structure; K^+ will maintain a certain ionic strength and keep the enzyme in its native structure; NH_4^+ has the same function as a salt and it is also a substrate of the enzyme and thus retains an active form of the enzyme. As $(NH_4)_2SO_4$ is used in the purification and precipitation of the enzyme, KCl is preferred in the gradient buffer in purification. The purified enzyme is stable for several months in cold storage at -20°C in the presence of 50% glycerol and ammonium sulphate.

A method of purifying this enzyme to electrophoretic homogeneity has been developed.

In the purification, different methods, such as buffer, pH, and media were tried. The columns used included Sephadex G 150, Sephadex G 200, Sephadex 6B, DEAE-cellulose, TEAE-cellulose, A25 DEAE-sephadex, ß-NAD-agarose and Cibacron Blue 3 GA-agarose. The enzyme was too large and eluted out in the void volume in both Sephadex G 150 and Sephadex G 200, making these two columns ineffective in the purification. The Sepharose 6B column fractionated the enzyme from many other proteins, but destroyed much of the enzyme activity. DEAE-cellulose and TEAE-cellulose were able to bind glutamate dehydrogenase and fractionate it from most other proteins with significant retention of enzyme activity; thus they were valuable in enzyme purification. In addition, the enzyme adhered more tightly to these two columns than to A25 DEAE-Sephadex which was of great value because it caused the least destructive effect on the enzyme. The affinity column of ß-NAD-agarose was not able to bind glutamate dehydrogenase, even by incubation overnight. This is interesting in view of the coenzyme specificity of the enzyme.

In $(NH_4)_2SO_4$ fractionation, 40% $(NH_4)_2SO_4$ saturation could recover 100% of GDH activity but only salt out half the proteins. Instead, 42% $(NH_4)_2SO_4$ saturation was used which could salt out 63% of proteins and lose only 12.5% of the enzyme. Another saturation of 60% $(NH_4)_2SO_4$, left nearly half of the protein in the supernatant and recovered more than 90% of the remaining enzyme. Both of the $(NH_4)_2SO_4$ fractionations had purification factor greater than 1.

A25 DEAE-sephadex was an excellent column in the enzyme

purification. Diluted enzyme solution was easily absorbed and concentrated by A25 DEAE-sephadex column. The result in Fig 6 shows that the protein concentration is somewhat proportional to enzyme activity. Purification factor in this column was 2.6. In the second A25 DEAE-sephadex column, conditions were changed. A slightly different pH changed the binding behaviour of many contaminating proteins and their removal from the enzyme.

The Cibacron Blue 3 GA-agarose column has been successfully used in the purification of alcohol dehydrogenase (Low *et al.*, 1986; Ostrove, 1990). This modified agarose was used and found to be the crucial matrix in the complete purification of this enzyme. The purification factor in this step was 2.06.

The purified enzyme has interesting features.

Once purified, the enzyme was characterized in several ways.

The subunits of the enzyme were determined to be identical in size; M_r value of 125,000 by SDS-PAGE with gel concentrations of 7, 8, 10 and 12% respectively. When estimated by gel filtration, the M_r of the native enzyme was 470,000. When electrophoresed in a non-denaturing gradient gel (3-19.6%), the enzyme had an M_r value of 500,000. It is known that M_r determinations by gel filtration are imprecise. It is also difficult to determine accurately the M_r of a protein as large as this enzyme on a gradient gel. From these results, a reasonable conclusion is that the enzyme is a tetramer of four identical M_r polypeptides with M_r value of 125,000.

Subtilisin was used to modify the enzyme. When the enzyme was limitedly modified by subtilisin, it was desensitized to NADP(H)

activation. This is an interesting feature that could aid in our understanding of how this activator ($NADP^+$/NADPH) interacts with the enzyme. The results in Fig 15 show that when the enzyme was mildly treated with subtilisin, the M_r of the enzyme subunit (125,000 M_r) changed to 110,000 M_r. Whether the decrease in M_r was due to removal of a small peptide or oligopeptides from one or both ends of the enzyme subunit or to a change in conformation by cleavage without separation of the disrupted chains, is unknown. What is clear is that limited modification of the enzyme by subtilisin destroys the activator binding site while facilitating better interaction between the enzyme and its substrate. Limited cleavage of beef liver GDH by chymotrypsin also enhanced the enzyme activity (Place and Beynon, 1983).

Ways of Isolating the Gene

The rationale for complete enzyme purification was either to raise antibody to the enzyme and use the antibody to isolate the gene, or to determine the amino acid sequence of an appropriate segment of the enzyme protein and make a DNA probe to isolate the gene for the enzyme. Due to the large M_r of the enzyme, it was hard to obtain sufficient amounts of the enzyme from which an amino end terminal sequence of the subunit could be determined, even by micro-sequencing methods. The alternative method of fragmenting the enzyme into small peptides, and, using a couple of these peptides to determine their amino acid sequences and make DNA probes was not done. I opted for the antibody approach which, in retrospect, was a serious mistake but serendipitous in certain respects as will become clear later.

Polyclonal Mono-specific Antibody to NAD-GDH Raised?

The purity of the antigen used for raising antibody is a critical factor in these studies because the antibody raised with impure antigen will be a mixture of proteins with very different specificities and, hence, will identify a variety of positive cDNA expressive clones, some of which are irrelevant to the targeted gene. Therefore, after the 5 steps of enzyme purification, even though the enzyme was shown pure by dual cycles of silver staining, it was felt that it would be unsafe to directly use the protein eluted from the column to raise the necessary antibody. At that time, the purification procedure was still in progress. The yield of NAD-GDH was only 10% of what is shown in Table 3. The products of Step 4 in the purification process were deemed sufficiently rich in NAD-GDH and relatively poor in contaminations. Therefore, these proteins were electrophoresed in SDS-PAGE to separate NAD-GDH subunit from other protein contaminants, and it is this enzyme subunit that was used to raise antibody in rabbits. Antibody raised against this protein is unlikely to be contaminated in any major way with antibodies to other proteins that have significantly lower molecular masses. It was therefore quite surprising to discover that the anti-NAD:GDH polyclonal antibody raised in this way interacted with a protein of M_r 74,000 in addition to the 125,000 M_r enzyme subunit (Figs 21, 25, 27, 29 etc.).

To ascertain that the second protein detected was not a "carry-through" contaminant of the purification procedure, immunoblot analysis of samples from representative steps in the purification protocol was carried out. As shown (Fig 18), the 74,000 M_r protein was detected only in the cell free extract not after Step 3 in the purification process. Here then was

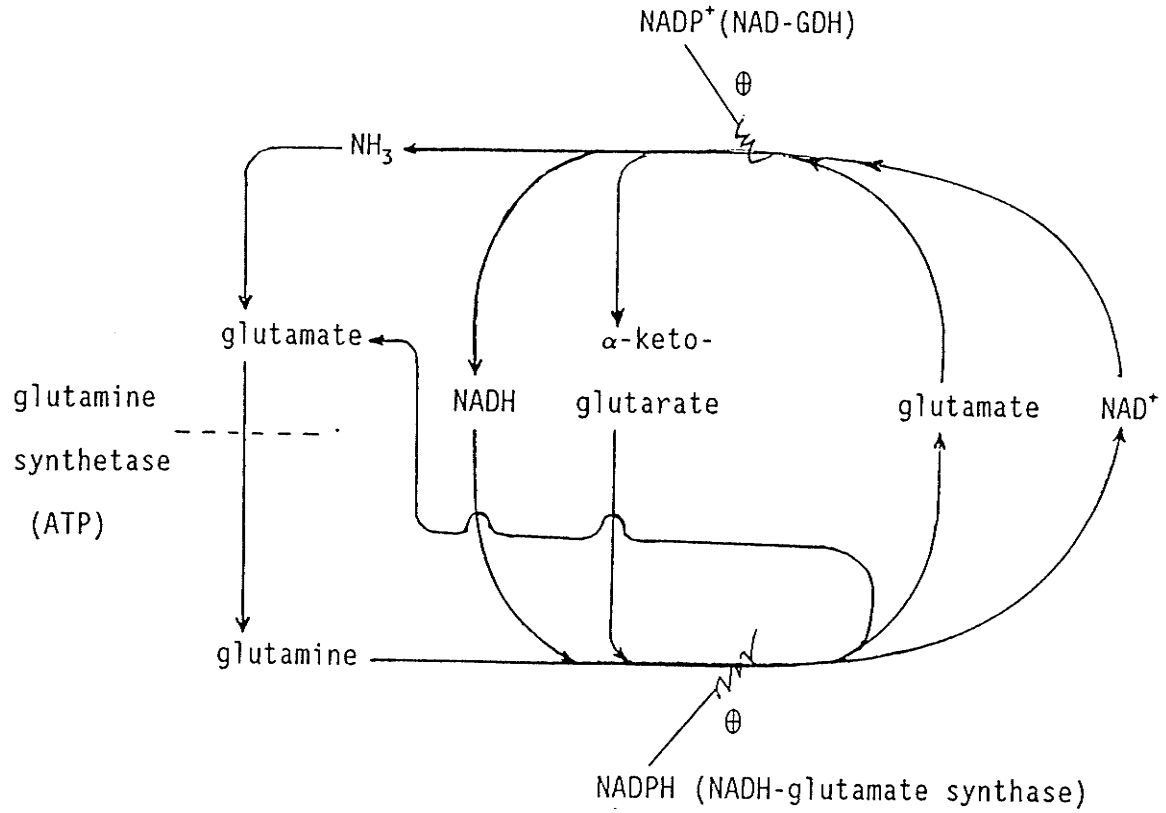
a perplexing case of an antibody against NAD-GDH cross-reacting with a protein that is clearly not a component of the enzyme that was purified, characterized and studied kinetically. It could not even explain the $NADP^+$ activation characteristics of the enzyme. The cross-reactivity between the two proteins implies similar amino acid sequences, hence DNA sequences. As will be addressed later, much effort was expended in designing and conducting experiments to determine whether this is so. The other possibility is that one antibody can recognize two proteins. As it was impossible to unequivocally decide which is correct, both possibilities had to be kept in mind when succeeding experiments were carried out.

NAD-GDH is Synthesised *De Novo*. In studies of enzyme induction, it is sometimes necessary to distinguish between activation of a pre-existing enzyme and actual synthesis of the enzyme. This was particularly important in the case of *Achlya* which has an NAD-GDH that is activated by $NADP^+$. $NADP^+$ does not function as an electron acceptor in the reaction. Both the reductive amination and oxidative deamination reactions are activated by $NADP^+$. Because it is conceivable that the induction observed could be due to either (a) enzyme modification for activation by $NADP^+$ (b) appearance of a new protein that responds to $NADP^+$ as activator, exclusively (c) appearance of a new coenzyme-specific enzyme (d) conversion of a pro-enzyme to an active one or (e) *de novo* synthesis of the same enzyme, it was necessary to conduct immunological analysis of the protein during its induction. The kinetic assays coupled with the immunological analyses support the idea that induction is due to *de novo* synthesis of only one type of NAD-GDH (Fig 26, 29). But these results bring into focus another feature; that of the co-induction of a 74,000 M_r

polypeptide with the 125,000 M_r NAD-GDH subunit by glutamine, and co-repression of the two proteins by glucose (Fig 25, 27). Both proteins cross-react immunologically even though the polyclonal antibody was raised against NAD-GDH. This subject is addressed later in this Discussion.

Is Glutamine an Inducer?

The efficient induction of NAD-GDH by glutamine compared to glutamate is probably due to the relatively low capability of the cell to transport glutamate (Singh and LéJohn, 1975). Glutamine is transported at least 10 times as effectively as glutamate. And glutamine is readily converted to glutamate by acting as a nitrogen donor for *de novo* biosynthesis of purine and pyrimidine nucleotides (LéJohn *et al.*, 1980) under the conditions used for NAD-GDH induction. Thus, even though glutamate synthase has not been formally detected in this organism, glutamate could still be formed from glutamine. Preliminary studies (B. Yang and LéJohn, unpublished data) have provided some experimental support for the presence of an NADH-linked glutamate synthase that is activated by NADPH. If this turns out to be true, then both NAD-GDH and NADH-glutamate synthase (with their respective activators, $NADP^+$ and NADPH) could effectively regulate the level of cell glutamate and ammonia through subtle adjustments in its pyridine nucleotide levels as follows:



⊕ is activation. (This model was devised by Dr. H. B. LéJohn.)

If glutamine synthetase is present, glutamate and ammonia would regenerate glutamine which could fuel the cycle all over again.

Does Glucose Repress?

Repression of NAD-GDH synthesis is mediated by glucose or a catabolic product thereof. The results of Fig 23 and 24 show clearly that the immuno-detectable level of NAD-GDH subunit is a function of glucose concentration; with increase in glucose, there is a corresponding decrease in the level of the subunit (Fig 24).

The results of Fig 23 are somewhat more informative in that they show that metabolism of glucose is essential for repression to occur. Under nutrient starvation conditions, even though excessive amounts of

glucose are supplied, the level of repression quickly plateaus at a relatively low concentration of glucose. A maximum of 20% repression is reached quickly and sustained even though the concentration of glucose increased more than 100-fold. Presumably, the starvation conditions restrict the provision of nutrients essential for glucose metabolism which, in turn, would provide the catabolite(s) that cause repression.

The general conclusion reached from these studies is that organic nitrogen promotes induction and glucose carbon facilitates repression of *Achlya* NAD-GDH.

The 74,000 M_r Polypeptide Is not a Degradation Product of NAD-GDH.

An unexpected development arising from this study is the finding of a 74,000 M_r polypeptide that is in a similar manner as the 125,000 M_r subunit of NAD-GDH, and was immunostained by the antiserum raised against the 125,000 M_r subunit. Even its decay kinetics followed that of the enzyme (Fig 32). During the repression and decay of the enzyme, the 74,000 M_r polypeptide and 125,000 M_r subunit are sustained at the same relative levels, albeit the 125,000 M_r subunit level being much higher than the 74,000 M_r one) (Fig 25, 27, 29, and 32). The function of the 74,000 M_r polypeptide is unknown. These studies have shown (enzyme purification and kinetic assays) that the 74,000 M_r polypeptide may not be a component of the active glutamate dehydrogenase because the purified native enzyme which uses NAD^+ as coenzyme and is activated by $NADP^+(H)$ has been shown to physically consist of 4 identical subunits. This was supported further by the observation that the 74,000 M_r polypeptide was found only in cell-free extracts and at no other stages in the purification of the enzyme.

74,000 M_r Polypeptide Is Part of a 220,000 M_r Protein

Immunoblot analysis (Fig 35) showed clearly that the 74,000 M_r polypeptide is part of a native protein that has a molecular mass of 220,000. Denatured, the 125,000 and 74,000 M_r proteins were immunodetected in the cell-free extracts. Undenatured, native proteins of 500,000 and 220,000 were immunodetected. The 125,000 M_r protein is NAD-GDH subunit. Therefore, the 74,000 M_r polypeptide has to be a constituent of the 220,000 M_r protein. Because this protein has not been purified, it is impossible to say whether the 220,000 M_r protein is made up entirely of 74,000 M_r polypeptides or of different types of polypeptides. In view of the relative intensity of immunostaining of the native NAD-GDH and 220,000 M_r proteins, the latter is a likely possibility. If so, then there must be another gene(s) coding for the non-74,000 M_r component(s), and this sequence has no homology to the 74,000 M_r one.

Antibody Detected only One cDNA Species.

It was reported that rabbit serum usually contained anti-*E. coli* antibodies (Snyder *et al.*, 1987). In this research, preliminary cDNA library screening always resulted in all of the plaques being dark purple in colour. This may be due to the anti-*E. coli* antibodies. After the procedure of "Removing anti-*E. coli* antibodies by pseudoscreening" (Sambrook *et al.*, 1989) was used, the background of plaques was still high probably because the anti-GDH antiserum still contained abundant amounts of anti-*E. coli* antibodies. This was shown to be the case (Figure 38). After using the procedure of " Preparation of *E. coli* lysates for absorption of anti-*E. coli* antibodies" (Sambrook *et al.*, 1989), a marked improvement was still not evident. This may have been due to the use of

improper concentration (e.g., too high or too low) of anti-GDH antiserum and the *E. coli* proteins. However, it is impossible to adjust a correct concentration between the *E. coli* proteins and the anti-*E. coli* antibodies in the anti-GDH antiserum, since different anti-*E. coli* antibodies exist in the antiserum. The affinity chromatography method was expected to remove the anti-*E. coli* antibodies but it did not do so satisfactorily. Why not? The following is a reasonable explanation. Anti-*E. coli* antibodies are mostly "anti" the outer proteins of *E. coli* cells. The proteins used to couple to the cyanogen bromide-activated Sepharose 4B are the total proteins of *E. coli* cells. Therefore, only a small part of the affinity column had the capacity to remove anti-*E. coli* membrane protein antibodies from the anti-GDH antiserum. Nevertheless, the affinity column helped in antibody purification. The results shown in Fig 39 support the notion that the anti-GDH antiserum indeed contained large amounts of anti-*E. coli* antibodies and was only anti to certain *E. coli* proteins (maybe outer proteins). The purification of the antibody, therefore, was a critical step in the cDNA library screening.

The anti-GDH antibody obtained was very active. A dilution of antiserum at 1:5,000 was routinely used in NAD-GDH staining. The purified antibody (in 25 mL) used in cDNA library screening was an amount equal to 2.7 μl of the original anti-GDH antiserum. This was a dilution of 1:10,000 approximately. The 1:10,000 diluted antibody was used 15 times (membranes) in cDNA library screening without an appearent decrease in its immunological activity.

As the antiserum obtained can react with two proteins, NAD-GDH subunit and the 74,000 M_r polypeptide, two possibilities exist; either the

antiserum contains two antibodies or one which can immunostain two proteins. Whether there are two antibody species or one, it stands to reason that the antiserum should immunodetect two distinct cDNA clones corresponding to the 2 proteins; the 125,000 M_r and 74,000 M_r protein genes. When the four cDNAs isolated were analyzed electrophoretically, they were shown to belong to the same gene (Fig 49, 50) by virtue of their overlapping sequences. The detection of one type of cDNA for two distinct proteins means that either only one species of mRNA corresponding to one gene was cloned because the other was under-represented (lack of poly(A)$^+$ RNA or instability) or else both genes share a common sequence found in the cDNAs. The former possibility was examined in Northern blotting experiments while the latter depends on knowledge of the sequences of the two genes.

Northern blot hybridization studies of (a) poly(A)$^+$ RNA and (b) total cell RNA (Fig 42) revealed that there are 2 potential transcripts that could be translated to polypeptides of 125,000 and 74,000 M_r, but poly(A)$^+$ RNA transcripts contained RNA that can only translate to a polypeptide of M_r 74,000. The possibility therefore exists that cDNAs for the 125,000 M_r polypeptide mRNA were not generated because the mRNAs are poly-A tail poor. Thus the cDNAs may derive from the mRNA for the 74,000 M_r protein.

All Genomic Clones Harbour the 74,000 M_r Protein Gene.

All 9 positive genomic clones were shown unequivocally to be from the same region of the DNA by their restriction endonuclease maps (Fig 47) and hybridization patterns against the cDNA that was used to select them (Fig 48). Because the 4 cDNAs were from the same gene and the 9 genomic clones were also from the same DNA fragment spanning a total of 20-25 kb,

it stands to reason that only one of the genes for the 125,000 M_r or 74,000 M_r polypeptide was isolated. Which one? The prevailing evidence, considering the RNA hybridization data as well, is that it is the gene for the 74,000 M_r protein that was isolated. This could not have been anticipated when the antibody to the 125,000 M_r polypeptide was being prepared. It is conceivable that neither the cDNA nor the genomic DNA encoding NAD-GDH has been isolated in these studies even though there is an ample supply of antibody to this protein at hand.

The 74,000 M_r Protein Dilemma.

Two polypeptides, one of M_r 125,000, the other of M_r 74,000 are immunologically related. But the 125,000 M_r protein belongs to NAD-GDH protein which is 500,000 in size whereas the 74,000 M_r protein is part of a protein that is 220,000 in size. Therefore these two polypeptides must have similar amino acid sequences in some section of their overall structure. By extension, the genes are likely to have a similarity in those areas coding for the amino acid sequences. Can these 2 genes be on the same stretch of genomic DNA that has been isolated? It is possible, but only complete sequencing of the appropriate region(s) will reveal this. The DNA sequence of the longest cDNA isolated showed no similarity to NAD-GDHs from *Neurospora crassa* (Vierula and Kapoor, 1989) or yeast (Miller and Magasanik, 1990), and even the non-specific human GDH (Mavrothalassitis *et al.*, 1988) as well as *Chlorella* NADP-GDH (Schmidt, 1988). This should not be surprising if the cDNA is for the 74,000 M_r protein. Being polyclonal, the population of antibody detecting the 125,000 M_r protein could be different from that recognising the 74,000 M_r polypeptide. There should not be exact correspondence between antibody,

amino acid sequence and DNA sequence in this case. It should therefore not be a surprise if the gene for NAD-GDH is outside the 20-25 kb DNA region that has been subcloned and undergoing sequencing.

The future experiments will include the sequencing of the gene for the 74,000 M_r polypeptide and its analysis. The analysis of the structural gene will lead to a better understanding of the gene and its protein. From the sequence of the gene, the amino acid sequence can be deduced. Based on the amino acid sequence, it will be possible to have some ideas about the native structure of the protein. For example, whether this protein is a membrane protein. The gene sequence and amino acid sequence can also be compared to others. This may lead to an understanding of the functions of the gene and the protein.

To obtain the gene for NAD-specific glutamate dehydrogenase, *Achlya* genomic DNA can be digested with EcoRI and fragments ranged from 1 kb to 4 kb recovered. These fragments can be put into λZap II expression vector and the genomic expression library can be screened with purified anti:NAD-GDH antibody. Theoretically, half of the positive clones will contain all or part of the gene for the 220,000 M_r protein. The other half of the positive clones will contain whole or part of the gene for NAD-specific glutamate dehydrogenase. The two genes will be very easy to tell from each other by comparing their restriction enzyme maps.

Another way to obtain the gene of NAD-GDH may be carried out by using one of the cDNA inserts as a probe to screen the cDNA library which may contain NAD-GDH cDNA but not expressed at all or not expressed as antigen determinants. All positive clones will be analysed by hybridisation and mapping. The clones which are different from the genomic

DNA may be those derived from the NAD-GDH gene and therefore can be used to screen the *Achlya* genomic library to get the gene for NAD-GDH.

Once the gene for NAD-GDH is obtained, the analysis of the gene will be carried out by restriction enzyme mapping and sequencing. The promoter region will be analysed in order to investigate transcriptional control of induction. The amino acid sequence will also be deduced to examine the similarity of the protein within *Achlya* with that in other organisms. The gene will also be put into an expression vector to investigate the expression of the gene.

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